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FOREWORD

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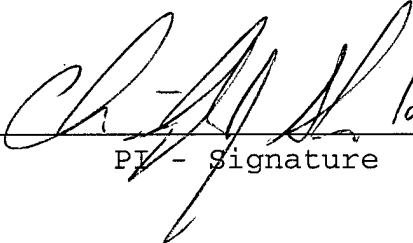
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INTRODUCTION

Breast cancer arises through multiple mutations in a single mammary epithelial cell, particularly those involving tumor suppressor genes, proto-oncogenes, and genes involved in DNA repair. The most rational approach to the treatment of breast cancer would therefore rely on knowledge of the particular molecules mutated or deleted in each clonogenic tumor. One of the molecules most commonly mutated in hereditary breast cancer is the tumor suppressor gene, *BRCA2*. *BRCA2* is mutated in approximately 35% of all multiple case, early-onset hereditary female breast cancers as well as in a significant portion of hereditary male breast cancers. These tumors frequently occur with a higher grade and larger degree of aneuploidy than sporadic tumors. Recent evidence has suggested that *BRCA2* may play a role in regulating genomic integrity of cells during proliferation and following DNA damage. Intriguingly, the properties of the familial breast cancer tumor suppressor, *BRCA1*, in spite of its lack of homology to *BRCA2*, have been demonstrated to be similar to those of *BRCA2* in almost every other respect. Moreover, *BRCA1* has been shown to physically interact with *BRCA2* *in vivo*. Due to the striking convergence of these two different breast cancer predisposition genes, we hypothesize that the association of *BRCA2* with *BRCA1* comprises a critical part of *BRCA2*'s role as a genomic caretaker and breast tumor suppressor. In this proposal we will determine if this hypothesis is correct by developing an animal model in which *BRCA2* is deficient in its ability to interact with *BRCA1*. Specifically, we will: 1) Determine whether *Brca1* and *Brca2* interact in murine cells; 2) Map the domain(s) of *Brca2* necessary for such an interacting with *Brca1*; and 3) Determine whether mice bearing a *Brca2* allele defective in binding to *Brca1* are genomically unstable or susceptible to tumorigenesis.

BODY

The goals of my 1999 pre-doctoral fellowship “Role Of *Brca2* In Cell Cycle Progression And The Response To DNA Damage In Mammary Epithelial Cells”, were to 1) Verify and Map the *Brca2* and *Brca1* Interaction *In Vivo* and 2) Determine if the *Brca2* interaction with *Brca1* is necessary for DNA Repair and Tumor Suppression *In Vivo*. We have published results addressing aim 1 in the manuscript, “Analysis Of Murine *Brca2* Reveals Conservation Of Protein-Protein Interactions But Differences In Nuclear Localization Signals” (Appendix 1) (1). In this manuscript we have addressed aim 1 by verifying the stable interaction between murine *Brca2* and *Brca1* (please see attached manuscript for details, Fig 4) and by demonstrating that the amino-terminus of murine *Brca2* is sufficient for interaction with human BRCA1 (please see attached manuscript for details, Fig 6). Also in this manuscript, we have characterized the murine *Brca2* protein. We find that *Brca2* stably interacts with murine Rad51 (please see attached manuscript for details, Fig 4). We demonstrate that the physical association of *Brca2* with Rad51 requires exon 11 of murine *Brca2*, but not its carboxyl-terminus (please see attached manuscript for details, Fig 5). We also show that murine *Brca2* differs from human BRCA2 in that carboxyl-terminal truncations of murine *Brca2* localize to the nucleus (please see attached manuscript for details, Fig 7). Collectively, our findings suggest that multiple functional interactions of *Brca2* have been evolutionarily conserved with the notable exception of those signals required for its nuclear localization.

To further define the region of murine *Brca2* required for interaction with *Brca1*, we have generated an amino-terminal *Haemagglutinin* (HA)-tagged full-length murine *Brca2* cDNA. Using this HA-tagged full-length *Brca2* cDNA, we have also cloned three *Brca2* mutants containing in-frame deletions of the amino terminus, exon 11, and the carboxyl-terminus of *Brca2*, respectively. We will determine regions of *Brca2* required for interaction with *Brca1* by transfecting in the HA-tagged deletion constructs, performing anti-HA immunoprecipitations, and immunoblotting for co-precipitating *Brca1*. We have also cloned 19 overlapping GST-*Brca2* fusion polypeptides, each containing approximately 230 amino acids of *Brca2* sequence, collectively spanning the entire *Brca2* protein. We will perform GST pulldowns from mammary epithelial cell

line protein extracts and immunoblot for co-precipitating Brca1 to more precisely identify regions of Brca2 critical for interaction with Brca1.

KEY RESEARCH ACCOMPLISHMENTS

- Murine Brca2 interacts with murine and human BRCA1 *in vivo*
- The amino-terminus of murine Brca2 is sufficient to interact with human BRCA1
- Murine Brca2 protein levels are cell cycle regulated such that it is upregulated at the G1/S cell cycle boundary
- Murine Brca2 is nuclear and localizes to subnuclear foci in mammary epithelial cell lines
- The majority of murine Brca2 protein is stably associated with Rad51 *in vivo*
- Exon 11 of murine Brca2 is required for association with Rad51, and the carboxyl-terminus of Brca2 is dispensable for this interaction
- Unlike human *BRCA2*, carboxyl-terminal truncating mutations of *Brca2* generate polypeptides that retain nuclear localization

REPORTABLE OUTCOMES

Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) *J. Biol. Chem.* **276**, 37640-37648

CONCLUSIONS

We have shown that the interaction between murine Brca2 and Brca1 exists *in vivo*. Furthermore, we have shown that the amino terminus of murine Brca2 is sufficient to interact with human BRCA1. We have generated reagents such as epitope tagged murine Brca2 deletion constructs and GST fusion proteins to more completely map this interaction.

We have also generated other new information regarding murine Brca2. We have demonstrated that the murine Brca2 protein is similar to human BRCA2 with regard to its nuclear localization, cell cycle regulation, binding to Brca1, and binding to Rad51. In addition, we shown that exon 11 of Brca2 is required for its interaction with RAD51. Finally, despite low overall homologies between the murine and human orthologs of BRCA2 and BRCA1, we have demonstrated that murine Brca2 is capable of stably interacting with human BRCA1 *in vivo*. This indicates that the interaction between Brca1 and Brca2 has been conserved evolutionarily and suggests that this interaction is functionally important. In aggregate, our data are consistent with the hypothesis that murine and human BRCA2 have largely equivalent functions.

Future experiments will include the generation of a Brca2 targeting construct that contains a Brca2 mutant lacking the Brca1 binding domain. Embryonic stem cells will be electroporated with this construct, screened to verify that a correct recombination event has occurred, and injected into blastocysts for the generation of Brca2 knock-in mice. We will determine that these mice lack a stable association between murine Brca2 and Brca1, and will determine their susceptibility to spontaneous and induced tumorigenesis, as well as genotoxic insult. These experiments will determine the significance of the Brca1-Brca2 interaction in the role of Brca2 as a tumor suppressor and guardian of the genome.

REFERENCES

1. Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) *J. Biol. Chem.* **276**, 37640-37648

APPENDICES

Appendix 1 (Attached) - Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) *J. Biol. Chem.* **276**, 37640-37648

Analysis of Murine *Brca2* Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals*

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In this report, we have analyzed the protein encoded by the murine *Brca2* locus. We find that murine *Brca2* shares multiple properties with human BRCA2 including its regulation during the cell cycle, localization to nuclear foci, and interaction with *Brcal* and *Rad51*. Murine *Brca2* stably interacts with human BRCA1, and the amino terminus of *Brca2* is sufficient for this interaction. Exon 11 of murine *Brca2* is required for its stable association with RAD51, whereas the carboxyl terminus of *Brca2* is dispensable for this interaction. Finally, in contrast to human BRCA2, we demonstrate that carboxyl-terminal truncations of murine *Brca2* localize to the nucleus. This finding may explain the apparent inconsistency between the cytoplasmic localization of carboxyl-terminal truncations of human BRCA2 and the hypomorphic phenotype of mice homozygous for similar carboxyl-terminal truncating mutations.

Women inheriting mutations in the *BRCA2* tumor-suppressor gene have up to an 84% lifetime risk of developing breast cancer (1), and these tumors account for ~35% of inherited breast cancers in women (2). *BRCA2* encodes a 3418-amino acid nuclear protein of a predicted molecular mass of 384 kDa. Most disease-causing *BRCA2* alleles contain truncating mutations that result in deletion of the three characterized nuclear localization signals present at the extreme carboxyl terminus of *BRCA2* (3, 4). Because these signals are required for the nuclear localization of human *BRCA2*, it has been postulated that truncating alleles of *BRCA2* are functionally equivalent to null alleles of this tumor suppressor gene (3).

Though its exact cellular role remains unclear, a growing body of evidence indicates that *BRCA2* is involved in DNA damage-response pathways shared with *BRCA1* and *RAD51*. *BRCA2*, *BRCA1*, and *RAD51* are each co-regulated with highest levels of expression occurring during the S and G₂/M phases of the cell cycle, and these proteins co-localize to discrete foci within the nucleus (5–11). Furthermore, human *BRCA2* has been shown to physically interact with both *RAD51* (12–16) and *BRCA1* (16).

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Human *BRCA2* binds to *RAD51* via eight BRC repeats, each 30–80 amino acids in length, that are located within exon 11 of *BRCA2* (17, 18). These repeats have been demonstrated by yeast two-hybrid analysis to be both necessary and sufficient for stable binding of human *BRCA2* to *RAD51* (12, 14, 15). The region(s) of *BRCA2* that are required for binding to *BRCA1* have been less clearly defined, though the carboxyl-terminal third of *BRCA2* has been shown to be dispensable for this interaction (16). Nevertheless, despite the identification of *BRCA2*-*RAD51* and *BRCA2*-*BRCA1* protein-protein interactions, the contribution of these interactions to the tumor-suppressor functions of *BRCA2* remains uncertain.

Mice bearing homozygous mutations in *Brca2* that yield truncations of all eight BRC repeats uniformly die *in utero* between embryonic day 6.5–8.5, with elevated levels of p53 and p21 (19–21). Notably, this phenotype is similar to that of mice homozygous for null mutations in either *Rad51* or *Brcal* (22–26). Whereas mice bearing truncating alleles of *Brca2* that remove only a subset of BRC repeats also die *in utero*, a fraction of homozygotes survive to birth with the survival rate being roughly proportional to the number of BRC repeats left intact (27–29). Surviving homozygotes invariably succumb to thymic lymphomas, and cells from these mice exhibit increased genotoxin sensitivity and chromosomal instability, as well as an impaired ability to form *Rad51* nuclear foci in response to DNA damage (27–30). In contrast, mice homozygous for truncating mutations in *Brca2* that leave exon 11 intact exhibit a more limited sensitivity to genotoxins, are 100% viable, and do not appear to develop spontaneous tumors (31). These data argue for a central role of exon 11 in the genomic surveillance and tumor-suppressor functions of *Brca2*.

Whereas murine knockout models support a role for *BRCA2* as a tumor suppressor, the increasingly severe defects observed in mice as larger amounts of the *Brca2* carboxyl terminus are truncated appear inconsistent with reports that even small carboxyl-terminal truncations in human *BRCA2* result in its cytoplasmic localization. That is, essentially all truncating alleles might be expected to behave similarly to null alleles, because carboxyl-terminal truncation would ostensibly lead to cytoplasmic localization and preclude *Brca2* from participating in nuclear functions (3, 4). This apparent discrepancy could be because of differences in the subcellular localization signals of human and murine *Brca2* or to differences in the functions of murine and human *BRCA2* in the cytoplasm. In this regard, another apparent functional difference between murine and human *BRCA2* is suggested by the mapping of a murine *Brca2*-*Rad51* interaction to the carboxyl terminus of murine *Brca2*, because similar approaches have shown that the corresponding region of human *BRCA2* lacks significant affinity for *RAD51* (12, 14, 15). Further complicating the direct comparison of

murine and human BRCA2 is the fact that the overall amino acid homology between these orthologs is only 59%, a relatively low degree of evolutionary conservation compared with other tumor-suppressor genes. Together, these data have called into question the applicability of murine models for understanding the function of human BRCA2.

In this report, we characterize the murine Brca2 protein. We find that Brca2 stably interacts with murine Brca1 and Rad51. We demonstrate that the physical association of Brca2 with Rad51 requires exon 11 of murine Brca2 but not its carboxyl terminus. We also show that murine Brca2 differs from human BRCA2 in that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. Collectively, our findings suggest that multiple functional interactions of Brca2 have been evolutionarily conserved with the notable exception of those signals required for its nuclear localization.

EXPERIMENTAL PROCEDURES

Isolation of Murine Brca2 cDNA—Poly(A)⁺ RNA isolated from day 14 murine embryos was used to generate a cDNA library in lambda ZAP using the ZAP-cDNA synthesis and ZAP-cDNA Gigapack II Gold packaging kits according to manufacturer's instructions (Stratagene). 5 × 10⁶ plaques from each library were screened by standard methods using [³²P]dCTP-labeled random-primed cDNA fragments (BMB) corresponding to nucleotides 2–221, 798–2932, and 9033–9972 of murine Brca2. Hybridization was performed at a concentration of 10⁶ cpm/ml in 48% formamide, 10% dextran sulfate, 4.8× SSC, 20 mM Tris, pH 7.5, 10× Denhardt's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42 °C overnight. Filters were washed twice in 2× SSC/0.1% SDS at room temperature for 20 min and twice in 0.2× SSC/0.1% SDS for 20 min at 50 °C and subjected to autoradiography on XAR-5 film (Eastman Kodak Co.). Phage clones were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions. Sequence analysis identified three overlapping clones that together spanned the entire Brca2 coding sequence, with the exception of an internal deletion of nucleotides 454–672. This region was replaced with a polymerase chain reaction product generated from murine testis first-strand cDNA and primers 5'-GAATTCATGCCCGTTGAATACAAAAGGAGAC-3' and 5'-CTCGAGGCAGATTTCTCATTCTGCTG-3'. After sequencing to verify the absence of additional mutations, the overlapping clones were assembled to generate a full-length murine Brca2 cDNA.

Generation of Antisera—Using primers 5'-CATCCGAATTCGACACACAGCGATTAGGAC-3' and 5'-CATCCCTCGAGGCACCGCAGAGTAAGAGGG-3' (Brca2A), and 5'-CATCCGAATTCGATGAAGAAGCAGCAGCTC-3' and 5'-CATCCCTCGAGACTGCATTTTCACAGTGGC-3' (Brca2B), polymerase chain reaction products corresponding to amino acids 19 to 135 (Brca2A) and 206 to 566 (Brca2B) were generated from a partial Brca2 cDNA, ligated into pGEM-T vector (Promega), and subcloned in-frame into pGEX-6P-1 (Amersham Pharmacia Biotech). GST¹ fusion peptides were purified from BL21 *Escherichia coli* according to manufacturer's instructions. Brca2 peptides were cleaved from the GST domain using a site-specific protease, gel-purified by SDS-PAGE, and injected into rabbits using standard immunization protocols (Cocalico Biologicals). Sera from immunized rabbits were affinity-purified on columns containing immunogen bound to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) according to published methods (32).

Transfection of Cells—293T cells were transiently transfected using a standard calcium-phosphate protocol (33). For co-immunoprecipitation experiments, 2.5 × 10⁶ cells on 150-mm dishes were transfected with 25 μg of DNA. For subcellular localization studies, 1 × 10⁶ cells on 100-mm dishes were transfected with 5 μg DNA, and cells were split onto culture slides at 24 h post-transfection. All analyses were performed at 48 h post-transfection.

Cell Culture—All cells were grown at 37 °C in a humidified incubator supplemented with 5% CO₂. 293T, NMuMG, and 16MB9A cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Gem Cell), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. HC11 cells were cultured in RPMI supplemented with 10% bovine calf serum (Gem Cell), 1 mM L-gluta-

mine, 100 units/ml penicillin, 100 units/ml streptomycin, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin.

Immunoblotting and Immunoprecipitation—Cells were harvested by lysis in EBC Buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Igepal CA-630 (Sigma)), supplemented with phosphatase inhibitors (50 mM NaF and 1 mM β-glycerol phosphate) and protease inhibitors (100 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepabloc). Following removal of insoluble debris by centrifugation, extracts were either boiled in 1× (final) Laemmli sample buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8, 5% β-mercaptoethanol, 250 mM dithiothreitol, 0.005% bromophenol blue) or subjected to immunoprecipitation. For immunoprecipitation studies, 1.0–1.5 mg of protein extract was incubated with 4 μg of affinity-purified antibody or, in the case of RAD51, 1 μl of polyclonal antiserum (Ab-1; Oncogene Science) for 1 h at 4 °C in a total volume of 1 ml of EBC plus inhibitors. Protein A-Sepharose (25 μl of a 50% slurry in PBS; Life Technologies, Inc.) was added, and incubation was continued for 1 h. The Sepharose beads were pelleted and washed three times in EBC plus inhibitors, resuspended, boiled in 14 μl of 2× (final) Laemmli sample buffer, and loaded for SDS-PAGE.

Except as noted, all protein samples were separated by 5% SDS-PAGE in 50 mM Tris base, 192 mM glycine, and 0.1% SDS. For immunoblotting, electrophoresed proteins were transferred onto nitrocellulose (Schleicher & Schuell) in 50 mM Tris base, 192 mM glycine, and 20% methanol in a submerged tank apparatus (Bio-Rad) for 12 h at 20 V. Blotted membranes were rinsed twice in PBS and blocked for 1 h at room temperature in PBS containing 5% nonfat milk and 0.05% Igepal CA-630 (MPBS-I). All affinity-purified rabbit polyclonal primary antibodies were used for immunoblotting at a final concentration of 2 μg/ml. Commercial antibodies, including anti-human BRCA2 Ab-2 (Oncogene Science), anti-RAD51 Ab-1 (Oncogene Science), anti-RAD51 Ab-1 (NeoMarkers), anti-BRCA1 MS110 (Oncogene Science), anti-β-tubulin N357 (Amersham Pharmacia Biotech), and anti-RAD50 R75020 (Transduction Laboratories) were used at the concentrations recommended by the manufacturer. Blots were incubated with primary antibodies diluted in MPBS-I for 1 h at room temperature and were subsequently washed three times in MPBS-I for 10 min each. Peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch) were incubated at a 1:5000 dilution for 1 h at room temperature in MPBS-I. Blots were washed three times in MPBS-I for 15 min each and rinsed four times with PBS, and antibody complexes were detected by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech) on XAR-5 film (Kodak).

Cell Cycle Synchronization and Analysis—HC11 cells were synchronized by serum starvation for 48 h and were subsequently restimulated with growth medium containing 20% serum. At 4-h intervals, cells were trypsinized and washed in PBS, and approximately two-thirds of cells were pelleted and snap-frozen for subsequent protein harvest. The remaining cells were pelleted, resuspended in PBS, and fixed in 70% ethanol. Following fixation, cells were pelleted, resuspended in PBS supplemented with 10 μg/ml propidium iodide and 100 μg/ml RNase A, and sorted by DNA content using a Becton Dickinson FACScan flow cytometer. The program ModFit was used to quantify percentages of cells in each phase of the cell cycle.

Subcellular Fractionation—Nuclear and cytoplasmic fractionation was performed as described previously (34). Briefly, 16MB9A cells were harvested by trypsinization, pelleted, and washed in PBS. Cells were washed in ice-cold hypotonic buffer (30 mM HEPES, pH 7.5, 5 mM KCl, 1 mM MgCl₂), resuspended in three packed cellular volumes of hypotonic buffer supplemented with protease inhibitors, and incubated on ice for 30 min. Cells were homogenized in a Wheaton Dounce with 25 strokes of a type B pestle. An equal volume of Nonidet P-40 lysis buffer (0.1% Igepal CA-630, 250 mM sucrose, 1 mM MgCl₂, 10 mM Tris, pH 7.5) was added dropwise, and cells were lysed using another 10 strokes. Nuclei were pelleted at 1300 × g at 4 °C for 5 min. Following removal of the cytoplasmic supernatant, nuclei were washed twice in 1:1 hypotonic buffer/Nonidet P-40 lysis buffer and resuspended in an amount of 1:1 hypotonic buffer/Nonidet P-40 lysis buffer equal to the extract volume prior to centrifugation of nuclei. Nuclear and cytoplasmic fractions were diluted with 6× EBC to a final concentration of 1×, centrifuged to remove insoluble debris, and boiled in 1× (final) Laemmli sample buffer prior to SDS-PAGE.

Immunofluorescence—Cells were cultured in 2-well culture slides (Falcon), rinsed in PBS, and fixed for 10 min in 3% paraformaldehyde/2% sucrose/PBS. Cells were rinsed twice in PBS and permeabilized for 5 min in ice-cold buffer (0.5% Triton, 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose). Following five rinses in PBS, cells were incubated at 37 °C for 20 min with anti-Brca2A (2 μg/ml in 3% bovine serum albumin/PBS). Cells were rinsed twice in PBS and

¹ The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

To address this, we performed reciprocal co-immunoprecipitations of Rad51 and Brca2 from HC11 cells (Fig. 4A). This

(mAb-1) and residues 995–1244 (mAb-3) (36), we performed reciprocal co-immunoprecipitations of Brca1 with Brca2. Low

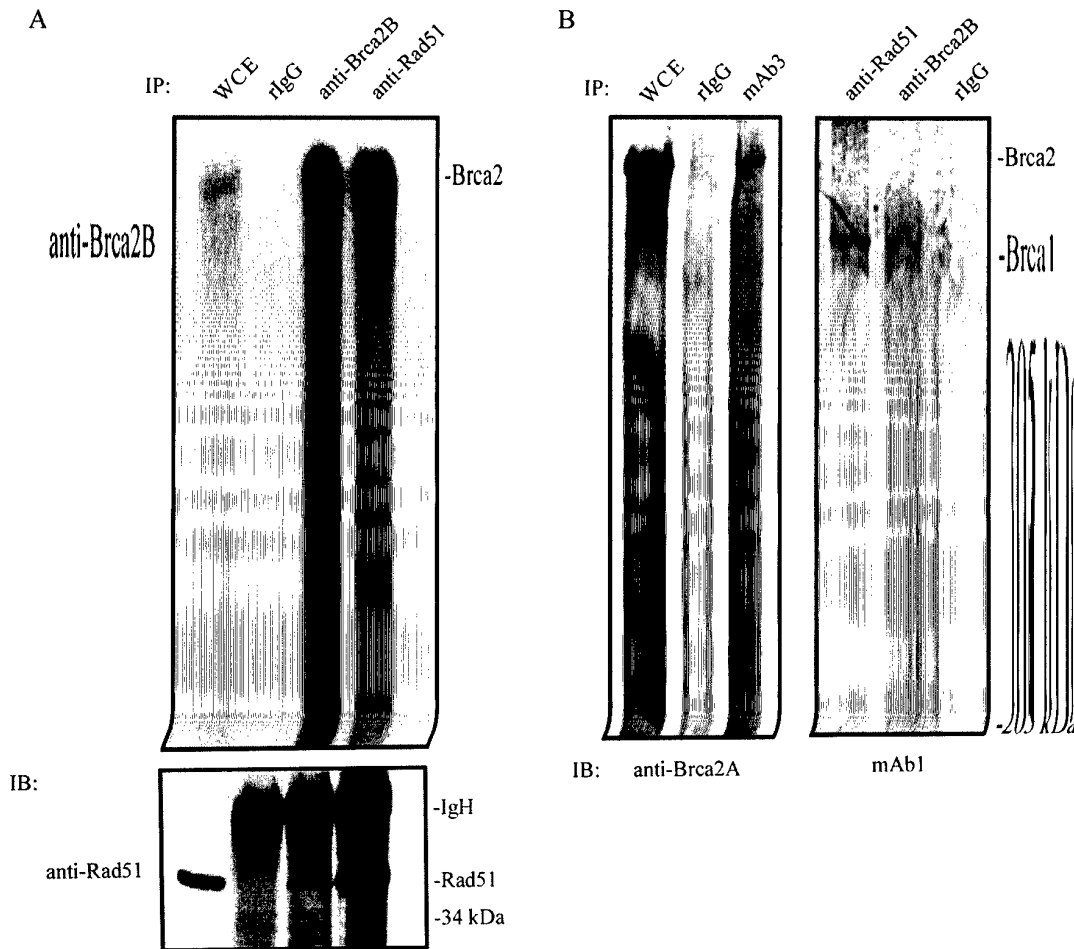


FIG. 4. **Brca2 interacts with Rad51 and with murine Brca1 *in vivo*.** A, asynchronously growing HC11 cells were harvested and immunoprecipitated using anti-Brca2B, anti-RAD51 Ab-1 (Oncogene Science), or rabbit IgG. Precipitates were separated by SDS-PAGE and immunoblotted with anti-Brca2A (top panel) or with anti-RAD51 Ab-1 (Oncogene Science; bottom panel). The whole cell extract (WCE) represents 1/30 of the input protein. B, Brca1 (left panel), Brca2 (right panel), or Rad51 (right panel) were immunoprecipitated (IP) from HC11 whole cell extracts using the indicated antibodies. Precipitates were immunoblotted (IB) with anti-Brca2A (left panel) or anti-Brca1 mAb-1 (right panel). The whole cell extract represents 1/20 of the input protein.

but significant levels of Brca2 were detected in Brca1 immunoprecipitates (Fig. 4B). Based on comparisons to input cellular extracts, less than 5% of total Brca2 polypeptides appear to be stably bound to Brca1 under these conditions (Fig. 4B). This finding is consistent with the fraction of total BRCA2 molecules that have been reported to be bound to BRCA1 in human cells (16). Conversely, we detected Brca1 in Brca2 immunoprecipitates at levels approximately equivalent to those present in Rad51 precipitates (Fig. 4B). No Brca1 or Brca2 was detected in rabbit IgG control precipitates. Taken together, our data indicate that the proteins with which murine Brca2 interacts are similar to those with which human BRCA2 interacts and that the stoichiometries with which these interactions occur may also be similar. Of note, however, we cannot rule out the possibility that our antibodies have incomplete access to cellular Brca2-Brca1 complexes or that the immunoprecipitation conditions employed in these experiments cause disruption of Brca2-Brca1 complexes.

Exon 11 of Murine Brca2, but Not Its Carboxyl Terminus, Is Required for Interaction with Rad51—The domain(s) with which murine and human BRCA2 each interact with RAD51 have been noted previously to differ (37). Specifically, murine Brca2 has been shown to interact with Rad51 via its carboxyl terminus by yeast two-hybrid and *in vitro* GST pulldown assays (19, 38). However, despite the high degree of evolutionary conservation of this domain, similar approaches have indicated that the corresponding domain of human BRCA2 does not

control vector, and harvested cell extracts were subjected to immunoprecipitation for Brca2 and RAD51. This analysis revealed that full-length Brca2 and Brca2 Δ C were comparable in their ability to co-precipitate RAD51 (Fig. 5B). In contrast, no RAD51 was detected in anti-Brca2 immunoprecipitates from cells transfected with *Brca2* Δ Bst or *Brca2* Δ Bst-C, despite the fact that these Brca2 mutant polypeptides were expressed at levels that exceeded those of full-length Brca2 and Brca2 Δ C (Fig. 5B). Performing the reciprocal co-immunoprecipitation experiment in 293T cells yielded similar results, as full-length Brca2 and Brca2 Δ C were found to co-immunoprecipitate with RAD51, whereas Brca2 Δ Bst and Brca2 Δ Bst-C failed to co-precipitate with RAD51 (Fig. 5C). These results were also observed when Brca2 Δ Bst and Brca2 Δ Bst-C were expressed at levels comparable with those of full-length Brca2 and Brca2 Δ C (data not shown) indicating that the failure of Brca2 Δ Bst and Brca2 Δ Bst-C to interact with RAD51 is not an artifact of their higher expression levels. Moreover, the fact that human and murine RAD51 are identical at the amino acid level, and that the BRCA2-RAD51 interaction has been shown to be direct, suggests that the inability of Brca2 Δ Bst and Brca2 Δ Bst-C to co-precipitate RAD51 is not due to differences in RAD51 sequences or bridging molecules present in human cells (12, 14, 15). Collectively, these data strongly suggest that exon 11 is the principal RAD51 interaction domain contained within murine Brca2.

The Amino Terminus of Murine Brca2 Is Sufficient for Inter-

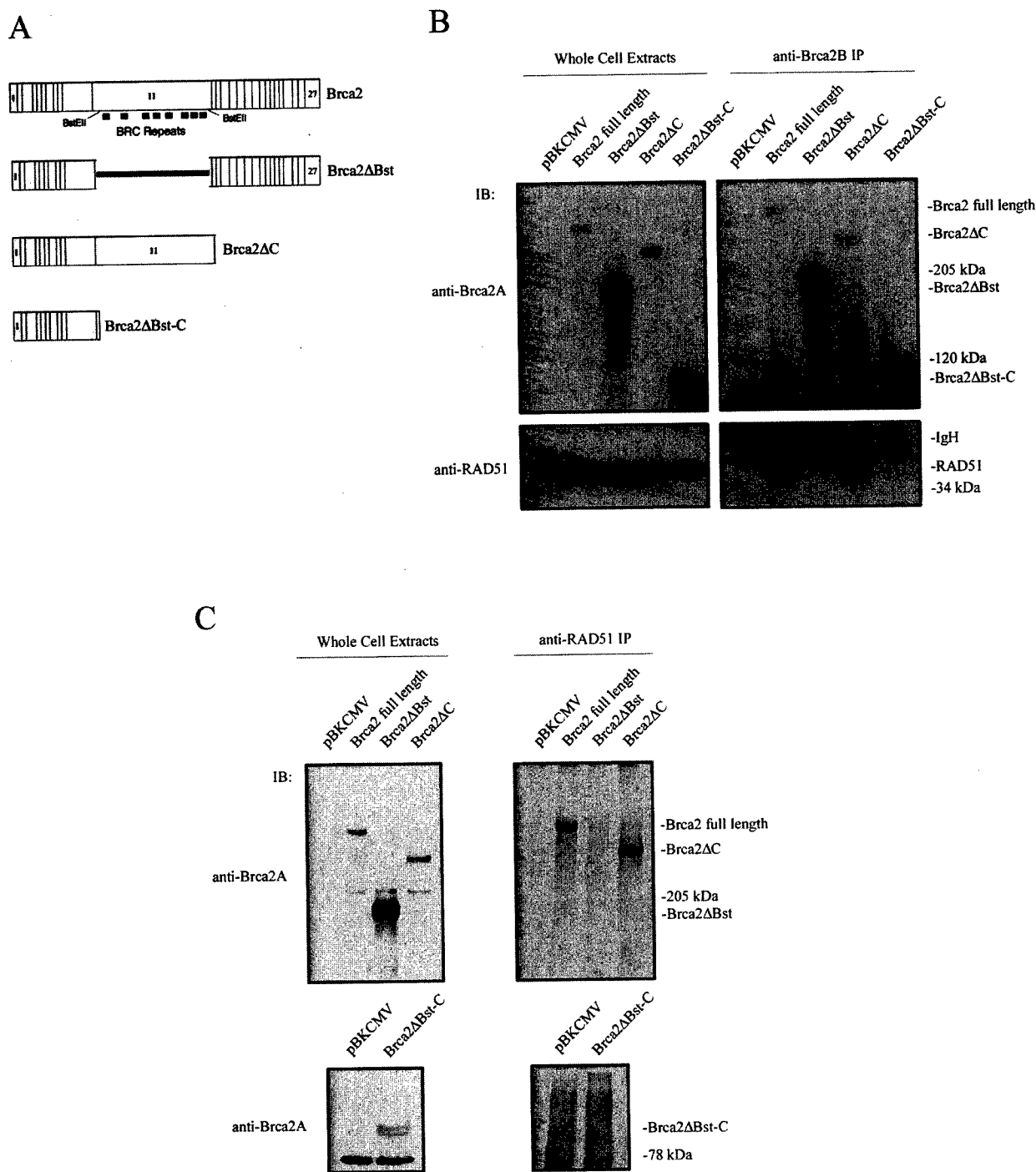
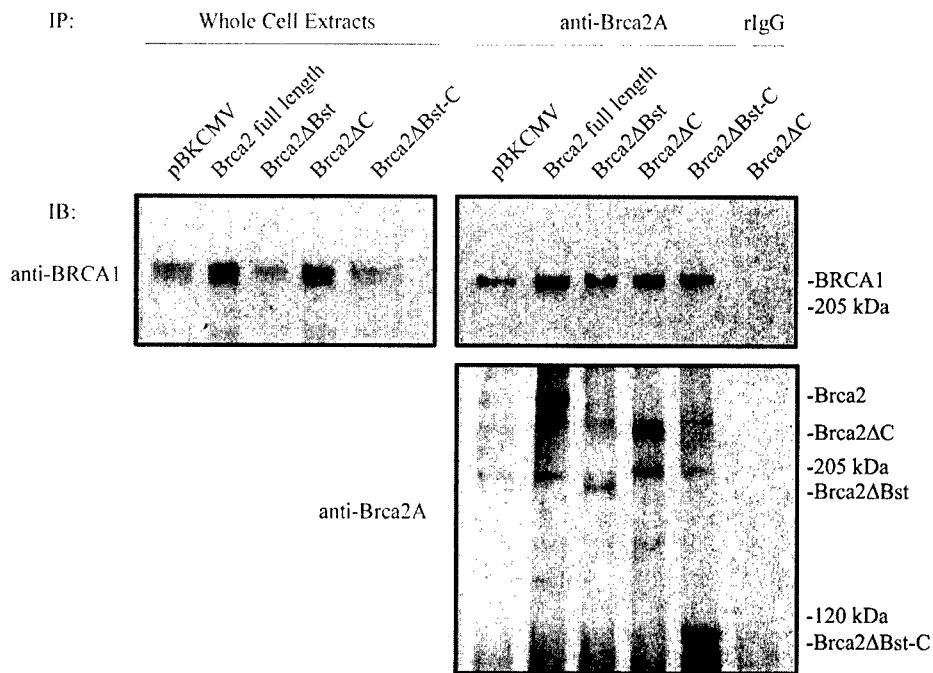


FIG. 5. Exon 11, but not the carboxyl terminus, of murine *Brca2* is required for interaction with *RAD51*. *A*, schematic depicting deletion mutants of *Brca2* generated from a full-length *Brca2* cDNA. *Brca2*Δ*Bst* represents an internal deletion mutant that encodes a polypeptide lacking amino acid residues 738–2278 from exon 11. *Brca2*Δ*C* lacks sequence from the second *Bst*EII site, at residue 2280, to the carboxyl terminus of the protein, and *Brca2*Δ*Bst-C* lacks sequence from the first *Bst*EII site, at residue 742, to the carboxyl terminus of the protein. *B*, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Cellular extracts (left panels) or cellular extracts immunoprecipitated (IP) with anti-Brca2B (right panels), were separated by SDS-PAGE and immunoblotted with either anti-Brca2A (top panel) or anti-RAD51 Ab-1 (NeoMarkers; bottom panel). *C*, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Whole cell extracts (left panels) or whole cell extracts immunoprecipitated with anti-RAD51 Ab-1 (Oncogene Science; right panels) were immunoblotted (IB) with anti-Brca2A. The bottom panels were separated by 15 rather than 5% SDS-PAGE to enhance the resolution of *Brca2*Δ*Bst-C*.

with the control vector, *pBKCMV*, suggesting that anti-Brca2A may cross-react, albeit weakly, with human BRCA2 (Fig. 6). In aggregate, our findings suggest that sequences within the amino-terminal 738 residues of *Brca2* are sufficient for interaction with BRCA1, although additional domains of *Brca2* may contribute to the stability of this association. These findings are consistent with experiments demonstrating that the interaction of human BRCA2 and BRCA1 is preserved in CAPAN-1

cells, which express a BRCA2 protein that lacks the carboxyl-terminal third of BRCA2 (16). To date, the lack of an antibody to the carboxyl terminus of *Brca2* and inefficient expression of epitope-tagged *Brca2* deletion mutants (data not shown) have prevented us from testing whether the amino terminus of *Brca2* is required for its interaction with BRCA1. Nevertheless, we conclude that the amino terminus of murine *Brca2* is sufficient to stably interact with human BRCA1 and that the se-

FIG. 6. The amino terminus of *Brca2* is sufficient for interaction with *BRCA1*. 293T cells were transfected with 12.5 μ g of *pBKCMV*, 12.5 μ g of full-length *Brca2*, 2 μ g of *Brca2 Δ Bst*, 12.5 μ g of *Brca2 Δ C*, or 2 μ g of *Brca2 Δ Bst-C*. All transfections included 12.5 μ g of a *BRCA1* expression plasmid and sufficient *pBKCMV* to bring the total DNA to 25 μ g per transfection. Whole cell extracts (left panel) or anti-*Brca2A* immunoprecipitates (right panels) from transfected cells were immunoblotted (IB) with anti-*BRCA1* MS110 (top panels) or anti-*Brca2A* (bottom panel). IP, immunoprecipitated.



quences mediating this interaction have been evolutionarily conserved.

The Amino Terminus of Murine *Brca2* Is Sufficient for Nuclear Localization—Human *BRCA2* has been shown to contain three nuclear localization signals at its extreme carboxyl terminus (3, 4). Accordingly, human *BRCA2* alleles that harbor truncating mutations anywhere within the gene result in *BRCA2* polypeptides that localize to the cytoplasm and that are therefore presumed to be nonfunctional (3). If the signals directing nuclear localization in murine *Brca2* are similarly positioned, even small carboxyl-terminal truncations in murine *Brca2* should result in a cytoplasmic gene product. These findings predict that truncating mutations anywhere within *Brca2* would result in equally severe phenotypes. In contrast to this prediction, the viability of mice bearing truncations in *Brca2* directly correlates with the length of the resulting protein, in that amino-terminal truncations have more severe phenotypes than carboxyl-terminal truncations. We reasoned that it was more likely that murine and human *BRCA2* would have conserved functions but different nuclear localization signal locations than have conserved nuclear localization signal locations and different functions. We tested this hypothesis using immunofluorescence to analyze the subcellular location of *Brca2* polypeptides in 293T cells transiently transfected with the above murine *Brca2* deletion constructs.

As expected, exogenously expressed full-length murine *Brca2* was observed to localize exclusively to the nucleus, as demonstrated by co-fluorescence with ECFP-Nuc (CLONTECH), a control for nuclear localization (Fig. 7). Similarly, *Brca2 Δ Bst* was also shown to localize to the nucleus of transfected cells, indicating that exon 11 is not required for the nuclear localization of murine *Brca2*. Surprisingly, *Brca2 Δ Bst-C* was also shown to localize to the nucleus despite its deletion of more than three-fourths of the full-length protein, including the carboxyl terminus. *Brca2 Δ Bst-C* encodes a polypeptide with a predicted molecular mass of 82 kDa that is significantly greater than the 65-kDa molecular mass cutoff for passive diffusion through nuclear pores (39). As such, the nuclear localization of *Brca2 Δ Bst-C* cannot be explained by simple diffusion. Moreover, no fluorescent signal was detected in control cells transfected with the empty vector, indicating that the apparent localization of *Brca2 Δ Bst-C* is not the result of

antibody cross-reactivity with endogenous human *BRCA2*. These findings suggest that the amino terminus of murine *Brca2* is sufficient to direct the nuclear localization of this protein.

DISCUSSION

We have demonstrated that the murine *Brca2* protein is similar to human *BRCA2* with regard to its nuclear localization, cell cycle regulation, binding to *Brca1*, and binding to *Rad51*. In addition, we have defined further the domains of *Brca2* that are required for its interaction with *RAD51* and *BRCA1*. Finally, despite low overall homologies between the murine and human orthologs of *BRCA2* and *BRCA1*, we have demonstrated that murine *Brca2* is capable of stably interacting with human *BRCA1* *in vivo*. This indicates that the interaction between *Brca1* and *Brca2* has been conserved evolutionarily and suggests that this interaction is functionally important. In aggregate, our data are consistent with the hypothesis that murine and human *BRCA2* have largely equivalent functions.

One notable difference that we observed between murine and human *BRCA2* was the finding that the amino terminus of murine *Brca2* appears to be sufficient for its nuclear localization. In contrast, analysis of human *BRCA2*-GFP fusion proteins has demonstrated that truncating even 155 residues from the carboxyl terminus of *BRCA2* completely abrogates its nuclear localization (3). Similarly, endogenous *BRCA2* in CAPAN-1 cells, which lacks the carboxyl-terminal third of *BRCA2*, has been shown to localize to the cytoplasm by biochemical fractionation (3). As such, we believe that our findings reflect differences in the placement of nuclear localization sequences within human and murine *Brca2*. A potential caveat to this interpretation is that our localization studies were performed on *Brca2* polypeptides expressed ectopically in human 293T cells rather than in murine cells. However, our conclusions are supported by the finding that a targeted deletion of the final 566 coding nucleotides of *Brca2* results in a polypeptide that localizes to the nucleus in murine cells (40). Paradoxically, the difference in positioning of nuclear localization signals that we have identified between murine and human *BRCA2* strengthens the hypothesis that murine and human *BRCA2* are functionally equivalent. If, as for human *BRCA2*, truncation at the

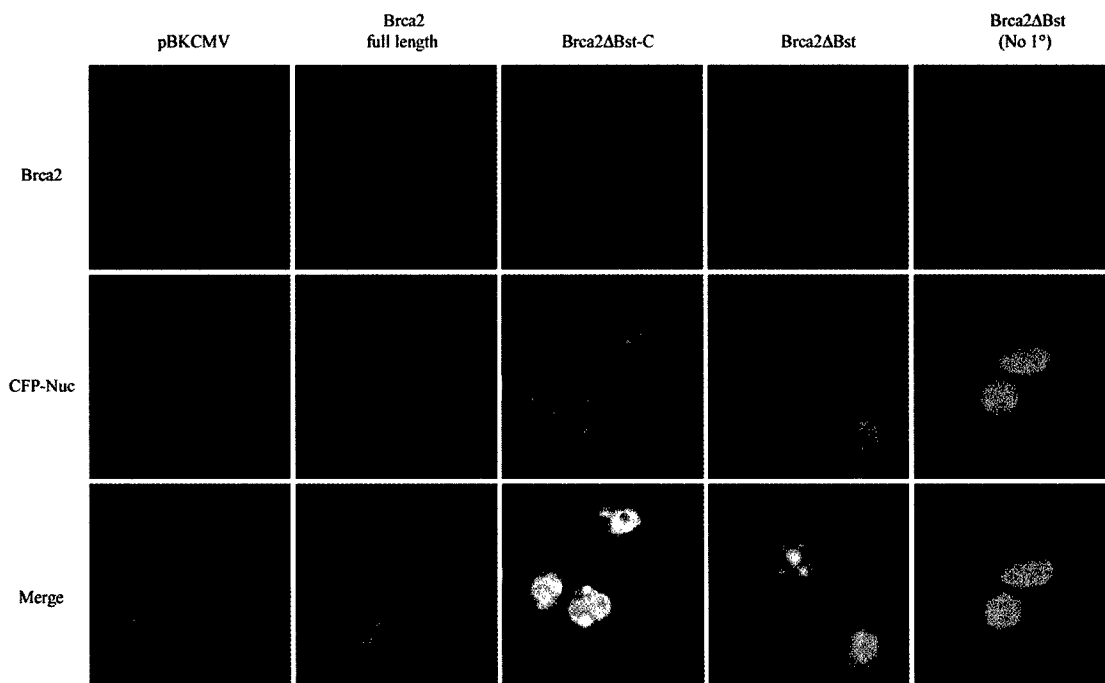


FIG. 7. The amino terminus of murine *Brca2* is sufficient for nuclear localization. 293T cells were transiently transfected with 4.5 μ g of the indicated *Brca2* deletion constructs and 0.5 μ g of *pECFP-Nuc*. Cells were analyzed for *Brca2* subcellular localization by indirect immunofluorescence with anti-*Brca2A* and co-fluorescence with CFP-Nuc.

extreme carboxyl terminus of murine *Brca2* resulted in cytoplasmic localization, we would have been forced to conclude that the more severe phenotype of mice bearing amino-terminal compared with carboxyl-terminal truncating *Brca2* mutations reflected differences in the functions of murine and human BRCA2. Our demonstration that carboxyl-terminal truncations of murine *Brca2* remain nuclear resolves this dilemma.

Mice homozygous for truncating mutations within exon 11 exhibit reduced embryonic survival, spontaneous tumorigenesis, genomic instability, and reduced Rad51 nuclear foci formation following DNA damage (27–30). Our finding that *Brca2* polypeptides lacking exon 11 are incapable of co-immunoprecipitating RAD51 constitutes the first biochemical evidence that exon 11 is required for the interaction of *Brca2* with RAD51. This, along with our observation that *Brca2* mutants lacking the carboxyl terminus retain their capacity to bind to RAD51, is consistent with yeast two-hybrid studies of the human BRCA2-RAD51 interaction and suggests that exon 11 is the principal domain of murine *Brca2* required for binding to RAD51. Nevertheless, given that interactions of the carboxyl terminus of murine *Brca2* with Rad51 have been detected by two different approaches, we favor the possibility that this region may contribute to the interaction of *Brca2* with Rad51. The targeted, in-frame deletion of exon 11 in mice would permit a more accurate assessment of the role of the BRC repeats in binding to Rad51 and of the impact that this interaction has on DNA repair and tumor susceptibility.

The multiple similarities between human and murine *Brca2* that we have demonstrated in this report shed new light on observations made in mice bearing targeted mutations in *Brca2*. Mice lacking the small carboxyl-terminal domain of *Brca2* shown to interact with Rad51 have not been reported to develop tumors, although cells from such mice exhibit premature senescence and decreased efficiency in homology-based DNA repair (31, 40). Our data predict that mice bearing carboxyl-terminal deletions would have at most only slightly impaired binding of *Brca2* to Rad51. This, in turn, may explain

the more modest phenotype of mice bearing such mutations. In support of this hypothesis, Moynahan and colleagues (40) have shown recently that the amount of *Brca2* bound to Rad51 in murine embryonic stem cells is unaffected by deletion of the carboxyl-terminal domain of *Brca2*. As such, the premature senescence and decreased DNA repair phenotypes observed in these mice may be due either to an uncharacterized defect in the Rad51 pathway or to the disruption of interactions with other proteins involved in homology-based DNA repair.

Finally, our finding that the amino terminus of *Brca2* is sufficient to interact with BRCA1 suggests that *Brca2*-*Brca1* complexes may be maintained in all *Brca2* knockout mouse models generated to date; however, whether such *Brca2*-*Brca1* complexes retain their function is unknown. Both *Brca2* and *Brca1* mutant cells have defects in Rad51 focus formation following DNA damage (36, 41). We have recently demonstrated that murine *Brca1*, like murine *Brca2*, localizes to nuclear foci (36). As peptides bearing consensus BRC repeat sequences can inhibit the polymerization of RAD51 onto DNA substrates *in vitro* (42), these data collectively suggest a role for *Brca2*, and potentially *Brca1*, in recruiting or preparing Rad51 for subsequent recombination events at sites of DNA damage. Nevertheless, it has yet to be demonstrated how *Brca2* and *Brca1* orchestrate Rad51 nuclear focus formation following DNA damage, and it has not been determined whether the disrupted regulation of Rad51 nuclear focus formation is ultimately responsible for the malignant transformation of *Brca1* and *Brca2* mutant cells. Such studies should enhance our understanding of the mechanism by which *Brca1* and *Brca2* gene products suppress tumor formation.

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