Interaction with BRCA2 Suggests a Role for Filamin-1 (hsFLNa) in DNA Damage Response*

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Yuan Yuan‡§ and Zhiyuan Shen‡¶

From the ‡Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131 and the §Graduate Program, Department of Molecular Genetics, School of Medicine, University of Illinois, Chicago, Illinois 60607

The BRCA2 tumor suppressor plays significant roles in DNA damage response. The human actin binding protein filamin-1 (hsFLNa, also known as ABP-280) participates in orthogonal actin network, cellular stress responses, signal transduction, and cell migration. Through a yeast two-hybrid system, an in vitro binding assay, and in vivo co-immunoprecipitations, we identified an interaction between BRCA2 and hsFLNa. The hsFLNa binding domain of BRCA2 was mapped to an internal conserved region, and the BRCA2-interacting domain of hsFLNa was mapped to its C terminus. Although hsFLNa is known for its cytoplasmic functions in cell migration and signal transduction, some hsFLNa residues in the nucleus, raising the possibility that it participates in DNA damage response through a nuclear interaction with BRCA2. Lack of hsFLNa renders a human melanoma cell line (M2) more sensitive to several genotoxic agents including γ irradiation, bleomycin, and ultraviolet-c light. These results suggest that BRCA2/hsFLNa interaction may serve to connect cytoskeletal signal transduction to DNA damage response pathways.

Inherited BRCA2 mutations confer profound susceptibility to human breast and ovarian cancers (1, 2). BRCA2 participates in DNA repair through interactions with other proteins, notably RAD51 and BRCA1, that mediate homologous recombination (3–6) but is apparently not required for repair of DNA double-strand breaks by non-homologous end joining (7). The BRCA2 gene encodes a 3418-amino acid protein with very little homology to other known proteins (1, 2). The overall identity of BRCA2 is 58–59% between rodents (rat and mouse) and human, which is an uncommonly low level of conservation for a tumor suppressor (8, 9). By comparing the amino acid sequences of mouse and human BRCA2, five highly conserved regions have been identified within BRCA2 and designated as Domain I through Domain V (8). It is expected that important functions of BRCA2 reside in these conserved regions.

Domain I resides in amino acids (aa) 1–100 and is a putative transcriptional activation domain (10). Domain II (aa 1001–1051 of human, aa 980–1030 of mouse) and Domain III (aa 1089–1138 of human, aa 1072–1120 of mouse) reside in exon 11 (8), which also contains the eight loosely conserved BRC motifs (11) that mediate interaction with RAD51 (12, 13). The first BRC motif overlaps Domain II. Domain IV (aa 2479–3157 of human; aa 2400–3075 of mouse) is coded by exons 14–24 (8). Domain V (aa 3267–3316 of human; aa 3190–3238 of mouse) is encoded by exon 27 and also mediates BRCA2/RAD51 interaction (14). A putative nuclear localization signal also resides in exon 27 (15).

Domain IV is the longest of the conserved domains, yet the least understood. In mice, two mutations delete some of the BRCA repeats (truncation at aa 1492 deleting BRCA repeat 4–8 and truncation at aa 2014 deleting BRCA repeat 8), together with conserved Domains IV and V. Both result in partial lethality of embryos and infertility, as well as enhanced expression of p21 and p53 (4, 16–18). However, truncation of mouse BRCA2 at aa 3140, which preserves all of the BRC repeats plus Domain IV and deletes only Domain V, results in no p21 and p53 induction (19). Mice with this truncation have much higher rate of viability and are fertile.2 The difference in phenotype between truncation at aa 2014 and truncation at aa 3140 indicates that important functions might reside in Domain IV and its flanking BRCC8 repeat.

Recently, a few protein interaction partners of Domain IV have been identified. A protein named DSS1 (Deleted in Split hand/Split foot) interacts with aa 2472–2957 within Domain IV (20). The function of human DSS1 is uncertain. The yeast DSS1 homologue (also termed SEM) plays roles in cell growth and differentiation (21). A second protein named hUBR1 interacts with aa 2867–3176 of Domain IV and affects the phosphorylation status of BRCA2 (22); hUBR1 and its yeast homologues contain a kinase domain, and may participate in mitotic checkpoint control (22–24). We have also identified a novel protein, designated BCCIPα (Brca2, and cip1 interacting protein) that interacts with Domain IV (25). BCCIPα is an evolutionarily conserved protein that may participate in the regulation of tumorigenesis (25).

Here, we report an interaction of the BRCA2-conserved Domain IV with the actin-binding protein hsFLNa (human non-muscle filamin-1). hsFLNa (also known as ABP-280) is an actin cross-linking protein (26) that participates in cytoskeletal remodeling, signal transduction, and protein nuclear translocation (for a review see Ref. 27). hsFLNa interacts with several partners, including β1 and β2 integrins, Rho GTPase, SEK-1, and human androgen receptor (28–33). In addition, we report that hsFLNa-deficiency in human cells is associated with cel-

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‡ To whom correspondence should be addressed: Dept. of Molecular Genetics and Microbiology, Univ. of New Mexico School of Medicine, 915 Camino de Salud, NE, Albuquerque, NM 87131; Tel.: 505-272-4291; Fax: 505-272-6029; E-mail: zshen@salud.unm.edu.

§ The abbreviations used are: aa, amino acids; UV-C, ultraviolet-c; GST, glutathione-S-transferase; Co-IP, co-immunoprecipitation; HA, hemagglutinin; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase.

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lular sensitization to DNA-damage agents such as γ irradiation, bleomycin, and UV-C. These data suggest a functional affiliation of hsFLNa with the DNA damage response pathway.

MATERIALS AND METHODS

**Yeast Two-hybrid System**—The yeast Matchmaker two-hybrid system (CLONTECH Laboratory, Palo Alto, CA) was used for a library screen as described in a previous publication (25). In brief, BRCA2H (aa 2883–3053), a BRCA2 fragment, was cloned into the Gal4-DB DNA binding domain (Gal4-DB) vector (pS2-1) to screen a library of human lymphocyte cDNAs fused to the Gal4-DNA activation domain (Gal4-DA) vector (pACT). MV103 cells were used as the host strain (34). An independent yeast host strain (SFY526) and vectors (pGBT9 and pGAD424) were used to confirm the interaction identified in the library screen. Yeast two-hybrid filter assays and liquid quantitative assays were performed according to the Matchmaker manual. pGBT9 and pGAD424 were used as negative controls.

**Recombinant Proteins**—To express a His-tagged hsFLNa fusion protein, an hsFLNa C-terminal fragment (aa 2250–2647) was cloned into pET28a (Novagen) and transformed into Escherichia coli BL21 (DE3). His/hsFLNa fusion protein expression and purification was performed as described previously (25, 35, 36). To fuse glutathione S-transferase (GST) with fragments of BRCA2, various coding regions of BRCA2 were amplified by PCR and cloned into pGEX-5X-1 or pGEX-5X-2. plasmids were transformed into competent E. coli BL21, and the expression and purification of GST fusion proteins were performed as previously described (25, 35, 36).

**In Vitro Protein Binding Assay**—GST or GST/BRCA2 fragments bound to glutathione-Sepharose beads were incubated with 10 μg of purified His/hsFLNa (aa 2250–2647) in binding buffer (100 mM KCl, 50 mM HEPES/KOH, pH 7.6, 250 mM NaCl, 20 mM β-mercaptoethanol) for 1 h at 4 °C. Beads were washed three times with wash buffer I (100 mM NaCl, 50 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Nonidet P-40) and three times with wash buffer II (200 mM NaCl, 50 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Nonidet P-40). The beads were collected and resuspended in 2× sample buffer. Samples were boiled for 5 min and separated by SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membrane, and co-purified His/hsFLNa proteins were detected by immunoblotting with anti-His antibody and anti-GST antibody (Santa Cruz). Proteinase inhibitor mixture (Sigma) was used in the lysis, binding, and washing buffers.

**Co-immunoprecipitation (Co-IP)**—Two BRCA2 fragments, BRCA2F (aa 2883–3418) and BRCA2B (aa 2883–3194), were cloned into pHACMV vector to tag them with the Myc epitope. The C terminus of hsFLNa (aa 2250–2647) was cloned into pMyc-CMV vector to tag it with the Myc epitope. Plasmids were transfected into 293 human kidney cells using GenePorter transfection reagent (GeneTherapy Systems). Forty-eight hours after transfection, cells were collected, treated with lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1× protease inhibitors (Boehringer), sonicated, and centrifuged for 10 min at 10,000 rpm. The supernatant (whole cell lysate) was used for immunoprecipitation. Forty microliters of anti-HA antibody and 1 μg of anti-Myc monoclonal antibody (clone 9E10) and HA monoclonal antibody (clone 12CA5) were added to 1 ml of whole cell lysate. Then beads were resuspended in lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) for 2 h at 4 °C. Beads were washed three times with wash buffer I (100 mM NaCl, 50 mM Tris, pH 7.9, 1 mM EDTA, 10% glycerol) for 1 h at 4 °C. The beads were washed three times with wash buffer II (200 mM NaCl, 50 mM Tris, pH 7.9, 1 mM EDTA) for 1× and resuspended in 2× SDS sample buffer for 10 min at 100 °C. Samples were analyzed by SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membrane, and co-purified His/hsFLNa proteins were detected by immunoblotting with anti-His antibody and anti-GST antibody (Santa Cruz). Proteinase inhibitor mixture (Sigma) was used in the lysis, binding, and washing buffers.

**Genotoxin Sensitivity Assays**—The number of cells to be plated for each assay was determined by a pilot experiment so as to yield 50–100 surviving colonies per 100-mm plate. For radiation sensitivity assay, 18 h after the cells were plated, surviving cells were irradiated with a Cs-137 γ rays (dose rate: 1.05 Gray/min). For UV-C sensitivity assay, the UV-C exposure was achieved at 1.3 J/m2/s dose rate. For bleomycin sensitivity assay, exponentially growing cells were treated with bleomycin for 2 h, trypsinized, and plated. For all the assays, cells were cultured for 12–14 days for colony formation. The colonies were stained with crystal violet and counted to determine cell viability. The number of colonies was normalized to the number of plated cells to calculate the survival fraction.

RESULTS AND DISCUSSION

**A C-terminal Domain of hsFLNa Interacts with BRCA2 in a Yeast Two-hybrid System**—A portion of BRCA2 Domain IV comprising amino acids 2883–3053 (designated BRCA2H) was used as “bait” for a yeast two-hybrid screen. BRCA2H is 77% identical between human and mouse, compared with the overall identity of 58–59% (8). Nine independent clones containing the C terminus of hsFLNa were isolated from a total of 4 × 105 independent clones. The interaction of these nine clones with BRCA2H was confirmed using a second, independent yeast two-hybrid system (SPY526 instead of MV103 as host yeast, pGB7T9 instead of pAS2-1 and pGAD424 instead of pACT vectors).

To further map the region of hsFLNa required for interaction with BRCA2H, defined C-terminal portions of hsFLNa were tested in the yeast two-hybrid system. hsFLNa is an elongated homodimeric, Y-shaped structure (26). Each monomer consists of four domains with the actin binding domain followed by 24 tandem repeats, each ~96 amino acids in length. The last 65 amino acids of its C terminus contain a self-assembly sequence that allows dimerization. This repeat is separated from the previous 23 repeats by an ~34-amino acid “hinge” domain. Ten hsFLNa constructs containing tandem repeats and/or the hinge region, as described in Fig. 1, were cloned into pGAD424. Each of the ten constructs was co-expressed with Gal4-DB/BRCA2H fusion protein in SPY526 cells and tested for interaction. The largest construct tested, comprising aa 2250–2647, represents the nate-conjugated anti-mouse IgG secondary antibody (Pierce) for 30 min. The stained cells were mounted under coverslips with Vectashield mounting medium containing DAPI (Vector Laboratories). Immunofluorescence was recorded using the Zeiss confocal microscope LSC 510 with Ar/Kr and UV laser sources.

**Cellular Protein Fractionation**—To prepare whole cell protein extracts, HeLa cells were trypsinized, washed in ice-cold PBS, pelleted, resuspended in lysis buffer (50 mM HEPES/KOH, pH 7.5, 250 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM NaF, 0.1 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin) and sonicated for 30 s. Insoluble debris was removed from centrifugation at 14,000g for 5 min, and the soluble fractions were retained as whole cell extracts. To prepare nuclear and cytoplasmic extracts, HeLa cells were trypsinized, washed in PBS and then in buffer X (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1× protease inhibitors (Boehringer)), pelleted, and resuspended in buffer X containing 0.5% Nonidet P-40 for 10 min on ice. The lysate was centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatant was taken as the cytoplasm/membrane fraction. The remaining nuclear pellet was washed with buffer X containing 0.5% Nonidet P-40 and resuspended in the same buffer containing 1× loading buffer. The samples were then sonicated and boiled before loading onto the SDS-PAGE gel.

**Cell Culture**—Human M2 and A7 cells were kindly provided by Drs. T. P. Stossel and Y. Ohta (Brigham and Women’s Hospital, Harvard Medical School). M2 is a human melanoma cell line with tagged hsFLNa expression. The A7 cell line was derived from M2 by stably transfecting with a plasmid expressing full-length hsFLNa cDNA (37). Cells were subcultured twice a week in minimum essential medium Eagle with Earle’s salt (EMEM) (M2) or EMEM with 0.5 mg/ml G418 (A7). MCF-7 cells and Capan-1 cells were obtained from ATCC and cultured as recommended. All media contained 10% fetal bovine serum and 1% penicillin/streptomycin.
minimum region of hsFLNa required for interaction with BRCA2. This region is also known to interact with several other proteins (Fig. 1B) (Please see Ref. 27 and citations therein).

Mapping of the hsFLNa-interacting Domain in BRCA2—To map the hsFLNa binding domain in BRCA2, four BRCA2 fragments were constructed: BRCA2H (aa 2883–3053), BRCA2Q (aa 2883–3036), BRCA2S (aa 2883–3001), and BRCA2N (aa 2973–3053) (Fig. 2A). These constructs were tested for interaction with hsFLNa (aa 2250–2647) in a filter assay (Fig. 2A). BRCA2H, BRCA2Q, and BRCA2S were also tested in a quantitative yeast two-hybrid assay (Fig. 2B). Based on these data, the putative hsFLNa binding site can be assigned to a small region common to these constructs, namely aa 2973–3001 of BRCA2.

Although the yeast two-hybrid assays demonstrate that hsFLNa and BRCA2 interact, it was not clear whether this interaction is a direct BRCA2/hsFLNa binding or mediated by other protein(s) in yeast. To further test whether hsFLNa directly binds to BRCA2, an in vitro protein-binding assay using purified recombinant proteins was performed. The BRCA2-interacting domain of hsFLNa (aa 2250–2647) was fused with a His_S tag. Various regions of BRCA2 were fused with GST and summarized in Fig. 3C. The BRCA2/GST fusion proteins were purified to near-homogeneity and used to precipitate His/hsFLNa (aa 2250–2647) fusion protein (Fig. 3, A and B). None of the negative controls bound to His/hsFLNa (aa 2250–2647) (Fig. 3, A and B, lanes 2, 3). GST/BRC2A2S (aa 2883–3001) and GST/BRC2A2N (aa 2973–3053) co-precipitate His/hsFLNa (aa 2250–2647). Since aa 2973–3001 is the region shared by BRCA2N and BRCA2S, we conclude that BRCA2 (aa 2973–3001) is the minimal domain for interaction with hsFLNa, consistent with the yeast two-hybrid data.

We noticed that BRCA2Q (aa 2883–3036) interacts with hsFLNa in the yeast two-hybrid system but fails to bind hsFLNa in vitro and that BRCA2P (aa 2973–3041) contains aa 2973–3001 but fails to bind hsFLNa in vitro (Fig. 3C). This inconsistency may be caused by the different protein contexts of Gal4 versus GST fusions or the folding of the recombinant GST/BRC2A2Q and GST/BRC2A2P proteins such that the interaction domain is covered.

BRCA2 Interacts with hsFLNa in Vivo—To confirm that complex formation between BRCA2 and hsFLNa occurs in vivo, Co-IP was performed in mammalian cells. Both exogenously expressed and endogenous proteins were tested. Because smaller fragments of BRCA2 (BRCA2H, BRCA2Q, BRCA2S, and BRCA2N) exhibited limited solubility when tagged with an HA epitope (data not shown), longer BRCA2 constructs, BRCA2B (aa 2883–3184) and BRCA2F (aa 2883–3418), were used for exogenously expressed proteins. Both of the latter contain the minimal hsFLNa-interacting domain of BRCA2, aa 2973–3001. A C-terminal hsFLNa fragment (aa 2250–2647) was tagged with a Myc epitope. Myc/hsFLNa (aa 2250–2647) was co-expressed in 293 human kidney cells with HA/BRC2A2B.
or HA/BCCIP, or with empty HA vector or a non-relevant protein, HA/BCCIPα (25), as negative controls. The HA-tagged proteins were immunoprecipitated with anti-HA affinity matrix, and co-precipitated Myc/hsFLNa (aa 2250–2647) was detected by anti-Myc antibody. As shown in Fig. 4 (top panel), HA/BCCIPα (lane 6) or HA vector (lane 9) did not co-precipitate Myc/hsFLNa (aa 2250–2647). On the other hand, HA/BCCIP (lane 7) and HA/BCCIPα (lane 8) co-precipitated Myc/hsFLNa (aa 2250–2647). These data suggest a stable complex between BRCA2 and hsFLNa in vivo.

To investigate the endogenous protein complex, MCF-7 and Capan-1 cells were used. MCF-7 expresses the wild type BRCA2 protein, while Capan-1 cell contains the 6174delT mutation that produces an ~230-kDa C-terminally truncated protein lacking the hsFLNa interaction domain. We used an anti-BRCA2 antibody (Ab-1, Oncogene) that reacts with both the full-length and the 230-kDa-truncated BRCA2 protein for immunoprecipitation. The immunoprecipitated BRCA2 protein complex was immunoblotted with anti-hsFLNa and anti-BRCA2. As shown in Fig. 4B, both the wild type BRCA2 from MCF-7 cells and the truncated BRCA2 from Capan-1 cells were precipitated with the anti-BRCA2 antibody (lanes 3 and 4 of bottom panel). Both MCF-7 and Capan-1 cells express hsFLNa protein (lanes 1 and 2 of top panel). However, only the endogenous hsFLNa from MCF-7 was co-precipitated with BRCA2 (lane 3, top panel), but not from Capan-1 cells (lane 4, top panel). These further indicate that a stable complex exists in vivo between full-length BRCA2 and hsFLNa and that the C-terminal region deleted in Capan-1 cells is required for the interaction.

A Fraction of hsFLNa Resides in the Nucleus—BRCA2 has been described as a predominant nuclear protein (39). hsFLNa is thought to execute most of its functions in the cytoplasm (26, 27, 29–33, 40–42), although it may participate in nuclear-related functions (28). It might be argued that the interaction between hsFLNa and BRCA2 is not physiologically relevant since they apparently reside in different cellular compartments. To address this issue, we performed immunostaining of hsFLNa in human skin fibroblast and HeLa cells to observe the distribution of hsFLNa with confocal microscopy, which detects fluorescent signals from a thin intersection of 0.5 μm within the cells. The majority of hsFLNa was seen in the cytoplasmic compartment of the cells (Fig. 5, B, C, E, F), which is consistent with previous reports (26, 27, 29–33, 40–42). However, a moderate level of hsFLNa signal was seen in the nucleus as well, suggesting that a fraction of hsFLNa resides in the nucleus. To further demonstrate that hsFLNa exists in the nucleus, the cytoplasmic and nuclear proteins were extracted from HeLa cells and analyzed by Western blot. As shown in Fig. 5G, hsFLNa protein was detected in both the cytoplasmic and nuclear fractions. YY1 and paxillin are used as nuclear and cytoplasmic controls, respectively.

Lack of hsFLNa Does Not Affect Cell Growth but Renders Cells More Sensitive to Genotoxic Agents—The diverse interaction partners of hsFLNa suggest its multiple functions in different cellular processes (27). Characterization of these interacting proteins has provided major insights into the roles of hsFLNa. For instance, hsFLNa was related to integrin signaling pathways through its association with β1, β2, and β3 integrins (31, 32, 43, 44). The physiological significance of hsFLNa was suggested by the discovery of an X-linked dominant human disorder, periventricular heterotopia, caused by mutations in the hsFLNa gene. Mutations are embryonic lethal in males; in females, they are characterized by defects in the migration of cerebral cortical neurons and vascular abnormalities (45, 46). These results are consistent with the previous observation that hsFLNa is essential for melanocyte migration in vitro (37), suggesting that hsFLNa might be a key protein in cell migration. Similarly, hsFLNa was connected to the MAPK cascade through its associations with SEK-1 and TRAF2 (tumor necrosis factor receptor-associated factor 2) (33, 47). Taking the above examples together, the association of hsFLNa with BRCA2 suggests its involvement in BRCA2-mediated DNA damage response.

BRCA2 plays important roles in DNA damage response, possibly by participating in homologous recombination repair and/or by mediating signal transduction that affects other cellular responses to DNA damage. To evaluate whether hsFLNa is also involved in DNA damage response, we used the hsFLNa-deficient human M2 melanoma cell line and an isogenic hsFLNa-proficient derivative line, A7, M2 (hsFLNa−/−) cells have impaired locomotion and display circumferential blebbing of the plasma membrane. The hsFLNa expression has been restored to approximately normal level in A7 cells by stable transfection with an hsFLNa expression vector. A7 cells have restored translocational motility and reduced membrane blebbing (37, 42). As confirmed in Fig. 6A, A7 cells express hsFLNa, but M2 cells do not. Lack of hsFLNa does not affect the growth...
of M2 cells in culture (Fig. 6B). A7 (hsFLNa+1) and M2 (hsFLNa−) cells have similar plating efficiencies (50–70%). However, when treated with γ radiation, bleomycin, and UV-C light, M2 cells (hsFLNa−) were markedly more sensitive than A7 cells (hsFLNa+1), as assessed in colony formation assays (Fig. 6C–E).

DNA double-strand break is the major damage caused by ionizing radiation, which is repaired by a variety of pathways including recombinational repair. Bleomycin is believed to generate oxygen radicals that damage DNA in a similar manner to ionizing radiation. Whereas pyrimidine dimer is the major damage caused by UV-C light, which is predominantly repaired by nucleotide excision repair. As the results shown, deletion of hsFLNa causes increased sensitivity to several distinct genotoxic agents, establishing that hsFLNa plays an essential role for cells to survive DNA damage.
In summary, we have demonstrated an interaction between BRCA2 and hsFLNa by yeast two-hybrid analyses, an in vitro binding assay, and an in vivo Co-IP. The interaction domains of BRCA2 and hsFLNa for this interaction have been mapped. The identified hsFLNa binding domain of BRCA2 resides in the longest-conserved region of BRCA2 encoded by exons 14–24. This conserved Domain IV of BRCA2 interacts with several other proteins as well, including DSS1 (20), BCCIP (19), and the longest-conserved region of BRCA2 interacts with several other proteins as well, including DSS1 (20), BCCIP (19), and the hsFLNa level in A7 and A, M2 cells. M2 lacks hsFLNa expression. The identified hsFLNa binding domain of BRCA2 resides in the conserved Domain IV of BRCA2 encoded by exons 14–24. This longest-conserved region of BRCA2 interacts with several other proteins as well, including DSS1 (20), BCCIP (25), and hUBR1 (22). These proteins are involved in a variety of cell functions, such as growth control, cell cycle regulation, and signal transduction. Therefore, Domain IV may be a particularly important region for the functions of BRCA2 in tumorigenesis. We have also found that deficiency of hsFLNa renders cells more sensitive to γ radiation, bleomycin, and UV-C light. The results suggest that the interaction between hsFLNa and BRCA2 may represent an important link between BRCA2-mediated DNA damage response and cytoskeletal signal transduction pathways.

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