Structural analysis of BRCA1 reveals modification hotspot

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Cancer cells afflicted with mutations in the breast cancer susceptibility protein (BRCA1) often suffer from increased DNA damage and genomic instability. The precise manner in which physical changes to BRCA1 influence its role in DNA maintenance remains unclear. We used single-particle electron microscopy to study the three-dimensional properties of BRCA1 naturally produced in breast cancer cells. Structural studies revealed new information for full-length BRCA1, engaging its nuclear binding partner, the BRCA1-associated RING domain protein (BARD1). Equally important, we identified a region in mutated BRCA1 that was highly susceptible to ubiquitination. We refer to this site as a modification “hotspot.” Ubiquitin adducts in the hotspot region proved to be biochemically reversible. Collectively, we show how key changes to BRCA1 affect its structure-function relationship, and present new insights to potentially modulate mutated BRCA1 in human cancer cells.

INTRODUCTION

The breast cancer susceptibility protein (BRCA1) coordinates DNA repair through a variety of mechanisms designed to protect genetic material (1–5). BRCA1 performs these duties in association with its binding partner, the BRCA1-associated RING domain protein (BARD1). In the nucleus, the BRCA1-BARD1 heterodimer interacts with other repair proteins at DNA lesions to function as an E3 ubiquitin ligase (6–8). Through a series of exquisitely controlled steps, BRCA1-BARD1 facilitates the transfer of ubiquitin moieties to a variety of nuclear protein substrates (9). These ubiquitin adducts direct their bound substrates toward different fates, one of which involves correcting DNA damage.

Base excision repair (BER) is a process that corrects non–helix-distorting damage to DNA caused by conditions such as oxidation. BRCA1 plays an essential role in helping cells deal with oxidative conditions by triggering BER pathways through ubiquitin signaling (10, 11). Cells harboring inherited mutations in the BRCA1 gene cannot adequately deal with increased levels of reactive oxygen species (ROS) arising from estrogen metabolism. These inadequacies lead to functional deficiencies in BER, an accumulation of DNA insults, and widespread genomic instability—a known hallmark of cancer induction (12–15). Ultimately, the weakened state of mutated BRCA1 in oxidative environments supports disease progression.

We recently reported that a prevalent clinical mutation, BRCA15382insC, influences the manner in which BRCA1 itself is modified in cancer cells (16). The main type of modification identified on BRCA15382insC was K48-linked ubiquitin chains. In the nuclei of cancer cells, higher levels of ubiquitination correlated with lower levels of active BRCA15382insC and changes in its biochemical properties. These results complemented the work of other colleagues who identified ubiquitin attachment site on BRCA1 in ovarian cancer cells. This site, termed the “degron sequence,” is proximal to the BRCA1 N-terminal RING domain (17).

Although many studies have connected modifications in BRCA1 to changes in cellular activity, what remains missing from these analyses is the face of BRCA1.

Increasing our knowledge of BRCA1’s three-dimensional (3D) structure can provide new insights into rational drug design and precision medicine. Structural information is currently available for the BRCA1-BARD1 RING domains (18) and the BRCA1 C-terminal (BRCT) region (19). However, the molecular architecture of full-length BRCA1 has not been determined. Current outstanding questions in the field that structural studies can address include the following: (i) How do clinical mutations in BRCA1 affect its structure-function relationship? (ii) Can mutated forms of BRCA1 be rescued or restored to normal? Similarly, the manner in which BRCA1 adapts to environmental changes or stressful conditions is poorly understood at the molecular level.

To begin tackling these issues, we used a combination of biochemical and structural biology tools. Specifically, we used single-particle electron microscopy (EM) to investigate differences among BRCA1-BARD1 structures derived from human breast cancer cells. We found a high degree of similarity between wild-type and mutated assemblies under normal growth conditions. During chemically induced oxidative stress, we found that a lysine-rich “hotspot” region on mutated BRCA15382insC was readily ubiquitinated. Structural evidence suggests that this hotspot encompasses the documented degron sequence of BRCA1. Excessive ubiquitination in this area of the protein may be related to the previously noted decreases in BRCA15382insC activity (16, 17). Significantly, the enzymatic removal of ubiquitin moieties in the hotspot region restored the overall structure of mutated BRCA1 assemblies. Together, our work provides a new lens to view BRCA1 along with the opportunity to transform its molecular properties.

RESULTS

Wild-type BRCA1-BARD1 forms a stable clamp-like motif

To determine the 3D architecture of BRCA1-BARD1 natively formed in breast cancer cells (Fig. 1A), we used biochemical tools and single-particle EM imaging technology. Here, we focused on visualizing differences between wild-type and genetically mutated or modified BRCA1. Wild-type BRCA1-BARD1 heterodimers (~300 kDa) produced in the nucleus of primary ductal carcinoma cells [HCC70 line (20); American...
Type Culture Collection (ATCC) were enriched by incubating nuclear extracts with nickel–nitrilotriacetic acid (Ni-NTA)–coated agarose beads. Phosphorylated BRCA1-BARD1 heterodimers involved in DNA damage response naturally bound to the beads and eluted in early fractions. The phosphorylated form of BRCA1 migrated at ~260 kDa on SDS–polyacrylamide gel electrophoresis (PAGE) analysis and BARD1 migrated at ~87 kDa (Fig. 1B).

To verify interactions between BRCA1 and BARD1, we performed coimmunoprecipitation (co-IP) experiments. Antibodies against BARD1 [Santa Cruz Biotechnology (SCBT)] or BRCA1 (C-20; SCBT)
were decorated onto protein G–labeled magnetic beads, and the protein fractions were incubated with the beads. The magnetically separated material was analyzed using Western blot detection. We identified BRCA1-BARD1 interactions by probing the blots with antibodies against the BRCA1 RING domain or against BARD1 (Fig. 1B). After confirming protein associations, we examined the BRCA1-BARD1 complexes using single-particle EM.

Low-dose images (<5 electrons/Å²) were acquired for BRCA1-BARD1 specimens using an FEI Spirit BioTWIN transmission electron microscope (TEM) operating at 120 kV (Fig. 1C). Individual complexes were selected from the images using the SPIDER (System for Processing Image Data from Electron microscopy and Related fields) software package (21). Selected particles were subjected to standard reference-free alignment techniques also implemented in SPIDER. Class averages for wild-type BRCA1-BARD1 showed clamp-like structures with a diameter of ~120 Å (Fig. 1C). The particle images were imported into the RELION (REgularised LIkelihood OptimisatioN) software package (22) that was used to reconstruct and refine an EM density map (see Materials and Methods and table S1).

The 3D structure of wild-type BRCA1-BARD1 confirmed a clamp-like motif that was ~120 Å across its long axis and consistent with the class averages (Fig. 1D and movie S1). 2D projections of the 3D structure agreed with the class averages (Fig. 1C). Examining the density map in various orientations provides a conformational snapshot of the heterodimer in solution. The general molecular architecture of the complex resembled another recently determined E3 ubiquitin ligase structure of comparable molecular mass to BRCA1 (23).

To distinguish the BRCA1 RING domain from the BRCT region, we used EM affinity grids (24, 25). Affinity grids were separately decorated with antibodies against each component (fig. S1). Atomic models of the RING [Protein Data Bank (PDB) code, 1JM7 (18)] and the BRCT [PDB code, 1JNX (19)] domains were placed in the density map according to positions defined by antibody-labeling results. The respective models could only fit in the density maps as indicated due to their unique features (Fig. 1D). The quality of the model fit is demonstrated in cross-sectional views shown in movie S1. The particles did not show limited orientations in their angular distribution, and the structure was refined to 14.5 Å according to the 0.5 Fourier shell correlation (FSC) criteria in RELION (fig. S1). The resolution of the map was verified using the RMEASURE application (26). The calculated molecular volume of the density map accommodates one BRCA1-BARD1 dimer.

Because expression levels and cellular stress can affect the functional response of BRCA1-BARD1 to DNA lesions, we tested for protein stability in breast cancer cells under stressful conditions. We induced oxidative stress by incubating cells with culture medium containing 1 mM hydrogen peroxide (H₂O₂) for up to 60 min, as previously described (16). Fluorescence microscopy was used to detect antibodies against 8-oxo-guanine (8-OxoG; SCBT) accumulation in genomic DNA. The formation of 8-OxoG is a direct marker for oxidative DNA damage in the nucleus.

After a 40-min incubation with H₂O₂, 8-OxoG signal (red fluorescence) increased in and around the nucleus of treated cells (blue fluorescence) (Fig. 1E). Control cells received culture medium lacking H₂O₂ and showed no signal for 8-OxoG during the incubation period. After 60 min of treatment, viability issues in treated cells limited measurements. As an additional control, HCC70 cells that experienced mild thermal stress before H₂O₂ treatment were also included in our analysis. These cells (HCC70-R) were primed to deal with cellular stress conditions and provided a model for oxidative resistance (16). Western blot comparisons of protein levels in treated cells showed that BRCA1 and BARD1 modestly decreased (~10 to 20%) in replicate experiments. As an independent control, we also assessed nuclear RAD51 levels and found little to no change in protein quantities during treatment. Nuclear β-actin served as a loading control for Western blot analyses. Overall, these results suggested that wild-type BRCA1 and BARD1 levels were relatively stable in the nucleus during oxidative conditions and DNA damage response.

How does the BRCA15382insC clinical mutation affect protein structure?

After gaining insight into wild-type BRCA1-BARD1, we focused on learning more about the BRCA15382insC cancer-related mutation. We hypothesized that the mutated BRCA15382insC protein may adopt a slightly different architecture. A frameshift mutation in the BRCA15382insC-BARD1 complexes from HCC1937 cells (ATCC) (27) that naturally express the mutated protein. According to SDS-PAGE analysis, BRCA15382insC migrated at ~260 kDa, similar to wild-type BRCA1. Subtle differences in protein conformation may account for the higher-than-expected mobility of mutated BRCA1. BARD1 migrated at ~87 kDa, and co-IP experiments confirmed BRCA15382insC-BARD1 interactions (Fig. 2B).

To determine the 3D structure of mutated BRCA15382insC-BARD1, we used the same imaging and computing procedures described for the wild-type assemblies. Individual particles were selected from images, and class averages were calculated using the SPIDER software package. The EM structure of the mutated BRCA15382insC-BARD1 complex revealed the same clamp-like motif seen in the wild-type structure (Fig. 2, C and D). The dimeric RING domain fits well within the N-terminal density, and a homology model of the mutated BRCT domain (25) was placed in the C-terminal region of the map. The BRCT density was somewhat reduced in the mutated structure, which is expected considering the truncation (Fig. 2D). Cross sections through the RING domain and EM density indicate the quality of the model fit from multiple views (movie S2). The particles did not show limited orientations in their angular distribution, and the structure was refined to 14.7 Å according to the 0.5 FSC criteria in RELION (fig. S2). The resolution was verified using the RMEASURE application.

A “modification hotspot” is identified on mutated BRCA1

Our recent biochemical studies showed that mutated BRCA1 was highly susceptible to ubiquitination under oxidative stress conditions (16). Comparatively, wild-type BRCA1 was not as susceptible to this effect. To understand the structural consequences of oxidative stress on mutated BRCA1, we performed EM analysis on protein assemblies isolated from HCC1937 cells treated with H₂O₂ (Fig. 3A). Images and class averages showed a clamp-like conformation, and particle dimensions were generally conserved in the treated BRCA15382insC-BARD1 complexes. One difference noted in the treated assemblies was a more compact shape than the untreated structures (Fig. 3A).

Taking a closer look at the protein components isolated from H₂O₂-treated cells, we found differences in SDS–PAGE and Western blot analysis. Mutated BRCA15382insC migrated at ~270 kDa after H₂O₂ treatment, whereas BARD1 primarily migrated at ~87 kDa (Fig. 3B). Another form of BARD1 was detected (~120 kDa), but it did not associate with BRCA1. We further examined the nuclear material of treated cells expressing BRCA15382insC or wild-type BRCA1 from two different...
sources (HCC70 and HCC70-R cells). Western blots revealed a major decline in the detection of BRCA1\(^{15382\text{ins}C}\) using antibodies against the RING domain (Fig. 3C). This result may be due to decreased protein levels or limited accessibility near the BRCA1\(^{15382\text{ins}C}\) RING epitope.

We calculated an EM density map for the BRCA1\(^{15382\text{ins}C}\)-BARD1 complex isolated from H\(_2\)O\(_2\)-treated cells. The same imaging and computing methods were implemented in the SPIDER and RELION software packages. Models for the dimeric RING domain and mutated BRCT region fit well within the density as illustrated in Fig. 3D, fig. S3, and movie S3. Similar to the structures of wild-type and untreated complexes, particle orientations were not limited, and the map was refined to 15.6 Å according to the 0.5 FSC criteria determined in RELION and RMEASURE (fig. S3). The BRCA1\(^{15382\text{ins}C}\)-BARD1 structure produced under oxidative stress conditions was more compact and had extra density adjacent to the RING domain (Fig. 3D).

To better understand these physical changes, we calculated a difference map between the H\(_2\)O\(_2\)-treated BRCA1\(^{15382\text{ins}C}\)-BARD1 structure and the untreated BRCA1\(^{15382\text{ins}C}\)-BARD1 structure. Difference densities at or above a 3σ threshold are considered statistically significant (28). On the basis of these criteria, conformational changes in the central portion of the structure and the BRCT regions were smaller in comparison to differences near the RING domain but were visibly present. We found a significant difference in the region proximal to the RING domain, which we refer to as a modification hotspot (Fig. 3D, fig. S3, and movie S3). The volume of the difference peak in the hotspot area (Fig. 3D, yellow) can accommodate a protein density of ~12 kDa, which is sufficient to contain at least one ubiquitin moiety.

Previous studies identified this region on BRCA1 to contain a degron sequence (17). This degron site is a known target for K48 ubiquitination that can lead to proteasomal degradation of the protein. Therefore, we tested for BRCA1 stability in the nucleus of H\(_2\)O\(_2\)-treated cells. Cells were incubated with 1 mM H\(_2\)O\(_2\) for up to 40 min, and fluorescence microscopy was used to detect 8-OxoG formation in and around the nucleus of the cells. Untreated control cells expressing BRCA1\(^{15382\text{ins}C}\) contained 8-OxoG foci, a signature of oxidative DNA damage, at the start of the experiment (Fig. 3E, red foci). The 8-OxoG signal in the untreated cells persisted throughout the experiments but did not increase. Treated cells accumulated greater levels of 8-OxoG during the 40-min incubation. These results suggested that cells expressing mutated BRCA1\(^{15382\text{ins}C}\) were not well equipped to deal with...
Fig. 3. Changes in the BRCA1<sup>5382insC</sup>-BARD1 EM structure under oxidative pressure. (A) Image (left) and class averages (center) of mutated BRCA1<sup>5382insC</sup>-BARD1 isolated from HCC1937 cells treated with 1 mM H<sub>2</sub>O<sub>2</sub>. Scale bar, 50 nm. Projections of the 3D structure (right) agree with the class averages. Box size of averages, 25 nm. (B) Under oxidative conditions, BRCA1<sup>5382insC</sup> migrates at ~270 kDa, and BARD1 migrates at ~87 kDa according to SDS-PAGE and Western blot analysis. (C) After H<sub>2</sub>O<sub>2</sub> treatment, the RING domain of BRCA1<sup>5382insC</sup> was difficult to detect compared to wild-type BRCA1 (WT). Wild-type BRCA1 (WT-R) from treated HCC70-R cells is resistant to oxidative damage. Nuclear β-actin served as a loading control. (D) The BRCA1<sup>5382insC</sup>-BARD1 structure shows a clamp-like motif with extra density adjacent to the RING domain (black circle). Scale bar, 1.5 nm. Atomic models for the RING domain [magenta; PDB code, 1JM7 (18)] and a homology model of the BRCT domain (25) fit in the molecular envelope (fig. S3 and movie S3). Difference peak (yellow) indicates the additional mass present in the hotspot region of BRCA1<sup>5382insC</sup> under oxidative conditions. This additional mass was not present in the untreated BRCA1<sup>5382insC</sup>-BARD1 density map (gray). The red star indicates the mutation site. Scale bar, 1.5 nm. Cross sections through the RING domain are indicated (movie S3). (E) 8-OxoG formation (red) in the nuclei (blue) of HCC1937 cells treated for 40 min with 1 mM H<sub>2</sub>O<sub>2</sub>. Untreated cells (− H<sub>2</sub>O<sub>2</sub>) had inherent 8-OxoG foci. Scale bar, 50 μm. (F) Western blots indicated unstable BRCA1<sup>5382insC</sup> and BARD1 compared to RAD51 in treated HCC1937 cells. Nuclear β-actin served as a loading control.
oxidative conditions. This finding is important because ROS are produced during estrogen metabolism, giving rise to oxidized DNA lesions (10, 11). Because cells expressing mutated BRCA1 5382insC had inherent 8-OxoG accumulation, unlike cells expressing wild-type BRCA1, there may be differences in the wild-type and mutated proteins that influence these processes.

To learn more about the biochemical differences between wild-type BRCA1 and mutated BRCA1 5382insC under oxidative conditions, we accessed protein levels in nuclear extracts. Western blot analysis performed on replicate experiments showed that BRCA1 5382insC and BARD1 levels were reduced in H2O2-treated cells. Wild-type proteins from two different cell sources (HCC70 and HCC70-R lines) were robust to the H2O2 treatment. Polyclonal antibodies against the BRCT domain of BRCA1 were used for detection to ensure an adequate comparison of protein signals, considering that the RING domain may be less accessible, according to results in Fig. 3C. BRCA1 5382insC migrated at ~270 kDa, slower than the phosphorylated form of wild-type BRCA1 (Fig. 3F). As an independent control, we evaluated RAD51 levels, which were stable in the nucleus during treatment. Together, the data indicate that oxidative stress alters the structure and the function of BRCA1 5382insC in breast cancer cells. These observations were consistent with other studies on mutated BRCA1, which show that its DNA repair function is reduced during stressful situations (10–14). We then questioned, can we better manage inadequacies in mutated BRCA1?

**Modified BRCA1 5382insC-BARD1 is altered by deubiquitination treatment**

The biophysical evidence presented here shows that cellular stress changes the molecular properties of mutated BRCA1. The evidence includes (i) a shift in the mobility of BRCA1 5382insC in SDS-PAGE and Western blot analysis, (ii) limited accessibility of the BRCA1 5382insC RING domain, and (iii) extra density in the BRCA1 5382insC-BARD1 structure proximal to the RING domain. To further test whether ubiquitination accounts for these changes, we evaluated protein fractions from cells undergoing oxidative stress and treated with deubiquitinas (DUB) enzyme, ubiquitin-specific protease 2 (USP2). USP2 removes a variety of ubiquitin adducts from protein substrates, generating monoubiquitin (~8 kDa) upon removal. Protein fractions of BRCA1 5382insC-BARD1 isolated from H2O2-treated cells were incubated with catalytically active USP2 (500 nM; Boston Biochem) in Heps buffer (pH 7.5) for 30 min at 37°C. Control fractions received a Heps buffer solution lacking USP2.

The biochemical analysis of USP2-treated fractions across replicate experiments showed a shift in BRCA1 5382insC mobility from ~270 kDa back to ~260 kDa. This band shift was consistent across multiple Western blots probed with antibodies against the BRCT and RING domains of BRCA1 (Fig. 4A). Treated and control samples contained equal quantities of BRCA1 5382insC, as indicated by equal detection of the BRCT domain. USP2-treated samples further showed a marked increase in the detection of the BRCA1 5382insC RING domain. Ubiquitinated products were also present in the control sample at ~270 kDa, and these bands were generally reduced in the USP2-treated fractions. Moreover, we detected a significant increase in monoubiquitin at the expected molecular weight of ~8 kDa in the treated fractions compared to the control samples (Fig. 4A).

To understand these biochemical changes in the context of the 3D structure, we used single-particle EM to examine deubiquitinated BRCA1 5382insC-BARD1. Images and class averages of USP2-treated BRCA1 5382insC-BARD1 (Fig. 4B) closely resembled the unmodified form of the heterodimer. The particles were less compact in nature and maintained the conserved clamp-shaped architecture. Again, particle orientations were not limited in the USP2-treated structure, and the density map was refined to 15.4 Å using RELION and verified by RMEASURE (fig. S4).

The 3D structure of deubiquitinated BRCA1 5382insC-BARD1 lacked the extra density in the hotspot region adjacent to the RING domain (Fig. 4C and movie S4). To better visualize this change, we calculated a difference map between comparable regions of the H2O2-treated BRCA1 5382insC-BARD1 structure and the USP2-treated BRCA1 5382insC-BARD1 structure (Fig. 4D). We implemented the same density threshold procedures described for Fig. 3 to highlight changes in the BRCA1 hotspot area. The resulting difference peak (Fig. 4D, yellow) shows the additional density that is present in the BRCA1 5382insC-BARD1 structure upon HO2 treatment but is lacking in the same region of the USP2-treated BRCA1 5382insC-BARD1 structure.

The lack of density in the deubiquitinated BRCA1 5382insC-BARD1 structure is a clear indicator that ubiquitin moieties were distinctly removed from the BRCA1 hotspot area. There were also minor differences found in the molecular structure at the BRCT domain. However, these differences are likely due to enhanced flexibility or conformational variability in the mutated BRCT domain during oxidative stress and showed no signs of ubiquitin density. Overall, both the 2D averages and the 3D structure of USP2-treated BRCA1 5382insC-BARD1 demonstrate that a modified, less functional form of BRCA1 can be modulated to restore its structural integrity.

**DISCUSSION**

In summary, we present the first structural information for full-length BRCA1-BARD1 isolated from human breast cancer cells. Structures formed under a variety of cellular conditions allowed us to directly compare wild-type and mutated complexes. Each of the 3D structures adopted a conserved clamp-like motif with characteristic features found in other E3 ubiquitin ligases (29–31). Under normal growth conditions, there were subtle differences between wild-type and mutated structures. For example, the BRCT domain of mutated BRCA1 was slightly truncated, resulting in less density in this region of the reconstruction. In general, E3 ubiquitin ligases bring E2-conjugating enzymes in proximity to a substrate. The substrate-binding region of BRCA1 resides in the BRCT domain. Hence, mutations that affect the structural properties of the BRCT can influence BRCA1’s ability to transfer ubiquitin moieties to its substrates.

Under oxidative conditions, the mutated BRCA1-BARD1 structure showed attributes that were not present in the wild-type or untreated structures. To better understand these changes, we performed additional biochemical experiments. These studies revealed that BRCA1 5382insC migrated more slowly than wild-type BRCA1 on Western blot analysis, and the RING domain was less exposed in the mutated protein. Work by others determined that residues surrounding the BRCA1 RING domain can be ubiquitinated, phosphorylated, or sumoylated (32–34). Ubiquitin adducts were present in this region on mutated BRCA1 under oxidative conditions. This observation agrees with the work of Lu et al. (17) who first discovered the degron sequence of BRCA1 in ovarian cancer patients.

The general mechanisms by which ubiquitination is involved in the DNA damage response is dynamic and complex. BRCA1 is one of many players that orchestrate protective measures against genotoxic
Fig. 4. DUB treatment of BRCA1<sup>5382insC</sup>-BARD1 restores structural integrity. (A) Western blot analysis of USP2-treated protein fractions isolated from HCC1937 cells experiencing oxidative stress. The band shift for BRCA1<sup>5382insC</sup> to ~260 kDa in USP2-treated samples was confirmed by probing the BRCT and RING domains of BRCA1. Greater signal for the RING domain was detected in the USP2-treated samples along with a reduced signal for ubiquitin attachments at ~260 kDa. Increased levels of monoubiquitin (~8 kDa) were found in USP2-treated samples. (B) Image (left) and class averages (center) of mutated BRCA1<sup>5382insC</sup>-BARD1 treated with 1 mM H<sub>2</sub>O<sub>2</sub> and USP2. Scale bar, 50 nm. Projections of the 3D structure (right) agree with the class averages. Box size of averages, 25 nm. (C) The EM structure of BRCA1<sup>5382insC</sup>-BARD1 shows a clamp-like motif lacking extra density adjacent to the RING domain (black circle) (fig. S4 and movie S4). Scale bar, 1.5 nm. (D) Difference peak (yellow) indicates the additional mass present in the hotspot region of BRCA1<sup>5382insC</sup> under oxidative conditions. This area of extra mass is lacking in the USP2-treated BRCA1<sup>5382insC</sup>-BARD1 structure (green).
insults. Other examples of ubiquitination playing a role in DNA repair involve regulatory events surrounding histone H2A modifications. USP51 was recently shown to deubiquitinate H2A at Lys13 and Lys15 during double-stranded breaks resulting from ionizing radiation. This loss of ubiquitin signal on H2A prevented the proper recruitment of repair proteins to DNA lesions (35). Another recent study on H2A ubiquitination during ultraviolet-induced nuclear excision repair processes pinpoints the biochemical players and steps involved in chromatin remodeling through the zbtu-related factor 1 (ZRF1) molecular switch (36). A complementary role for BRCA1 in ubiquitinating H2A at sites of DNA damage has also been well established (37). However, BRCA1’s ability to perform this important task is reduced as protein levels are diminished, or its functional N- and C-terminal domains are compromised.

Our recent biochemical studies demonstrated that increases in K48 ubiquitination can lower functional levels of mutated BRCA1 in cellular assays (16). The structural analysis presented here further explains how mutated BRCA1 is affected by detrimental ubiquitination events. Because irregularities in the BRCA1 structure were linked to functional deficiencies in cancer cells, it is intriguing to think that the restoration of BRCA1’s structural properties may improve its cellular activity. Ongoing efforts to test this idea are promising but fall outside the scope of the current report.

Overall, our results provide a unique outlook on the structure-function relationship of BRCA1 that is currently missing in the field. We found that deficiencies in mutated BRCA1 were related to un-warranted ubiquitination in cells experiencing oxidative stress. Cells deficient in BRCA1 activity tend to accumulate DNA insults that provide a tipping point toward cancer induction (2, 14). In contrast, we show that detrimental changes to mutated BRCA1 can be biochemically tempered to renew its structural integrity. Further investigation of this paradigm on a functional level can provide new mechanistic insights into therapeutic intervention.

MATERIALS AND METHODS

Authentication of cells, cell culture, and protein enrichment

Breast cancer cells (HCC70 and HCC1937 lines) used in this study were purchased from ATCC and independently characterized by ATCC as triple-negative primary ductal carcinoma cells. For all experiments, cells were promptly used within 6 months of resuscitation. Cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (ATCC) and 0.5x penicillin-streptomycin (Thermo Fisher Scientific) at 37°C and 5% CO2. BRCA1-BARD1 complexes were enriched as previously described (25). Briefly, ~1 million cells were collected using trypsin-EDTA (Thermo Fisher Scientific) followed by centrifugation (500 g; 5 min). For experiments involving H2O2, cells were collected with cell scrapers after treatment with 1 mM H2O2 (Sigma-Aldrich) for 40 min at 37°C and 5% CO2. Subcellular fractions (cytoplasmic and nuclear) were separated using the NE-PER kit (Thermo Fisher Scientific). The soluble nuclear material was incubated with Ni-NTA agarose beads (Qiagen) and incubated with rotation for 1 hour at 4°C. The beads were washed with five bed volumes of 20 mM Heps buffer (pH 7.2; 140 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, and 5 mM imidazole). Phosphorylated BRCA1-BARD1 naturally bound to the Ni-NTA column matrix and was eluted in the same Heps buffer, supplemented with 150 mM imidazole. Protein concentrations were determined using the standard Pierce Bradford assay (Thermo Fisher Scientific).

Coomassie blue staining and immunoblot analysis

Protein fractions were analyzed by SDS-PAGE followed by either Coomassie blue staining or Western blotting. Proteins were separated on 3 to 8% tris-acetate NuPAGE mini gels (Thermo Fisher Scientific) and stained by SimplyBlue SafeStain solutions (Invitrogen) for 60 min. Gels were washed with deionized water for 60 min and then 3% NaCl for an additional 2 hours—overnight to achieve maximum sensitivity. Western blots were performed as described previously (16). The following primary antibodies were used in our analysis: BRCA1-C20 (SCBT, sc-642; α-BRCT), BRCA1-Ab1 (Calbiochem, OP92; α-RING), BRCA1-A8X9F (Cell Signaling Technology, #14823; α-RING), BARD1 (SCBT, sc-11438), ubiquitin-pAb (Enzo Life Sciences, ADI-SPA-200), RAD51 (SCBT, sc-8349), and β-actin (Sigma-Aldrich, A5441).

Co-IP analysis

To detect protein-protein interactions, co-IP experiments were performed on isolated BRCA1-BARD1 protein fractions using previously described procedures (16). Antibodies used for IP experiments included BRCA1-C20 (5 μg; SCBT, sc-642) and BARD1 (5 μg; SCBT, sc-11438).

DUB assay

BRCA1K538Rmcs-,BARD1 protein fractions isolated from H2O2-treated cells were used for DUB assays. The reaction mixture (total volume, 200 μl) contained 180 μl of the protein fraction and 20 μl of 10× USP2 catalytic domain (final concentration, 500 nM; UbiCREST, K-400; Boston Biochem). Control mixtures were prepared with 180 μl of the protein fraction and 20 μl of 1× DUB reaction buffer (UbiCREST, K-400; Boston Biochem) lacking the enzyme. Both reaction and control tubes were incubated in a water bath at 37°C. After 30 min, samples were directly analyzed by either EM imaging or SDS-PAGE and Western blot analysis. Before EM specimen preparation, free ubiquitin was removed from the samples using Pierce Concentrator (100K MWCO, 0.5 ml; Thermo Fisher Scientific).

EM specimen preparation and imaging

Samples of isolated wild-type, mutated, and modified BRCA1-BARD1 assemblies [0.02 mg/ml in 20 mM Heps buffer (pH 7.2), 150 mM NaCl, 10 mM CaCl2, 10 mM MgCl2] were applied to glow-discharged, continuous carbon support films on copper grids (Ted Pella) or to EM specimen preparation and imaging

Image processing

Image processing procedures are summarized schematically in table S1. Individual particles were selected from the EM images using the SPIDER software package (21). Selected particles were subjected to reference-free alignment routines implementing k-means classification to compute 2D class averages. Particles contained in the averages were grouped into image stacks and exported to the RELION software.
Each structure was rotated about the x axis by 1° per frame for 90 frames. The slicing procedure was repeated and then reversed again. For rotated views, each structure was rotated about the y axis by 2° per frame for 45 frames (90°). The labels to indicate the hotspot region along with the RING and BRCT domains appear accordingly.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/9/e1701386/DC1

fig. S1. Biochemical characterization of wild-type BRCA1-BARD1.

fig. S2. Biochemical characterization of mutated BRCA15382insC-BARD1.

fig. S3. Changes in the properties of the BRCA15382insC-BARD1 under oxidative conditions.

fig. S4. The BRCA15382insC-BARD1 structure is restored following USP2 treatment.

movie S1. Movie of the wild-type BRCA1-BARD1 structure.

movie S2. Movie of the mutated BRCA15382insC-BARD1 structure.

movie S3. Movie of mutated BRCA15382insC-BARD1 isolated from H2O2-treated cells.


table S1. Flow chart of image processing procedures including steps for assessing particle heterogeneity during 2D averaging and 3D classification procedures.
31. A. M. Simons, A. A. Horwitz, L. M. Starita, K. Griffin, R. S. Williams, J. N. Glover, J. D. Parvin, BRCA1-BARD1 (EMD-8833) and mutated BRCA1-BARD1 (EMD-8834) are being deposited in the EMDataBank and can be freely downloaded at www.emdatabank.org.
37. A. M. Simons, A. A. Horwitz, L. M. Starita, K. Griffin, R. S. Williams, J. N. Glover, J. D. Parvin, BRCA1-BARD1 (EMD-8833) and mutated BRCA1-BARD1 (EMD-8834) are being deposited in the EMDataBank and can be freely downloaded at www.emdatabank.org.

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