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Correcting Errors in the BRCA1 Warning System

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Abstract

Given its important role in human health and disease, remarkably little is known about the full-length three-dimensional (3D) molecular architecture of the breast cancer type 1 susceptibility protein (BRCA1), or its mechanisms to engage the tumor suppressor, TP53 (p53). Here, we show how a prevalent cancer-related mutation in the C-terminal region of the full-length protein, BRCA1^{5382insC}, affects its structural properties, yet can be biochemically corrected to restore its functional capacity. As a downstream consequence of restoring the ubiquitin ligase activity of mutated BRCA1^{5382insC}, the DNA repair response of p53 was enhanced in cellular extracts naturally deficient in BRCA1 protein expression. Complementary structural insights of p53 tetramers bound to DNA in different stage of the repair process support these biochemical findings in the context of human cancer cells. Equally important, we show how this knowledge can be used

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Data sharing / availability

Density maps were refined and reconstructed with RELION software package (<https://www2.mrc-lmb.cam.ac.uk/relion>). The Chimera software was used for the visualization and analysis of the density maps (<https://www.cgl.ucsf.edu/chimera>). The resolution of each density map was verified by RMEASURE (<http://grigoriefflab.janelia.org/rmeasure>). EM density maps for wild-type p53 (EMD-8927) and p53^{R306} (EMD-8926) are publically available for download from the EM databank (<http://www.emdatabank.org/>). All data is available in the main text or the Supplemental data.

Conflict of interest

Authors declare that there are no conflicts of interest.

to lower the viability of breast cancer cells by modulating the stability of the BRCA1 protein and its associated players.

Graphical abstract

Keywords

BRCA1; p53; ubiquitin; breast cancer; Electron Microscopy; Structural biology

1. Introduction

Cells with mutations in the breast cancer type 1 susceptibility protein (BRCA1) have difficulty protecting their genetic material from repeated daily stressors (1, 2). These deficiencies create an unstable environment in the nucleus as DNA insults accumulate. Scientific evidence points to BRCA1's ubiquitin ligase activity as a key element in safeguarding the genome (3, 4). Consistent with this idea, disruptions in the function of BRCA1, particularly in the nucleus, impact normal protective events needed for cellular maintenance. Over time, these compromised activities can contribute to the development of disease, including aggressive forms of cancer (5, 6). In general, ubiquitin ligases act as warning systems inside cells. Ubiquitin signals are passed along from molecule to molecule coordinating protein turnover, nuclear escape, or repair processes. In this capacity, BRCA1 systematically adds ubiquitin tags to protein substrates supporting a complex signalling network in response to DNA damage (7). A recurring lapse in these stepwise procedures can impact genomic stability.

As BRCA1 represents one important factor in the DNA repair cascade, another significant player is the tumor suppressor protein TP53 (p53) (8, 9). Mutations in the p53 gene occur in ~50% of all human tumors and ~80% of BRCA1-related cancers (8, 10, 11). Other studies suggest that p53 mutations are more prevalent in BRCA1-related breast cancers than in sporadic breast cancers, building a strong case for the collective dysfunction of DNA repair processes contributing to cancer induction (10-12). *Ouchi et al.* showed that the p53-mediated transcriptional activation is enhanced in the presence of BRCA1, suggesting that BRCA1 serves as a p53 coactivator (13). In alignment with these results, BRCA1 is known

to ubiquitinate p53 during DNA repair events. Hence, mutations that interfere with these processes can leave portions of the genomic in a state of disrepair (12, 14).

To learn more about the BRCA1 warning system and its role in cancer initiation, we began investigating the structural properties of BRCA1. We recently reported the first structure of full-length BRCA1 interacting with its binding partner, the BRCA1-associated RING domain protein 1 (BARD1) (15). We found that BRCA1's role in the nucleus was disrupted by changes in a conserved "hotspot" region of the mutated protein but not the wild-type protein. Under stressful cellular conditions, excessive ubiquitination in the hotspot area inappropriately targeted mutated BRCA1 for degradation (16). We posited that a decrease in BRCA1 levels may limit essential ubiquitination steps involving p53 and DNA repair events. Interestingly, we could remove ubiquitin tags from BRCA1 using deubiquitinase enzymes (DUBs) (15). Although DUB treatment successfully rescued the structure of mutated BRCA1, burning question remained. *Can we successfully restore the function of mutated BRCA1? If so, how can we use this information to better manage disease processes?*

Here, we show that the ubiquitin ligase function of mutated BRCA1^{5382insC} can be greatly restored by enzymatic treatment with deubiquitinases. Restoring the actions of mutated BRCA1^{5382insC} elevated downstream activities of p53. To better understand these biochemical findings and their relationship to the p53 structure, we isolated native p53 assemblies naturally formed in cancer cells and examined their attributes using single-particle electron microscopy (EM). Although the p53 structure has been widely studied using NMR, small angle X-ray scattering (SAXS), and cryo-EM, since the protein was discovered three decades ago (17-25), our results provide the first insights for wild-type and mutated p53 naturally produced in human cancer cells. These assemblies were captured and analysed while poised on native DNA fragments. Collectively, our work shows the BRCA1 warning system can be exquisitely modulated while offering new strategies to mechanistically target cancer cells. Developing new tools to chemically nudge tumor suppressors in human cells holds great potential to improve disease management or preventative measures.

2. Material and Methods

2.1. Authentication of cells and cell culture conditions

Human breast cancer cells (HCC1937 line) were purchased from American Type Culture Collection (ATCC) and independently characterized by ATCC as triple-negative primary ductal carcinoma cells. Human glioblastoma multiforme (GBM) cells (U87MG, T98G, and A172 lines) and normal human astrocytes were kindly provided by Dr. Zhi Sheng at Virginia Tech Carilion Research Institute. For all experiments, cells were promptly used within 6 months of resuscitation. HCC1937 cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (ATCC) and 0.5X penicillin-streptomycin (Life Technologies). Glioma cells (U87MG, T98G, and A172 lines) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (Atlas Biologies), streptomycin (100 ug/ml, Gibco), and penicillin (100 IU/ml, Gibco). Normal human astrocytes were cultured in MCDB-131 Medium (Sigma) containing 3% fetal bovine serum (Peak Serum), 10X G-5 Supplement (Gibco), 100 µg/mL

streptomycin and 100 IU/mL Penicillin (Gibco). Cells were free of contamination and cultured at 37°C with 5% CO₂.

2.2. Protein isolation procedures

BRCA1-BARD1 complexes were isolated as described (15). Briefly, ~1 million cells were treated by adding hydrogen peroxide (1 mM H₂O₂, Sigma-Aldrich) to the culture media for various time points at 37°C and 5% CO₂. Control cells received culture media lacking H₂O₂. Following the incubation period, cells were collected using cell scrapers. Cytoplasmic and nuclear fractions were separated using the NE-PER kit (Thermo Fisher Scientific). The soluble nuclear material was further incubated with Nickel-Nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen) with rotation for 1 hour at 4°C. The beads were washed with five bed volumes of 20 mM HEPES buffer (pH 7.2) containing 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 HEPES buffer, supplemented with 150 mM imidazole. Protein concentrations were determined using the standard Pierce Bradford assay (Thermo Fisher Scientific). The same cell lysis and Ni-NTA enrichment steps were used to isolate p53 from HCC1937 cells (p53^{R306}) and U87MG cells (p53^{WT}), except the protein assemblies were eluted with HEPES buffer (pH 7.2) supplemented with 60 mM imidazole.

2.3. Cell lysate preparations

For whole cell lysate preparations, HCC1937 cells were treated by adding 1mM H₂O₂ to the culture media for 10 - 20 minutes, while incubating cells at 37°C and 5% CO₂. Following each incubation time, cells were washed with 1X PBS (Sigma) to eliminate residual H₂O₂. The cells were then treated with 7 µM - 9 µM ML364 (Axon Medchem) for up to 48 hours at 37°C and 5% CO₂. Control cells were cultured in media lacking H₂O₂ and / or ML364 during the same time period. Cells were collected and washed with ice-cold 1X PBS once followed by centrifugation at 500 xg for 5 minutes. Cell pellets were lysed by resuspending the pellets in buffer solution containing 20 mM HEPES (pH 6.8), 150 mM NaCl, 2.5 mM CaCl₂, 2.5 mM MgCl₂, 1 mM EDTA, 2% Nonidet-P40 (NP40), 1% Nadeoxycholate, 2X protease inhibitors (Sigma), 1X phosphatase inhibitors (Sigma), and 8% glycerol. The lysis mixture was incubated on ice for 30 minutes and centrifuged at 21,000xg for 15 minutes at 4°C. The supernatants were collected and protein concentrations were quantified using the standard Pierce Bradford assay.

2.4. Native gel and immunoblot analysis

For native gel analysis, protein fractions of purified p53 were combined with 4X NativePAGE sample buffer (Thermo Fisher Scientific). Native sample preps were separated using light blue cathode running buffer on a NativePAGE 3-12% Bis-Tris gel (1 hr at 150V then ~1 hour at 250V; Thermo Fisher Scientific). For coomassie staining, gels were fixed then stained with 0.02% Coomassie Brilliant Blue R-250 (Fisher Scientific). For immunoblotting, gels were placed in 2X NuPAGE transfer buffer (Thermo Fisher Scientific) for 10 minutes before transfer onto PVDF membranes (Millipore) in 1X NuPAGE transfer buffer at 25V for 1 hour at 4°C. The membrane was fixed in 8% acetic acid for 15 minutes followed by staining for 5 minutes in 0.1% coomassie R-250 in 50% methanol to visualize

the NativeMark Unstained Protein Standards (Thermo Fisher Scientific). The blot was destained in 50% methanol/10% acetic acid solution three times for 5 minutes, rinsed with several changes of purified water and allowed to air dry. The membrane was re-wet then blocked with 1% non-fat dry milk (NFDM) in TBS-T (0.05%) for 1 hour with gentle rocking. Anti-p53 (DO-1, Santa Cruz Biotechnology) primary antibody was diluted in blocking solution and incubated overnight at 4°C. Blots were washed three times with TBS-T (0.05%). Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch) were incubated for 1 hour followed by additional washing. ECL Prime western blotting reagent (GE Healthcare) or West Femto (Thermo Fisher) was used for detection. A ChemiDoc MP (Bio-Rad) was used for imaging.

2.5. SDS-PAGE, immunoblotting, and densitometry measurements

Protein fractions were also analyzed by SDS-PAGE denaturing gels followed by staining with SimplyBlue SafeStain solution (Invitrogen) or Western blotting as described (15). For Western blot analysis, the following primary antibodies were used: BRCA1 (C-20; Santa Cruz Biotechnology, sc-642), ubiquitin-pAb (Enzo Life Sciences, ADI-SPA-200), p53 (DO-1; Santa Cruz Biotechnology, sc-126), K63-linkage specific polyubiquitin (D7A11; Cell Signaling, #5621) and β -actin (Sigma-Aldrich, A5441). Western blot quantification and densitometry measurements were performed using Image Lab™ Software (Bio-Rad). The band intensities were selected using the volume tool. Local subtraction and linear regression methods were implemented to eliminate the local background values and to quantify the band intensities.

2.6. Deubiquitinase (DUB) assay

Purified BRCA1^{5382insC}-BARD1 fractions (0.1 - 0.2 mg/ml) were incubated with 500 nM USP2 catalytic domain (Boston Biochem) in a water bath at 37°C. USP2 is a general deubiquitinase enzyme (DUB). Control mixtures were prepared using the same protein fractions and concentrations, along with 1X DUB reaction buffer (Boston Biochem) that lacked USP2. These reaction mixtures were incubated in a water bath at 37°C along-side the enzymatically-treated material. The inactive samples were incubated in parallel at 4°C. Following a 30-minute incubation period, samples were analyzed by EM imaging, SDS-PAGE, and Western blot analysis. Protein fractions designated for EM were concentrated using a Pierce Concentrator (100 KDa MWCO, 0.5ml; Thermo Fisher Scientific) to remove free ubiquitin from the samples.

2.7. Ubiquitin ligase assay

Aliquots of DUB-treated or “restored” BRCA1-BARD1 (0.2 mg/ml) were incubated with 10 μ M ML364 (USP2 inhibitor) at 37°C for 1 hour to halt USP2 activity. Samples were then stored overnight at 4°C. The restored BRCA1-BARD1 fractions were added in varying amounts to soluble nuclear extracts prepared from T98G cells. T98G cells naturally produced low levels of BRCA1. Control samples lacked restored BRCA1-BARD1. Reaction mixtures were incubated in a water bath at 37°C for 1 hour.

2.8. Fluorescence microscopy

The production of 8-Oxo-Guanine (8-Oxo-G) DNA lesions was detected by fluorescence imaging as described (15, 16). Briefly, HCC1937 cells (~40,000 cells / chamber) were plated in an eight-chamber slide and incubated at 37 °C and 5% CO₂ overnight. Cells were treated with complete culture media containing 1mM H₂O₂ for 10 - 20 minutes to induce oxidative damage in the nucleus. Cells were then wash with 1X PBS and treated with ML364 (7μM - 9 μM) for up to 48 hours at 37°C and 5% CO₂. Control cells were cultured in media without H₂O₂ and ML364 during the same time period. Cells were washed with standard PBS solution and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes. The fixed cells were permeabilized by PBS solution containing 0.5% Triton X-100 (Sigma) for 10 minutes. After 1-hour blocking step with PBS supplemented with 10% normal goat serum (Jackson Immuno Research) and 0.2% Triton X-100, cells were incubated with anti-8-OxoG DNA Lesion (483.15; Santa Cruz Biotechnology) at 4°C overnight. The 8-OxoG DNA lesions were detected with goat anti-mouse IgM-TR (Santa Cruz Biotechnology) and nuclei were stained with Hoechst 33342. An inverted fluorescence microscope (Zeiss Axio Vert.A1; Carl Zeiss Microscopy) was used to image the cells.

2.9. Cell viability and proliferation assays

To determine cellular viability, we used the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (MTS; Promega). HCC1937 were seeded (~4000 cells per well) in a 96-well plate at 37°C and 5% CO₂ overnight. Following a 10-minute incubation with culture media containing 1mM H₂O₂, cells were washed with PBS solution and further treated with 7μM ML364 for 24 hours at 37°C with 5% CO₂. Control cells were cultured in media lacking H₂O₂ and / or ML364 during the same time period. Culture media and containing MTS reagent was added to each well. The titer plate was incubated 37°C and 5% CO₂ for 1-4 hours. Absorbance values were measured at 490 nm using iMark microplate reader (Bio-Rad). MTS was also used to determine cell growth following ML364 treatment. HCC1937 cells were plated (~1500 cells per well) in a 96-well plate at 37°C and 5% CO₂ overnight. Cells were treated with DMSO and ML364 at the different doses (2.5 μM - 80 μM). After 3 days of incubation, cell viability was measured using the MTS reagent. Cellular survival was calculated by dividing the absorbance of the treated groups by those of the untreated groups (DMSO). Values for the half-maximal inhibitory concentration (IC₅₀) were obtained using Prism software package (GraphPad).

2.10. EM specimen preparation and data collection

Aliquots of purified proteins (~3 μl each of 0.02 mg/ml) were added to the surface of glow-discharged silicon nitride (SiN) microchips or 400-mesh copper EM grids (Ted Pella, Inc.) that were coated with carbon support films. After a 1-minute incubation, the samples were washed with purified water, stained with 0.75% uranyl formate, and allowed to air dry. The EM specimens were viewed using a Spirit BioTwin TEM (Thermo Fisher Scientific) equipped with a LaB6 filament and operating at 120 kV under low-dose conditions (<5 electrons/Å²). Images were recorded using an Eagle 2k HS CCD camera having a pixel size of 30-μm (Thermo Fisher Scientific) at a magnification of ~68,000x for a sampling of 4.4 Å / pixel.

2.11. Image processing routines and difference mapping

A full description of the BRCA1-BARD1 reconstructions was recently reported (15). Briefly, individual particles were selected from EM images and the RELION software package was used to refine and reconstruct density maps using a spherical model. The model aided in allocating alignment parameters to each particle in the first round of refinement. Ten subsequent iterations relied on the experimental data using a regularization parameter of $T=4$. Standard procedures were used employing a pixel size of 4.4 Å / pixel and a mask of 120 Å. EM maps were examined at similar significance levels and threshold values set in the Chimera program. The FSC-0.5 criteria provided an estimate the resolution of each density map, verified by the RMEASURE program. The structure of the non-ubiquitinated BRCA1^{5382insC}-BARD1 (14.7 Å) contained 4222 particles; ubiquitinated BRCA1^{5382insC}-BARD1 (15.6 Å) contained 4103 particles; restored BRCA1-BARD1 (15.4 Å) contained 4000 particles (15). Difference densities were calculated between the following EM maps: 1) non-ubiquitinated BRCA1^{5382insC}-BARD1 and ubiquitinated BRCA1^{5382insC}-BARD1; 2) ubiquitinated BRCA1^{5382insC}-BARD1 and restored BRCA1-BARD1. The density values for each map were normalized to a common range and differences maps were derived using the DIFFMAP executable, which is publically available. The difference peak in the hotspot region exceeded the 3-threshold level and was considered significant.

For mutated and wild-type p53 complexes, the same reconstruction and refinement procedures were implemented in the RELION software package, using a resolution-filtered (50 Å) model of the p53 core bound to DNA (pdb code, 2AC0). Class averages and reconstructions for the mutated p53 structure contained 747 particles and the wild-type p53 structure contained 704 particles. C2-symmetry was imposed during refinement bringing the total number of particles to an equivalency of 1494 for the mutated structure and 1408 for the wild-type structure. The maps were masked at ~80 Å in diameter. The Chimera software package was used to visualize the density maps at equivalent contour values. Threshold values are included in each map deposition. We used the 0.5-FSC criterion to determine the resolution of each density map. Particle data was divided into two halves and resolution values for each half converged to a common value. The resolution of the mutated p53 structure was estimated to be 15.5 Å, while the resolution of the wild-type structure was estimated to be 20 Å. These values are in good agreement with independent measurements performed using the RMEASURE program.

2.12. Movie production

Movies were produced using the Chimera software package. For each movie, the EM reconstructions and relevant models were imported and aligned manually within each session. Separate files containing Chimera commands were generated for the reconstructions and models were rotated, rocked, and cross-sectioned. Labeled sites indicate the location of protein or DNA features. For the BRCA1^{5382insC}-BARD1 structures, labels included the ubiquitin site in the hotspot region, along with the RING, and BRCT domains. For the p53^{R306} and p53^{WT} structures, protein domains and DNA are shown in different stages of repair. To produce cross-section views, camera slices were generated using 110 frames and reversed as frame slices were replaced. Each structure was rotated about the x- or y- axis by 1 degree per frame for up to 90 frames. Chimera output movies in .mov format.

3. Results and Discussion

3.1. Structurally correcting mutated BRCA1

To gain insight of how mutations in BRCA1 affect its molecular properties, we first utilized breast cancer cells that express BRCA1^{5382insC} (HCC1937 line; (26)). This mutation truncates the C-terminal domain of BRCA1 by ~10 kDa and it is one of the most common aberrations in hereditary breast cancer worldwide. Cells were cultured under normal conditions at 37°C with 5% CO₂. Prior to harvesting, cells were treated with 1 mM hydrogen peroxide (H₂O₂) for various time intervals at 37°C to induce oxidative stress (15, 16)(Material and Methods). Stressful culture conditions enhanced the formation of ubiquitinated-BRCA1 for downstream analysis.

We compared the molecular features of mutated BRCA1-BARD1 purified from breast cancer cells under different treatment conditions. The recently reported electron microscopy (EM) structures (15) were calculated using the RELION software package (27). Under oxidative stress, mutated BRCA1-BARD1 (Fig. 1, gray mesh) gained ubiquitin adducts in a defined hotspot region on the protein. Upon treating ubiquitinated BRCA1 (Fig. 1, yellow mesh; Movie S1) with the deubiquitinating enzyme, Ubiquitin Specific Peptidase 2 (USP2), the bulky density in this hotspot region disappeared. We refer to this treated structure as “restored” BRCA1 (Fig. 1, green mesh; Movie S2). The difference density in the hotspot region can accommodate a model for the ubiquitin monomer. The orientation of the model presented here permits it to attach to BRCA1 in a biologically-relevant manner.

The presence of ubiquitin on mutated BRCA1 was confirmed by SDS-PAGE and Western blot analysis. Ubiquitinated-BRCA1 migrated at ~270 kDa, a rate slower than predicted for the truncated protein (Fig. S1). Ubiquitinated samples treated with USP2 showed a shift in the mobility of BRCA1 to ~260 kDa. This change in mobility corresponded with an increase in free ubiquitin at ~8 kDa (Fig. S1) as noted (15). We refer to this rescued form of the protein as “restored BRCA1”. Taken together, these results suggested that we can successfully alter the properties of mutated BRCA1 *in vitro*. This exciting discovery established the foundation for new functional studies on restored BRCA1.

3.2. Functionally correcting mutated BRCA1

In the nucleus, p53 is a key participant in DNA damage response – frequently referred to as the “guardian of the genome”. During the life cycle of the protein, modifications to p53 can either stimulate its repair activity or trigger its degradation (28). One of these influential modifications is ubiquitination. As p53 is ubiquitinated by BRCA1 during DNA damage response, we tested whether restored BRCA1 could function in this capacity. We prepared nuclear extracts from HCC1937 cells that contained restored BRCA1 and monitored for p53 activation using Western blot detection and quantification. During repair, p53 forms tetramers upon DNA lesions. Hence, we looked for p53-tetramer formation in cells receiving oxidative reagents (Material and Methods). The HCC1937 cells express a truncated form of p53 (p53^{R306}) with known repair function (29). We compared the ratio of p53^{R306} tetramers/monomers (T/M ratio) in nuclear extracts and used this signature to assess DNA engagement (Fig. 2A, 2B). The T/M ratio increased by ~3-fold or nearly 20% in samples containing

restored BRCA1 compared to controls. These results show p53^{R306} tetramer formation was enhanced in the presence of restored BRCA1.

To better understand the physical nature of the p53^{R306} assemblies, we purified the complexes from H₂O₂-treated HCC1937 cells for additional characterization. Coomassie-stained denaturing gels revealed the purified p53^{R306} monomer (Fig. 2C) and active tetramers were present on Western blots and in EM images (Fig. 2C, 2D). The tetramer population was concentrated by using Pierce concentrators (PES, 100 kDa MWCO; Thermo Scientific). From the EM images of the tetramers, we selected protein complexes using the RELION software package. An initial model of the p53 tetramer core (pdb code 2AC0; (30)) was used to assist with reconstructing an EM density map that was refined using standard procedures in RELION (Material and Methods).

Cross-sections through the 3D reconstruction and model revealed p53^{R306} engaging a native DNA strand having double-stranded breaks (DSB) (Fig. 2E, Fig. S2; Movie S3). Projections of the density map were in good agreement with class averages, and the angular orientations of the particle population were not limited in the ~15.5-Å structure (Fig. 2D, Fig. S2). At the current resolution, we cannot distinctly assign ubiquitin density in the p53^{R306} map, although tetramer formation is supported by the biochemical data. We further evaluated the ubiquitin ligase activity of restored BRCA1 on wild-type p53 using a different cellular system.

3.3. Wild-type p53 – caught in the act of repair

We tested the extent to which the addition of restored BRCA1 could enhance the ubiquitin ligase activity of cells with inherently low levels of BRCA1 expression (T98G line) (Fig. S3). Proteins receiving K63-linked ubiquitin adducts are often involved in autophagy and DNA repair (31-33). Quantities of restored BRCA1-BARD1 were added to nuclear reaction mixtures prepared from T98G cells, and changes in ubiquitination were assessed over multiple experiments (Fig. 3A). To simplify the results, we first focused on modifications to the p53 monomer, a known substrate of BRCA1 ubiquitination. According to Western blot analysis, full-length p53 migrated at ~50 kDa while ubiquitinated monomers migrated close to ~68 kDa, showing multiple bands typical of ubiquitinated proteins (Fig. 3B, Fig. S3). The intensities of both bands in the 68 kDa region were added together during densitometry measurements and used to evaluate differences between BRCA1 addition (+) and control lanes (–) (Fig. 3, Fig. S3). Duplicate experiments were performed and reaction mixtures receiving restored BRCA1-BARD1 showed increased levels of K63-type ubiquitin adducts on p53 (up to 18%) compared with control samples, as summarized in Figure 3C. As the T98G cells naturally produced little BRCA1, we attributed the increase in ubiquitin ligase activity to the restored BRCA1-BARD1 supplement. By comparison, wild-type BRCA1-BARD1 supplementation increased the ubiquitination of p53 by ~24%. Hence, restored BRCA1 could operate up to 75% of its full capacity.

To visualize differences in the wild-type and mutated p53 structures, we biochemically purified wild-type p53 assemblies from human cancer cells (U87MG line; Material and Methods). Monomeric p53 migrated at ~50 kDa on denaturing gels (Fig. 3D). To ensure the presence of p53 tetramers, we performed native gel electrophoresis, which confirmed the

tetramers migrated at ~220 kDa. The purified tetramers were concentrated and imaged using EM. Class averages of the wild-type assemblies were larger in diameter (~80 Å) than their mutated counterparts (~70 Å), but showed similar overall features. Particles were selected from EM images using the same criteria, model, and reconstruction procedures.

The resulting EM density map (~20 Å) accommodated the p53 tetramer model with a notable difference in the wild-type structure. The density surrounding the DNA strand was continuous in the wild-type map. (Fig. 3E, Fig. S4; Movie S4). This continuity of density in the wild-type structure suggests an intact DNA strand, consistent with a state of repair (Fig. 3E; Movies S5, S6). The same region of density was fragmented in the structure of the mutated complex (Fig. 2). Projections of the density map were consistent with experimentally-determined class averages calculated from the overall particle population (Fig. 3D). Although biochemical results suggested the assemblies contained ubiquitin to some extent, we were unable to assign ubiquitin density in the EM map. One reason for this observation is conformational flexibility at ubiquitin attachment sites. Another issue is the fact that ubiquitin moieties can attach to p53 at multiple locations. Although we are working to address these factors, our present EM reconstructions show the first p53 structures derived from human cancer cells. High-resolution cryo-EM studies may reveal molecular details beyond the current observations, however, this work demonstrates an essential first step toward understanding the physical differences in native DNA repair assemblies. Our results also provide new technical advances that can be used to assess active complexes formed in other types of aggressive cancers, such as ovarian or pancreatic cancer. Moreover, as the structures portrayed here represent different mechanistic stages in DNA engagement, they may also offer new insights for targeting p53-related processes at the molecular level.

3.4. Targeting cancer cells that express mutated BRCA1

As we can successfully fine-tune mutated BRCA1 *in vitro*, how can we use this knowledge to eliminate cancer cells? Enhancing the activity of tumor suppressors in healthy cells may facilitate their growth and resilience. Conversely, treating cancer cells with drugs that limit DNA repair may lessen their survival. This philosophy is the inspiration behind the use of Poly(ADP-Ribose) Polymerase (PARP) inhibitors to treat BRCA1-related cancers (34, 35). To test this limited-repair paradigm, we treated breast cancer cells (HCC1937 line) with the DUB inhibitor, ML364 (Axon Ligands™). ML364 is a USP2-specific drug that interferes with cell cycle progression and homologous recombination in colorectal cancer and lymphoma models (36). The half maximal inhibitory concentration (IC₅₀) range for ML364 in HCC1937 cells is 7-10 μM (Fig. 4A). Cells were treated with 1 mM H₂O₂ for 10 minutes, after which time, the oxidizing agent was removed and cells were then incubated with ML364 in the IC₅₀ range up to 48 hours (Fig. S5). Untreated control cells simply received culture media and experiments were performed using four replicates. Recent results showed that oxidative damage spurred the degradation of BRCA1 (16). As such, we predicted the addition of ML364 would enhance these effects by negatively impacting BRCA1 and p53. The viability of treated cells and untreated cells was quantified using MTS assay measurements. Cells treated with ML364 and H₂O₂ separately showed a decrease in viability by ~50%, while those receiving the combined treatment declined by ~70% (Fig. 4B).

To determine the effect of ML364 on genomic stability, we performed immunofluorescent imaging on HCC1937 breast cancer cells to detect 8-Oxo-Guanine (8-Oxo-G) (Fig. 4C). The nuclear accumulation of 8-Oxo-G in cells indicates deficiencies in DNA damage repair (16). Cells were treated with H₂O₂ to induce oxidative DNA damage. The ML364 drug was then administered to the cells in the IC₅₀ range. The accumulation of 8-Oxo-G was detected using monoclonal antibodies against the substrate and fluorescently-labeled secondary antibodies. Control samples received culture media lacking H₂O₂ or ML364 and showed some inherent DNA damage as anticipated (16). A higher level of 8-Oxo-G accumulation (red punctate) was noted in and around the nucleus (blue fluorescent signal) of cells treated with ML364. Under oxidative stress conditions, there was a minor degree of 8-Oxo-G detected in nuclear region following 10 minutes of treatment. Cells treated with a combination of H₂O₂ and ML364 showed the highest degree of 8-Oxo-G accumulation and a reduced capacity for DNA damage repair (Fig. 4C, Fig. S5). To better understand the impact of these treatments on the BRCA1 system, we examined changes in repair proteins under each condition.

According to Western blot analysis, BRCA1 and p53 levels decreased by ~30% in ML364-treated cells (Fig. 4D, E). H₂O₂-treated cells lacking ML364 treatment showed a decrease in both proteins by ~15 – 20%. Cells treated with a combination of H₂O₂ and ML364 showed a remarkable decline (~50%) in BRCA1 and p53. This trend continued up to 48-hours, although, protein recovery was stronger in H₂O₂-treated cells lacking ML364 (Fig. S5). These results suggested that both tumor suppressors were naturally sensitive to the ML364 treatment, particularly under oxidative stress. Our data is consistent with other studies on cancer cells involving the use of DUB inhibitors to promote protein degradation and decrease cellular vitality (36).

4. Conclusions

Overall, the imaging results were in good agreement with our biochemical and structural findings. As deficiencies in BRCA1 and p53 compromised the cellular warning system, DNA repair response was limited. The build-up of 8-Oxo-G and other toxic species in the nucleus contributed to cellular demise. As cancer cells grow, divide, and produce proteins more rapidly than healthy cells, limiting their repair options poses a powerful therapeutic approach. This notion has proven beneficial in a clinical setting through the use of PARP inhibitors to treat patients having BRCA1 mutations (37). Likewise, inducing oxidative stress suggests a rational strategy to potentially manage BRCA1-mutated cancers (38). Our results are consistent with these ideas as DUB inhibitors demonstrated a remarkable ability to kill breast cancer cells heavily reliant upon robust repair activities. These findings also contribute fresh design concepts for small molecules or DUB inhibitors to combat hereditary cancers. Collectively implementing new mechanistic-based strategies introduces promising opportunities to manage disease conditions for individuals with errors in tumor suppressor genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Functional properties of mutated BRCA1 can be restored biochemically
- Modulating BRCA1 affects the downstream activities of p53
- Structural results show the first p53 assemblies isolated from human cancer cells
- Deubiquitinase inhibitors provide a new means to reduce viability of breast cancer cells

Fig. 1. Mutated BRCA1 can be modified through ubiquitination.

Mutated BRCA1-BARD1 (gray, mesh) lacks density in a lysine-rich hotspot region in comparison to ubiquitinated BRCA1-BARD1 (yellow, mesh). Upon enzymatically removing ubiquitin adducts from the complex, the bulky density in the hotspot area disappears. We refer to the treated structure as “restored” BRCA1-BARD1 (green, mesh). An atomic model of the ubiquitin monomer (pdb code, 1UBQ (39)) fits well within the difference density. Scale bar is 15 Å.

Fig. 2. p53^{R306} forms active tetramers on native DNA.

(A) Western blot analysis under denaturing conditions shows differences in the presence of p53^{R306} tetramers (~160 kDa) and monomers (~40 kDa) in nuclear extracts of HCC1937 cells. Cells were treated with H₂O₂ (Ox) and nuclear fractions were supplemented with USP2 and incubated at 4°C or 37°C for activation. Control cells lacked H₂O₂ treatment. (*) indicates activated samples with restored BRCA1. (B) Quantification of p53 tetramer to monomer (T/M) ratios in active and inactive extracts. (C) Coomassie-stained SDS-PAGE gel shows the purified p53^{R306} monomer obtained from H₂O₂-treated cells. Western blot shows the p53^{R306} tetramer assembly (~160 kDa) and monomer (~40 kDa). (D) EM image of the purified p53^{R306} assemblies along with class averages and corresponding projections of the 3D density map. Scale bar is 200 Å. Box size is 250 Å. (E) EM map (white) and homology model of the p53^{R306} tetramer (yellow) bound to DNA (blue) containing a double-stranded break (DSB). Scale bar is 15 Å.

Fig. 3. Restored BRCA1 collaborates with p53 in cancer cells.

(A) Restored BRCA1 migrates at ~260 kDa and BARD1 migrates at ~87 kDa on a denaturing gel. (B) Western blot analysis and densitometry measurements showed changes in ubiquitinated-p53 at ~68 kDa in reaction mixtures receiving restored BRCA1-BARD1 (μg). Controls (–) lacked restored BRCA1-BARD1. (C) Graphical summary of the quantitative increases (μg) noted in the total signal for both bands representing ubiquitinated-p53 compared with control samples (–). (D) Wild-type p53 (p53^{WT}) isolated from U87MG cells migrated at ~50 kDa according to SDS-PAGE and Western blot analysis. p53^{WT} tetramers migrated at ~220 kDa on a native (non-denaturing) gel. EM image and class averages of p53-DNA assemblies (~80 Å) showed similar features as the mutated assemblies (~70 Å). Calculated projections of the density map for p53^{WT} assemblies were in good agreement with experimental averages. Scale bar is 200 Å. Box size is 250 Å. (E) EM map (white) of the p53^{WT} tetramer (yellow) engaging DNA (blue) in a pre- or post-repair configuration. Scale bar is 15 Å.

Fig. 4. Breast cancer cells are weakened by DUB inhibitors.

(A) The IC_{50} value for the DUB inhibitor, ML364, use in HCC1937 cells is ~7-10 μ M. (B) Cancer cells (HCC1937 line) treated H_2O_2 , an oxidizing reagent (Ox), and subsequently with ML364 (+) showed a decline in viability. (C) The 8-Oxo-Guanine (8-Oxo-G) accumulation (red punctate) was more abundant in the nucleus (blue) of ML364-treated cells than in H_2O_2 -treated cells. Cells treated with H_2O_2 and ML364 showed the greatest 8-Oxo-G buildup in and around the nucleus. Scale bar is 50 microns. (D) Western blot analysis of cell lysates show a shift in the migration of BRCA1 along with changes in its band intensity following H_2O_2 and ML364 (Ox/+ML364) treatment. Corresponding changes in the levels of p53^{R306} tetramers were noted in lysate fractions of treated cells. (E) Quantification of

Western blot band intensities showed that dual treatment (Ox/+ML364) lowered mutated BRCA1 and p53^{R306} levels along with the propensity for DNA repair.

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