



# BARD1 is necessary for ubiquitylation of nucleosomal histone H2A and for transcriptional regulation of estrogen metabolism genes

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Missense mutations that disrupt the RING domain of the tumor suppressor gene *BRCA1* lead to increased risk of breast and ovarian cancer. The *BRCA1* RING domain is a ubiquitin ligase, whose structure and function rely critically on forming a heterodimer with *BARD1*, which also harbors a RING domain. The function of the *BARD1* RING domain is unknown. In families severely affected with breast cancer, we identified inherited *BARD1* missense mutations Cys53Trp, Cys71Tyr, and Cys83Arg that alter three zinc-binding residues of the *BARD1* RING domain. Each of these mutant *BARD1* proteins retained the ability to form heterodimeric complexes with *BRCA1* to make an active ubiquitin ligase, but the mutant *BRCA1/BARD1* complexes were deficient in binding to nucleosomes and in ubiquitylating histone H2A. The *BARD1* mutations also caused loss of transcriptional repression of *BRCA1*-regulated estrogen metabolism genes *CYP1A1* and *CYP3A4*; breast epithelial cells edited to create heterozygous loss of *BARD1* showed significantly higher expression of *CYP1A1* and *CYP3A4*. Reintroduction of wild-type *BARD1* into these cells restored *CYP1A1* and *CYP3A4* transcription to normal levels, but introduction of the cancer-predisposing *BARD1* RING mutants failed to do so. These results indicate that an intact *BARD1* RING domain is critical to *BRCA1/BARD1* binding to nucleosomes and hence to ubiquitylation of histone H2A and also critical to transcriptional repression of *BRCA1*-regulated genes active in estrogen metabolism.

*BRCA1/BARD1* heterodimer comprises the first ~100 residues of each protein, bringing their two RING domains into close proximity. As part of the heterodimer, the *BRCA1* RING domain interacts with an E2 enzyme to activate transfer of ubiquitin onto a substrate, but the role of the *BARD1* RING domain has remained unknown. Mutations that result in truncation of the *BARD1* protein are associated with increased risk of breast and ovarian cancer (11–15), but the functional and clinical consequences of missense mutations in the *BARD1* RING domain have not been characterized. To address these questions, we evaluated three missense mutations in the *BARD1* RING domain, newly discovered in families severely affected by breast cancer.

## Results

**Germline Mutations in the *BARD1* RING Domain in Breast Cancer Families.** Truncating mutations of *BARD1* are known to increase risk of breast and ovarian cancer (16, 17), but much less is known of the functional and clinical consequences of missense mutations in the *BARD1* RING domain. In the course of genomic analysis of inherited predisposition to breast cancer, we identified three

*BRCA1* | *BRCA1* | breast cancer | ubiquitin | transcriptional repression

Among inherited mutations of *BRCA1* that increase risk of breast and ovarian cancer are missense mutations that abrogate the function of the *BRCA1* RING domain. The *BRCA1* RING domain is a ubiquitin ligase (E3) enzyme, that catalyzes the covalent attachment of the signaling protein ubiquitin (Ub) onto protein substrates by binding to and activating E2 enzymes. As is true for most Ub ligases, *BRCA1* modifies numerous proteins, including histone H2A, estrogen receptor  $\alpha$  (ER $\alpha$ ), and progesterone receptor (1–3). Although the biological consequences of protein ubiquitylation by *BRCA1* are largely unknown, the *BRCA1*-dependent modification of histone H2A is critical for DNA damage repair and genome stability (4, 5). Cancer-predisposing missense mutations in the *BRCA1* RING domain cause loss of the domain's ligase activity, and hence a general defect in ubiquitylation. The mutations also lead to genomic instability (2, 6–8). Nevertheless, there are conflicting hypotheses regarding the role of *BRCA1* Ub ligase activity in tumor suppression (4, 8–10). The alternative hypotheses have been difficult to resolve because *BRCA1*-mutant RING domains are inactive toward all ubiquitylation substrates, so the specific target(s) involved in tumor suppression activity of *BRCA1* has not been identified.

Ub ligase activity of *BRCA1* depends on *BRCA1* forming a complex with *BARD1*, which also harbors a RING domain. The

## Significance

Loss-of-function mutations in *BRCA1* and its protein partner *BARD1* lead to high risks of breast and ovarian cancer. Both *BRCA1* and *BARD1* proteins harbor RING domains, and missense mutations in the critical residues of the *BRCA1* RING domain are among those known to predispose to cancer. The *BRCA1* RING domain is a ubiquitin ligase, but the function of the *BARD1* RING domain and the consequences of mutations in it are unknown. Evaluation of missense mutations at evolutionarily conserved zinc-binding residues of *BARD1*, each identified in a family severely affected with breast cancer, revealed that *BARD1* is necessary for two core functions of the *BRCA1/BARD1* complex: ubiquitylation of histone 2A on nucleosomes and transcriptional regulation of genes of estrogen metabolism.

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mutations in the RING domain of BARD1 in four families: BARD1 p.C53W (c.159T > G) at chr 2: 215,661,841 in families CF2947 and CF3031 (who are not related to each other); BARD1 p.C71Y (c.212G > A) at chr 2: 215,661,788 in family CF3490; and BARD1 p.C83R (c.247 A > G) at chr 2: 215,657,138 in family CF4638 (18) (Fig. 1A). Each of the three BARD1 mutations occurs at a residue completely conserved across all sequenced species. None of the three mutations appear in the Genome Aggregation Database (gnomAD) of 123,136 exome sequences (19) or among 10,000 cancer-free older female participants of the Women's Health Initiative ([whi.color.com/](http://whi.color.com/)). For five of the 12 breast cancers in the four families, hormone receptor information was available: one tumor was triple negative and four were estrogen receptor and progesterone receptor positive. Most breast cancers from patients with BRCA1 or truncating BARD1 mutations are triple negative (17). Whether triple negative breast cancer will also predominate among patients with missense mutations in the BARD1 RING domain awaits additional data. The observation in family CF3490 of two unaffected women, ages 67 and 68, who carry BARD1 p.C71Y suggests that BARD1 RING domain mutations may not convey as high a risk as mutations in the comparable residues of the BRCA1 RING domain (Fig. 1B) (20).

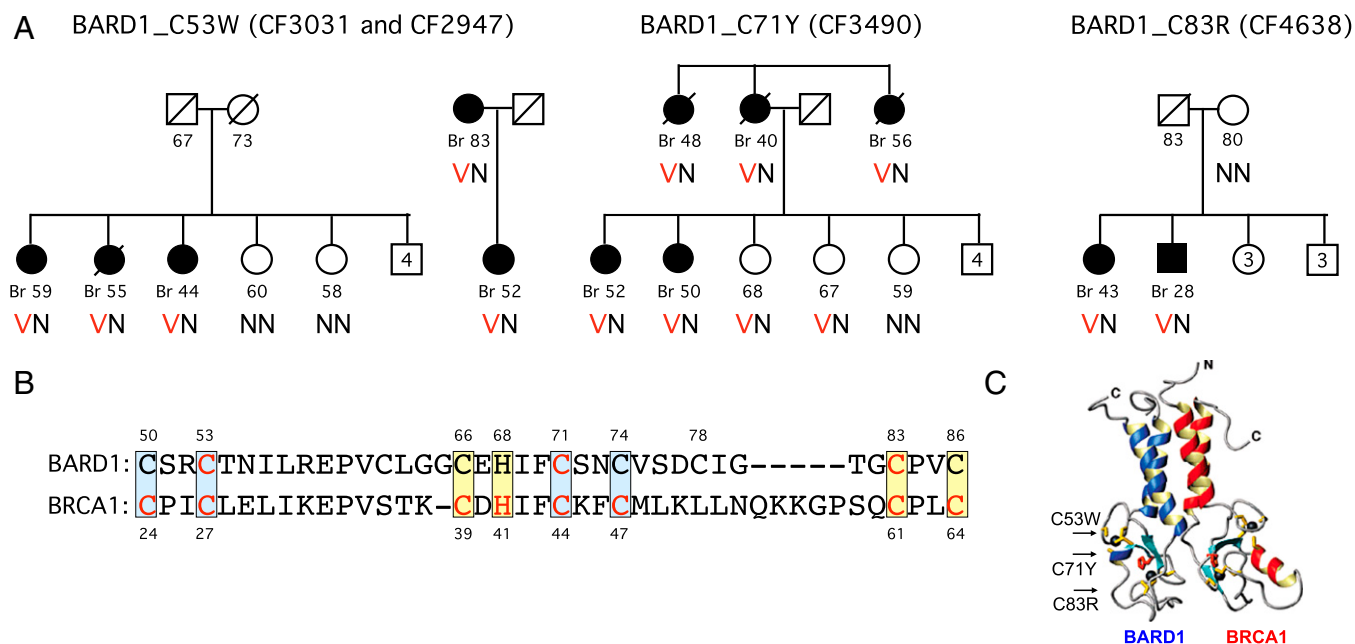
RING domain structures are stabilized by two zinc atoms that are bound by seven cysteine and one histidine residues. The three BARD1 RING domain mutations identified in breast cancer families alter cysteine residues that coordinate these zinc ions (Fig. 1B and C). The amino acids of the resulting mutant proteins (tryptophan, tyrosine, and arginine) are not chemically capable of coordinating zinc in a manner similar to cysteine. In BRCA1, missense mutations in zinc-binding cysteine residues of the RING domain were among the first cancer-predisposing mutations identified (21). In BRCA1, these mutations cause structural changes that abrogate BRCA1 binding to ubiquitin-conjugating (E2) enzymes, thereby destroying the ubiquitin ligase activity of the BRCA1/BARD1 heterodimer (22). Because

the BARD1 RING domain does not bind directly to E2 enzymes (22, 23), the consequences of comparable mutations in the BARD1 RING domain have remained undefined.

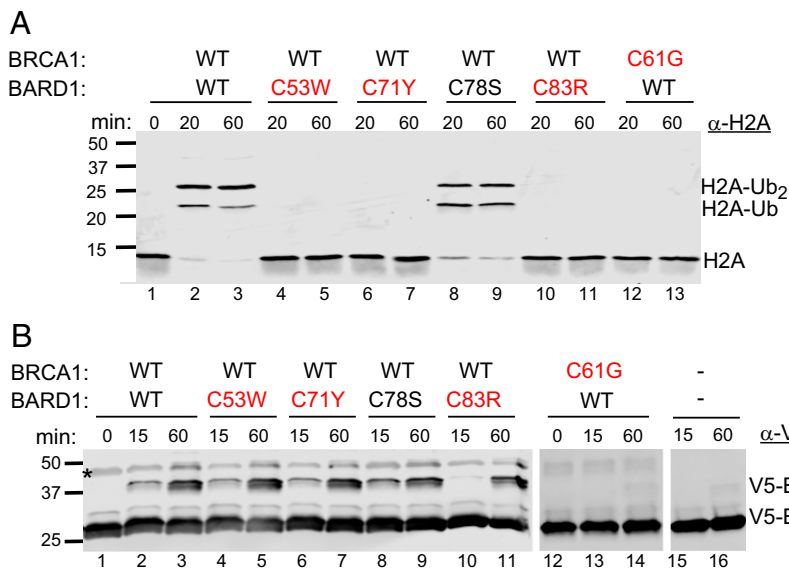
To better understand the effects of mutations in zinc-binding residues of the BARD1 RING domain, we evaluated BRCA1/BARD1 heterodimer complexes that contained each of the BARD1 missense mutations. For comparison, we similarly evaluated BARD1 p.C78S (c.233G > C) at chr 2: 215,657,152, which also lies in the BARD1 RING domain but alters a cysteine residue that is not conserved and does not coordinate zinc. BARD1 p.C78S has not been observed in any family in our study, but was reported as a "variant of unknown significance" in one individual of unknown phenotype in the ClinVar genetic database (NIH clinical variation database) (24).

**Effect of BARD1 Mutations on Ubiquitylation of Nucleosomal Histone H2A.** The BARD1 RING domain binds to BRCA1 and stimulates BRCA1 ubiquitin ligase activity (6, 25, 26). To determine the effect of BARD1 mutations, we carried out *in vitro* assays for these activities using constructs of BRCA1 and BARD1 that have been shown to exhibit binding and ubiquitin ligase function similar to the full-length proteins (1, 2, 22). All wild-type and mutant BARD1 RING domains copurified with BRCA1, confirming that all BARD1 variants retained their ability to form heterodimers with BRCA1 (*SI Appendix, Fig. S1*).

Ubiquitylation of lysine residues in the C-terminal tail of histone H2A by the BRCA1/BARD1 heterodimer plays an important role in DNA damage repair and gene silencing and is a possible explanation for the regulation of transcription of certain estrogen-metabolizing enzymes by BRCA1 (5, 27, 28). We therefore assessed the ability of BRCA1/BARD1 to modify H2A with ubiquitin in assays with recombinant mononucleosomes as the substrate. During reactions in which the wild-type BRCA1/BARD1 heterodimer served as the Ub ligase, ubiquitylated H2A products appeared as higher molecular weight bands, corresponding



**Fig. 1.** Missense mutations in the BARD1 RING domain in breast cancer families. (A) Families with inherited mutations in critical residues of the BARD1 RING domain. Breast cancer (Br) is indicated by filled symbols; ages represent age at breast cancer diagnosis for affected individuals, or current age or age at death for individuals without breast cancer. Numbers inside symbols indicate additional siblings. For each family, V indicates the variant allele, and N indicates the reference allele. (B) RING domain sequences of BARD1 and BRCA1. Zinc-binding sites I and II are indicated in light blue and yellow, respectively. Residues with cancer-predisposing mutations in BARD1 (reported here) or in BRCA1 (reported elsewhere) are indicated in red. (C) The BRCA1/BARD1 heterodimer (based on protein database entry 1JM7) (52) indicates the positions of the missense mutations in the BARD1 RING domain (shown as blue subunit).



**Fig. 2.** Nucleosome and ER $\alpha$  ubiquitylation activities of BARD1 mutant proteins. (A) Wild-type BRCA1 in complex with BARD1 p.C53W, BARD1 p.C71Y, or BARD1 p.C83R is deficient at *in vitro* ubiquitylation of nucleosomal histone H2A, as is BRCA1 p.C61G in complex with wild-type BARD1. In contrast, wild-type BRCA1 in complex with BARD1 p.C78S has normal ubiquitylation activity. (B) *In vitro* ubiquitylation of ER $\alpha$  is not altered by any mutant BARD1 proteins but is affected by BRCA1 p.C61G. ER $\alpha$  ubiquitylation was carried out for the specified amount of time with heterodimers incorporating the BRCA1 or BARD1 variant indicated and then Western blot probed for anti-V5 tag on the ligand-binding domain of ER $\alpha$ . The asterisk indicates nonspecific antibody binding to BRCA1. Mutations identified in breast cancer families are labeled in red.

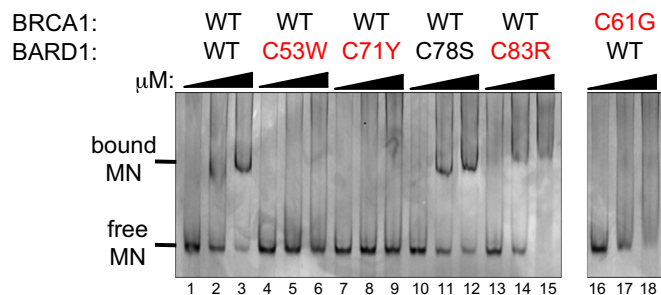
to H2A plus one or two ubiquitin molecules (Fig. 2A, bands H2A-Ub and H2A-Ub<sub>2</sub>), and the band for unmodified H2A disappeared (Fig. 2A, band H2A). As a positive control, the same reaction with BRCA1/BARD1 heterodimer containing the known loss-of-function mutation BRCA1 p.C61G had no detectable activity towards nucleosomes, consistent with its lack of ligase activity toward any substrate that has been measured to date. Reactions with the BRCA1/BARD1 heterodimer containing BARD1 p.C53W, BARD1 p.C71Y, or BARD1 p.C83R also yielded absence of ubiquitylated H2A product. In contrast, a BRCA1/BARD1 heterodimer containing BARD1 p.C78S yielded results similar to those of the wild-type heterodimer. These results indicate that the zinc-binding BRCA1 and BARD1 variants inherited in breast cancer families are defective in catalyzing ubiquitylation of H2A in nucleosomes.

To evaluate the generality of loss of ubiquitylating activity by BARD1 mutants, we tested ligase activity toward another known substrate, the ligand-binding domain of ER $\alpha$ . BRCA1/BARD1 activity toward ER $\alpha$  is important for regulation of estrogen signaling in cells, and this activity is defective in BRCA1 RING domain mutations (2, 29). *In vitro* ubiquitylation assays were carried out and the substrate and products were visualized by Western blot analysis for the V5 tag on the ER $\alpha$  ligand-binding domain (Fig. 2B). For wild-type BARD1 and all mutant BARD1 proteins, the BRCA1/BARD1 complex produced a product that corresponds to attachment of one ubiquitin to the ER $\alpha$  ligand-binding domain (Fig. 2B, band ER $\alpha$ -Ub), whereas the BRCA1/BARD1 complex including BRCA1 p.C61G was inactive. Similarly, when BRCA1/BARD1 complexes were assayed for autoubiquitylation activity, in which BRCA1 serves as a proxy substrate (6), heterodimers incorporating BARD1 mutant proteins produced products, whereas the heterodimer incorporating BRCA1 p.C61G did not (SI Appendix, Fig. S2). Together, the results of *in vitro* ubiquitylation assays indicate that while BRCA1 p.C61G causes a complete loss of Ub ligase function (due to its inability to bind an E2 ubiquitin species that brings ubiquitin to the substrate), the newly discovered BARD1 RING mutations specifically fail to ubiquitylate nucleosomal H2A.

BRCA1/BARD1 has been reported to ubiquitylate free H2A; that is, outside the context of a nucleosome (1, 30, 31). In assays containing free H2A (at much higher concentration than in the nucleosome reactions), ubiquitylated product is generated by heterodimers incorporating each of the BARD1 variants (SI Appendix, Fig. S3, band H2A-Ub). In contrast, heterodimers incorporating to BRCA1 p.C61G and wild-type BARD1 do not ubiquitylate free

H2A. Thus, while the BRCA1 p.C61G protein is inactive in both contexts, the BARD1 RING mutations only fail to ubiquitylate H2A on nucleosomes. Ubiquitylation of free H2A is far less efficient than ubiquitylation of H2A on nucleosomes; higher concentrations of free H2A must be used and most free H2A remains unmodified. It is possible that efficient histone 2A ubiquitylation by the BRCA1/BARD1 complex is orchestrated by recruitment of nucleosomes by BARD1 to target histone H2A as a substrate.

The simplest explanation for why mutant BARD1 RING domains are only inactive toward H2A in nucleosomes is that the mutant BRCA1/BARD1 complex is unable to recognize and bind nucleosomes. To test this hypothesis, we assessed the ability of BRCA1/BARD1 complexes to bind to nucleosomes in electrophoretic mobility shift assays, using mononucleosomes reconstituted from purified recombinant histones and DNA as substrates (Fig. 3). With increasing amounts of wild-type BRCA1/BARD1 complex, the band corresponding to unbound mononucleosomes decreased in intensity and a new band corresponding to a higher molecular weight species appeared (Fig. 3, lanes 1–3). Results similar to wild type were observed for BARD1 p.C78S (Fig. 3, lanes 10–12). In



**Fig. 3.** Mononucleosome binding activities of BARD1 mutant proteins. Mononucleosome binding by wild-type BRCA1/BARD1 and by complex with neutral BARD1 p.C78S is observed as the appearance of a distinct band (marked as "bound MN") at increasing concentration of added protein. In contrast, BARD1 and BRCA1 mutations identified in breast cancer families are deficient at binding mononucleosomes *in vitro*. Electromobility gel shift assays were carried out at increasing concentrations (10, 20, and 30  $\mu$ M) of the indicated BRCA1/BARD1 complexes and visualized by ethidium bromide staining of DNA incorporated into the mononucleosomes (MN). Mutations identified in breast cancer families are labeled in red.

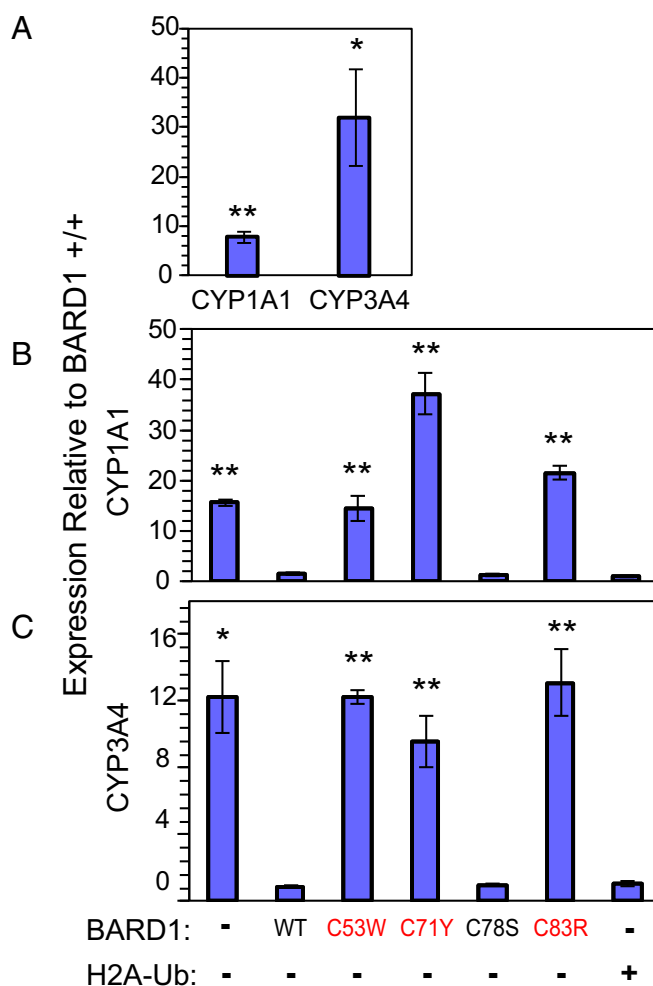
contrast, complexes incorporating BARD1 p.C53W (Fig. 3, lanes 4–6) or BARD1 p.C71Y (Fig. 3, lanes 7–9) did not appear to bind nucleosomes, as evidenced by no change in the band corresponding to unbound mononucleosomes. Complexes incorporating BARD1 p.C83R (Fig. 3, lanes 13–15) or BRCA1 p.C61G (Fig. 3, lanes 16–18) appeared to bind mononucleosomes, as evidenced by the disappearance of the free mononucleosome band, but the apparent binding does not result in a distinct bound species at the same molecular weight as seen with wild-type BRCA1/BARD1. Instead, binding by these complexes produced a diffuse staining that likely corresponds to a distribution of dynamically associated species. Notably, the mutated site in these two proteins, Cys83 of BARD1 and Cys61 of BRCA1, are structurally homologous within their corresponding RING domains (Fig. 1B), which may underlie their similar behavior on electrophoretic shift assays. Together with the mononucleosome ubiquitylation assay (Fig. 2A), these results indicate that both BRCA1 and BARD1 RING domains contain nucleosome-binding sites and that sites of both RING domains must be intact for formation of the nucleosomal complex required for ubiquitylation of H2A in chromatin.

**Effect of BARD1 Mutations on Transcriptional Regulation of Estrogen Metabolizing Genes.** Ubiquitylation of the C-terminal tail of histone H2A is associated with gene silencing (4, 32, 33). We therefore hypothesized a link between regulation of gene expression by BRCA1/BARD1 and loss of H2A ubiquitylation activity due to mutation of *BARD1*. Appropriate genes to test this hypothesis are *CYP1A1* and *CYP3A4*, two members of the cytochrome P450 family that are transcriptionally regulated by BRCA1, and that catalyze transformation of estrogen into metabolites that generate DNA damage-inducing free radicals (28, 34, 35). Depletion of *BRCA1* results in increased *CYP1A1* and *CYP3A4* transcription and increased estrogen metabolite-mediated DNA damage (28).

To determine the consequences of loss of function of *BARD1* on *CYP1A1* and *CYP3A4* transcription, we created *BARD1* knockout alleles in MCF10A breast epithelial cells by CRISPR/Cas9 gene editing. Guide RNAs were developed that efficiently deleted *BARD1* exon 1 in cell pools (SI Appendix, Fig. S4A and Table S1). Of 182 clones screened, 64 were heterozygous, but none were homozygous for the *BARD1* deletion. We hypothesize that homozygous loss of *BARD1* is lethal. We therefore isolated several *BARD1*<sup>+/-</sup> heterozygous clones (SI Appendix, Fig. S4B). As expected, *BARD1*<sup>+/-</sup> cells have decreased levels of BARD1 RNA and protein (SI Appendix, Fig. S4C and E). Furthermore, *BARD1*<sup>+/-</sup> cells have higher levels of *BRCA1* RNA but lower levels of BRCA1 protein (SI Appendix, Fig. S4D and E), presumably due to the reliance of BRCA1 on BARD1 for stability (30).

We compared expression of *CYP1A1* and *CYP3A4* in *BARD1*<sup>+/+</sup> versus *BARD1*<sup>+/-</sup> MCF10A cells by quantitative real-time PCR (qRT-PCR). Expression levels of both *CYP1A1* and *CYP3A4* were significantly higher in *BARD1*<sup>+/-</sup> cells than in *BARD1*<sup>+/+</sup> cells (Fig. 4A); increases were 7.8-fold ( $P = 0.008$ ) for *CYP1A1* and 32-fold ( $P = 0.017$ ) for *CYP3A4*. By comparison, *CYP1A1* and *CYP3A4* expression increased 3-fold and 2-fold in similarly edited *BRCA1*<sup>+/-</sup> MCF10A cells (28). These data suggest that BARD1 plays a role in the repression of some genes associated with estrogen metabolism, and that haploinsufficiency of *BARD1* leads to a substantial increase in the transcription of these genes.

To test whether mutant BARD1 proteins could rescue loss of BARD1 transcriptional regulatory function, we transiently transfected *BARD1*<sup>+/-</sup> cells with plasmids carrying either wild-type *BARD1* or one of the mutant *BARD1* genes, with simultaneous coexpression of wild-type *BRCA1* to ensure formation of BRCA1/BARD1 heterodimers, and again measured *CYP1A1* and *CYP3A4* expression by qRT-PCR (Fig. 4B and C and SI Appendix, Fig. S5).



**Fig. 4.** Expression of estrogen metabolism genes in *BARD1*<sup>+/-</sup> MCF10A breast epithelial cells. (A) Expression of *CYP1A1* and *CYP3A4* in *BARD1*<sup>+/-</sup> cells relative to *BARD1*<sup>+/+</sup> cells was measured by qRT-PCR. Transcriptional repression by the BRCA1/BARD1 complex is relaxed in *BARD1* haploinsufficient cells, leading to increased expression of *CYP1A1* and *CYP3A4*. For each gene, bar heights and error bars are means and SEs of relative expression levels derived from  $\Delta\Delta\text{Ct}$  measurements, with GAPDH standardization, from three replicate experiments. Comparisons were made by *t* tests for paired data. \* $P < 0.05$ ; \*\*\* $P < 0.001$ . Details are shown in SI Appendix, Table S2. (B) Effects on *CYP1A1* gene expression of introducing wild-type *BARD1*, one of the *BARD1* mutants, or H2A-Ub fusion by transient transfection into *BARD1*<sup>+/-</sup> cells, accompanied by cotransfection with wild-type *BRCA1*. *BARD1* mutants that fail to ubiquitylate nucleosomal H2A also fail to restore gene silencing of *CYP1A1*. Species introduced are noted below C. Mutations identified in breast cancer families are labeled in red. (C) Effects on *CYP3A4* gene expression of introducing wild-type *BARD1*, one of the *BARD1* mutants, or H2A-Ub fusion by transient transfection into *BRCA1*<sup>+/-</sup> cells, accompanied by cotransfection with wild-type *BRCA1*. *BARD1* mutants that fail to ubiquitylate nucleosomal H2A also fail to restore gene silencing of *CYP3A4*.

Introduction of either wild-type *BARD1* or BARD1 p.C78S resulted in *CYP1A1* and *CYP3A4* expression returning to the low levels measured in *BARD1*<sup>+/+</sup> cells. In contrast, the *BARD1* mutations with defects in H2A ubiquitylation failed to restore gene silencing of either *CYP1A1* or *CYP3A4*. These results indicate that BARD1 proteins that retain capacity to ubiquitylate H2A in nucleosomes in vitro are also able to restore repression of *CYP1A1* and *CYP3A4* in cells, whereas BARD1 proteins with a defect in nucleosome binding and ubiquitylation are unable to functionally restore *CYP1A1* or *CYP3A4* repression.

To test the relationship between BARD1 H2A ubiquitylation activity observed in vitro and gene expression changes in BARD1 haploinsufficient cells, we carried out a complementation experiment with ubiquitylated H2A. The BRCA1/BARD1 heterodimer attaches ubiquitin to the most C-terminal lysine residues on H2A, including the C-terminal residue, Lys129, of the histone tail (30). An H2A-Ub fusion expressed exogenously rescues heterochromatin silencing in *BRCA1*-negative cells (4) and homologous recombination in *BARD1*-silenced cells (5). Similarly, transfection of H2A-Ub fusion into *BARD1*<sup>+/-</sup> cells rescued *CYP11A1* and *CYP3A4* gene silencing, with their expression returning to levels similar to *BARD1*<sup>+/+</sup> cells (Fig. 4 B and C and *SI Appendix, Fig. S5*). The ability of a mimic of BRCA1/BARD1-ubiquitylated H2A to rescue the increase in *CYP11A1* and *CYP3A4* expression observed in the *BARD1*<sup>+/-</sup> cells is strong, if indirect, evidence that ubiquitylation of H2A is the BARD1 function responsible for transcriptional regulation.

## Discussion

We identified three missense mutations in highly conserved zinc-coordinating cysteine residues of the BARD1 RING domain in families severely affected with breast cancer. BRCA1/BARD1 heterodimers incorporating any of these mutations fail to ubiquitylate nucleosomal histone H2A. The same mutations are defective in mononucleosome binding, indicating that the BARD1 RING domain is instrumental in anchoring the BRCA1/BARD1 complex to chromatin. Engineered *BARD1* haploinsufficiency in MCF10A cells led to increased expression of BRCA1-regulated estrogen metabolism genes *CYP11A1* and *CYP3A4* (28, 36).

A potential role of BRCA1 ubiquitylation of H2A in transcriptional regulation is based on the observation that expression of H2A with ubiquitin fused at its C terminus rescues heterochromatin silencing in a *BRCA1*-deficient cell line (4). Here we report that in *BARD1* haploinsufficient cells, loss of transcriptional regulation of certain cytochrome P450 genes is rescued by reintroduction of wild-type *BARD1*, or of a BARD1 mutant protein with no effect on H2A ubiquitylation, or of H2A-Ub fusion, but is not rescued by BARD1 mutant proteins that fail to ubiquitylate H2A. Altogether our results suggest that the BARD1 RING domain binds a substrate required for nucleosome ubiquitylation, and that this function is linked to transcriptional regulation by BRCA1/BARD1 in breast epithelial cells.

Our results also indicate that BARD1 plays a major role in formation of a BRCA1/BARD1/nucleosome complex. BRCA1 ubiquitylates H2A on its C-terminal tail at Lys127 and Lys129 both in vitro and in vivo (1, 31). Another heterodimeric RING Ub ligase, RING1B/BMI1 of the polycomb repressive complex 1, also ubiquitylates H2A on the C-terminal tail but at a different position, Lys119 (32). While both RING1B/BMI1 and BRCA1/BARD1 activities lead to gene silencing, the two enzymes are not redundant (27) and the different sites of ubiquitylation play different roles in the cell (37). Binding of the RING1B/BMI1 complex to a nucleosome involves contact between the nucleosome and BMI1, the subunit of the heterodimer analogous to BARD1 (38). The contact is made by an extended loop on BMI1 that does not have a structural homolog in BARD1. But in contrast to the BARD1 mutants tested here, mutation of BMI1 at residues in the nucleosome interface did not affect nucleosome binding or activity (38), even though mutation of a conserved positively charged patch in RING1B or in BRCA1 resulted in similar losses of activity (38). Because the binding region on the ligase-active subunits (i.e., RING1B and BRCA1) is conserved, while the nucleosome binding sites on BMI1 and BARD1 are not, we propose that BARD1 and BMI1 function to position their ligase-active partners in specific orientations on the nucleosome to produce different ubiquitylated H2A products.

The observation that BARD1 mutants that show loss of activity toward H2A in vitro are the same BARD1 mutants that are unable to rescue gene repression lends further support to the hypothesis that BRCA1/BARD1 acts as a transcriptional repressor through its activity toward H2A. H2A with ubiquitin attached to a lysine on its C-terminal tail is a known repressive marker found in compact chromatin (4, 32, 33). BRCA1 recruitment to promoter regions of progesterone-responsive genes is correlated with appearance of ubiquitylated H2A in the promoter region and subsequent termination of progesterone signaling (3). BRCA1 activity toward H2A has also been associated with heterochromatin silencing in neuronal tissue culture (4, 27), suggesting that the role of BRCA1 as a transcriptional repressor via H2A ubiquitylation may not be limited to epithelial cells. The demonstration that BARD1 plays an active role in transcriptional repression, and the demonstration of a link between Ub ligase activity of BRCA1/BARD1 and transcriptional repression, add to the substantial body of evidence that BRCA1 acts as a transcriptional repressor.

The three familial *BARD1* variants presented here exhibit loss of nucleosomal H2A ubiquitylation, but maintain activity toward other substrates such as ER $\alpha$  and BRCA1 autoubiquitylation (Fig. 2 and *SI Appendix, Figs. S2 and S3*). This contrasts with RING missense mutations in BRCA1 that are inactive against all known substrates. That BARD1 variants in complex with BRCA1 are active toward nonnucleosomal substrates is consistent with the established role of the BRCA1 RING domain as the site to which E2 Ub-conjugating enzymes bind (22). While we cannot exclude additional undiscovered substrates responsible for BRCA1 and BARD1 tissue-specific tumor suppression, nucleosomal H2A was the only substrate tested that shows a loss of activity for both BARD1 and BRCA1 variants, strongly implicating H2A as an important BRCA1/BARD1 substrate for tumor suppression activity. We note that the importance of BRCA1 ligase function for tumor suppression remains a topic of debate (4, 8–10, 39). The *BARD1* mutations presented here provide a powerful opportunity to better define the functions and substrates of BRCA1 involved in tumor suppression through identification of similar dysfunction in vivo for mutations of *BRCA1* and *BARD1*.

We propose that the BRCA1/BARD1 complex acts as a tumor suppressor in estrogen-dependent tissues through H2A ubiquitylation, by suppressing the expression of genes that encode estrogen-metabolizing enzymes. Loss-of-function mutations of *BRCA1* and *BARD1* lead to increased risk of breast, ovarian, and fallopian tube cancer (11–15, 40), tissues that are exposed to high levels of estrogen and estrogen metabolites over a woman's lifetime. Prophylactic removal of the ovaries and fallopian tubes well before menopause (by about age 40) significantly reduces risk of breast cancer in *BRCA1* mutation carriers (41–43), reflecting the strong influence of estrogen level in tumor formation in these women. Cytochrome P450 enzymes transform estrogen into metabolites that produce free radicals that induce double-stranded DNA breaks (28, 34). BRCA1 binds to promoters of a subset of P450 genes, and *BRCA1* haploinsufficiency results in increased transcription of the P450 genes *CYP11A1* and *CYP3A4* (28). In addition, carriers of *BRCA1* mutations have higher concentrations of DNA-damaging estrogen metabolites in their serum and urine (44, 45). Our results indicate that *BARD1* haploinsufficiency also results in increased transcription of *CYP11A1* and *CYP3A4*, and that introduction of cancer-predisposing *BARD1* mutations cannot restore P450 gene repression in *BARD1* haploinsufficient cells. Together with comparable findings for *BRCA1* (28), our results indicate that two functional copies of both *BRCA1* and *BARD1* are required for proper transcriptional control of estrogen-metabolizing enzymes.

A role for BRCA1/BARD1 in DNA damage repair is well established. Ubiquitylated H2A is a DNA damage repair signal, and ubiquitylation of H2A by BRCA1/BARD1 is essential for

efficient homologous recombination (5), reflected in the observation that two of the *BARD1* mutations identified in severely affected families, *BARD1* p.C53W and *BARD1* p.C71Y, are defective in homologous recombination *in vitro* (46). This link suggests that *BRCA1* or *BARD1* mutations deficient in H2A modification are also likely to be defective in repair of double-stranded breaks induced by estrogen metabolites. In consequence, carriers of these mutations would accumulate more estrogen-induced DNA damage over time than would other women. The convergence of damaging effects of *BRCA1* and *BARD1* mutants both on transcriptional repression activity and on DNA damage repair activity, mediated by a shared H2A ubiquitylation mechanism, begins to reveal the molecular basis of the high risks of breast and ovarian cancer among women who carry these mutations.

## Materials and Methods

A delineation of methods used, including genomics (47), protein constructs and purification (2, 23, 48, 49), ubiquitylation assays, nucleosome binding assays, gene editing (50), transfection assays (5), and RNA assays (51), is available in *SI Appendix, SI Methods*. This project was approved by the Human Subjects Division of the University of Washington (study 1583). Written informed consent was obtained from all participants.

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