SUPPORTING INFORMATION: BARD1 is necessary for ubiquitylation of nucleosomal histone H2A and for transcriptional regulation of estrogen metabolism genes

SUPPLEMENTAL METHODS

Genomics. DNA was isolated from blood donated by patients and their unaffected relatives from families severely affected with invasive ductal breast cancer. DNA from all participants was sequenced for 27 genes by hybridization to the BROCA gene panel and multiplex sequencing on an Illumina HiSeq instrument, as previously described (1). Variants were filtered using public databases (ExAC, gnomAD, and whi.color.com) and ~10,000 sequences of these genes previously obtained in our lab.

Protein Constructs and Purification. The N-terminal domains of BRCA1 (residues 1-304) and BARD1 (residues 26-140) were co-expressed in *E. coli* and co-purified as previously reported (2), using nickel affinity resin to capture the Histidine-tag on BRCA1. All mutations were created using the protocol from QuikChange site-directed mutagenesis (Agilent). The estrogen receptor α ligand-binding domain, ubiquitin E1 and ubiquitin E2 (UBE2D3) were expressed and purified as described previously (2, 3, 4). Histones and 147-base pairs of Widom 601 DNA was purified and assembled into mono-nucleosomes as established previously (5).

Ubiquitylation Assays. All ubiquitylation assays were carried out in 25 mM sodium phosphate pH 7.0, 150 mM sodium chloride at 37ºC with shaking. H2A ubiquitylation was measured in reactions that contained 1 μM E1, 4 μM E2 (Ube2d3), 8 μM BRCA1/BARD1, 20 μM Ub, 0.12 μM mono-nucleosomes or 2 μM free H2A, 5 mM ATP, and 5 mM $MgCl_2$. Time-points were taken at 0, 20, and 60 minutes after addition of ATP and the reaction was stopped by boiling in SDS sample load buffer. Samples were analyzed by gel electrophoresis followed by western blotting with an antibody to H2A (Cell Signaling #3636).

Ubiquitylation of ERα ligand-binding domain (LBD) was measured in reactions containing 2 μM ERα LBD, 1 μM E1, 2 μM E2 (Ube2d3), 5 μM BRCA1/BARD1, 20 μM Ub, 5 mM ATP, and 5 mM $MgCl_2$. Time points were taken at 0, 15, and 60 minutes after addition of ATP and the reaction was stopped by boiling in SDS sample load buffer. Samples were analyzed by gel electrophoresis followed by western blotting with an antibody against the V5 tag on the ERα LBD using an antibody from Invitrogen (46-0705). Auto-ubiquitylation was measured in reactions containing 1 μM E1, 2 μM E2 (Ube2d3), 10 μM BRCA1/BARD1, 40 μM Ub, 5 mM ATP, and 5 mM $MgCl_2$. Time-points were taken at 0, 10, and 30 minutes after addition of ATP and the reaction was stopped by boiling in SDS sample load buffer. Samples were analyzed by gel electrophoresis followed by western blotting with an antibody against the FLAG-tag on BRCA1 (Sigma-Aldrich (F3165)).

Nucleosome binding. BRCA1/BARD1 binding to nucleosomes was evaluated by electromobility shift assays. The indicated concentration of BRCA1/BARD1 heterodimer was incubated with 50 nM mono-nucleosomes on ice for 30 minutes in 10 mM Tris, pH 7.5, 150 mM NaCl. Samples were run on 5% TBE gels in pre-chilled running buffer and stained with ethidium bromide.

Gene editing and transfection assays. MCF10a cells were cultured in DMEM/F12K containing 5% horse serum, insulin, epidermal growth factor, cholera toxin, hydrocortisone, and antibiotics (penicillin and streptomycin). BARD1 heterozygous knockout cells were generated using CRISPR/Cas9 approach (6). Guide sequences (Table S1 and Figure S4) were used to target the BARD1 exon1. Pre-assembled ribonucleoprotein complex (RNP) of Cas 9 and sgRNA was introduced into the cells by nucleofection. Nucleofected cells were single cell sorted, analyzed by PCR using primers listed in Table S1, and screened for BARD1 expression by immunobloting using an antibody against BARD1 (Bethyl Lab A300-263A) (Figure S5).

PCDNA3.1 vectors were used for transfection of either HA-tagged H2A-Ub fusion or cotransfection of N-terminal FLAG-tagged full length BARD1 and N-terminal HA-tagged full-length BRCA1. The H2A-Ub fusion construct (a gift from the Morris Laboratory, University of Birmingham) contained modifications described previously (7). Cells were plated in a six well plate at 80% density and allowed to grow one day before transfection. Room temperature OptiMEM (155 µl) was mixed with 5.4 µg of DNA before adding 9.9 µl of Fugen HD reagent (Promega) and mixed by pipetting 15 times. After 10 minutes of incubation at room temperature, 150 µl of the mixture was added to each well and mixed gently. This protocol produces ~50% transformation efficiency as measured by fluorescence 48 hours after transfection with GFP in a PCDNA3.1 vector. After 48 hours of transfection, cells were lysed using RIPA buffer (Bio-Rad). 20 µg of protein extracts were loaded per lane and analyzed by SDS-PAGE and western blot using anti-BARD1 (Bethyl Lab A300-263A), anti-GAPDH (Cell Signaling #2118), and anti-HA (Sigma H336) antibodies.

RNA assays. Total RNA was isolated using the Direct-zol RNA miniprep kit (Zymogen). 1 µg of RNA was reverse transcribed by SuperScript III (Invitrogen), and FastSYBR Green Master mix (Applied Biosystems) was used to prepare reaction mixes. Relative quantities of the tested transcripts were normalized against GAPDH and evaluated by standard methods (8). Primer sequences for *BARD1, CYP1A1,* and *CYP3A4* are listed in Table S1.

SUPPLEMENTAL FIGURES

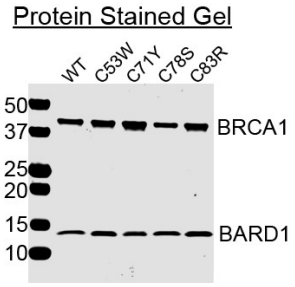

Fig. S1. BARD1 variants bind to BRCA1. Coomassie-stained SDS-PAGE gel of co-expressed and co-purified RING domains of BRCA1 and wildtype and mutant BARD1 proteins

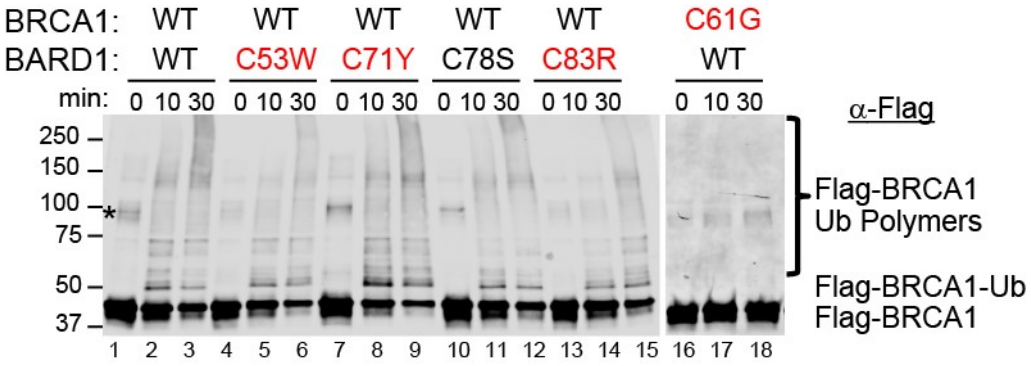

Fig. S2. BARD1 mutations do not inhibit auto-ubiquitylation of BRCA1. Western blot of ubiquitylation reactions carried out using complexes of BRCA1 and various BARD1 constructs probed for the Flag tag present on BRCA1.

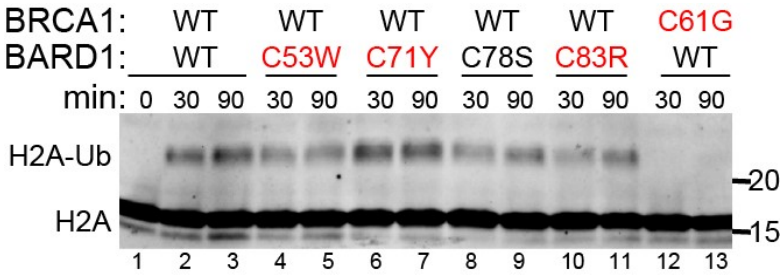

Fig. S3: Ubiquitylation of free H2A (not in the context of nucleosomes) is not impaired by BARD1 RING domain missense mutations.

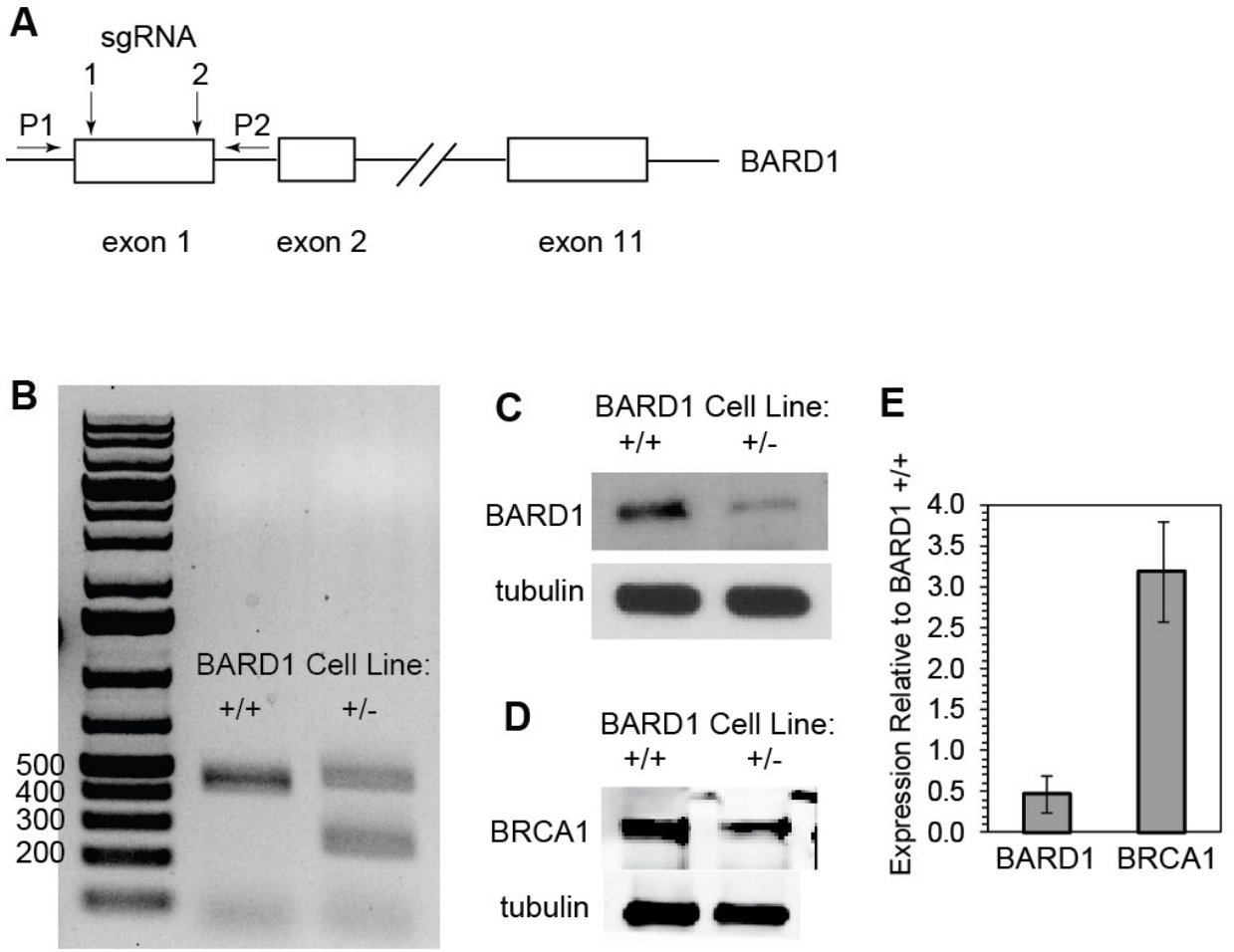

Fig. S4. Creation and characterization of the *BARD1 +/-* clone (D8) of MCF10A cells used for Figures 4 and S5. (A) Two sgRNA were designed to delete exon 1 of *BARD1* with a CRISPR/cas9 strategy. The knockout was verified using primers that anneal to the locations P1 and P2. Sequences of sgRNA and primers are shown in Table S1. (B) Ethidium bromide staining of PCR products generated using primers P1 and P2. The parent MCF10a cells (*BARD1 +/+*) generate a ~500 base pairs product, while the clone generated by CRISPR/cas9 (*BARD1 +/-*) produces an additional truncated product at ~250 base pairs. (C) Western blot for BARD1 shows a decrease in BARD1 protein levels in the CRISPR/Cas9 generated clone to 19% of the level of WT cells (quantification using ImageJ (1)). (D) Western blot for BRCA1 shows a decrease in BRCA1 protein levels in the CRISPR/Cas9 generated clone to 79% of the WT cells (quantification using ImageJ (9)). E) Relative RNA expression of *BARD1* and *BRCA*1 in *BARD1* +/- cells (clone D8) compared to *BARD1 +/+* cells, measured by qRT-PCR with ΔΔCt calculations and standardized by GAPDH. For three biological replicates, mean and standard error of relative expression of *BARD1* were 0.48 ± 0.14, P = 0.14. For two biological replicates, mean and standard error of relative expression of *BRCA1* were 3.18 ± 0.61, P = 0.11.

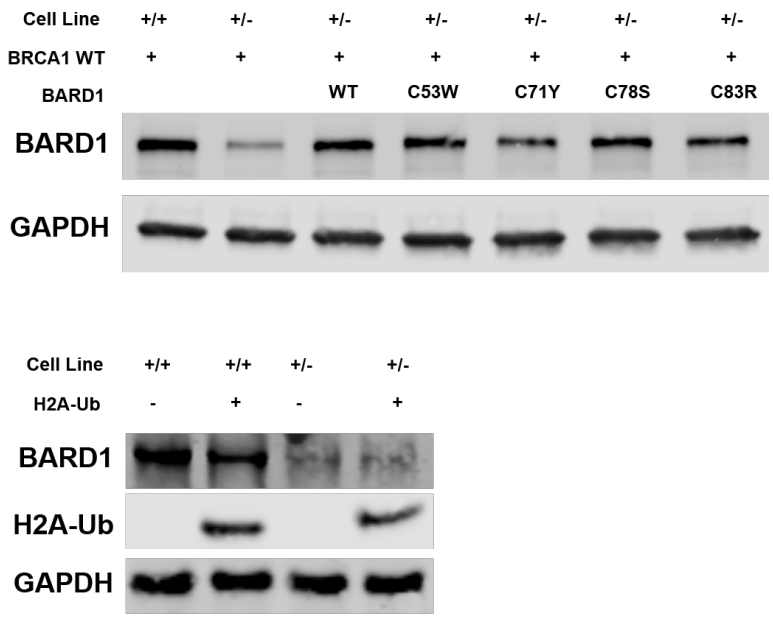

Fig. S5. Verification of BARD1 and H2A-Ub fusion protein levels for transfections presented in Fig. 4B and 4C. The antibodies used for western blots were to BARD1, GAPDH, and the HA-tag on H2A-Ub fusion.

SUPPLEMENTAL TABLES

Table S1. Primer sequences.

sgRNA_1	CTTGGCCGGTTTCGAGTCGCTGG
sgRNA_2	CGCGGCGCGACTGTGGGCCCAGG
PCR primer-F (P1)	GGGACTTTGGCAAGTTTCAG
PCR primer-R (P2)	AAAGACGGAGCTTCTTGGG
qRTPCR BARD1-F	CTGCTCGCGTTGTAATTGTGT
qRTPCR BARD1-R	TATCCAGGCCGGGGTGTAAC
qRTPCR CYP1A1-F	GGCCACATCCGGGACATCACAGA
qRTPCR CYP1A1-R	TGGGGATGGTGAAGGGGACGAA
qRTPCR CYP3A4-F	GGTCCAGTGGGATTTATG
qRTPCR CYP3A4-R	TTGGAGACAGCAATGATC
qPCR GAPDH-F	TGCACCACCAACTGCTTAG
qPCR GAPDH-R	GGATGCAGGGATGATGTTC

Table S2. Expression of *CYP1A1* or *CYP1A4* in BARD1 +/- cells relative to expression in BARD1 +/+ cells.
Data for Figures 4 and S4.

	Mean	Standard Error	P-value
Figure 4A			
CYP1A1	7.79	1.17	0.008
CYP3A4	32.07	9.68	0.017
Figure 4B			
BARD1 +/-	15.65	0.59	0.0002
WT	1.44	0.33	0.382
C53W	14.40	2.53	0.004
C71Y	37.23	4.16	0.001
C78S	1.16	0.29	0.833
C83R	21.48	1.32	0.0004
H2A-Ub	1.06	0.18	0.87
Figure 4C			
BARD1 +/-	12.22	2.15	0.006
WT	0.91	0.07	0.336
C53W	12.29	0.42	0.0002
C71Y	9.58	1.51	0.006
C78S	1.01	0.06	0.989
C83R	13.11	1.99	0.003
H2A-Ub	1.10	0.16	0.71
Figure S4			
BARD1	0.48	0.23	0.140
BRCA1	3.18	0.61	0.110

SUPPLEMENTAL REFERENCES

1. Walsh T, et al. (2017) Genetic predisposition to breast cancer due to mutations other than BRCA1 and BRCA2 founder alleles among Ashkenazi Jewish women. *JAMA Oncol* doi: 10.1001/jamaoncol.2017.1996. [Epub ahead of print]
2. Eakin CM, Maccoss MJ, Finney GL, Klevit RE (2007) Estrogen receptor α is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci USA* 104(14):5794-5799.

3. Christensen DE, Brzovic PS, Klevit RE (2007) E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages. *Nat Struct Mol Biol* 14(10):941-948.
4. Pickart CM, Raasi S (2005) Controlled synthesis of polyubiquitin chains. *Methods Enzymol* 399:21-36.
5. Dhall A, et al. (2014) Sumoylated human histone H4 prevents chromatin compaction by inhibiting long-range internucleosomal interactions. *J Biol Chem* 289:33827-33837.
6. Lin S, Staahl BT, Alla RK, Doudna JA (2014) Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* 3:e04766.
7. Densham RM, et al. (2016) Human BRCA1-BARD1 ubiquitin ligase activity counteracts chromatin barriers to DNA resection. *Nat Struct Mol Biol* 23(7):647-655.
8. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25(4):402-408.
9. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9(7):671-675.