Investigating the functional conservation of BRCA1/BARD1 in *Caenorhabditis elegans*

By

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Introduction

Many factors influence a person's chances of developing breast cancer. Studies in the early 1990s began to reveal an association between an individual's genetic history and breast cancer centering around one gene called *BRCA1*.[1] Further research has shown that hereditary mutations in the *BRCA1* gene can result in an eighty percent increased risk of being diagnosed with breast cancer by age seventy.[2] The reason this gene heavily influences breast cancer risk is because it codes for a protein (by the same name) that plays a role in several cellular functions critical to the body's defense against cancer. These include DNA damage repair[3], control of cell division[4], and transcriptional regulation.[5-6] The BRCA1 protein performs these tumor-suppressive functions by partnering with several other proteins. One of them, called BARD1, physically associates with BRCA1 at a region called the RING domain (which is shared by both proteins).[7] This BRCA1-BARD1 (BCBD) heterodimer acts as a E3 ubiquitin ligase contingent upon the RING domain.[8-9] This E3 ligase plays a specific role in a process known as ubiquitination. Ubiquitination is a complex process that requires several enzymes to attach the small peptide, ubiquitin, to a substrate. Specifically, the E3 ligase stabilizes an intermediate step in the reaction known as the E2-ubiquitin conjugate. [10] This action promotes a reactive conformation of the E2-ubiquitin conjugate that favors covalent attachment of ubiquitin to specific target proteins by the E2 enzyme.[10] The E3 ligase therefore acts as a catalyst for the ubiquitination reaction. Once attached, ubiquitin may alter the chemical properties of its targets or modify the way in which other proteins/molecules interact with those substrates. This in turn can shift metabolic pathways and bring about significant changes in cellular activity. The BCBD complex has been shown to ubiquitinate estrogen receptor α[11], progesterone receptors[12], and histone H2A.[13] Both hormone receptors have been extensively studied in breast cancer

progression/suppression.[14-15] However, the ubiquitination of histone H2A has only recently been implicated in tumor suppression. The BCBD complex ubiquitinates histone H2A most efficiently when the protein is in the nucleosome conformation at Lys 127 and 129.[13] To do so, the BCBD complex binds to the nucleosome at specific points relative to each member of the heterodimer. BRCA1 attaches directly to histone H2A at the H2A/H2B acidic patch using a stretch of residues that include Lys 70 and Arg 71,[16] while BARD1 binds to the H2B/H4 cleft using a stretch of residues that includes Pro 89 and Trp 91.[17] Binding at these sites is critical since mutations at these residues decreased H2A ubiquitination *in vitro*.[17] Furthermore, specific mutations inhibit the enzymatic function of the BCBD complex towards all substrates. Mutations in the RING domain of BRCA1 inhibit E3 ubiquitin ligase activity of the heterodimer and are associated with cancer development.[18] Likewise, *in vitro* studies determined that a positive charge at residue 99 of BARD1 is essential for E3 ligase activity of the heterodimer.[19] The previously described mutations inhibit the intrinsic E3 ligase function of the BCBD complex and are therefore relevant in understanding BCBD activity towards histone H2A. H2A ubiquitination by the BCBD complex promotes cell survival following DNA damage and is also associated with the homologous repair (HR) pathway.[19] Additionally, BCBD ubiquitination of H2A is associated with transcriptional regulation, since mutations in BARD1 that inhibit the process are associated with over-expression of CYP estrogen catabolism genes.[6] It is thought that ubiquitination of histone H2A results in chromosomal changes that suppress the expression of genes; thus, acting as a form of transcriptional repression. Reducing expression of these catabolic CYP genes may decrease cellular exposure to genotoxic and carcinogenic substances that form as a byproduct of estrogen breakdown. Therefore, research devoted to understanding the E3 ligase function of the BCBD complex is necessary to understand an individual's risk for developing breast cancer.

Yet barriers exist to this research. Most studies conducted on the BCBD complex have used human breast-cancer cell lines, but there are downsides with this approach. Cells in culture behave differently than cells in the context of the organism[20], and data from different cell lines are not always comparable to each other.[20] Moreover, use of immortalized cell lines does not allow researchers to study the process of transformation, since those cells already possess the many mutations that made them cancerous. To circumvent these drawbacks, some researchers are looking to the round worm *Caenorhabditis elegans* as a model organism for studying the BCBD complex and the role it plays in breast cancer. There are several reasons why *C. elegans* is a promising model organism for BRCA1 and breast cancer studies. The worms share many orthologous genes with humans.[21] These include orthologs for BRCA1 and BARD1 referred to as Ce-*brc*-1 and Ce-*brd*-1 respectively.[22] These worm homologs share roughly fifty percent sequence similarity with the human genes and possess similar tertiary structures like the RING domain.[22] Additionally, testing cancer-causing mutations in worms is easier than in humans since they fully develop from eggs to adults in just days[23] and possess easy to manipulate genomes.[24] However, research needs to establish that the BCBD complex functions the same in *C. elegans* as it does in humans before widespread testing of the genes may occur in worms.

To that end, researchers have shown that the Ce BCBD complex colocalizes with the synaptonemal complex during meiosis and inhibition of the worm heterodimer increases the lethality of *C. elegans* embryos that possess other deficiencies in chromosomal synapsis.[25] These results indicate that the Ce BCBD complex functions as a checkpoint for cell division. Additionally, the Ce BCBD complex possesses E3 ubiquitin ligase capabilities and ubiquitinates chromatin-associated substrates following DNA damage indicating that the Ce BCBD complex plays a role in DNA damage repair.[26] Together these results show that the Ce BCBD performs

two of the three known roles of the human BCBD complex. Yet no study has determined if the *C. elegans* orthologues also play a role in transcriptional regulation. For that reason, the overall goal of this project is to determine if the Ce BCBD heterodimer has conserved enzymatic function towards histone H2A, which as previously stated is important for transcriptional regulation in humans. If Ce BCBD maintains E3 ligase activity towards histone H2A in worms, then it serves as a powerful indicator that Ce BCBD also plays a role in transcriptional regulation. We expect that the Ce BCBD complex ubiquitinates histone H2A in worms in the same manner as human BCBD. To achieve this goal, we outlined two specific objectives: 1) Classify the C-terminal lysine residue on histone H2A that Ce BCBD ubiquitinates in worms. 2) Determine if residues in Ce BCBD (conserved from human BCBD) are important for E3 ligase activity towards histone H2A.

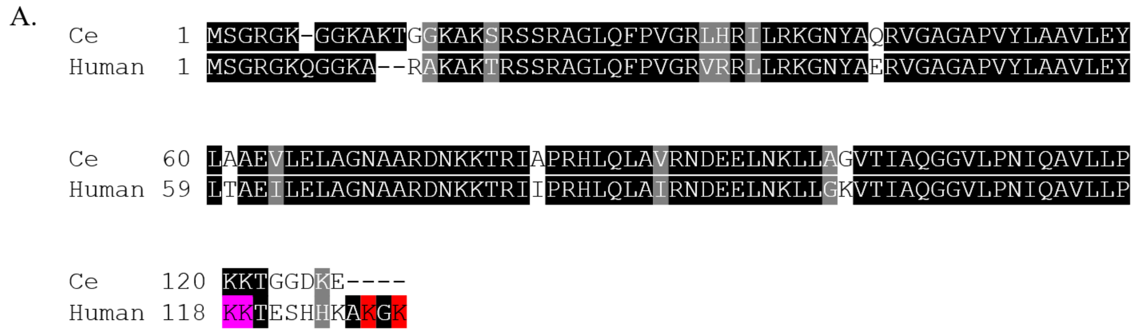
Results

Ce BCBD does not primarily target Lys 126 on histone H2A

We hypothesized that Ce BCBD ubiquitinates the far C-terminal Lys residue 126 in *C. elegans* H2A just as human BCBD ubiquitinates the far C-terminal Lys residues in human H2A. Ubiquitination of Lys residues on histone H2A has been implicated in transcriptional repression by the BCBD complex.[5-6] Therefore, identifying which Lys residue is targeted by Ce BCBD is important in showing that this function is also conserved in *C. elegans*. The chief difference between the histones of the two species lies at the C-terminal tail. Human H2A contains two ubiquitin attachment sites at Lys residues 127 and 129,[13] whereas *C. elegans* H2A contains one Lys residue 126 of unknown significance at the far C-terminal tail. We set out to synthesize a *C. elegans*-human H2A chimeric protein to identify if this is the Lys residue targeted by Ce BCBD ubiquitination. This is possible, because the *C. elegans* H2A construct is eighty-seven percent

identical and ninety-two percent similar to human H2A (figure 1.A). Importantly residues necessary for BRCA1 binding to H2A are also conserved in *C. elegans* H2A[17] (figure 1.A).

A.



B.

H2A Chimera

MSGRGKQGGKARAKAKTRSSRAGLQFPVGRVRRLLRKGNYAERVGAGAPVYLAAVLEYLT
AEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLPKK
TGGDKE

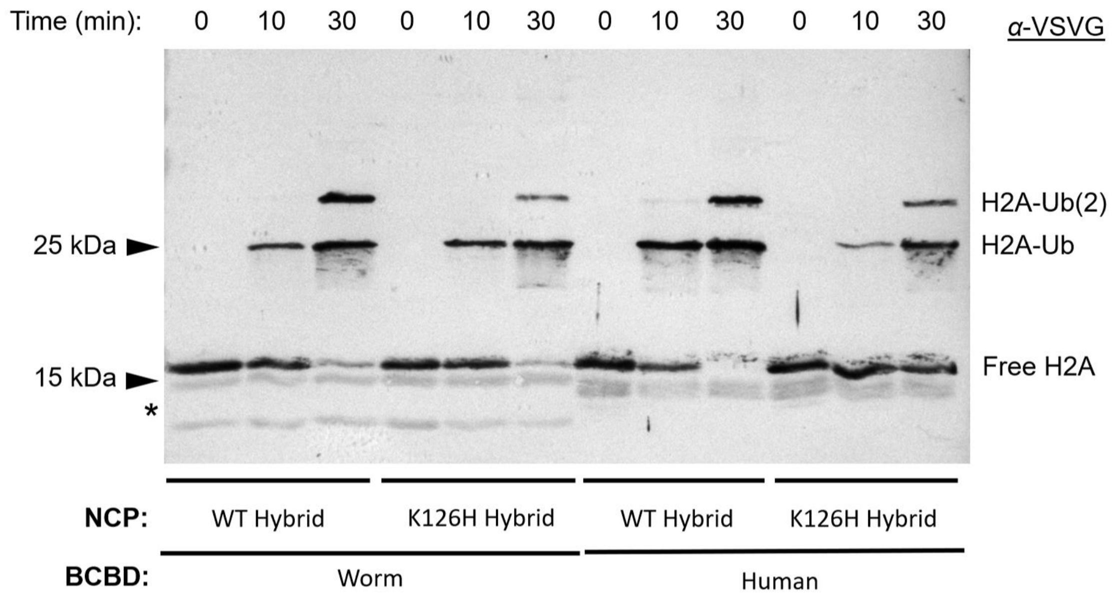
H2A K126H Chimera

MSGRGKQGGKARAKAKTRSSRAGLQFPVGRVRRLLRKGNYAERVGAGAPVYLAAVLEYLT
AEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLPKK
TGGDHE

Figure 1. Sequence homology between Ce H2A and human H2A as well as chimeric H2A constructs. A) Amino acid sequence alignment of *C. elegans* and human histone H2A. Residues highlighted in black are the same in both proteins, while residues highlighted in grey maintain similar chemical properties. Dashes indicate gaps in the sequence. Lys residues highlighted in purple are ubiquitinated by the E3 ligase complex RING1b/BMI1. Lys residues highlighted in red are ubiquitinated by BRCA1/BARD1 complex. B) Amino acid sequence of the two chimeric H2A constructs synthesized for this study. Residues highlighted in green indicate *C. elegans* sequence. Residues highlighted in yellow indicate mutations introduced into the chimeric construct.

Furthermore, human mono-nucleosome reconstitution is a well-defined process with a higher probability of success than reconstituting *C. elegans* mono-nucleosomes. Taken together, these factors made synthesizing a *C. elegans*-human H2A chimeric protein advantageous for our study.

To that end, we employed mutagenesis to attach the *C. elegans* H2A C-terminal tail sequence to the body of human H2A (termed 'WT Hybrid' figure 1.B). Once we achieved this chimeric construct, we used mutagenesis again to remove the predicted ubiquitination site (Lys 126) from the C-terminal tail of the chimera and replace it with a His residue (termed 'K126H Hybrid' figure 1.B). This allowed us to test if loss of Lys 126 had any effect on H2A ubiquitination by Ce BCBD. We performed functional assays to test the efficiency of both the *C. elegans* and human E3 complexes at ubiquitinating the WT Hybrid and K126H Hybrid H2A constructs in the context of nucleosomes. The western blot of these assays can be seen in figure 2.A with an accompanying quantification in figure 2.B



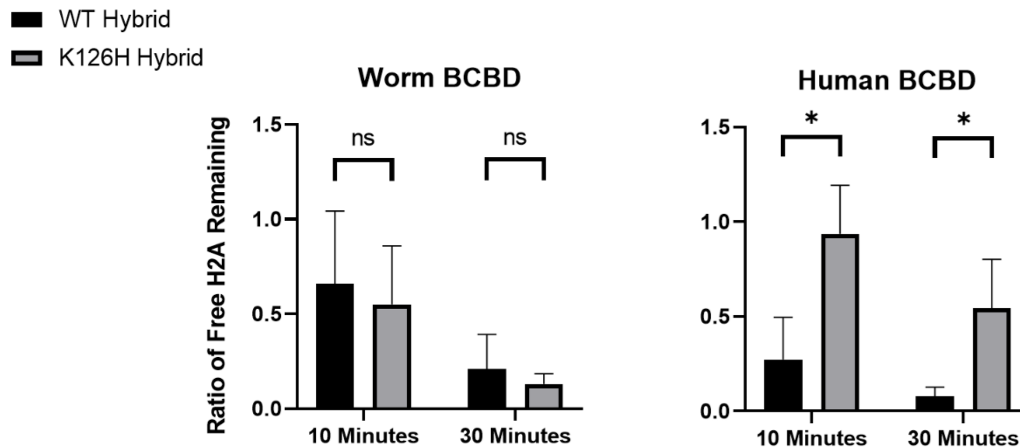


Figure 2. Lys 126 is not the primary residue ubiquitinated by Ce BCBD. A) Representative western blot of two biological and two technical replicates of *in-vitro* H2A ubiquitination assays. E3 ligase activity of either human or *C. elegans* BCBD was tested against nucleosome core particles (NCP) containing either chimeric hybrid WT VSVG-H2A or K126H chimeric VSVG-H2A. Note: asterisks denotes non-specific activity of the α-VSVG antibody towards the BCBD complex. The *C. elegans* BCBD complex appears as two bands with distinct molecular weights, while the human BCBD complex appears as a single band. B) Quantification of western blots of two biological and two technical replicates of *in-vitro* H2A ubiquitination assays. The amount of free H2A in solution remaining at 10 and 30 minutes was quantified using ImageJ. These values were normalized to the quantified amount of free H2A at 0-minutes for each assay. The average amount of free H2A was calculated using values obtained from each blot (n=4), and these averages were compared using an unpaired two-sample t-test and Welch's t-test. Statistical significance was determined using a P-value < 0.05.

These results show that human BCBD is a more efficient E3 enzyme than Ce BCBD since the WT Hybrid assays containing Ce BCBD have a greater ratio of non-ubiquitinated free H2A remaining on average at both the ten- and thirty-minute time points than the same assays containing human BCBD (figure 2.B). We also see that human BCBD appears to have a preference for ubiquitinating Lys residue 126 compared to Ce BCBD. Statistical analyses were carried out on repeated and quantified blots of the Human BCBD assays. The quantified amounts of free H2A remaining in solution at 10 and 30 minutes was divided by the quantified amount of free H2A in solution at time 0 for each reaction. This yielded ratios that could be averaged and compared using t-tests. These tests showed that there was a statistically significant difference in the average ratio of free H2A remaining in solution between assays containing WT hybrid H2A and K126H H2A (figure 2.B). The data indicate that the assays containing K126H H2A on average had more free H2A remaining in solution at both the 10-minute and 30-minute time points when compared to the assays containing WT hybrid H2A. These results indicate that the K126H mutation decreases the efficiency of H2A ubiquitination by human BCBD, as would have been predicted based on studies in Witus et al.[17] Surprisingly, the K126H mutation does not seem to significantly affect the efficiency of H2A ubiquitination by Ce BCBD; the ratio of free H2A remaining in the ten- and thirty-minute time points are very similar between the assays using WT and K126H Hybrid H2A (figure 2.B). Statistical analyses were performed in the same manner as described previously for repeated and quantified western blots of assays containing Ce BCBD, and they showed no significant difference between the ratio of free H2A remaining in solution in assays containing WT or K126H hybrid H2A (figure 2.B). From these results we may conclude that Lys residue 126 is not the only target of ubiquitination by Ce BCBD; indicating

that Ce BCBD primarily targets other Lys residues on histone H2A. This shows that Ce BCBD has different Lysine specificity than human BCBD when interacting with H2A.

Conservation of substrate binding is conserved for BRC1, but varies in BRD1

We hypothesized that Ce-*brd*-1 and Ce-*brc*-1 function in a mechanistically similar manner to that of their human orthologs. The proteins share 50% sequence similarity and functional motifs including the RING domain necessary for E3 ubiquitin ligase activity.[22] A sequence alignment shows that BRD-1 has a conserved Pro residue at the 69th position and a conserved positively charged His at the 79th position, which align with the Pro at the 89th and Arg at the 99th positions of BARD1 respectively (Figure 3.A).

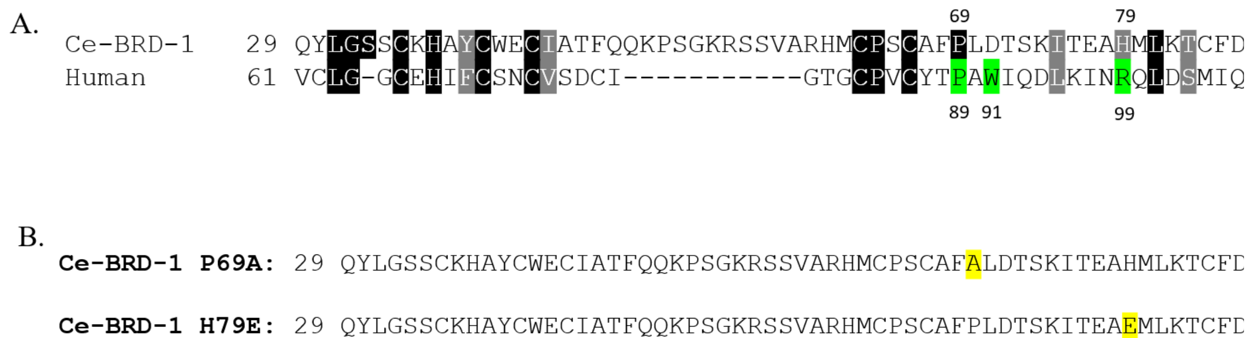


Figure 3. Synthesizing Ce-*brd*-1 mutants. A) Amino acid sequence alignment between Ce-*brd*-1 and BARD1 RING domains. Residues highlighted in black are the same in both proteins, while residues highlighted in grey maintain similar chemical properties. Dashes indicate gaps in the sequence. Residues highlighted in green are important for human BARD1 binding to H2A and E3 ligase functions. Note that Trp 91 is not conserved in *C. elegans* BRD-1. B) Sequences of the CE-*brd*-1 mutant constructs hypothesized to inhibit E3 ligase activity in *C. elegans.*

Pro 89 is thought to be important for BARD1 binding to the nucleosome during E3 ubiquitin ligase activity, since loss of this residue decreases H2A ubiquitination *in vitro*.[17] Positive charge at the 99th position is critical for BARD1 E3 ubiquitin ligase activity, since loss of that charge

inhibits E3 ubiquitin chain building *in vitro*.[19] To determine if these two functions of BARD1 are conserved in *C. elegans* we performed mutagenesis to create two Ce-*brd*-1 mutant constructs: P69A and H79E. Site-directed mutagenesis was also utilized to produce the Ce-*brc*-1 mutants: R67A and K66/R67E (KREE). These basic residues are conserved on BRCA1 and are critical for human BCBD E3 ligase activity; disruption of this "arginine anchor" on BRCA1 inhibits ubiquitination of H2A *in vitro*.[16]

We performed functional assays to identify if conserved residues from BARD1 are necessary for BRD-1 function and determine if BRD-1 behaves in a similar manner to the human ortholog. The western blot of the assay is found in figure 4.

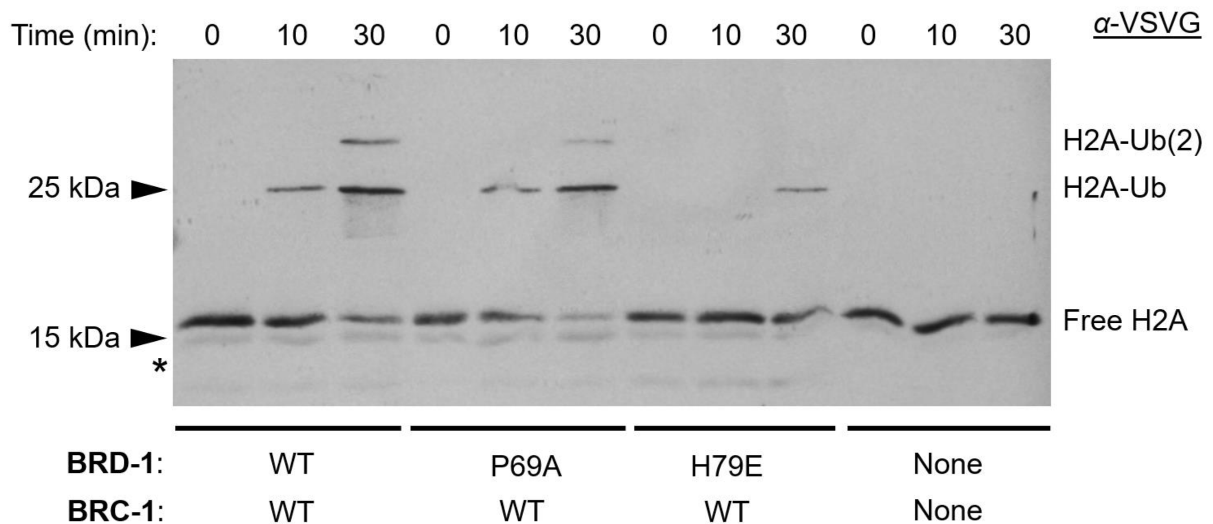


Figure 4. BRD-1 uses a conserved positive residue to catalyze ubiquitin transfer. Western blot of *in-vitro* H2A ubiquitination assays. E3 ligase activity of either WT or mutant *C. elegans* BCBD constructs was tested against NCP containing the previously synthesized chimeric Hybrid WT VSVG-H2A. Note: asterisks denotes non-specific activity of the α-VSVG antibody towards the *C. elegans* BCBD complex appearing as two district bands.

The no E3 control assays show that Ub-H2A is not formed in the absence of the E3 enzyme. This indicates that the results are not due to non-specific activity of the E2 enzyme towards free (non-nucleosomal) H2A in the reactions. These results show that mutation P69A had little to no effect

on the E3 ligase activity of Ce BCBD. The disappearance of free H2A by the thirty-minute time point and the synthesis of Ub-H2A at the ten-minute time point occur at roughly the same rate for both the WT and P69A Ce BCBD assays. However, mutation H79E did appear to significantly decrease the rate of H2A ubiquitination as compared to WT Ce BCBD. The intensity of the free H2A band appears to be roughly the same between the ten- and thirty-minute time points and the zero-time point for the H79E Ce BCBD assay indicating that little Ub-H2A synthesis had occurred. Moreover, the Ub-H2A band does not begin to appear until the thirty-minute time point and has a very low intensity compared to the WT Ce BCBD assays in which the Ub-H2A band appears at the ten-minute time point and increases in intensity by the thirty-minute time point. These results indicate that Ce BCBD, like most other heterodimeric E3 ligases, requires positive charge at a conserved position to perform ubiquitination. The results also show that the Pro conserved in BRD1 from BARD1 is not necessary for the E3 ligase function of Ce BCBD. It stands to reason that BRD1 binds in a different manner to the nucleosome than BARD1, since this conserved Pro is implicated in BARD1-nucleosome interaction.

We performed functional assays to determine the effects of the mutations to BRC-1 to determine if BRC-1 functions in a mechanistically similar manner to BRCA1 and show that key residues for this function are conserved in the worm protein. The western blot of the assay is found in figure 5.B. Again, a no E3 control assay was performed to verify that the results shown accurately reflect Ce BCBD E3 ligase activity towards H2A in the context of nucleosomes. These results show that the mutation R67A moderately decreases the E3 ligase activity of Ce BCBD. This is evidenced by the synthesis of Ub-H2A only by the thirty-minute time point, while Ub-H2A synthesis can be seen in the ten-minute time point in the WT assay. The mutation

K66R67/EE has an even greater inhibitory effect on the E3 ligase activity of Ce BCBD than the single mutation. This is because Ub-H2A synthesis is only seen in the thirty-minute time point, and the intensity of this band is less than the Ub-H2A band seen in the WT assay at the same time point. These findings show that conserved basic residues from BRCA1 are necessary for E3 ligase activity of the Ce BCBD complex towards nucleosomes. In BRCA1, these residues form an "arginine anchor" that interacts with the acidic patch of histone H2A.[17] Ultimately indicating that BRC-1 behaves in a similar manner to the human ortholog when binding to the nucleosome.

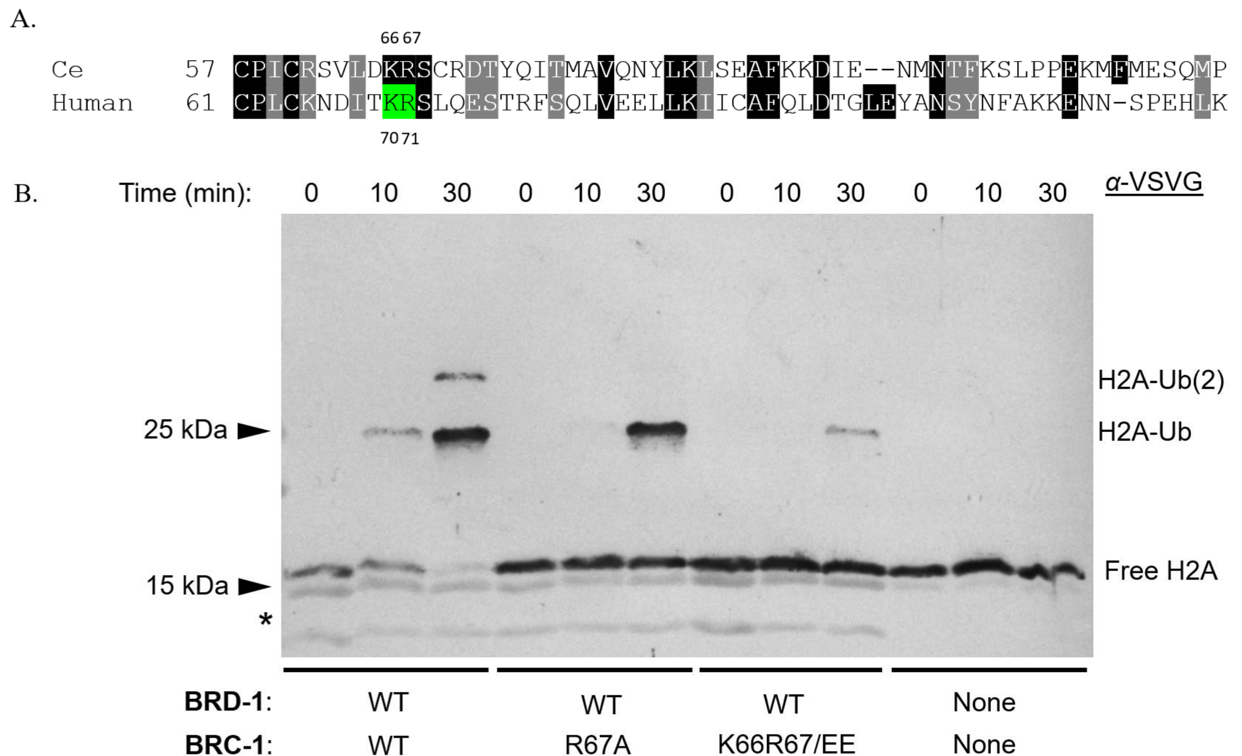


Figure 5. BRC-1 uses a conserved basic residues to catalyze ubiquitin transfer. A) Amino acid sequence alignment between Ce-*brc*-1 and BRCA1 H2A binding interfaces. Residues highlighted in black are the same in both proteins, while residues highlighted in grey maintain similar chemical properties. Dashes indicate gaps in the sequence. Residues highlighted in green are important for human BRCA1 binding to H2A and E3 ligase functions.
B) Western blot of *in-vitro* H2A ubiquitination assays. E3 ligase activity of either WT or mutant *C. elegans* BCBD constructs was tested against NCP containing the previously synthesized chimeric Hybrid WT VSVG-H2A. Note: asterisks denotes non-specific activity of the *α*-VSVG antibody towards the *C. elegans* BCBD complex appearing as two district bands.

Discussion

Our study employs *in-vitro* biochemical assays to better understand the intrinsic functions of BRC-1 and BRD-1 in *C. elegans*. These proteins are orthologous to the human tumor suppressor proteins BRCA1 and BARD1, which presents an intriguing opportunity to use *C. elegans* as a model organism for studying the BRCA1/BARD1 system and the role it plays in preventing human breast cancer. BRCA1/BARD1 form a heterodimeric complex (BCBD) possessing E3 ubiquitin ligase capabilities.[8-9] The human BCBD complex ubiquitinates Lys 127 and 129 on the c-terminal tail of histone H2A[13] resulting in the transcriptional repression of CYP estrogen metabolism genes.[5-6] It is hypothesized that this relationship to estrogen metabolism is a crucial element linking mutations in BRCA1/BARD1 to breast cancer. While previous work indicates that BRC-1 and BRD-1 also form a heterodimeric complex (Ce BCBD) possessing E3 ubiquitin ligase capabilities,[26] no study has shown that the Ce BCBD complex possesses conserved enzymatic function towards histone H2A or identified specific amino acids critical for this function in *C. elegans*. These questions needed to be answered before thee*C. elegans* can be employed as a model system for studying the relationship between BRCA1/BARD1 mediated transcriptional regulation and breast cancer development. Our results indicate that the Ce BCBD complex ubiquitinates histone H2A in *C. elegans* using conserved charges at specific locations within the E3 complex. The specific residues include a positively charged His at the 79th position in BRD-1 and the basic amino acids Arg and Lys at the 66th and 67th positions in BRC-1 respectively. Interestingly, we found that Ce BCBD does not exhibit a high degree of specificity for targeting the far C-terminal Lys 126 of histone H2A in *C. elegans*. This is a key distinction between the *C. elegans* and human systems.

Our findings agree with Densham et al 2016 revealing that positive charge at a position equivalent to Arg 99 on human BARD1 is required for E3 ligase function in Ce BCBD. These results indicate that the Ce BCBD complex functions in a mechanistically conserved manner to other RING E3 ubiquitin ligases (including human BCBD) by utilizing positive charge to catalyze the transfer of ubiquitin from the E2 enzyme to the substrate.[10,19] Our results also shed light on the potential binding mechanism of Ce BCBD towards nucleosomes. We found that loss of basic residues at positions 67 and 68 on BRC1 result in abrogation of E3 ligase activity towards histone H2A. These residues are equivalent to basic residues at positions 70 and 71 in BRCA1 (Figure 5.A) that interact with acidic residues on histone H2A and align the human BCBD complex so that ubiquitination of the proper Lys residues may take place.[16-17] Our results are consistent with the model that BRC1 utilizes these basic residues to bind to the nucleosome and ubiquitinate histone H2A in *C. elegans* like BRCA1. However, unlike BRC-1, BRD-1 may behave differently than BARD1 when binding to the nucleosome. This is because removal of Pro 69 had negligible effect on E3 ligase activity of the Ce BCBD complex. These results are surprising given that Pro is directly conserved in BRD-1 from BARD1, implicated in human E3 ligase activity, and associated with BCBD-nucleosome interaction[17] (see figure 3.A). Additional evidence for the idea that BRD-1 behaves differently than BARD1 is the fact that Trp 91, also shown to be critical for BARD1-nucleosome binding,[17] is neither directly or functionally conserved in the BRD-1 primary sequence. A possible explanation for these results is the presence of an insertion of 10 residues from the 49th to the 59th positions in BRD1 that has no equivalent in BARD1. It is possible that these residues form a structure that is necessary for BRD-1 interaction with the nucleosome. Indeed, the presence of this insertion in BRD1 may also explain why Ce BCBD does not exhibit human-like site-specific ubiquitination towards Lys 126

on histone H2A. Our findings show that removal of this Lys has negligible effects on Ce BCBD E3 ligase activity, while it does reduce human BCBD activity towards histone H2A. The differences in sequence and Lys preference between BRD1 and BARD1 suggest that BRD-1 may not utilize equivalent residues to BARD1 when interacting with the nucleosome and directing site-specific ubiquitination of histone H2A. This hypothesis is reasonable when we consider the structure and function of each partner protein in heterodimeric E3 ligases that target histone H2A. Structural data indicates that one partner protein interacts directly with the E2 enzyme and the nucleosome acidic patch, while the other is responsible for positioning the E2 enzyme over specific Lys residues. Cryo-EM structures of the BCBD complex reveal that BRCA1 contacts the E2 enzyme and nucleosome acidic patch, while BARD1 interacts with the nucleosome specifically at the H2B/H4 cleft.[17] This model holds true for another human heterodimeric E3 ligase called RING1b/BMI1. In this heterodimer, RING1b is the BRCA1-like partner and BMI1 is the BARD1-like partner. RING1b/BMI1 are known to ubiquitinate Lys 118/119 on the C-terminal tail of histone H2A,[13,27-28] and this process is also related to gene silencing.[29] Structural studies have shown that BMI1 does not have the same binding residues as BARD1 and instead relies on an unstructured loop to interact with the nucleosome.[17] This results in the H2A C-terminal tail possessing less flexibility when bound to RING1b/BMI1 and accounts for the specific range of Lys residues hit by RING1b/BMI1 complex when compared to the range of Lys residues hit by the BCBD complex. Indeed, future research should seek to analyze if the Ce BCBD complex behaves in a similar manner to RING1b/BMI1 complex based off the potential structural similarities between BRD-1 and BMI1. If they are similar, then the Ce BCBD complex may primarily ubiquitinate Lys 118/119 of H2A in *C. elegans*.

In summary, our study shows that several aspects of human BCBD E3 ligase activity towards histone H2A are conserved in *C. elegans* including residues necessary for site-specific ubiquitination of histone H2A by the Ce BCBD complex as well as residues necessary for BRC-1 interaction with nucleosomes. We also demonstrate that the mechanisms by which BRD1 binds to the nucleosome are likely different from BARD1, and these distinctions manifest themselves as differences in target Lys residues between the two BCBD complexes. Ultimately, our work establishes a plausible functional mechanism by which Ce BCBD could regulate transcription in *C.* elegans. These results indicate that use of Ce BCBD as a model system for studying the relationship between human BCBD E3 ligase activity, transcriptional regulation, and breast cancer is possible. However, future research should focus on identifying the exact interaction between BRD-1 and the nucleosome as well as identifying which Lys residue on histone H2A is ubiquitinated by Ce BCBD. Future research could also study the link between Ce BCBD ubiquitination and changes in gene transcription in *C. elegans*.

Methods

Creating the H2A Chimeric Mutants

Synthesis of the H2A chimeric protein began with the full length human H2A construct on the pHIS-Vesicular stomatitis virus glycoprotein (VSVG)-Ampicillin resistance plasmid. This plasmid was obtained from the Klevit Laboratory at the University of Washington. The H2A chimera was made using New England Biolabs (NEB) Q5 Site-directed mutagenesis protocol. Briefly, 10 uM of the appropriate forward and reverse primers (described in table 1) and the NEB specified amount of the pHIS-VSVG-Human H2A template were added to a PCR reaction containing NEB Q5 High-fidelity Hot-Start Polymerase. A PCR reaction was carried out according to the specified protocol and annealing temperature shown in table 1. Successful PCR was confirmed with DNA gel electrophoresis (on 1.5% agarose gel). The resulting PCR reaction

mixture was treated with NEB KLD solution according to manufacturer specifications to ligate the PCR amplified plasmids containing the chimeric construct and degrade the template plasmid. Then 5 μL of the KLD treated PCR solution was added to 50 μL of DH5-α chemically competent *E. coli*. The *E. coli* were purchased from NEB, but grown and treated to generate in house stocks of competent cells. The DH5-α *E. coli* were transformed with the chimeric construct according to the strain specific NEB transformation protocol and grown on LB agar in the presence of the appropriate antibiotic. Select resultant colonies were isolated and allowed to be grown in a LB broth solution containing a final concentration of 1 mM ampicillin for 14-16 hours at 37°C with shaking. After their growth, the cells were harvested (3000 rpm for 10 minutes). These pellets were then treated with a QIAgen mini-prep kit to isolate the plasmid containing the H2A chimeric construct. Presence of the chimeric mutations were confirmed using a Hitachi Genetic Analyzer 3130XL according to the protocol outlined in ThermoFisher BigDye Terminator v3.1 Cycle Sequencing Kit. Sequence ends were trimmed with Sequencher and sequences were confirmed to be successfully mutated using Expasy. This same protocol was followed with different primers (Table 1) to synthesize and confirm the presence of the of the K126H Chimeric mutant H2A construct also located on the pHIS-VSVG-Ampicillin resistance plasmid.

Creating Ce-BRD-1 P69A and H79E Mutants

WT Ce-BRD-1 (Met1-Glu107 with extra Gly at position 2: see table 2) constructs were obtained from the Klevit Laboratory at the University of Washington. These constructs were generated in pET28N-Kanamycin resistant plasmids. The specified mutations in the Ce-BRD-1 construct were synthesized using the Agilent QuikChange Site-directed Mutagenesis protocol with modifications described in Edelheit et al. 2009.[30] Briefly, two separate PCR reactions were

carried out using either the forward or reverse primers with annealing temperatures listed in table 1 in the specified reactions. The PCR products were combined and slowly annealed by cooling at 95°C for 5 minutes, 90°C for 1 minute, 80°C for 1 minute, 70°C for 30 seconds, 60°C for 30 seconds, 50°C for 30 seconds, 40°C for 30 seconds, and 37°C indefinitely. Then the plasmids were digested with Dpn1 to degrade the parent strands. Finally, the plasmids were transformed into DH5-α chemically competent *E. coli*, and the bacteria were grown on LB agar in the presence of the appropriate antibiotic. The plasmids were recovered and purified using the Qiagen Mini-Prep protocol. Mutations were confirmed via genetic sequencing analysis using a Hitachi Genetic Analyzer 3130XL. The processes of transformation, plasmid isolation, sequencing, and mutation confirmation as the same as previously described in the *Creating the H2A Chimeric Mutants* section.

Creating Ce-BRC-1 R67A and K66/R67E Mutants

WT Ce-BRC-1 (Met1-Lys100 with an N-terminal His tag: see table 2) constructs were obtained from the Klevit Laboratroy at the University of Washington. These constructs generated in PCOT7-Chloramphenicol resistant plasmids. The specified mutations in the Ce-BRC-1 construct were synthesized by C. Skalley of the Stewart Laboratory using the Agilent QuikChange site-directed mutagenesis protocol with modifications described in Edelheit et al. 2009 as described previously in the *Creating Ce-BRD-1 P69A and H79E Mutants* section.[30] The processes of transformation, plasmid isolation, sequencing, and mutation confirmation as the same as previously described in the *Creating the H2A Chimeric Mutants* section.

Ce BCBD Expression

Specified Ce BCBD constructs were co-transformed into BL21 (DE3) *E. coli* according to the NEB strain-specific protocol. The transformed cells were grown and the Ce BCBD

constructs were synthesized as described in Brzovic et. al 1998 and Meza et al.[31,7] 1999 with minor changes. BL21 *E. coli* were grown in a solution of LB broth containing a final concentration of 1 mM appropriate antibiotic(s) and 1 mM ZnCl. Expression of the Ce BCBD constructs was induced using IPTG at a final concentration of 0.2 mM-0.25 mM. Once induced, cells grew for a minimum of 16 hours at 16°C with shaking.

Ce BCBD Purification

Once expressed, the Ce BCBD constructs were purified in a similar manner to Brzovic et. al 1998 with some modifications. Induced cells were spun down at 3000 rpm at 4°C for 25 minutes, resuspended in nickel column binding buffer containing: 500 mM NaCl, 20 mM TRIS, 5 mM Imidazole, pH 7.4, and either directly sonicated or frozen at -80°C for use later. Prior to lysis cells were incubated with protease inhibitor, egg-white lysozyme, DNase, and 1 mM final concentration of DTT. Cells were lysed using sonication on ice at 60 MHz on 85% amplification in 10 second pulses with 30 seconds of rest between pulses for a total pulse time of 10 minutes. A saturated solution of PMSF in 100% ethanol was added three times in 50 µL portions before, during, and after sonication to prevent protein degradation. Lysed cells were then spun down at 14000 rcf for 25 minutes at 4°C and purified by Histidine tag affinity chromatography using a HiTrap Talon Crude cobalt column (GE) on an Äkta Start GE system. DTT was added to the protein fractions as they came off the column, so that each fraction had a final DTT concentration of 1mM. Fractions containing the BCBD constructs were collected and concentrated using PALL 10k centrifugation concentrators. The concentrated protein solution was purified again by size exclusion chromatography on a NGC Quest 10 Plus Bio-Rad chromatography system. The size exclusion chromatography buffer used in the final purification of the CE BCBD constructs consisted of 25 mM HEPES, 150 mM NaCl, 1 mM TCEP-HCl, pH

7.0. Fractions containing the proteins of interest were collected and concentrated using PALL 10 kDa cutoff centrifugation concentrators, aliquoted, and stored at -80°C for use later. The final Ce BCBD concentrations were determined using absorbance at 280 nm and an extinction coefficient of 15.00 mM cm^{-1}. Construct expression and purity was confirmed with 15% SDS-PAGE.

Nucleosome Core Particle (NCP) Reconstitution

Both Chimeric H2A constructs were sent to S. Witus at the University of Washington where they were expressed and assembled into octamers as described in Witus et al. 2021. S. Witus and R. Klevit also provided Widom 185 BP DNA. NCP reconstitution was followed according to Witus et al. 2021 with minor changes. Briefly, Widom 185 BP DNA and histone octamers were added to a dialysis solution containing 2 M NaCl and 20 mM TRIS. This solution was added to 100 µL Slide-A-Lyzer™ MINI Dialysis Devices (ThermoFisher Scientific) and the devices placed in 200 mL of buffer solution containing 2 M NaCl and 20 mM TRIS. The octamer solution was dialyzed against this buffer for a period of 36 hours during which 1800 mL of buffer containing 20 mM TRIS was slowly added to the buffer containing NaCl. This reduced the concentration to roughly 400 mM NaCl. Slowly reducing the NaCl concentration allowed the Widom 185 BP DNA to bind to histone octamers to form nucleosomes. Afterwards, the octamer solution was subjected to a secondary dialysis against buffer containing 50 mM NaCl and 20 mM TRIS. This acted as a storage buffer for the newly formed NCPs. Proper NCP reconstitution was verified with 5% polyacrylamide TBE gel electrophoresis.

H2A Ubiquitination Assays

H2A ubiquitination assays were carried out to measure the effects that the specified mutations in Ce-*brc*-1, Ce-*brd*-1, and histone H2A had on E3 Ligase activity. This assay was performed as previously described in Stewart et al. 2018 with minor changes. Briefly, all assays

were performed at 37°C with shaking in 40 µL reactions containing a final concentration of 25 mM HEPES, 150 mM NaCl, 20 µM Ubiquitin, 8 µM Ce BCBD, 4 µM Ce-LET70 (E2), 0.5 µM Human UBA1 (E1), 0.3 µM nucleosomes, 5 mM ATP, 5 mM $MgCl_2$, 0.1-0.2 mM TCEP-HCl at pH 7.0. The nucleosomes were stored in the buffer solution described in the section *NCP Reconstitution*. The E2 and E1 enzymes as well as the WT-human ubiquitin were stored in a buffer containing 25 mM sodium phosphate, 150 mM NaCl at a pH of 7.0. These proteins were synthesized, purified, and shipped to the Stewart Laboratory from the Klevit Laboratory at Washington University. The 0 minute time point was taken prior to the addition of ATP. Time points were taken at 10 and 30 minutes after the addition of ATP, and the reaction was stopped by the addition of SDS-PAGE BME load dye at the given time intervals. Samples were run on 15% SDS-PAGE and analyzed with western blotting. Blotting was carried out using a 1:10000 dilution of a rabbit primary antibody specific against the VSVG-tag on Histone H2A (Millipore Sigma Corp.). A 1:5000 dilution of a goat-anti-rabbit secondary antibody conjugated to alkaline phosphatase (Rockland Immunochemicals Inc.) was used for detection. The secondary antibody was detected using BCIP/NBT Color Development Substrate (Promega Corporation) according to manufacturer specified protocols.

Quantification of Western Blots

Western blots of two biological and two technical replicates (n=4) of *in-vitro* H2A ubiquitination assays in figure 2 were used for quantification. The amount of free H2A in solution remaining at 10 and 30 minutes was quantified using ImageJ (NIH). These values were divided by the quantified amount of free H2A at 0-minutes for each assay. The average amount of free H2A remaining at 10 and 30 minutes was calculated using values obtained from each blot. Averages of the 10- and 30-minutes time points from the Ce BCBD assays were compared using an

unpaired two-sample t-test. Averages of the 10-minute time point from the human BCBD assay was compared using an unpaired two-sample t-test. Averages from the 30-minute time point from the human BCBD assay was compared using the Welch's t-test. Statistical significance for all statistical comparisons was determined using a P-value < 0.05.

Tables

Table 1. Primer Sequences and Annealing Temperatures. Nucleotides highlighted in red indicate the codon(s) changed to obtain the desired mutations. Note that both the Chimera and Chimera K126H reactions used the same reverse primer with reaction specific forward primers. "F" refers to the forward primer and "R" refers to the reverse primer.

Primer Name and Mutation	Annealing Temperature	Sequence
F_Ce-BRD-1_P89A	61°C	5'GTCCGAGTTGTGCTTTCGCGCTAGACACATCCAA AATC 3'
R_Ce-BRD-1_P89A		5'GATTTTGGATGTGTCTAGCGCGAAAGCACAACTC GGAC 3'
F_Ce-BRD-1_H79E	60°C	5'GACACATCCAAAATCACAGAAGCTGAAATGCTG AAAACGTGC 3'
R_Ce-BRD-1_H79E		5'GCACGTTTTCAGCATTTCAGCTTCTGTGATTTTGG ATGTGTC 3'
F_H2A_Chimera	63.5°C	5' AAGGAATAGGTCGACGAGCTCAACTAGTGCGGC 3'
F_H2A Chimera_K126H		5'CACGAATAGGTCGACGAGCTCAACTAGTGCGGC 3'
R_H2A_Chimera	63.3°C	5'GTCTCCTCCGGTTTTTTTCGGCAGCAGAACCGCC 3'
F_Ce-BRC-1_R67A	56°C	5'GTGTACTGGATAAGGCTAGTTGTCGAGATAC 3'
R_Ce-BRC-1_R67A		5'GTATCTCGACAACTAGCCTTATCCAGTACAC 3'
F_Ce-BRC-1_K66/R67E	56°C	5'GTAGAAGTGTACTGGATGAAGAAAGTTGTCGAG ATAC 3'
R_Ce-BRC-1_K66/R67E		5'GTATCTCGACAACTTTCTTCATCCAGTACACTTCT AC 3'

Table 2. Constructs and Sequences This is a list of the DNA sequences of the constructs used in this study

Construct	Sequence
Human Histone H2A	GGTTACGGCTTTCTTGGAGCCCTTTTTGGGGGCAGGAGCAGATTTA GCCGGGTCGGGCATGGCGGAGGAGAGTGAAAGAGCACTCAAATA ACAAGAATGAGGACAAGCTGGGTTATGCCCTATTTATTTACAAGT CTATATGCAGATGAGATTGAGAAATTCTTCTGTCTGATTGGAAGTT ACTCAGATGACGTTAATATCAATATAGTCCAATCAAAACACGTAT ATTCAGAAAGCTCATTTGCATTAAGAGGAAGCTGCAAGCTTAGCC AATGGCCCAGCTTCTTTTTCGCGCCCAGCAGCTGCTATAAAATGCG CGTCCCTGTAGGTTCCTTTCACTCACTTTCTGACTTAGGCCACAGG TCGTTTTACCATGTCTGGACGTGGCAAGCAGGGCGGCAAGGCTCG CGCCAAGGCCAAAACCCGCTCCTCTAGAGCTGGGCTCCAATTTCCT GTAGGACGAGTGCACCGCCTGCTCCGCAAGGGCAACTACGCTGAG CGGGTCGGGGCCGGCGCGCCGGTTTACCTGGCGGCGGTGCTGGAG TACCTAACTGCCGAGATCCTGGAGCTGGCGGGCAACGCAGCCCGC GACAACAAAAAGACCCGCATCATCCCGCGCCACTTGCAGCTGGCC ATCCGCAACGACGAGGAGCTCAACAAGCTGCTTGGTAAAGTTACC ATCGCTCAGGGCGGTGTTCTGCCTAACATCCAGGCCGTACTGCTCC CCAAGAAGACTGAGAGCCACCACAAAGCTAAGGGCAAGTAAGGG CTGAACTTTAAAAATGTAAACTTACAAGACAAAAGGCTCTTTTCA GAGCCACCCACCATTTCTACGGAAGAACTGAGCACTCTGTTCTCCA AACCTATCAGAAATTTGTGGCCGAGTTCAAGCACTGAGGCCATTA CTTTCCTATTGGGTAAAATAAAAGTATTGAATCAGGCCTAGTAAAT ACTATCCAACTGCTACCTTATAACATGAAGGAACCTCCTTAATTGT CCCCTCCCCACAAACTGTCCTGGGTCTTTAAAAAACTTGGGGCCGG GCGCGGTGGTTTATGCGTCCTTAGCATTTTGGGAAACTGAGGCAG GTGGGTGGGGAGGGGAGAAATCTCTAGGGAC
Ce-*brd*-1	ATGGGATTTGAAAACACTAAAAAAGCATTGGAAACGTTTCGAACA GCTATCGAGTGTGTTAAATGCAAAAAACCGAGGGGAGACTTGCAA TATCTCGGATCATCTTGCAAACATGCTTATTGCTGGGAATGTATCG CCACATTTCAACAGAAACCGTCTGGAAAACGCTCTTCAGTGGCTC GACACATGTGTCCGAGTTGTGCTTTCCCACTAGACACATCCAAAAT CACAGAAGCTCATATGCTGAAAACGTGCTTTGATACTTTGTCTGAA CTAAACGACCTTTTACAGAAAGTCGGAACAACATCTCTAACTCAA GCAGAGTTTAA
Ce-*brc*-1	ATGGCAGATGTTGCACTGAGGATCACAGAAACAGTGGCACGACTG CAAAAAGAACTGAAATGTGGAATTTGCTGTTCAACATACAAAGAT CCAATTCTGTCCACATGTTTCCATATTTTCTGTCGTTCTTGCATTAA CGCTTGCTTCGAACGAAAACGAAAAGTTCAATGCCCAATTTGTAG AAGTGTACTGGATAAGCGAAGTTGTCGAGATACTTATCAAATTAC AATGGCTGTGCAGAACTATTTAAAGCTATCAGAAGCATTTAAAAA AGATATTGAGAATATGAATACGTTCAATAA

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VITAE

Russell Lloyd Vahrenkamp was born February 21, 1997, in Fort Worth, Texas. He is the son of Lloyd and Anna Vahrenkamp. A 2015 graduate of Christian Life preparatory School in Fort Worth, Russell received a Bachelor of Arts degree with a Major in Biology and a minor in History from Texas Christian University in 2019. In August 2019, Russell enrolled in graduate studies at Texas Christian university receiving a Master of Science in Biology in 2021.

ABSTRACT

Breast cancer susceptibility gene 1 (BRCA1) and its partner BARD1 function in genomic stability by regulating DNA repair, cell-cycle checkpoints, and transcriptional regulation. Mutations in these genes expose individuals to a higher risk of breast and ovarian cancer. The *Caenorhabditis elegans* orthologs, BRC-1 and BRD-1, also regulate DNA repair and cell-cycle checkpoints. Yet, how these proteins regulate gene transcription is unknown. Previous work shows that the worm orthologs function in a homologous manner to their human counterparts forming a heterodimeric complex (CeBCBD) that acts as an E3 ligase. We hypothesize that this CeBCBD complex ubiquitinates histone H2A resulting in epigenetic transcriptional regulation in *C. elegans*. Using biochemical ubiquitylation assays, we show that mutations in the CeBCBD complex inhibit ubiquitination of H2A in nucleosomes. These mutations are analogous to inhibitory and loss-of-function mutations in the human proteins. Our results suggest that transcriptional regulation is a conserved function of BRC-1 and BRD-1.