

CHARACTERIZING THE SUBSTRATE TARGET OF BRCA1/BARD1 IN *C. ELEGANS*

by

Caitlin Lightle

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CHARACTERIZING THE SUBSTRATE TARGET OF BRCA1/BARD1 IN *C. ELEGANS*

Project Approved:

Supervising Professor: Mikaela Stewart, Ph.D.

Department of Biology

Matthew Hale, Ph.D.

Department of Biology

Heidi Conrad, Ph.D.

Department of Chemistry

ABSTRACT

BRCA1 and BARD1 are proteins involved in the repression of genes associated with increased risk for breast and ovarian cancers. This is accomplished through ubiquitination of H2A and subsequent changes in chromatin compaction. BRCA1 and BARD1 form an E3 ligase (BCBD complex), and mutations affecting the enzymatic functions of this complex can predispose women to these cancers. The model organism *C. elegans* contains orthologs of these proteins, BRC-1 and BRD-1, which makes it a useful organism for studies of protein function; however, little is known about the mechanism of ubiquitination in *C. elegans* as compared to humans. This project used nucleosome assays to provide more insight on the ubiquitination of H2A by the BCBD complex in *C. elegans*. The objectives of this project included characterizing the interaction of the BCBD complex with H2A and identifying a specific lysine target in *C. elegans*. The conserved lysine residues (potential targets) were mutated out of H2A and nucleosome assays were performed to identify potential reductions in ubiquitination activity. The H2A nucleosome assays showed that the mutations of conserved lysines in the H2A N-terminus and C-terminus in *C. elegans* did not significantly reduce ubiquitination activity, and a definitive target could not be identified through these methods. Further studies are needed to determine if *C. elegans* has any preferential lysine targets at a non-conserved residue or if it is truly nonspecific in its activity. Currently, mass spectrometry analysis is being performed as a complementary method to attempt to pinpoint the location of lysine ubiquitination.

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INTRODUCTION

Mutations in the BRCA1 gene have been known to be implicated in the development of breast and ovarian cancers and have made their mark among the seemingly endless list of carcinogens and other factors predisposing humans to cancer. Notably, mutations in the BRCA1 gene are involved in up to an 80% increased risk of development of breast cancer and up to a 54% increased risk of development of ovarian cancer.¹ This is due, at least in part, to the involvement of BRCA1 in the transcriptional regulation and repression of CYP genes.

BRCA1 has many functions in the genome that contribute to tumor suppression, including DNA repair, cell cycle regulation, and transcriptional regulation. One of the things that BRCA1 does to stabilize the genome is regulate the activity of the cytochrome p450 (CYP) genes.² CYP genes play a large role in the risk of development of breast and ovarian cancers, which is why their repression is so important.² The function of CYP genes is to metabolize estrogen, which can result in the formation of unstable free radicals that can lead to DNA damage and an increased risk of developing breast and ovarian cancer.² While the goal is not complete repression of these genes, as estrogen is fat-soluble and must be metabolized into soluble molecules, fine-tuned regulation of these CYP genes is instrumental in order to mitigate the risk of breast and ovarian cancer.²

The BRCA1/BARD1 complex (BCBD) is involved in the regulation and repression of CYP genes through ubiquitination of histone H2A.^{2,3} Ubiquitination of histones plays an important role in many different aspects of gene expression, as they are the most commonly ubiquitinated protein, and are involved in processes including transcription, DNA repair, and cell cycle regulation.⁴ Ubiquitination of histone H2A is typically associated with repression of genes, which is important for regulation of potential oncogenes.⁴ While it is known that histone

ubiquitination is important for many aspects of regulation, the exact mechanism by which this occurs is not yet fully understood.⁴ However, it is known that the ubiquitination of histone H2A by the BCBD complex is not only associated with gene silencing, but also plays a role in repairing DNA damage that might have been caused by the estrogen metabolites from CYP genes.^{2,5} Ubiquitylation of lysine on the C-terminal tail of histone H2A is instrumental in repressing the cancer-predisposing CYP genes, and mutations in the BCBD complex can be highly detrimental with regards to cancer predisposition.²

Mutations that affect the function of BRCA1 and BARD1 predispose women to breast and ovarian cancers. These tissues are more susceptible to the damaging estrogen free radicals due to the fact that they are estrogen-regulated tissues and are therefore exposed to higher levels of estrogen compared to other tissues.² Mutations affecting the BCBD complex prevent it from carrying out its essential functions in repressing the CYP genes.³ Not only will this lead to an increased amount of damaging estrogen metabolites, but this can also hinder the DNA repair function of the BCBD complex.³ This creates an even larger problem, as increasing DNA-damaging metabolites with no way to repair them puts cells at an even greater risk of becoming cancerous.

C. elegans has been used as a model organism for studies of the BRCA1 gene and functions of BCBD.⁶ *C. elegans* contains a homolog for the *BRCA1* gene and produces orthologs of the BRCA1 and BARD1 proteins as well, called BRC-1 and BRD-1, with the BRC-1 protein having a 52% sequence similarity to human BRCA1 and the BRD-1 protein having a 41% sequence similarity to human BARD1.⁶ Like humans, *C. elegans* also has a RING domain and forms a BCBD complex (CeBCBD) that performs ubiquitination at sites with damaged DNA.^{6,7} While this is potentially a good system because the process of H2A ubiquitination and repression

of CYP genes is conserved in *C. elegans*, the target site seems to be different.⁸ This is due to the presence of additional amino acids that form an extra loop in BRD-1 that is not present in the human BARD1 protein.⁸ It has been proposed that this additional loop changes the position of the CeBCBD complex such that it is no longer aligned with the nucleosome in the same way as the human BCBD complex, resulting in the targeting of a different lysine on histone H2A.⁸

Ubiquitination is a very current and extensive topic in the field, and recently, there has been progress made with regards to understanding how E3 ligases target the nucleosome differently. There are many different E3 ligases that put ubiquitin in different places on the nucleosome, and depending on the placement of the ubiquitin, opposite results can occur. For example, while H2A ubiquitination is typically associated with gene repression, H3 ubiquitination is associated with histone acetylation leading to upregulation of gene expression.⁹ This project covers just one of the countless opportunities for research regarding ubiquitination. The purpose of this project is to study the ubiquitination process that is associated with gene regulation in *C. elegans*. Specifically, locating the amino acid that ubiquitin is added to will provide insight on the ability of BRCA1 and BARD1 to regulate the genes associated with an increased risk of breast and ovarian cancer. While the human BCBD complex typically ubiquitinates histone H2A at lysine residues 127 and 129, the lysine residue at which the *C. elegans* BCBD complex (CeBCBD) ubiquitinates histone H2A is currently unknown, but does seem to be a different location than in the human protein.^{8,10} With ubiquitination playing such an important role in many cellular processes, including gene regulation, locating the CeBCBD ubiquitination site will give more insight into the structural and functional differences of the BRCA1 and BARD1 proteins in humans and *C. elegans*, which will aid in future breast and ovarian cancer research that utilizes *C. elegans* as a model organism.

METHODS

Creating the H2A Chimera Lysine Mutations

The “C Tail” (K119/120R, K125H) H2A chimera mutant was obtained using the QuikChange Site-directed Mutagenesis protocol with the modifications as described in Edelheit et al. 2009.¹¹ Briefly, this mutation was created using a pHis vector already containing a K125H mutation, allowing for the creation of a mutant with all three lysines on the C-terminal tail mutated. Separate PCR reactions were run containing either the forward or reverse primers and annealed at 58 °C, followed by a Dpn1 digest to remove unmutated plasmids. Two other H2A chimera mutants, termed “K0” (K13/15R, K119/120R, K125H) and “13/15” (K13/15R), were obtained using the New England Biolabs (NEB) Q5 Site-directed mutagenesis protocol. The Q5 mutagenesis protocol, followed by a KLD digest to remove unwanted plasmids, was found to be more efficient than the QuikChange Site-directed Mutagenesis protocol and therefore was used for the majority of mutagenesis procedures. The Q5 mutagenesis protocol allows for one PCR reaction containing both forward and reverse primers, as opposed to two separate PCR reactions which are then followed by annealing. The PCR and annealing steps, whether by the QuikChange or Q5 protocol, were followed by a bacterial transformation into BL21 (DE3) *E. coli* cells. The bacteria were grown on LB agar in the presence of ampicillin, as the plasmid contains an antibiotic marker for ampicillin resistance. Select colonies were isolated and grown in LB broth and then purified using the QIAprep Spin Miniprep protocol. Genetic sequencing using the T7 forward and reverse primers was used to confirm the presence of the mutations.

Nucleosome Core Particle (NCP) Reconstitution and Protein Purification

The H2A chimera mutants were sent to Sam Witus at the University of Washington Rachel Klevit laboratory, where the histones were overexpressed and used to construct histone

octamers with human H2B, H3, and H4 as delineated in Witus et al. 2021.¹² Reconstitution was done by dialysis as described in Witus et al. 2021.¹² Briefly, 7 μM of Widom 601 DNA and 8 μM of the octamers were dialyzed in a solution containing 2 M NaCl and 20 mM TRIS. The NaCl solution was diluted with 20 mM TRIS to a final salt concentration of 180 mM over the course of 36 hours to allow the octamers to bind DNA. NCPs formed at a salt concentration of approximately 400 mM NaCl and were then subjected to a second dialysis in 20 mM TRIS and 50 mM NaCl for storage. Reconstitution was confirmed through TBE gel electrophoresis. Nucleosomes were stored on ice at 4 °C.

Purified forms of BRCA1 and BARD1, as well as all proteins in the nucleosomes, were utilized for this project. BRCA1 and BARD1 domains were overexpressed from a DNA plasmid in *E. coli* and purified away from other *E. coli* proteins. The purified protein was isolated via column chromatography as described in Thapa and Vahrenkamp et al. 2022.⁸ Specific construct links and enzymatic domains used can be found in Thapa and Vahrenkamp et al. 2022.⁸

Nucleosome Assays

Nucleosome assays were used to measure CeBCBD E3 ligase activity in the presence of different lysine mutations on the H2A chimera. The reactions each contained 25 mM HEPES, 150 mM NaCl, 0.3 μM nucleosomes, 20 μM ubiquitin, 0.5 μM human UBA1 (E1), 4 μM LET-70 (E2), 5 mM ATP, 5 mM MgCl_2 , and 8 μM CeBCBD (E3). The CeBCBD E3 ligase was either wild-type or contained one or more lysine mutations in the H2A chimera. The assays were run at 37 °C and 400 rpm shaking, with time points taken at 0, 10, and 30 minutes (the 0 time point was taken prior to the addition of ATP). Reactions were quenched via the addition of load dye containing SDS to denature the proteins to each time point sample.

Western Blotting Analysis

Time points taken from the nucleosome assays were analyzed via western blotting and then quantified using ImageJ. The samples were first run on a 15% polyacrylamide gel and then transferred to a nitrocellulose membrane. The samples were blocked with non-fat dry milk proteins at a 5% concentration and blotted using a 1:5000 dilution of a rabbit primary antibody (Millipore Sigma Corporation) to detect the VSVG-tag on histone H2A followed by a 1:10000 dilution of a goat anti-rabbit secondary antibody (Rockland Immunochemicals Incorporated). The H2A bands were visualized via a BCIP/NBT kit (Promega Corporation) according to manufacture instructions. The western blots were quantified using ImageJ (NIH), comparing unmodified H2A at each time point sample to the baseline time point 0. Means were compared using a Student's t-test and statistical significance was determined using a p-value of < 0.05 .

Mass Spectrometry

In preparation for mass spectrometry, a large-scale one-hour nucleosome assay was performed using the CeBCBD E3 ligase on wild type H2A chimera. This was done using the same procedure as the nucleosome assays described previously with a few modifications, as the assay was allowed to run for one hour and no time points were taken. This was done to allow for the maximum amount of H2A-Ub conjugate to be created. Following this, the proteins were cleaved into many peptides using the arginine protease Arg-C. This was done via an in-solution digest for one hour at a 1:50 enzyme to substrate ratio. The protein was identified using a combination of reverse-phase liquid chromatography and tandem mass spectrometry, which allowed the visualization of the lysine location at which CeBCBD is ubiquitinating histone H2A.

RESULTS

Mutation of conserved H2A lysine residues does not significantly affect BRCA1/BARD1 ubiquitination activity

It is known that the human BCBD complex ubiquitinates histone H2A primarily at the farthest C-terminal lysine residues, including K125, K127 and K129¹⁰ (figure 1.A). However, this pattern does not seem to hold for *C. elegans*, and it seems as though the CeBCBD complex ubiquitinates the histone at a different location. This was tested by mutating out potential lysine targets and performing assays to test for ubiquitination activity in the presence of these mutations. For the assay, all components needed to add ubiquitin to H2A were mixed together, and the reaction was run for 30 minutes, with samples taken at 0-, 10-, and 30-minute time points.

The time points were analyzed via western blotting. Gel electrophoresis was performed followed by transferring and visualizing the samples on a nitrocellulose membrane, as seen in figure 1.B. In this figure, a previous student, Russell Vahrenkamp, mutated the far C-terminal lysine in *C. elegans* (K125H), as this is the lysine closest to where human H2A is ubiquitinated (figure 1.A).⁸ Lanes 1-3 of the western blot in figure 1.B show normal ubiquitination activity from unmutated H2A in *C. elegans*. The lines dictating higher molecular weight are indicative of H2A with ubiquitin added to it, seen in lanes 2-3, and the lines dictating lower molecular weight are indicative of levels of free H2A present. Lanes 4-6 show ubiquitination activity in the presence of the K125H mutation. This mutation does not produce any significant change in ubiquitination activity by the CeBCBD complex, as lanes 5 and 6 clearly show evidence of ubiquitination in the presence of the mutation, and the disappearance of free H2A in these lanes is also indicative of ubiquitination by the CeBCBD complex (figure 1.B).⁸ Because

ubiquitination is such a critical process in the regulation of CYP genes by BRCA1/BARD1, determining the lysine residue at which histone H2A is ubiquitinated in *C. elegans* will aid in future related research that uses *C. elegans* as a model organism.

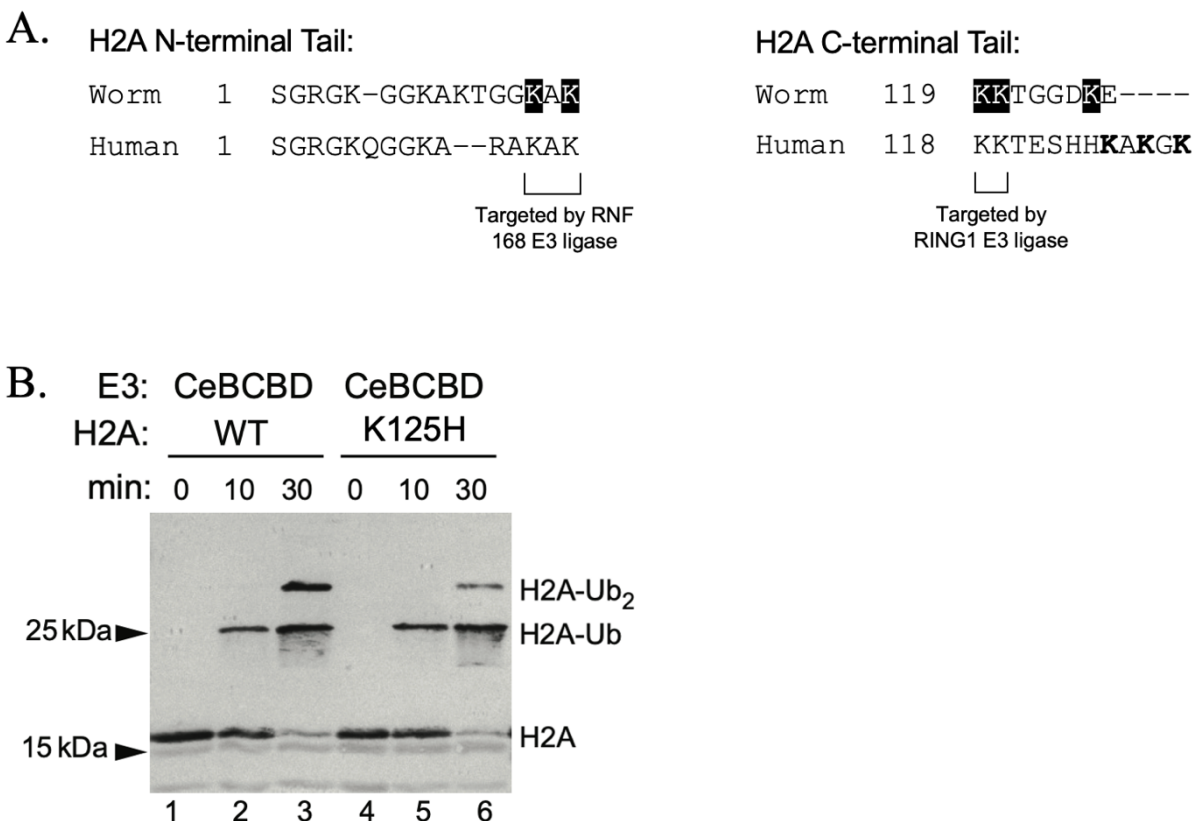


Figure 1: A) Locations of lysine mutation sites on N-terminal and C-terminal tails of worm H2A are highlighted. The lysine ubiquitination sites of human H2A on the C-terminal tail are bolded. B) Western blot showing ubiquitination activity for unmutated worm H2A (CeBCBD WT) and worm H2A with a K125H mutation (CeBCBD K125H). Ubiquitination activity was not significantly impacted by the K125H mutation, meaning that the far C-terminal lysine is likely not the preferred ubiquitination target in *C. elegans* as it is in humans. Data reproduced from Thapa et. al, Conservation of transcriptional regulation by BRCA1 and BARD1 in *Caenorhabditis elegans*, *Nucleic Acids Research*, 2022.⁸

To determine the lysine residue at which histone H2A is ubiquitinated in *C. elegans*, mutagenesis was used to create additional lysine mutations on the N-terminus and C-terminus of histone H2A in *C. elegans* (figure 1.A). The lysines targeted as potential ubiquitination sites in *C. elegans* were those conserved and known to be targeted in human H2A. Three different mutated H2A constructs were created, termed “13/15,” “C Tail,” and “K0.” In addition, three sets of nucleosomes were reconstituted, each containing one set of H2A mutations to be used in assays comparing H2A ubiquitination activity by CeBCBD in the presence of different lysine mutations (figure 2).

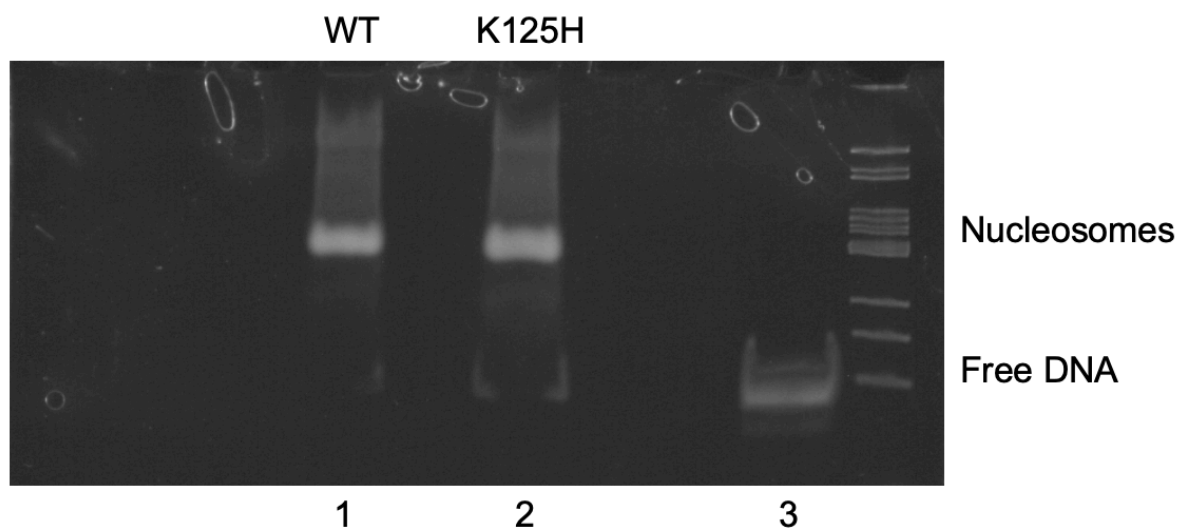


Figure 2: TBE gel demonstrating successful reconstitution of wild type and K125H mutated nucleosomes to be used in assays. Lane 3 contains free DNA and represents the control. Lanes 1 and 2 contain the reconstituted WT and K125H mutated nucleosomes, respectively. Reconstitution in lanes 1 and 2 is confirmed by the presence of the higher molecular weight nucleosome band as well as the absence of the low molecular weight free DNA band.

In this project, lysine residues 119 and 120 were the first targets for mutation in *C. elegans* due to their involvement with the RING1 E3 ligase in humans and proximity to BRCA1 targeted lysines. *RING1* encodes a human E3 ligase that ubiquitinates H2A at lysine residues 119

and 120, and there is known to be a *C. elegans* ortholog of this E3 ligase, called SPAT-3, which also ubiquitinates H2A.¹³ Lysine residues 119 and 120 are conserved in *C. elegans*, and because they were already known to be ubiquitinated in humans, these residues were the first targets for mutation. The “C Tail” mutant includes the mutations at lysine residues 119 and 120 as well as the 125 mutation previously tested, allowing for the creation of a mutant with all three lysines on the C-terminal tail mutated.

The “13/15” mutation was created by mutating lysine residues 13 and 15 on the N-terminal tail of *C. elegans*. In humans, lysine residues 13 and 15 are ubiquitinated by the RNF 168 E3 ligase, and although these lysine residues are conserved between humans and *C. elegans*, there is not an established ortholog for RNF 168 in worms.¹⁴ The “K0” mutation combines the mutations present in the “C Tail” and “13/15” mutants, creating a mutant in which all lysine residues known to be both ubiquitinated in humans and conserved in *C. elegans* have been mutated. This combination mutant provides insight on whether CeBCBD can hit different lysine sites depending on which lysines are present.

In order to determine if a lysine residue is at the position where H2A is ubiquitinated, nucleosome assays testing for ubiquitination activity and western blotting analysis were performed in the same manner as explained above, and the results were quantified using ImageJ. Figure 3 shows the results of the nucleosome ubiquitination assays with unmutated H2A (WT) as well as the three different sets of lysine mutations tested. For both the 10-minute and 30-minute time points of the assays, while there seems to be some decrease in ubiquitination activity, there is no statistically significant difference for any of the lysine mutations tested. Therefore, a definitive lysine ubiquitination target could not be identified. One hypothesis to explain these results is that *C. elegans* may be less specific in its ubiquitination target lysine, and when its

preferred lysine is mutated, it simply targets a different lysine. On the other hand, another hypothesis may be that *C. elegans* is specific to one of the lysines that is not conserved between humans and worms and therefore has not been tested. Both hypotheses would require further research and testing in order to characterize the substrate target of CeBCBD in *C. elegans*.

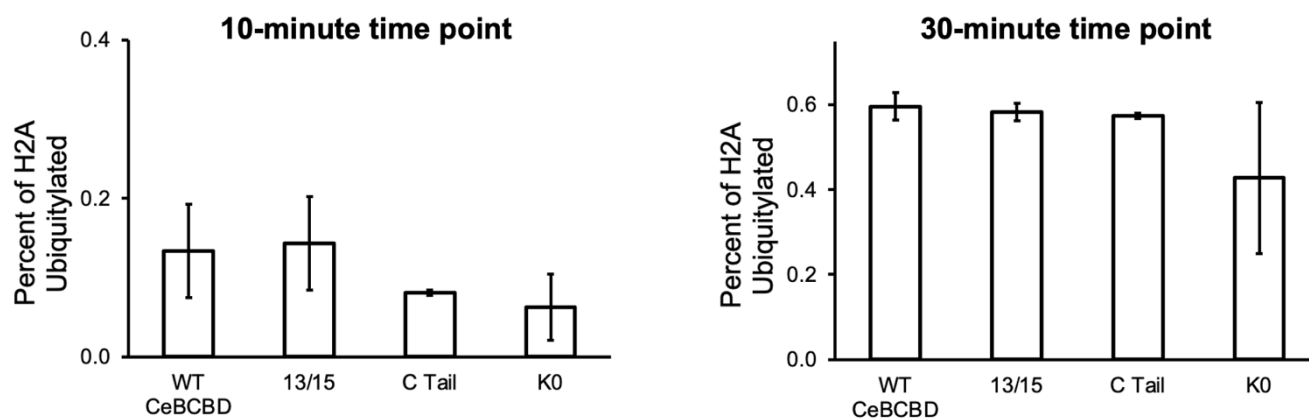


Figure 3: Graphs depicting percent of H2A ubiquitinated at two assay time points for wild type worm H2A and mutated worm H2A. Mutations include “13/15” (K13/15R), “C Tail” (K119/120R, K125H), and “K0” (K13/15R, K119/120R, K125H). While there seems to be some difference in ubiquitination activity for the C tail and K0 mutants at the 10-minute time point, it is not a statistically significant difference.

Because there was a possibility that mutagenesis causes loss of specificity for the worm enzyme, mass spectrometry was attempted to rule out this potential complication causing CeBCBD to be unspecific in the hopes of visualizing the precise location at which CeBCBD is ubiquitinating H2A in the absence of mutations. A large-scale ubiquitination assay was performed with the CeBCBD E3 ligase and unmutated H2A, and the proteins from the assay were gel purified and digested in preparation. During mass spectrometry, the proteins were cut with Arg-C, which cleaves after positively charged amino acids such as lysine and arginine. This allowed for the visualization of the specific site at which H2A is ubiquitinated in *C. elegans*.

CONCLUSIONS AND DISCUSSION

The ubiquitination of histone H2A by BRCA1/BARD1 is an important tumor-suppressive process that functions to silence genes and repair DNA damage caused by estrogen metabolites in humans.^{2,5} Here we report ubiquitination of histone H2A by BRC-1 and BRD-1 from *C. elegans*, orthologs for human BRCA1 and BARD1.⁶ Suspected lysine targets were mutated out of H2A and nucleosome assays were performed to identify the target attachment site on H2A. However, the mutations of conserved lysines in the N-terminal and C-terminal tails of *C. elegans* did not significantly reduce ubiquitination activity by the CeBCBD complex, suggesting ubiquitylation of H2A varies in target and/or mechanism in comparison to the human enzyme complex.

One explanation for this is that *C. elegans* may be less specific in its target lysine, which is surprising given that the human BCBD complex shows specificity for K125, K127, and K129.¹⁰ However, many of the interactions between E3 ligases and their substrates are low-affinity, and there have even been observations of E3 ligases transiently binding to substrates that do not possess a specific binding site for the enzyme, such as is the case with polyubiquitination by UBR5.^{15,16} Therefore, this could suggest that binding to the nucleosome during the ubiquitination process may be lower affinity in *C. elegans*, given that when lysines on the C-terminal tail are mutated, CeBCBD can target another lysine, presumably on the N-terminus. In humans, specific amino acid residues in the RING domain of BARD1 direct the BCBD complex to the target location at which the ubiquitin should be placed.³ But in *C. elegans*, the BRD-1 structure contains an extra, non-conserved loop in its RING domain with amino acid residues that perform the substrate interactions for directing ubiquitin placement.⁸ Differences in the binding residues could explain the differences in affinity and therefore specificity.

An alternative explanation for these results is that *C. elegans* is specific in its H2A ubiquitination site, but may target a lysine that is not conserved between humans and *C. elegans* and thus was not tested here. Future steps include specifically characterizing this interaction between BRCA1/BARD1 and the nucleosome in *C. elegans*. Further studies could potentially identify if *C. elegans* has any preferential lysine targets, or if it is truly nonspecific in its ubiquitination location. In addition, further studies could also include mutation of lysines that are not conserved between humans and *C. elegans*.

Although the H2A ubiquitination target in *C. elegans* appears to be different than human target, or even non-specific in its targeting, the opportunities stemming from the use of *C. elegans* as a model organism for the BRCA1/BARD1 system are still promising. Since BRCA1/BARD1 in worms has the ability to ubiquitinate the C-terminal tail, this opens up the possibility of using *C. elegans* as a model organism for studies that will inform on the function of BRCA1 in preventing breast and ovarian cancer. On the other hand, the fact that CeBCBD may ubiquitinate H2A on the N-terminus in addition to the C-terminus means that there could be a completely different mechanism determining ubiquitination target sites in *C. elegans*. In humans, the location of ubiquitination can play a large role in determining the outcome of gene regulation. For example, ubiquitination at H3 can lead to gene upregulation through histone acetylation, while H2A is typically associated with repression.⁹ In addition, ubiquitination of the conserved lysine residues at 13 and 15 on the N-terminus by a different E3 ligase is known to play a role in the repair of double-stranded DNA breaks in humans, but when mutated in *C. elegans*, CeBCBD ubiquitination activity is still present. This suggests the possibility that a non-conserved lysine on the N-terminus could perform this function in *C. elegans*, although it is unclear if these N-terminal lysines are also involved in gene repression in either humans or *C.*

elegans.¹⁴ In addition, although CeBCBD seems to target a different position on the nucleosome, it still results in gene repression of the conserved *cyp* gene family, which can be important in helping researchers determine why the targeting of different positions on the nucleosome leads to different outcomes.

It has been debated whether ubiquitination activity is necessary for tumor suppression by BRCA1 in humans. Characterizing the interaction between H2A and CeBCBD and understanding the specificity or non-specificity of ubiquitination in *C. elegans* will allow for more insights on the role of ubiquitination in gene regulation and tumor suppression. This will in turn allow for a greater understanding of BRC-1 and BRD-1 as they compare to human BRCA1 and BARD1, which will help further research mechanisms underlying the development of breast and ovarian cancer using *C. elegans* as a model organism.

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