Identifying the role of BRCA1 in transcriptional regulation using *Caenorhabditis elegans*

By

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This is the summary of my master's program:

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- Made quality American friends (Austin, Dani, Dalton, Davis, Evan, Emma, Katie, Olivia, MR, Patrick, Peyton, Russell, Vuong, and uncountable)
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- Pandemic brought even more fun
- Back home: grandfather died, family-owned restaurant for last 27 years ran out of business
- Mum diagnosed with stage 3C ovarian cancer (she will kill it I know)
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- Kidney stone (fun part: Doctor failed to prescribe my medication after surgery) and the medical bill was enormous
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Introduction

A variety of epidemiological factors are associated with an increased risk of developing breast and ovarian cancer. One of the prominent factors is an inherited mutation in the tumor suppressor gene *BRCA1* (Breast cancer susceptibility 1) which accounts for nearly 10-15% of all breast cancer cases. A person possessing a germline mutation in *BRCA1* has an 80-90% chance of developing breast cancer and a 40-65% life time risk of developing ovarian cancer [1]. Since its discovery, *BRCA1* has garnered attention in an effort to understand how its genetic mutation is linked with various forms of cancers, like breast and ovarian cancer, and fully understand its underlying molecular mechanism [2].

In humans, *BRCA1* encodes the tumor suppressor protein called BRCA1, with two functionally distinct domains: an N-terminal RING domain and a C-terminal BRCT domain. BRCA1 plays an important role in cancer prevention by maintaining genomic stability. In order to achieve– genomic integrity, BRCA1 partners with BARD1 (BRCA1 associated ring domain 1) via their respective RING domains [3] to regulate three key cellular activities: 1) DNA damage repair through homologous recombination, 2) cell-cycle checkpoint regulation, and 3) transcriptional regulation [4–7]. The loss of the BRCA1 function ramifies defectiveness in the DNA damage response pathway, decreases apoptosis [8], and impairs G2/M cell cycle-checkpoints [9], resulting in a higher chance of getting cancer. Therefore, elucidating the mechanisms of BRCA1 tumor prevention can increase our current understanding of breast and ovarian cancer derived from *BRCA1* mutations. One common approach to study genes like *BRCA1* and *BARD1* is through utilization of model organisms [4–7].

Caenorhabditis elegans (*C. elegans*) is a 1 mm long free-living nematode that emerged as a promising candidate for studying the genes, proteins and their associated pathways [10–12]. They are multicellular with differentiated cells, easy to cultivate, have a high rate of reproduction, and remain transparent in all stages of their development, which helps in tracking the changes occurring within them [11,12]. Furthermore, these hermaphrodite worms show nearly 40% genome similarity with humans [13]. In addition, worms can easily be genetically modified and have many genes including *BRCA1 and BARD1* homologs to humans [7]. For these reasons they have the potential to mimic genetic mutation in patients and its influence in the BRCA1/BARD1 mediated functions. Previous research suggests, like the human *BRCA1* gene, the *brc-1* gene of *C. elegans* is shown to be involved in DNA damage repair and cell cycle via its encoded brc-1 protein [14–16]; however, it is unknown if the third role of BRCA1 in transcription regulation is conserved by brc-1 (figure 1).

In humans, one of the key transcriptional regulatory functions of the BRCA1/BARD1 protein is to repress the expression of several estrogen metabolism genes in the *Cytochrome P450* (*CYP*) gene family. The superfamily of *CYP* genes produce monooxygenase enzymes involved in biosynthesis and metabolism of estrogen. The metabolism of estrogen occurs by two pathways involving different *CYP* genes (figure 3). One pathway involves the metabolism of estradiol via CYP1A2 and CYP3A1 to form 2-hydroxoyestradiol. Another pathway involves the metabolism of estradiol via CYP1B1 and CYP3A2 to form 4-hydroxyestradiol and this pathway is known to generate DNA-damaging free radicals. 2-hydroxoyestradiol and 4-hydroxyestradiol may be further metabolized to form semiquinones and quinones. Once the level of semiquinones and quinones is high, they bind to the purine bases of DNA to form either stable or unstable DNA adducts. If left

unrepaired, stable adducts in DNA may induce mutation whereas the unstable adducts results in loss of adenine or guanine from the DNA (depurination), leading to errors in repair and replication [17]. For example, several studies were conducted to access the risk of developing breast cancer by measuring estrogen-DNA adducts. These authors observed high levels of estrogen-DNA adducts in the urine and serum of high-risk individuals [18–20]. This suggests that along with reactive oxygen species (ROS), formation of the DNA damaging adducts is one of the causative factors in developing breast cancer.

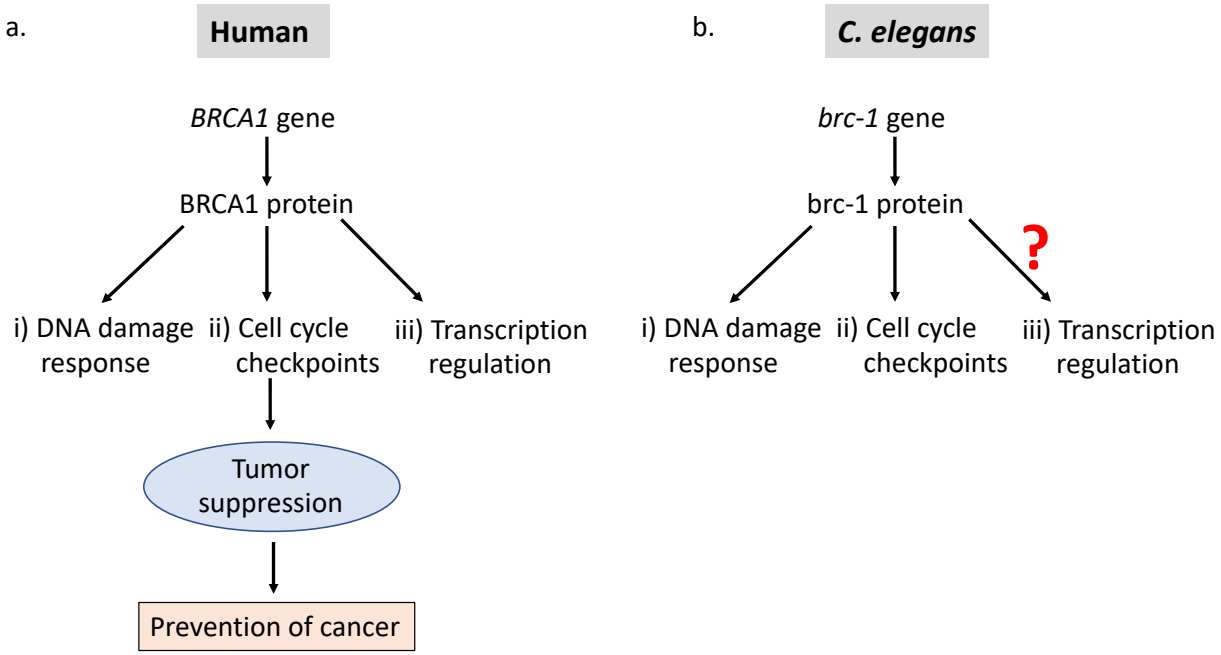

Figure 1: Schematic representation of known functions of the human *BRCA1 gene* and *C. elegans brc-1 gene*. BRCA1 of humans exhibits three crucial functions: DNA damage response, cell cycle checkpoints regulations, and, transcription regulation to prevent cancer (a). Similarly, brc-1 of *C. elegans* plays a role in DNA damage response and cell cycle checkpoint regulation, but the transcription regulation by brc-1 is yet to be reported (b).

The estrogen metabolism pathway needs to be tightly regulated as it is producing DNA damaging intermediates and ROS [21]. Savage et al. (2014) showed *BRCA1* haploinsufficiency in

breast cells leads to increased expression of *CYP3A4* and *CYP1A1*. Later this finding was further bolstered by Stewart et al. (2018), who demonstrated that BARD1 in the BRCA1/BARD1 complex is also necessary for transcriptional repression of *CYP3A* and *CYP1A1* genes in humans. *C. elegans* also possesses homologs of human estrogen metabolizing gene (*CYP3A4*) called the *cyp-13A* gene subfamily and consisting of the following genes: *cyp-13A1, cyp-13A2, cyp-13A3, cyp-13A4, cyp-13A5, cyp-13A6, cyp-13aA6, cyp13A7, cyp-13A8, cyp-13A9, cyp-13A10, cyp13A11*, and *cyp-13A12* (Figure 2). Studying the regulation of the *cyp-13A* gene subfamily by brc-1/brd-1 is necessary for establishing the conservation of brc-1 as a transcriptional regulator in *C. elegans*. This project hypothesizes that brc-1 represses the transcription of the *cyp-13A* gene subfamily in *C. elegans*, homologous to BRCA1/BARD1 induced transcriptional repression of *CYP3A4* gene in humans.

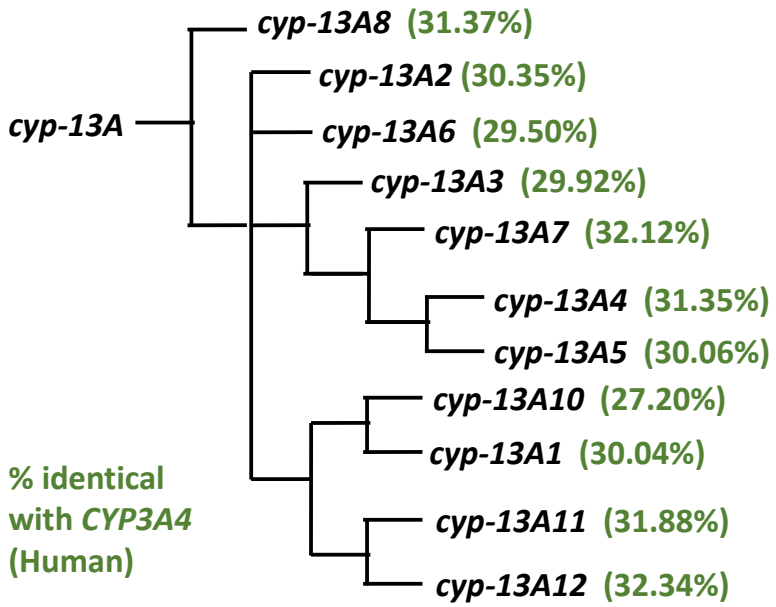

Figure 2: Phylogenetic tree of *C. elegans cyp-13A* gene subfamily showing the percentage identical of individual *cyp-13A* members with human estrogen metabolizing *CYP3A4.* *C. elegans cyp-13A* sequences were compared to sequences of human *CYP3A4* using NCBI protein blast. Phylogenetic tree of *cyp-13A* was generated using Łopieńska-Biernat et al (2020).

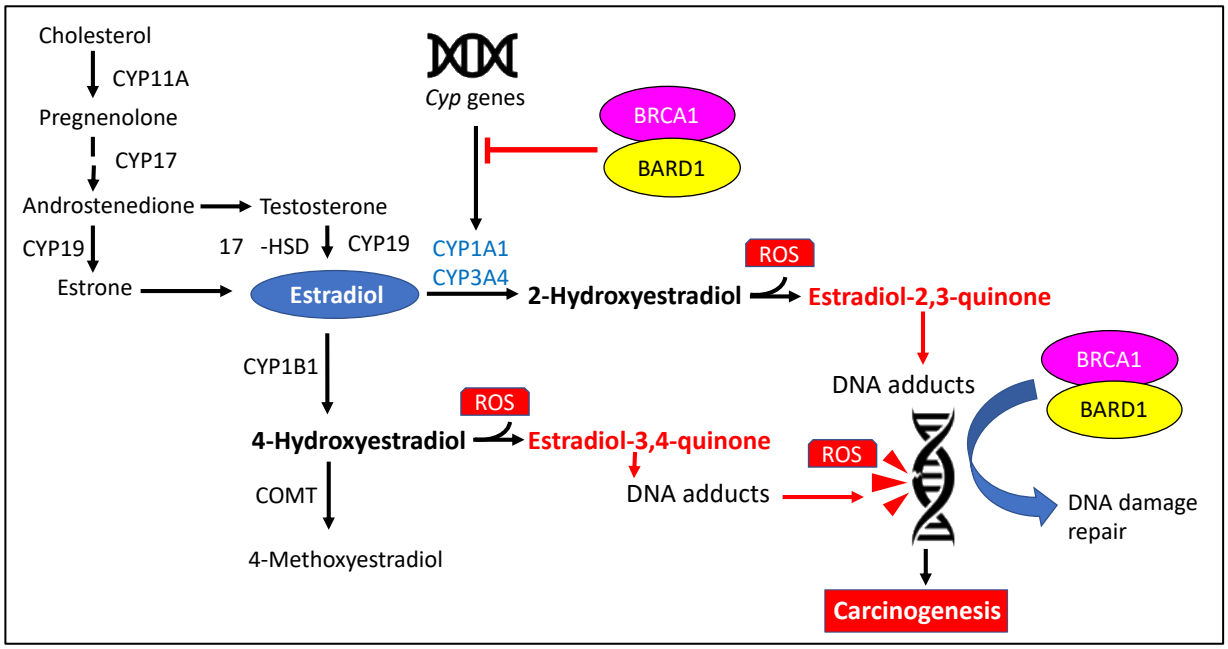

Figure 3: Schematic representation of estrogen metabolism pathway, associated with DNA damaging biproducts leading to carcinogenesis and their regulation by BRCA1/BARD1. Estrogen metabolism pathway is a complex process that begins with cholesterol and through the series of intermediate steps it forms androstenedione (androgens) which is a precursor for testosterone and estrone. Both testosterone and estrone are converted into estradiol catalyzed via 17β-HSD (17-hydroxysteroid dehydrogenase) and aromatase, respectively. Simply, estradiol (E_2) is hydroxylated to 2-hydroxyestradiol (2-OHE_2) by CYP1A1/CYP3A4, or in another pathway, estradiol is hydroxylated to 4-hydroxyestradiol (4-OHE_2) by CYP1B1. Both of these catechol estrogens, 2-OHE_2 and 4-OHE_2, undergo oxidation to form a quinone derivative catalyzed by oxidative enzymes [42]. During this redox-cycling of catechol estrogens, generation of reactive oxygen species mainly in the form of superoxide anions takes places, which causes the oxidative DNA damage [43]. Similarly, following the production of quinone derivatives, the breakdown biproduct of estradiol-2,3-quinone and estradiol-3,4-quinone binds directly to DNA producing adducts [44]. On the other hand, tumor suppressor proteins like BRCA1/BARD1 constantly check estrogen homeostasis to prevent cancer. BRCA1/BARD1 regulate the expression of estrogen metabolizing genes and they activate the homologous recombination mediated DNA damage repair pathway so as to eliminate the ROS induced DNA damage and repair DNA breaks caused by bulky adducts.

In summary, the overall goal of this research was to develop *C. elegans* as a model system to study brc-1. Our main objective was to determine if the transcriptional role of brc-1 is conserved in *C. elegans* like in humans. To accomplish this objective, we analyzed the differences in the expression pattern of *cyp-13A* gene subfamily to determine if there are any differences in wild-type (WT) and strains suffering mutation in *brc-1* gene or *brd-1* gene. We hypothesized that if brc-1 suppresses the *cyp* gene expression in worms, then deletion of *brc-1* will lead to increased expression of the *cyp-13A* subfamily.

Methods

C. elegans strains and culture

Wild-type (WT), N2 Bristol, worms were kindly gifted by Dr. Phil Hartman from Texas Christian University. The transgenic strains RB1209 [brc-1(ok1261)] and DW103 [brd-1(dw1)] were obtained from the *C. elegans* Genetics Center, University of Minnesota. CRISPR brc-1 knockout worms brc-1(xeo4)-were gifted by Lina Dahlberg from Western Washington University. Worms were cultured on nematode growth media (NGM) agar plates seeded with *Eshericia coli* strain OP50 and maintained at a room temperature of 21º C following the standard protocol [24]. L4 stage worms were used in all the experiments. Alkaline hypochlorite was used to obtain eggs from adult gravid worms. The eggs were allowed to hatch for 14-18 hours at 21º C in M9 buffer without *E. coli* with continuous shaking to obtain synchronous L1 worm populations. The pool of L1 larvae were grown in *E. coli* (OP50) seeded NGM plates for 36 hours until the worms reached L4 stage. L4 larvae were flash frozen using dry ice and stored at -80º C.

Isolation of RNA from *C. elegans*

RNA was isolated from a pool of 2000 flash frozen L4 worms for each biological replicate using the Maxwell1 16 LEV simplyRNA Tissue Kit connected with Maxwell 16 AS2000 instrument (both Promega Corporation, Madison, WI, USA). Total RNA was isolated according to the standard protocol provided in the kit with an additional sonication step after homogenization buffer was added. In brief, 200 μL of cold homogenization buffer was added to the sample and sonicated using VCX130 with CL-18 tip at 85% amplification set to six times pulse with three seconds on and three second resting followed by the addition of 200 μL of lysis buffer. A total of 400 μL of homogenized sample was used to extract RNA following the standard protocol provided by Promega. The quantification of RNA was performed using the NanoDrop ND-1000 Microvolume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity of RNA was determined by the absorbance ratio of 260/280 and 260/230. All samples had 260/280 and 260/230 ratios ≥ 2. The RNA samples were stored at -80°C until their conversion to cDNA.

cDNA synthesis

Quantabio qscript cDNA Superix was used to convert RNA to cDNA. To make a 50 ng/μL of working solution, the RNA samples were diluted using nuclease-free water. Then, the diluted RNA (12 μL) was added to 3 μL of cDNA super mix in small PCR tubes and reaction mixture were placed into a thermocycler (Bio-Rad). The thermocycler was programmed for in the following setup: 25°C (5 min), 42°C (30 min), 85°C (5 minutes) followed by an infinite hold at 4°C. After the reaction was complete, 15 μL of reverse transcription buffer. The synthesized cDNA was stored at -20°C.

Gene expression analysis

Out of twelve *cyp-13A* subfamilies, nine *cyp-13A* genes, including *cyp-13A2, cyp-13A4, cyp-13A5, cyp-13A6, cyp-13A7, cyp-13A8, cyp-13A10, cyp-13A11,* and *cyp-13A12* were selected for expression analysis. The expression level of two genes, *cyp-13A1* and *cyp-13A3,* were below quantification level so they were excluded from the study and *cyp-13A9* was also excluded from our study for being a pseudogene (as per NCBI database, GeneID: 13186601). We selected *tba-1* as a reference gene based on the findings by Zhang et al. (2012) where the authors found *tba-1* to be the most reliable and consistent in expression out of 13 common reference genes. Previous research by Hoogewijs et al. (2008) also suggested the use of *tba-1* as a suitable reference gene. The subfamily of *cyp-13A* genes shows high sequence similarity within themselves, we designed primers that target unique exon-exon junctions of each transcript. This also limits the binding of the primer to the genomic DNA. The information about the sequence of primer, annealing temperatures, reaction efficiencies are shown in table 1. To quantify the expression of *cyp-13A* subfamily genes, four biological replicates with three technical replicates of each worm strain were used. A CFX Manager Software 3.0 coupled with CFX-Connect real-time PCR detection system from Bio-Rad was used to perform qPCR reaction. Each 10 µL reaction mixture contained 0.4 µL of cDNA along with 5 µL of SYBR-GREEN (Quantabio) and 3 µL of primer. 96-well plates form Bio-Rad was used to carry all the reactions. The reactions were run in a thermocycler with a following program set up: an activation step (95 ◦C for 30 s) followed by 40 cycles of denaturing (95 ◦C for 10 s), and annealing (15 sec at a temperature specific to the primer as shown in table 1). A melting curve was generated between 65 ◦C and 95 ◦C in each plate to confirm primer specificity. Series of two-fold diluted standard cDNA samples were used to generate a standard curve, which ascertain the efficiency of the reaction and starting quantity

of target genes. The efficiency of all the primers ranged from 78.8 - 97% as shown in table 1. Standard deviation of standard deviation of obtained Ct values (SDofSD) for each biological and technical replicate were calculated and Ct values deviating by ± 2 SDofSD were considered outliers and removed.

Statistical analysis

Data analysis was done using GraphPad Prism software version 9.0.1. For analyzing the gene expression dataset of WT, brc-1 (ok1261), and brd-1 (dw1), one-way ANOVA (analysis of variance) followed by Dunnett's test was conducted. Starting quality (SQ) values of each gene in brc-1(ok1261) and brd-1 (dw1) strains were normalized to mean SQ values of WT. Similarly, for the expression dataset comparing WT and brc-1(xeo4), Student's t-test was performed. SQ values of each gene in brc-1 (xeo4) were normalized to mean SQ of WT. For all the tests, statistical significance was set at $\alpha = 0.05$.

Gene	Primer type	Primer Sequence	Annealing Temp	Reaction Efficiency
cyp-13A2	Forward	GATTTGCGATGGGTCAGACG	60°C	86.9%
	Reverse	TTGGTGGGAAGACTTGGCAG		
cyp-13A4	Forward	GGTCAAACCGAATCGCTGATG	60°C	89.5%
	Reverse	TTGGCGCATCAAAGTTCCTG		
cyp-13A5	Forward	GCATCCATCGTTCACAACCG	60°C	79.8%
	Reverse	TGCATCCTCTCCCCAAACTT		

cyp-13A6	Forward	CGTGATCTCCAGAGTTGCCA	57°C	88.6%
	Reverse	AACATCCAGATCTGCCAGCG		
cyp-13A7	Forward	TGTTAACGAGTTCAAGCCGGA	60°C	89.8%
	Reverse	ATTCTTGGTCCCATGCCGAA		
cyp-13A8	Forward	CGTGATCTCCAGAGTTGCCA	60°C	101.3%
	Reverse	AACATCCAGATCTGCCAGCG		
cyp-13A10	Forward	GTGGAAGACAGTGCGATGGA	62°C	92.8%
	Reverse	ACATTTGCGAGTCGGTCTGA		
cyp-13A11	Forward	ATACAGCAGGCGGGAAAGC	62°C	78.6%
	Reverse	CGTCTCGAGCACAAGGTAGT		
cyp-13A12	Forward	TGGGGAGAGGATGCTGAAGA	60°C	81.0%
	Reverse	CACTGTCGAGGTCCCAATCC		
tba-1	Forward	TCAACACTGCCATCGCCGCC	60°C	86.6%
	Reverse	TCCAAGCGAGACCAGGCTTCAG		

Table 1: Primer sequences (forward and reverse primer) used for RT-qPCR with their annealing temperatures and reaction efficiencies.

Results

Based on our proposed hypothesis that brc-1 and brd-1 in worms plays a similar role in repressing *cyp-13A* genes as in humans, we would expect to see higher expression of the *cyp-13A* gene subfamily in the absence of brc-1 and brd-1. The predicted protein constructs produced in the worm strains used for the experiments are shown in figure 4.

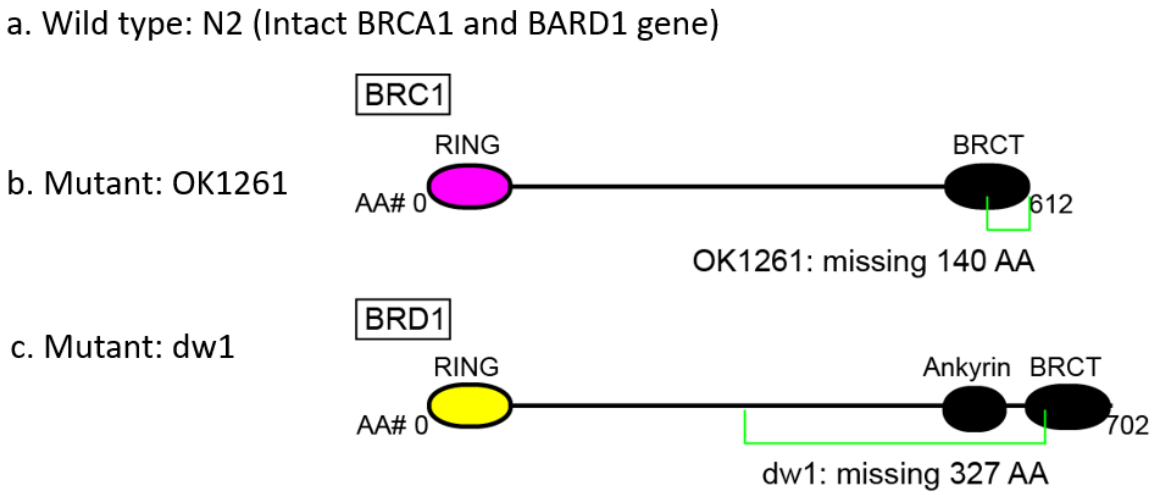

Figure 4: Cartoon depiction of conserved domains of the brc-1 and brd-1 and the predicted deletions produced in mutant worm strains: brc-1 (ok1261) and brd-1 (dw1). From the exon deletions present in mutant strains it is predicted that the proteins produced will contain partial deletion of brc-1 or brd-1 (shown with a green bracket in b or c, respectively). The pink and yellow ovals represent RING domains of brc-1 and brd-1 protein respectively. This domain is known to be necessary and sufficient for the enzymatic reaction required for gene repression. Black ovals represent the conserved ankyrin and BRCT domains with unknown relevance to gene repression functions.

The data from RT-qPCR revealed that only *cyp-13A5* was upregulated in brc-1 (ok1261), while the remaining genes (*cyp-13A2, cyp-13A4, cyp-13A6, cyp-13A7, cyp-13A10, cyp-13A11,* and *cyp-13A12*) were not affected by the loss of the exonic region of brc-1 (checkered bars figure 5). The brd-1 (dw1) strain showed significant upregulation of six *cyp-13A* genes, including *cyp-*

13A4, cyp-13A5, cyp-A6, cyp-13A8, cyp-13A11, and *cyp-13A12* (black bars figure 5). Out of six *cyp-13A* genes that were significantly upregulated in brd-1 (dw1) worms, *cyp-13A4* showed the largest change in expression of nearly 5.5-fold, while the other genes varied from 1.5 to 2.5-fold increases in expression. The upregulation of *cyp-13A* gene expression in brd-1 (dw1) worms demonstrated that brd-1 represses the expression of most of the cyp-13A subfamily of genes as shown in the figure 5.

In humans, BRCA1 and BARD1 work together to repress *CYP* genes, so it was surprising to not see more upregulated *cyp-13A* gene expression in brc-1 (ok1261) worms. This might suggest that brc-1 (ok1261) might be encoding for the truncated but partially functional brc-1. If this is the case, complete deletion of *brc-1* would result in increased expression of *cyp-13A* subfamilies. Therefore, we used CRISPR knockout worms, brc-1(xeo4), with complete deletion of *brc-1*. We accessed the level of *cyp-13A* subfamily expression in the brc-1 (xeo4) strain using RT-qPCR and compared to the control WT worms. Indeed, we observed four genes–*cyp-13A2, cyp-13A5, cyp-13A10,* and *cyp13A11*– are upregulated by 1.5 to 2.5-fold when brc-1 is knocked out (figure 6). This data revealed that brc-1 represses the *cyp-13A* subfamily of genes in worms. Together, our data shows how brc-1 and brd-1 significantly repressing the expression of *cyp-13A* subfamily suggesting both brc-1 and brd-1 to be the key players in regulating *cyp-13A* expression. Furthermore, this demonstrates the conserved role of brc-1 and brd-1 in repressing the expression of *cyp13-A* subfamily of genes in worms.

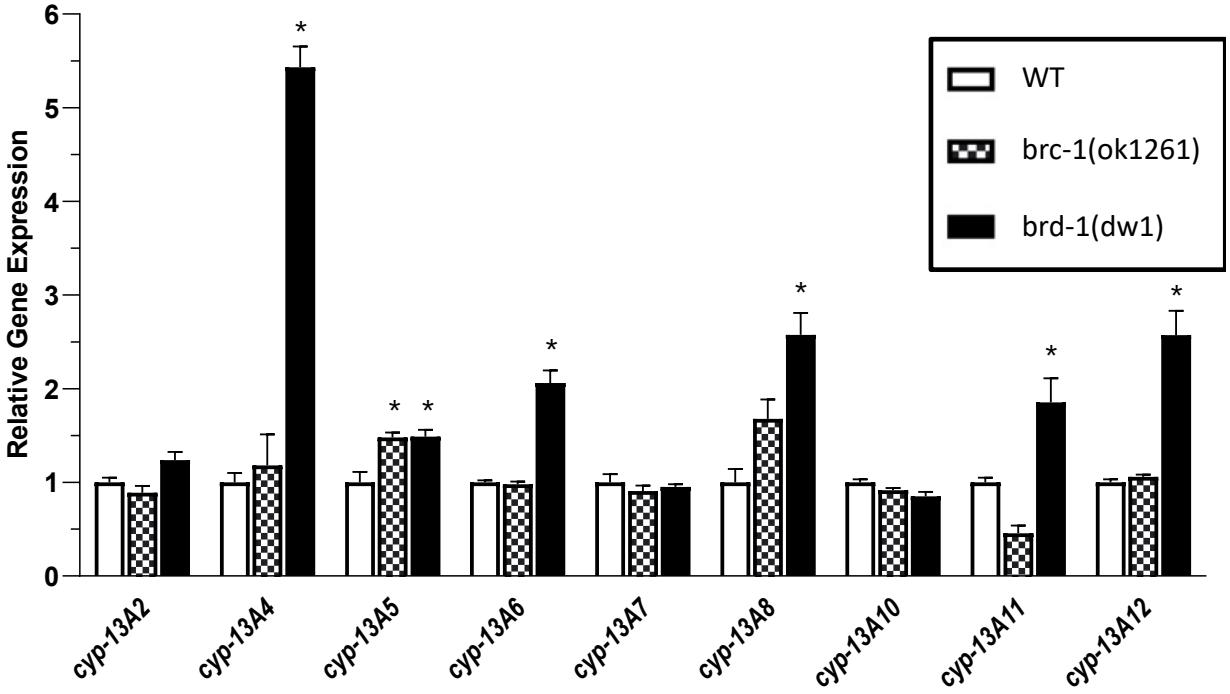

Figure 5: brd-1 represses *cyp-13A* genes in L4 worms. The expression of *cyp-13A2, cyp-13A4, cyp-13A5, cyp-13A6, cyp-13A7, cyp-13A8, cyp-13A10, cyp-13A11* and *cyp-13A12* in WT (control) and experimental brc-1 (ok1262) and brd-1 (dw1) as measured with RT-qPCR. White bar, checkered bar, and black bar represent relative *cyp-13A* gene expression of WT, brc-1 (ok1261), and brd-1 (dw1) strain respectively. The data are presented as the average fold change in gene expression normalized to reference gene *tba-1* and presented relative to WT. Each error bar represents ± SEM (standard error of means) from three or four biological replicates. * denotes statistically significant deviation from WT as determined by ANOVA followed by the Dunnett's multiple comparison test. p-values < 0.05 were considered as statistically significant.

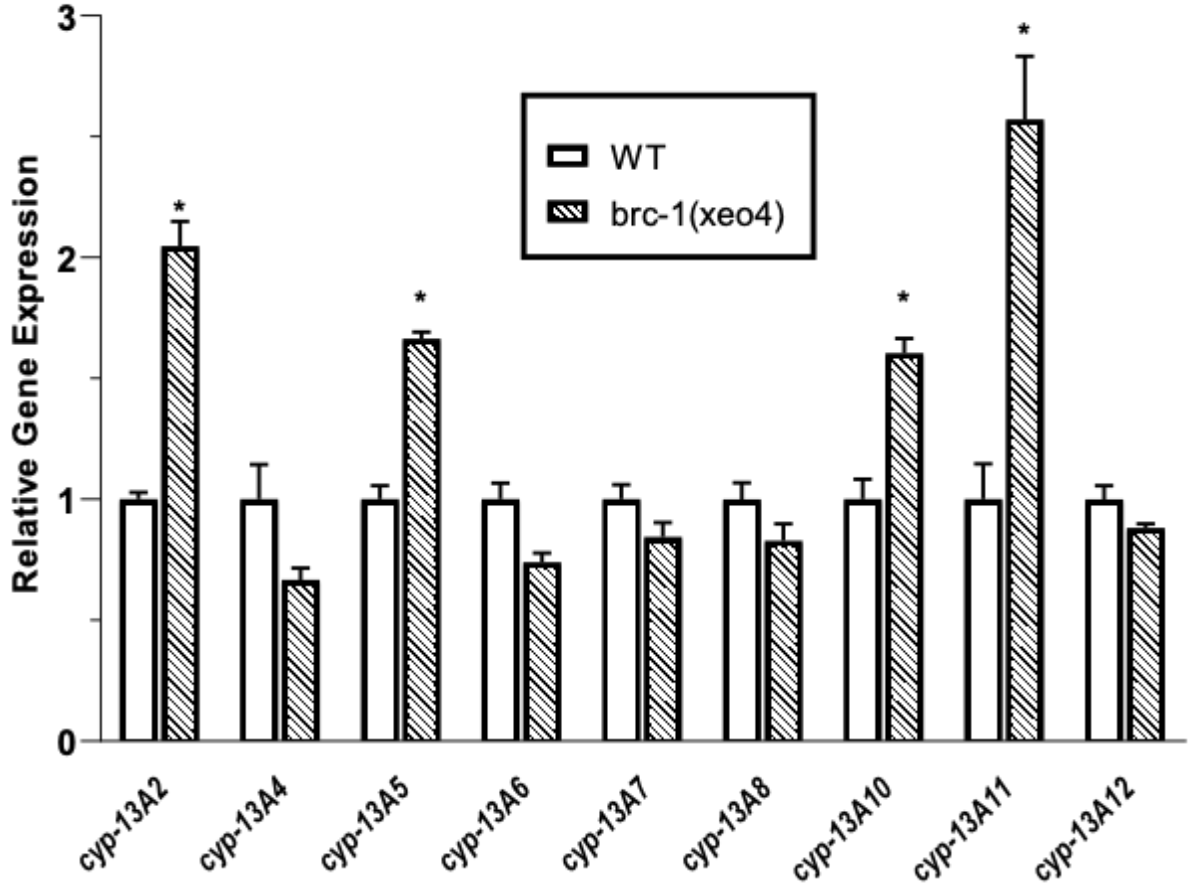

Figure 6: brc-1 represses *cyp-13A* genes in L4 worms. The expression of *cyp-13A2, cyp-13A4, cyp-13A5, cyp-13A6, cyp-13A7, cyp-13A8, cyp-13A10, cyp-13A11* and *cyp-13A12* in WT (control) and brc-1 (xeo4) (experimental) were measured using RT-qPCR. The white bar and crosses bar represent the relative cyp-13A gene expression in WT and brc-1 (xeo4), respectively. The data represent the fold change in gene expression normalized to the reference gene *tba-1*. Each bar represents ± SEM from three-four biological replicates and three technical replicates. Student's t-test was used to determine the differential gene expression and p-value < 0.05 was considered statistically significant.

Discussion

Though *C. elegans* is one of the most widely used model systems to study the biological functions of various molecules and related pathways we are yet to fully understand the molecular complexity within these worms. Our study employs *C. elegans* as a system to study brc-1/brd-1 and its functions. The DNA damage repair response, cell-cycle check point, and transcriptional regulation function of BRCA1/BARD1 ensures genomic stability in humans [4–7]. Similarly, previous research on *C. elegans* established the role of brc-1/brd-1in DNA damage repair and cell-cycle checkpoint regulation [14–16]. Here we show that both brc-1 and brd-1 regulate the expression of *cyp-13A* genes in *C. elegans.*

Our gene expression analysis demonstrated that brc-1/brd-1 of worms is necessary for repressing the expression of several *cyp-13A* gene subfamily members in worms. In humans, BRCA1/BARD1 transcriptionally regulates the expression of estrogen metabolizing genes (cytochrome P50 genes) via the mechanism of histone ubiquitination [23,27]. BRCA1/BARD1 mediated regulation prevents the accumulation of DNA damage caused by free radicals and bulky DNA adducts generated during the estrogen metabolism [28]. This transcriptional regulation function of BRCA1 is attributed to its N-terminal RING domain that interacts with the RING-domain of BARD1 to provide E3 ligase activity [29,30]. E3 ligases catalyze the attachment of ubiquitin to various substrates, including RNA polymerase II, p53, CtIP, progesterone receptor, and histones, which are crucial for genomic stability and anti-tumorigenic roles [29]. On the C-terminal side of BRCA1, the BRCT domain of BRCA1 helps in binding the phosphorylated proteins essential for restoring the stalled replication forks through HR (homologous recombination) mediated DNA damage repair [31](figure 3).

We found that knocking out brc-1 results in the increased expression of several *cyp-13A* subfamily members suggesting brc-1 represses the expression of *cyp-13A* genes. Similar results were observed in humans [28], where loss of BRCA1 leads to the upregulation of *CYP1A1* and *CYP3A4* which are the major regulators of estrogen metabolism in estrogen responsive tissues. Our *cyp-13A* expression data using brd-1 (dw1) strain also demonstrated the role of brd-1 in repressing the majority of *cyp-13A* genes. Similar findings were suggested by Stewart et al. (2018). The authors measured the expression of *CYP1A1* and *CYP3A4* in MCF10A breast epithelial cells with a heterozygous BARD1 mutation and showed substantial increased expression of *CYP1A1* and *CYP3A4*. Further, they were able to restore the *CYP3A4* and *CYP1A1* to wild-type levels upon transfecting BARD1 +/- cells with H2A-Ub fusion. This finding provided strong evidence in relating BARD1's H2A ubiquitination activity with the changes in the expression of estrogen-metabolizing genes. The genetic mutant brd-1 (dw1) encodes for brd-1 missing 327 amino acids (including ankyrin domain and some parts of BRCT domain); however, Boulton (2006) noted that brd-1 (dw1) worm extracts lack detectable levels of brd-1 and brc-1 protein in the western blots, indicating the mutated brd-1 is unstable and degraded which also causes degradation of brc-1. In humans, it has been established that BRCA1 and BARD1 are needed to stabilize each other [33,34]. This might be the reason why we observed the upregulation of *cyp-13A* genes in brd-1 (dw1) strain even though the RING domain is predicted to remain intact.

In the brc-1 (ok1251) genetic mutant, we only observed the upregulation of *cyp-13A5*. Loss of 140 amino acids from BRCT region might not have a significant effect in its transcriptional regulatory function as it has only regulates the HR mediated DNA damage repair, whereas the

same encoded brc-1 protein also has an intact RING domain. This might be sufficient enough to repress the expression of *cyp-13A* subfamily through ubiquitination. This would explain why we did not observe the upregulation of more *cyp-13A* genes in the brc-1 (ok1261) strain.

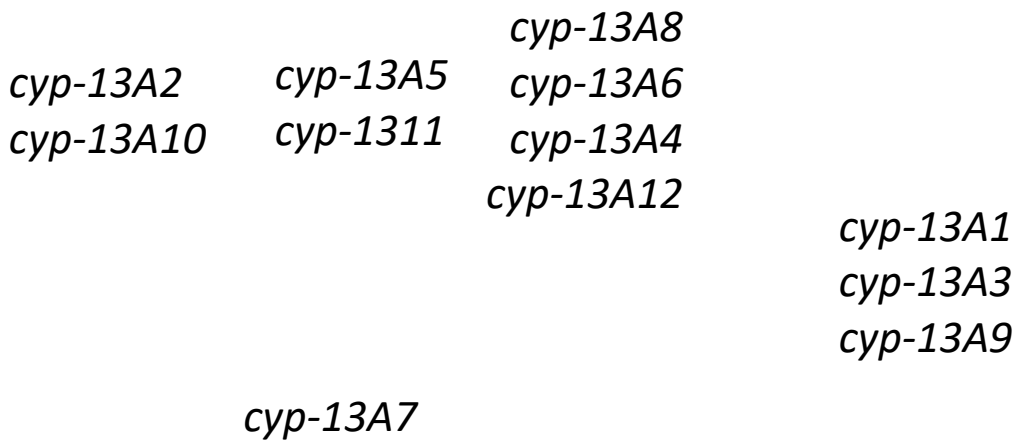

Figure 7: Venn diagram showing the upregulated *cyp-13A* genes in worm strains. Pink circle indicates *cyp-13A* genes that are significantly upregulated only in brc-1 (xeo4) strain and yellow circle lists *cyp-13A* genes that are significantly upregulated only in brd-1 (dw1) strain. Orange indicates genes that are upregulated in both strains and *cyp-13A7* is the only gene that was not regulated in either strains. Red circle indicates *cyp-13A* genes that are not included in our current study.

As shown in the figure 7, *cyp-13A5* and *cyp-13A11* were the common genes that were upregulated in both the brc-1 (xeo4) and brd-1 (dw1) strain and *cyp-13A7* was not upregulated in any strain. We also observed variation in the regulation by brc-1/brd-1 of expression within *cyp-13A* gene subfamilies which might suggest that not all genes within *cyp-13A* gene families are regulated equally by brc-1/brd-1. It is unknown why brc-1 and brd-1 is repressing *cyp-13A* in

worms, when worms do not use estrogen as a hormone. This also seems to be case in the few studies that have explored the possible role of *cyp-13A* subfamily of genes in *C. elegans*. Chakrapani et al. (2008) showed increased expression of *cyp-13A7* in worms treated with antibiotics (Rifampicin), indicating its possible role in drug metabolism. Its human homolog also plays a role in metabolizing antibiotics [36]. Research by Keller et al. (2014) demonstrated that *cyp-13A12* is an essential component for lipid biosynthesis that helps in metabolism of eicosapentaenoic acids (EPA) and arachidonic acids. Similarly, exposing worms to the heavy metal cadmium leads to the overexpression of *cyp-13A4, cyp-13A5, cyp-13A6,* and *cyp-13A7* [38]. Together with our results there is an indication that *cyp-13A* in worms functions in a variety of pathways and brc-1/brd-1 might influence their ability to metabolize drugs, lipids, and xenobiotics substances.

Overall, through our studies using *C. elegans* as a model system we were able to explore how brc-1/brd-1 influences the expression of *cyp-13A* subfamily of genes and show how the transcriptional regulation function of brc-1 is conserved in worms. This puts us one step closer to our goal of using worms as a model to study brc-1 functions and for testing human variants in BRCA1. Not only is brc-1/brd-1 in worms similar in sequence to human BRCA1/BARD1, but they also share the functions known to lead tumor suppression in humans. It enables researchers to look at the mechanism of how certain unknown genetic mutations in brc-1/brd-1 affects their normal functions in worms and these finding are highly applicable for humans. For example, in the past two decades the research in cancer biology using *C. elegans* has contributed to the discovery of RNAi, miRNA, and apoptosis [35]; all of which have since been used to make medical discoveries applicable to humans. Specifically, in the field of BRCA1/BARD1 recent research in

C. elegans has found new roles for these proteins in mediating meiotic recombination and axon regeneration [12, 36], proving work in *C. elegans* is applicable for discovering more about BRCA1 functions. As a new research topic in the field of *C. elegans* biology we are left with a plethora of exciting questions that can be addressed in the future work.

References

1. Fackenthal JD, Olopade OI. Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations. *Nat Rev Cancer*. 2007;7(12):937-948. doi:10.1038/nrc2054
2. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science (80-)*. 1994;266(5182):66-71. doi:10.1126/science.7545954
3. Wu LC, Wang ZW, Tsan JT, et al. Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nat Genet*. 1996;14(4):430-440. doi:10.1038/ng1296-430
4. Isono M, Niimi A, Oike T, et al. BRCA1 Directs the Repair Pathway to Homologous Recombination by Promoting 53BP1 Dephosphorylation. *Cell Rep*. 2017;18(2):520-532. doi:10.1016/j.celrep.2016.12.042
5. Wang L, Di LJ. BRCA1 and estrogen/estrogen receptor in breast cancer: Where they interact? *Int J Biol Sci*. 2014;10(5):563-573. doi:10.7150/ijbs.8579
6. Mullan PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. *Oncogene*. 2006;25(43):5854-5863. doi:10.1038/sj.onc.1209872
7. Polanowska J, Martin JS, Garcia-Muse T, Petalcorin MIR, Boulton SJ. A conserved pathway to activate BRCA1-dependent ubiquitylation at DNA damage sites. *EMBO J*. 2006;25(10):2178-2188. doi:10.1038/sj.emboj.7601102
8. Thangaraju M, Kaufmann SH, Couch FJ. BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. *J Biol Chem*. 2000;275(43):33487-33496. doi:10.1074/jbc.M005824200
9. Deng CX. BRCA1: Cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res*. 2006;34(5):1416-1426. doi:10.1093/nar/gkl010
10. Kaletta T, Hengartner MO. Finding function in novel targets: C. elegans as a model organism. *Nat Rev Drug Discov*. 2006;5(5):387-399. doi:10.1038/nrd2031
11. Zhang F, Berg M, Dierking K, et al. Caenorhabditis elegans as a model for microbiome research. *Front Microbiol*. 2017;8(MAR):1-11. doi:10.3389/fmicb.2017.00485
12. Meneely PM, Dahlberg CL, Rose JK. Working with Worms: Caenorhabditis elegans as a Model Organism. *Curr Protoc Essent Lab Tech*. 2019;19(1):1-35. doi:10.1002/cpet.35
13. Lai CH, Chou CY, Ch'ang LY, Liu CS, Lin WC. Identification of novel human genes

evolutionarily conserved in Caenorhabditis elegans by comparative proteomics. *Genome Res*. 2000;10(5):703-713. doi:10.1101/gr.10.5.703

14. Boulton SJ, Martin JS, Polanowska J, Hill DE, Gartner A, Vidal M. BRCA1/BARD1 Orthologs Required for DNA Repair in Caenorhabditis elegans. *Curr Biol*. 2004;14(1):33-39. doi:10.1016/j.cub.2003.11.029
15. Stergiou L, Hengartner MO. Death and more: DNA damage response pathways in the nematode C. elegans. *Cell Death Differ*. 2004;11(1):21-28. doi:10.1038/sj.cdd.4401340
16. Li Q, Saito TT, Martinez-Garcia M, et al. *The Tumor Suppressor BRCA1-BARD1 Complex Localizes to the Synaptonemal Complex and Regulates Recombination under Meiotic Dysfunction in Caenorhabditis Elegans*. Vol 14.; 2018. doi:10.1371/journal.pgen.1007701
17. Cavalieri EL, Rogan EG. Depurinating estrogen-DNA adducts, generators of cancer initiation: their minimization leads to cancer prevention. *Clin Transl Med*. 2016;5(1). doi:10.1186/s40169-016-0088-3
18. Tang et al. 2005. Cancer model in C.elegans. NIH Public Access. *Bone*. 2008;23(1):1-7. doi:10.1016/j.jsbmb.2012.02.002.Evaluation
19. Salama SA, Kamel M, Awad M, et al. Catecholestrogens induce oxidative stress and malignant transformation in human endometrial glandular cells: Protective effect of catechol-O- methyltransferase. *Int J Cancer*. 2008;123(6):1246-1254. doi:10.1002/ijc.23653
20. Yager JD. Catechol-O-methyltransferase: Characteristics, polymorphisms and role in breast cancer. *Drug Discov Today Dis Mech*. 2012;9(1-2):1-10. doi:10.1016/j.ddmec.2012.10.002
21. Blackburn HL, Ellsworth DL, Shriver CD, Ellsworth RE. Role of cytochrome P450 genes in breast cancer etiology and treatment: effects on estrogen biosynthesis, metabolism, and response to endocrine therapy. *Cancer Causes Control*. 2015;26(3):319-332. doi:10.1007/s10552-014-0519-7
22. Savage KI, Matchett KB, Barros EM, et al. Brca1 deficiency exacerbates estrogen-induced dna damage and genomic instability. *Cancer Res*. 2014;74(10):2773-2784. doi:10.1158/0008-5472.CAN-13-2611
23. Stewart MD, Zelin E, Dhall A, et al. BARD1 is necessary for ubiquitylation of

nucleosomal histone H2A and for transcriptional regulation of estrogen metabolism genes. *Proc Natl Acad Sci U S A*. 2018;115(6):1316-1321. doi:10.1073/pnas.1715467115

24. Brenner S. Nature's gift to science (Nobel lecture). *ChemBioChem*. 2003;4(8):683-687. doi:10.1002/cbic.200300625
25. Zhang Y, Chen D, Smith MA, Zhang B, Pan X. Selection of reliable reference genes in caenorhabditis elegans for analysis of nanotoxicity. *PLoS One*. 2012;7(3). doi:10.1371/journal.pone.0031849
26. Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. *BMC Mol Biol*. 2008;9:1-8. doi:10.1186/1471-2199-9-9
27. Thakar A, Parvin JD, Zlatanova J. Brca1/bard1 e3 ubiquitin ligase can modify histones h2a and h2b in the nucleosome particle. *J Biomol Struct Dyn*. 2010;27(4):399-405. doi:10.1080/07391102.2010.10507326
28. Savage KI, Matchett KB, Barros EM, et al. Brca1 deficiency exacerbates estrogen-induced dna damage and genomic instability. *Cancer Res*. 2014;74(10):2773-2784. doi:10.1158/0008-5472.CAN-13-2611
29. Chang S, Sharan SK. The role of epigenetic transcriptional regulation in BRCA1-mediated tumor suppression. *Transcription*. 2013;4(1):24-28. doi:10.4161/trns.22600
30. Mallery DL, Vandenberg CJ, Hiom K. Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J*. 2002;21(24):6755-6762. doi:10.1093/emboj/cdf691
31. Pietrasik S, Zajac G, Morawiec J, Soszynski M, Fila M, Blasiak J. Interplay between BRCA1 and GADD45A and its potential for nucleotide excision repair in breast cancer pathogenesis. *Int J Mol Sci*. 2020;21(3):1-22. doi:10.3390/ijms21030870
32. Boulton SJ. BRCA1-mediated ubiquitylation. *Cell Cycle*. 2006;5(14):1481-1486. doi:10.4161/cc.5.14.2930
33. Meza JE, Brzovic PS, King MC, Klevit RE. Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1. *J Biol Chem*. 1999;274(9):5659-5665. doi:10.1074/jbc.274.9.5659
34. Irminger-Finger I, Ratajska M, Pilyugin M. New concepts on BARD1: Regulator of BRCA pathways and beyond. *Int J Biochem Cell Biol*. 2016;72:1-17.

doi:10.1016/j.biocel.2015.12.008

35. Chakrapani BPS, Kumar S, Subramaniam JR. Development and evaluation of an in vivo assay in Caenorhabditis elegans for screening of compounds for their effect on cytochrome P450 expression. *J Biosci*. 2008;33(2):269-277. doi:10.1007/s12038-008-0044-5
36. Andrew Williams J, Ring BJ, Cantrell VE, et al. Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos*. 2002;30(8):883-891. doi:10.1124/dmd.30.8.883
37. Keller J, Ellieva A, Ma DK, et al. CYP-13A12 of the nematode Caenorhabditis elegans is a PUFA-epoxygenase involved in behavioural response to reoxygenation. *Biochem J*. 2014;464:61-71. doi:10.1042/BJ20140848
38. Cui Y, McBride SJ, Boyd WA, Alper S, Freedman JH. Toxicogenomic analysis of caenorhabditis elegans reveals novel genes and pathways involved in the resistance to cadmium toxicity. *Genome Biol*. 2007;8(6):1-15. doi:10.1186/gb-2007-8-6-r122
39. Tang et al. 2005. Cancer model in C. elegans. NIH Public Access. *Bone*. 2008;23(1):1-7. doi:10.1002/dvdy.22247.Cancer
40. Release TE, Attribution CC. BRCA1 – BARD1 regulates axon regeneration in concert with the Gqα – DAG signaling network concert with the Gq D – DAG signaling network. 2021.
41. Łopieńska-Biernat E, Stryiński R, Paukszto Ł, Jastrzębski JP. Correlation of NHR-48 transcriptional modulator expression with selected CYP genes' expression during thiabendazole treatment of Anisakis simplex (s.l.)?—An in vitro study. *Pathogens*. 2020;9(12):1-13. doi:10.3390/pathogens9121030
42. S M Gulliver L. Estradiol Synthesis and Metabolism and Risk of Ovarian Cancer in Older Women Taking Prescribed or Plant-derived Estrogen Supplementation. *J Steroids Horm Sci*. 2013;s12(01). doi:10.4172/2157-7536.s12-003
43. Cavalieri EL, Stack DE, Devanesan PD, et al. Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc Natl Acad Sci U S A*. 1997;94(20):10937-10942. doi:10.1073/pnas.94.20.10937
44. Convert O, Van Aerden C, Debrauwer L, et al. Reactions of estradiol-2,3-quinone with deoxyribonucleosides: Possible insights in the reactivity of estrogen quinones with DNA.

Chem Res Toxicol. 2002;15(5):754-764. doi:10.1021/tx015561y

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Identifying the role of BRCA1 in transcriptional regulation using *Caenorhabditis elegans*

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Breast cancer susceptibility gene 1 encoded BRCA1 and its heterodimeric partner BARD1 play an essential role in genomic stability by regulating DNA damage repair, cell-cycle checkpoints, and transcription regulation. Germline mutations in either of these genes exposes individuals to a higher risk of developing breast and ovarian cancer. The *Caenorhabditis elegans* orthologs, brc-1 and brd-1, also regulate DNA damage repair and cell cycle checkpoints; however, their role in regulating gene transcription is still unknown. We hypothesized that, similar to humans, brc-1 of *C. elegans* regulates expression of the *cyp-13A* gene subfamily, which are the homologs of the human estrogen metabolizing gene, *CYP3A4*. Here, we show the transcriptional regulation of brc-1 and brd-1 is conserved in worms. Using gene expression analysis, we found that knocking out brc-1 resulted in significant upregulation of four members of the *cyp-13A* subfamily, and loss of brd-1 function led to upregulation of six member of the *cyp-13A* subfamily. Our finding provides insights into how brc-1/brd-1 transcriptional regulation function is conserved in worms and further validates using *C. elegans* as a model system to investigate BRCA1 functions.