Investigating the Role of DEAD-box Helicase VBH-1 in *C. elegans*

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Abstract

VBH-1 is a DEAD-box RNA helicase found to co-immunoprecipate with CSR-1, an argonaute required for RNA-induced epigenetic activation (RNAa). Because most argonautes are thought to require an RNA helicase to function, this CO-IP data suggests that VBH-1 may be the helicase recruited by CSR-1. To test this hypothesis, experiments were conducted to assess CSR-1 function in *vbh-1* mutants. These experiments found that mutations in VBH-1 do not hinder CSR-1 localization, though do result in a reduction of brood size, as well as a reduction in protein expression of CSR-1 targets. Future experiments will be carried out to assess if mutations in VBH-1 affect CSR-1 targeting.

Acknowledgments

I would like to thank Dr. Craig C. Mello for giving me the opportunity of working on my MQP in his lab. I would also like to thank Professor Elizabeth Ryder for her guidance and support throughout the year. Finally, I would like to thank my mentor Krishna Ghanta for his tremendous support and teaching in the lab.

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Introduction

The study of epigenetics focuses on the heritable factors related to phenotypic expression that are not genomically encoded in the DNA of an organism. Classically, epigenetic factors were thought to relate to modifications made directly to DNA that affect how/when transcription can occur. These modifications do not alter the genetic code of an organism, though do modify the patterns of transcription, resulting in changes in protein expression. Discoveries made in the late 90's shed light on another method of epigenetic regulation – RNAi (Fire, 1998). RNAi is a system utilized by many eukaryotes and some archaea in which small RNAs are used to directly regulate translation of mRNAs. These small RNA pathways utilize several factors, mainly endogenously transcribed small RNAs, and argonautes proteins.

Argonaute proteins are specialized proteins which are able to bind small RNAs, such as micro RNAs (miRNAs) or small interfering RNAs (siRNAs) to regulate gene expression. These proteins are highly conserved amongst organisms (eukaryotic and prokaryotic alike) and can have many different roles in the regulation of gene expression. Two major classes of argonaute proteins have been identified within different organisms. AGO argonautes, which are similar in structure to the *Arabidopsis thaliana* argonaute AGO1, have been shown to interact with miRNAs and siRNAs, and have been implicated in post-transcriptional gene-silencing (Hutvagner & Simard, 2008). These small RNA sequences, when bound to AGO argonautes, guide the protein to specific endogenous mRNA targets. Once brought to an mRNA target, AGO argonautes are shown to cleave the target mRNA or simply bind to it, preventing further translation of the mRNA transcript (Tang, 2005). The other major class are PIWI argonautes, which bind to small RNAs called PIWI-interacting RNAs (piRNAs). PIWI argonautes function in similar fashion to AGO argonautes,

binding piRNAs which target specific mRNAs. Unlike AGO argonautes, PIWI argonautes have classically been shown to bind small RNAs which target exogenous rather than endogenous genes, resulting effectively in a defense mechanism against mRNAs foreign to the cell (Thomas et al., 2013).

PRG-1 is a PIWI argonaute found in *C. elegans* which has been found to play a role in argonaute-mediated gene silencing. In PRG-1 mediated silencing, PRG-1 first binds to endogenously transcribed 21 nucleotide sequences, called 21U RNAs (Batista et al., 2008). Once bound, PRG-1-21U complexes target RNA sequences and recruit RNA-dependent polymerases (RdRPs) to amplify 22 nucleotide segments antisense to the target RNA (Mani & Juliano, 2013). By creating these 22 nucleotide RNAs (called 22G RNAs), PRG-1 effectively facilitates the creation of small RNAs complementary to the target RNA, which can then be utilized to effectively silence the target RNA (Figure 1).

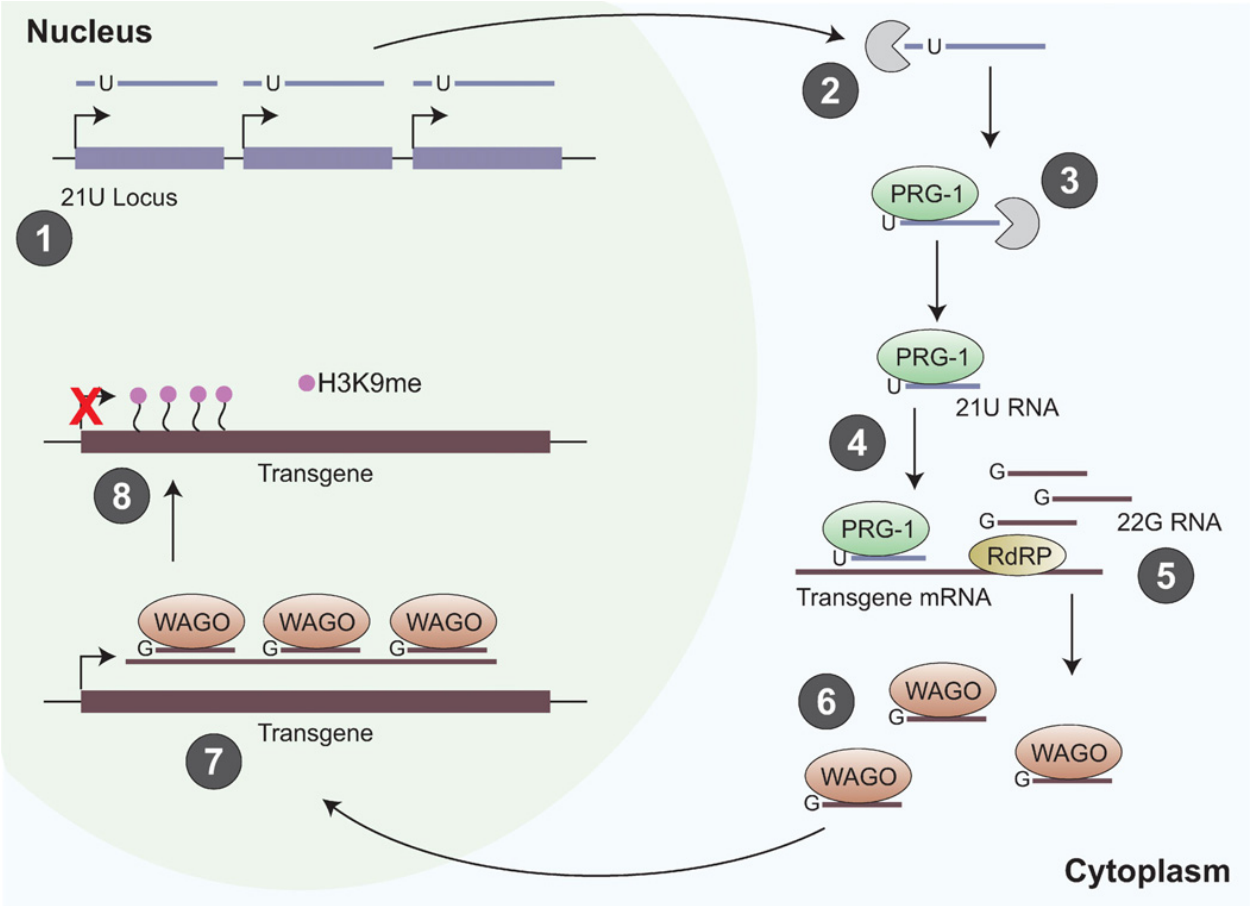

Figure 1. (Taken from Mani & Juliano, 2013) PRG-1 mediated mRNA silencing. Endogenously transcribed 21U's bind to PRG-1, targeting specific RNA transcripts. Once bound, PRG-1 complexed with a 21U recruits an RNA-dependent RNA polymerase to amplify 22G sequences, complementary to the target RNA. A WAGO argonaute then utilizes the 22G sequences complementary to the target RNA to bind to and silence it.

In order to silence the target RNA, PRG-1 recruits an argonaute belonging to the worm AGO (WAGO) argonaute family, a family of 12 argonautes found exclusively in *C. elegans* (Yigit et al., 2006). Once bound to a 22G produced by the RdRP, WAGO-22G complexes can bind to the target RNA sequence, silencing it (Mani & Juliano, 2013). Though classically thought to interact only with foreign transgenes, PRG-1 has been shown to bind the endogenous transcripts as well. From pull-down experiments of PRG-1, it has been shown that PRG-1-21U's are

complementary to endogenous gene transcripts (Shen et al., 2018), and likely recruit WAGO argonautes to silence these transcripts through the same pathway. This process of PRG-1 mediated silencing has been termed RNA-induced epigenetic silencing (RNAe), and functions as a powerful method to prevent the translation of RNA transcripts.

CSR-1 is a worm specific argonaute that has been found to counteract the PRG-1 silencing pathway (Figure 2). Though the pathway is not fully understood, it is thought that CSR-1 counteracts the PRG-1 silencing pathway by binding to the targets of the PRG-1 pathway, preventing WAGO-mediated silencing (Seth et al., 2014). In the proposed mechanism, CSR-1

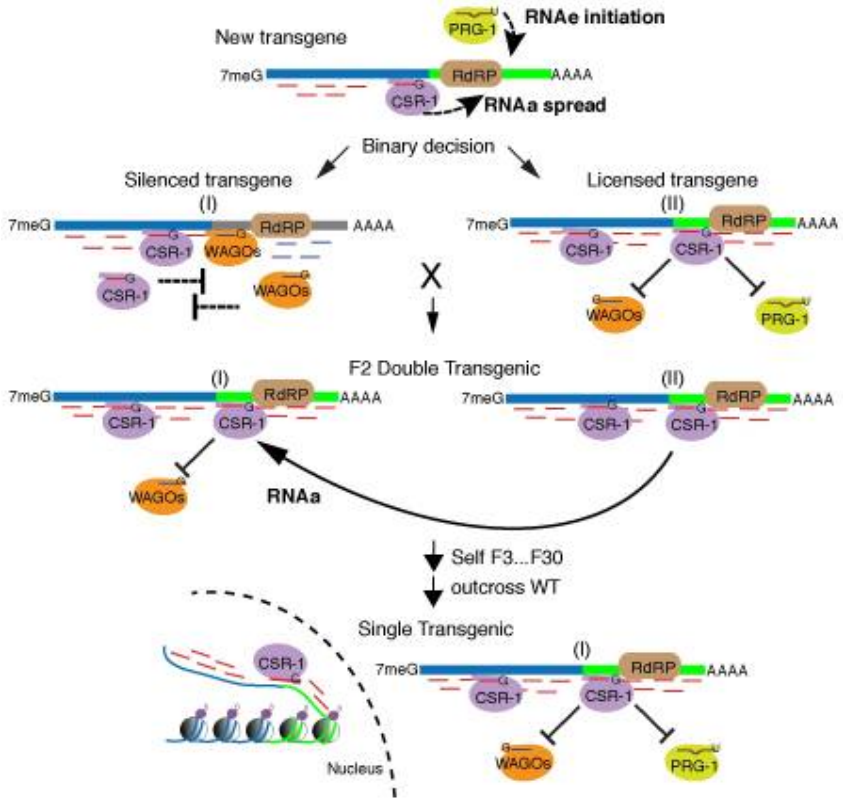

Figure 2. (Taken from Seth et al., 2013) CSR-1 mediated RNA-induced epigenetic activation (RNAa). In the RNAa CSR-1 mediated pathway, CSR-1 utilizes CSR-1 specific 22G's to bind to target RNA sequences. Once bound, CSR-1 recruits the RdRP EGO-1 to amplify these CSR-22G's. Amplified CSR-22G's can then bind to CSR-1, promoting continuous binding of CSR-1 to the target RNA transcript. This continuous binding prevents PRG-1 mediated silencing, likely by preventing PRG-1-21U binding to the target transcript (*see also Figure 1*).

binds to CSR-1 specific 22G's targeted to specific RNA transcripts. Once bound to a target transcript, CSR-1 recruits a specific RdRP, EGO-1, to amplify these CSR-22G sequences (Smardon et al., 2000). The growing number of CSR-22G's are then further utilized by CSR-1 to continue binding the target RNA transcript. This continuous binding is thought to protect the target transcript from PRG-1-WAGO mediated silencing, by disallowing the PRG-1-21U complex to bind to the target transcript (Seth et al., 2014, *see also Figure 1*). It is thought that if the PRG-1-21U complex cannot bind the target transcript, PRG-1 cannot recruit an RdRP to produce WAGO-22Gs, in turn preventing the downstream WAGO-22G mediated silencing. This process, which counteracts RNAe, is termed RNA-induced epigenetic activation (RNAa).

Because mRNA transcripts are often somewhat tangled, it is important in argonaute function to recruit the use of RNA helicases. All argonautes are thought to utilize RNA helicases to unwind target mRNAs (Ambrus & Frolov, 2009). Though no specific argonaute has been pinpointed in the CSR-1 RNAa pathway, the helicase VBH-1 has emerged as a possible candidate. From the pulldown of CSR-1, preliminary data has emerged suggesting that VBH-1 associates with CSR-1 in vivo.

VBH-1 is an RNA helicase belonging to the DEAD box family. Members of the DEAD box family are highly conserved proteins involved in the unwinding of RNA transcripts (Linder & Jankowsky, 2007). DEAD box proteins derive their name from a conserved amino acid sequence, D-E-A-D. Though VBH-1 has not been greatly studied in worms, data has shown that it co-precipitates with CSR-1, suggesting that it may play a role in CSR-1 meditated RNAa. Preliminary data shows that VBH-1 mutants are sterile under temperature stressed conditions (i.e when grown at 25 degrees Celsius), suggesting that VBH-1 has a vital role in germline function. Based on preliminary data acquired regarding the viability and fertility of VBH-1 mutants, and data hinting

at CSR-1 VBH-1 interactions, it is possible that VBH-1 plays a direct role in CSR-1 mediated RNAa, and perhaps may be the helicase that CSR-1 utilizes to unwind its target RNA transcripts.

To test this hypothesis, several mutations were knocked into *C. elegans* to elucidate the role of VBH-1 in CSR-1 function. Through the use of null alleles, as well as a DEAD box mutation, experiments can be carried out to assess worm viability with these various mutations. Furthermore, experiments can be carried to test protein levels of CSR-1 targets. CSR-1 targets all the endogenous transcripts in the germline and is thought to protect them from RNAe. As a result, it is hypothesized that protein levels of CSR-1 targets would go down in VBH-1 mutants. This can be tested through western blotting against the top targets of CSR-1. If the protein levels of CSR-1 targets decrease in VBH-1 mutants, this would support the hypothesis that VBH-1 is required for CSR-1 mediated RNAa.

Methods

Basic Maintenance

To propagate a colony of worms, one to two worms were plated on a 6 cm plate coated with a bacterial lawn, which serves as food for the worms. Depending on the age of the initial worms (see Figure 3) it can take up to 2 days for the worms to become gravid adults. Once the worms begin laying eggs, the plate will populate within ~10 hours. A healthy hermaphrodite can on average lay up to 280 eggs, and as a result within four to five days the plate will likely become starved of food. Before this occurs, worms were transferred to a new plate to prevent starvation. This process was repeated continuously to maintain a healthy population. Strains were grown at room temperature unless noted otherwise.

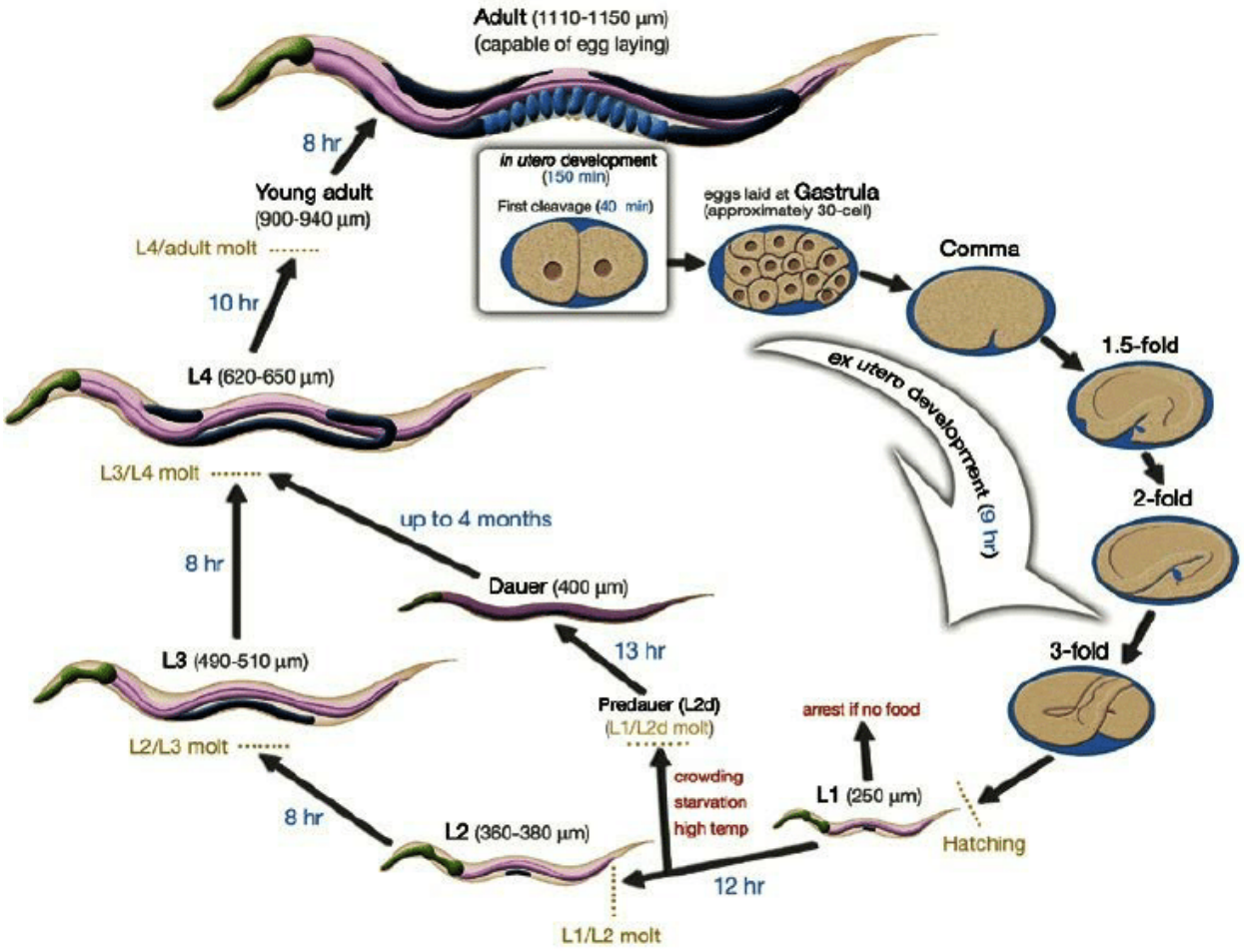

Figure 3. Life cycle of *Caenorhabditis Elegans*. *C. Elegans* typically take ~54 hours to become gravid adults, at which point they lay on average ~300 eggs. Several stages of development can be observed through a microscope, notable the L4 stage, during which the development of the vulva becomes noticeable. (Adapted from Cheong & Jaya, 2012).

Bleaching Worms

To obtain a synchronized population of worms, adult worms were bleached. During bleaching, a healthy population of adult worms were washed off of a plate into a solution of 10% Sodium Hypochlorite and 4% Potassium Hydroxide. These worms were then placed on a shaker for 13 minutes to allow for the breakdown of tissue surrounding the eggs. Once the eggs were

isolated, they were then spun down and washed 3 times with a buffer containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, and 0.5% NaCl in 1L H_2O (also called M9 buffer). After washing, the eggs carried by the worm remain. These eggs were then left in M9 overnight, allowing the worms to hatch into the L1 stage (Figure 1). Due to the lack of food, these L1 worms are not able to progress past this developmental stage, providing a synchronized L1 population.

Obtaining Males

C. Elegans are a hermaphroditic species; they produce both sperm and egg cells, and as a result the percentage of worms containing only sperm is often limited. Male worms are worms containing only one X chromosome (Figure 4) and are often a result of chromosome segregation errors. These errors can be induced by stress. To obtain males for crossing purposes, populations were starved for 4-5 days to induce chromosome segregation errors, resulting in larger male populations. Whereas normal plates may contain no males, progeny derived from a starved population have a much larger chance of being a male. Starved plates are divided into several sections, or 'chunk's', and plated onto new plates containing food. After 2 days, these new populations were scanned to find males. Males can be distinguished from females based on morphological differences (Figure 4).

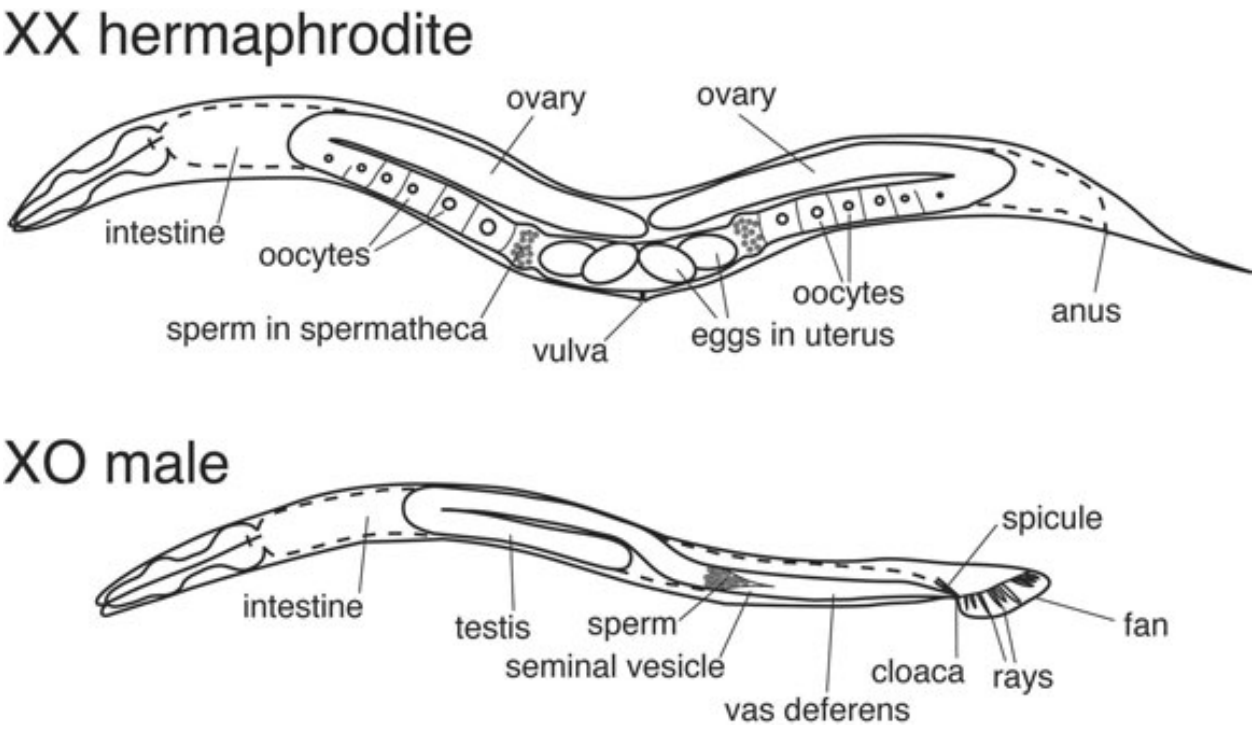

Figure 4. Hermaphrodite/Male characteristics. Males differ from hermaphrodites genomically by the lack of a second X chromosome. Morphologically, male worms have a distinct tail that can be easily distinguished from hermaphrodite tails. (Adapted from Zarkower, 2006)

CRISPR Injections/Strains Built

To create mutants, the CRISPR/CAS9 enzyme was used. Using CRISPR, four knockout mutations were made to disrupt the function of the DEAD-box helicase VBH-1. After creating these knockouts, names were assigned to the worms: ne4400, ne4401, ne4406, ne4406 (Table 1). CRISPR was also used to disrupt the DEAD-box helicase motif, to create the amino acid sequence DQAD. Three DQAD mutants were generated: ne4430, ne4434, ne4435.

CRISPR was also used to add a degron-tag onto the DEAD-box helicase LAF-1. This tag allows for the degradation of the tagged protein when the worm is placed on agar containing auxin.

Strain	Gene	Mutation
ne4400	*vbh-1*	8bp deletion
ne4401	*vbh-1*	127bp deletion
ne4406	*vbh-1*	683bp deletion
ne4407	*vbh-1*	1700bp deletion
ne4630	*vbh-1*	E237Q
ne4634	*vbh-1*	E237Q
ne4635	*vbh-1*	E237Q
NA	*laf-1*	235bp insertion

Table 1. Mutants created using CRISPR/CAS9 system.

Strain Crossing

Several methods can be used to build a strain. The primary method utilizes crosses between worms of two different strains (Figure 5). This method allows for the combining of two different unlinked alleles. If the allele being crossed into a strain is distinctly different in size from the wild type allele, a 2% agarose gel can be used to detect the introduction of the allele of interest. For alleles with no discernible difference from the wild type size, the PCR product is sent out for sequencing, and then analyzed to assess whether or not the desired allele was introduced. A demonstration of a cross can be seen in Figure 5, Panel D. The strains built can be seen in Table 1.

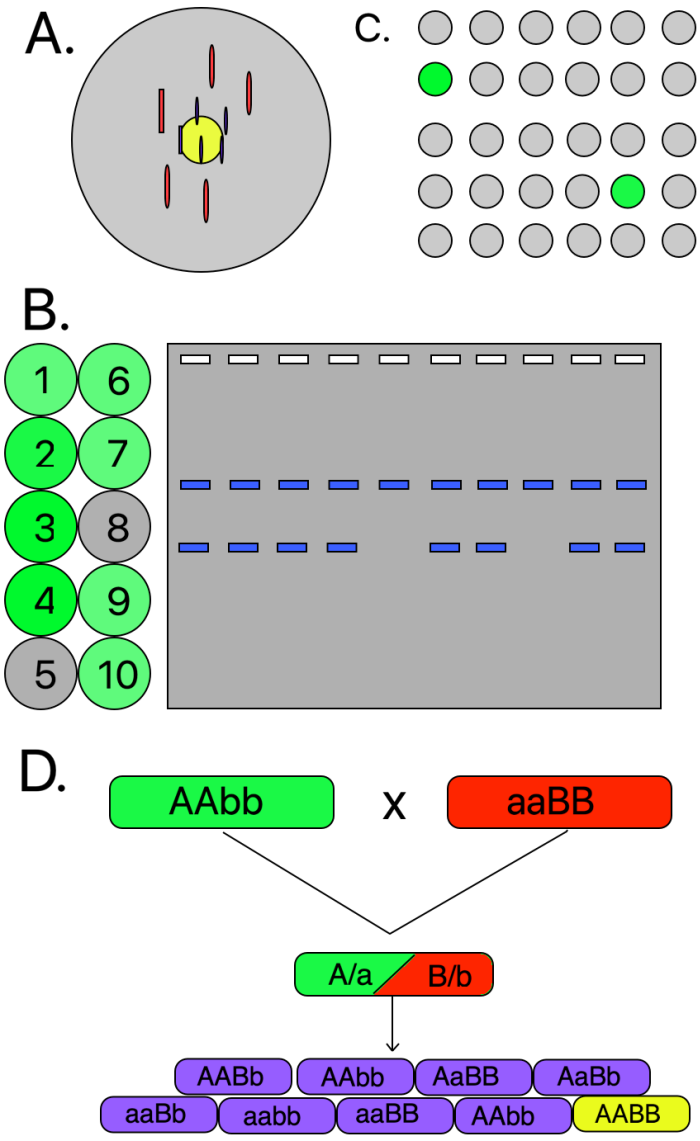

Figure 5. Strain Building Process. When building a unique strain by crossing two existing strains, the process follows several steps. (A) 5 males of one strain (black) are placed onto a plate with a very small amount of bacteria (yellow). On this same plate, 5 hermaphrodites (orange) of a different strain are placed. Due to the small amount of bacteria, the worms tend to congregate in a common location, promoting mating between strains. After 24 hours, the hermaphrodites are removed from the mating plate and placed onto individual plates, where they are allowed to lay eggs. After 48 hours each plate is observed to identify the presence of male progeny. Plates with males are likely contain mothers that are products of the initial cross, as a cross between a male and a hermaphrodite

produces 50% males. Plates that do not contain males likely do not contain cross-progeny. One plate containing males is then selected, and the rest are discarded. From the selected plate, 10 hermaphrodites are placed onto individual plates (B). After these 10 hermaphrodites lay eggs, each mother is genotyped utilizing a polymerase chain reaction (PCR), for one of the target alleles. If the mother is a heterozygote (has two DNA bands for the amplified allele, green plates) that plate is kept, as it is now certain that that mother was a product of the cross. Of all the heterozygous plates, only one is kept. From that one, 30 worms are picked onto individual plates. Once each worm lays eggs, the mother is genotyped for both alleles being crossed, to find a worm that is homozygous for both desired alleles. If the genes both follow Mendelian genetics, the chances of finding a homozygous double is 1/16. The full cross is as shown in Panel D. The final intended genotype is shown in yellow.

Strain 1	Strain 2	Notes
ne4400	Δ*glh-1*	Knockout mutation of Germ-Line-Helicase 1 (GLH-1)
ne4400	Δ*glh-4*	Knockout mutation of Germ-Line-Helicase 2 (GLH-2)
ne4400	*deg::glh-1*	Degron tag added to C terminus of GLH-1
ne4400	*flag::csr-1*	Flag tag added to C terminus of CSR-1
ne4406	*flag::csr-1*	Flag tag added to C terminus of CSR-1

Table 2. Strains built through crossing. To create new strains, crosses were carried out to combine alleles of two different strains.

Brood Counting and Percent Survival

Brood counts were performed on VBH-1 null mutants (ne4400, ne4406) and VBH-1 DQAD mutants (ne4430, ne4434). 20 worms at the L3 stage (Figure 3) were plated for each strain on individual 3 cm plates. For 24 hours each worm was allowed to lay eggs, and after 24 hours

each worm was transferred to a fresh plate, at which point eggs laid on the first plate were counted. After 24 hours, the worms were transferred again to a final third plate. Again, the number of eggs laid on the 2nd plate was counted. After another 24-hour period the worms were removed from the final plate, and the total number of eggs on the final plate was counted. After three days the number of eggs laid were totaled. To determine the percent of laid eggs that survived, 24 hours after each count the number of hatched worms was counted, and then compared to the number of eggs laid by each worm. Two wild-type controls were used in the assay (both isogenic, one derived from the ne4400 strain and one from ne4434).

***Unc-58* mutator assay**

The *unc-58* mutator assay assesses the role of a particular gene in DNA damage repair and general maintenance. The *unc-58* gene is a 40 kb gene important in muscle wall contraction. Specific mutations in this gene result in an uncoordinated phenotype which causes twitching and severely hinders movement. However, null mutations of the *unc-58* gene resulting in no protein product rescue the twitching phenotype observed in *unc-58* mutants. The large size of the gene lends itself to a higher chance of mutations during replication, and this principle can be used to track mutation rates over generations. If a mutation occurs in *unc-58* locus that shifts the frame of the coding region a null mutation will result, causing the loss of the twitching phenotype associated with *unc-58* mutants.

To assess if *vbh-1* has a role in DNA damage repair, *vbh-1* mutants were crossed into *unc-58* mutants, and after several generations these worms were observed to find worms rescued from the *unc-58* phenotype. Two *vbh-1* null mutants (ne4400 and ne4406) were crossed into *unc-58*

mutants and homozygosed to create double mutants (Figure 5). In each background, controls were created which exhibited homozygous alleles of the mutant *unc-58* gene alongside a wildtype allele of *vbh-1* (isogenic wildtype). For each strain (two *vbh-1* mutants and two controls), 200 worms were evenly distributed amongst 40 plates (6 cm diameter). These plates were stored at 20 degrees Celsius for 30 days, allowing for several generations of worms to propagate. After 30 days each of the 40 plates were chunked onto 40 new plates (10 cm diameter). After 3 days each plate was scored for positive or negative results. The presence of animals with wild-type movement on a plate indicated that mutations had occurred, and was scored as a positive result.

Western Blotting

Western blotting was used to determine protein levels of specific CSR-1 targets in *vbh-1* knockdown worms. Using an anti-gfp antibody, the protein levels of CSR-1 and KLP-7 were measured in a *gfp::csr*-1 and a *gfp::klp*-7 strain, respectively. 200 L4 worms were picked from worms grown at 25℃ on plates with bacteria expressing *vbh-1* RNAi and boiled for ten minutes in 50μL of a 10x NuPAGE reducing chemical. This step isolates and reduces the proteins present in the worms. 15μL of each sample were loaded into a 10% NuPAGE polyacrylamide gel, and run for 2 hours. The protein was then transferred onto a cellulose membrane, and then blotted against using an antibody targeted to GFP.

Results and Discussion

VBH-1 localizes to the P-granules of the germline and is required for proper fertility

A previously created *gfp::vbh-1* strain was used to characterize the localization of VBH-1 within the germline. VBH-1 is known to localize to the perinuclear nuage structures, called P-granules (Salinas et al., 2008). These granules are specific to the germline and have been shown to contain RNA as well as RNA-binding proteins: specifically, PGL-1, PGL-3, and DEAD-box proteins GLH-1— 4 (Wang & Seydoux, 2014).

To assess the role of VBH-1 in germline function, brood counts as well as viability assays were performed. Using one null allele and two DQAD mutants, it was shown that broods of the *vbh-1* null mutant were significantly reduced compared to wild-type (Figure 6, left), and were even more reduced in DQAD mutants, suggesting a possible dominant gain-of-function in the DQAD mutants. Survivability of this brood was further compromised in *vbh-1* mutants (Figure 6, right). These results were obtained with worms grown at 20℃.

This data affirms that VBH-1 is important for germline function, specifically for fertility. Because *csr-1* null mutants exhibit significant sterility, this may suggest a connection between VBH-1 and CSR-1, as both null mutants display a similar sterility. However, CSR-1 function is required for the transcription of all endogenous germline transcripts (Gerson-Gurwitz et al., 2016), so this connection must be considered with caution, as a mutation in CSR-1 likely has a cascade of varying phenotypes, as a result of all its downstream effects.

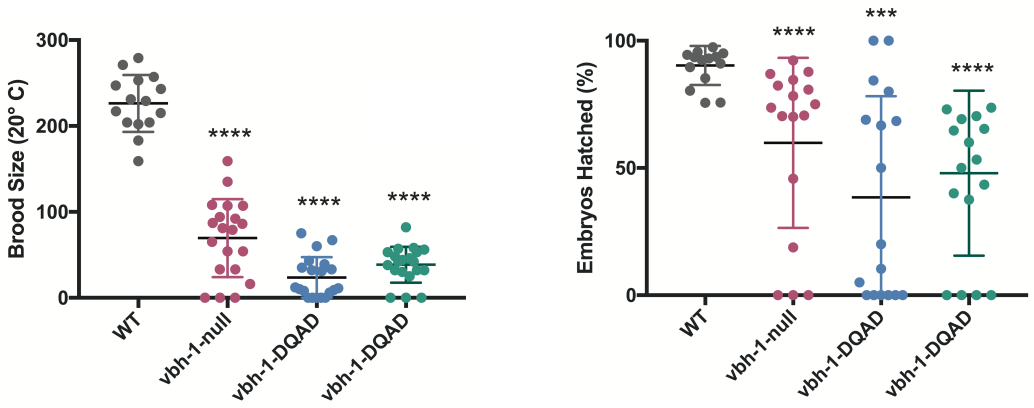

Figure 6. Brood size and percent of embryos hatched in *vbh-1* mutants. Brood counts (left) for *vbh-1* null mutants (ne4400) and DQAD mutants (blue – ne4630, green – ne4634) were performed at 20°C. Of the brood laid, the percent of hatched embryos was counted (right). Significance analyzed with two way T-test. **** - $P < 0.0001$; *** - $P < 0.001$.

VBH-1 is not required for maintenance of DNA

Vbh-1 mutants display a wide array of phenotypes, including short, wide worms (Dumpy), sterile worms, and twitching worms (Unc). However, these phenotypes are not seen in all *vbh-1* mutants, instead only arising at relatively small frequencies. This finding suggests that VBH-1 may play a role in DNA damage repair, or simply DNA maintenance.

Using the *unc-58* mutator assay, the importance of VBH-1 in preventing DNA mutations was assessed. After one month, plates containing *unc-58* X *vbh-1* mutants were scored, to assess the presence of revertant worms. Presence of any worms that did not display the *unc-58* twitching phenotype was scored as a positive, indicating a mutation occurring in the *unc-58* locus resulting in null protein formation. After 30 days, there was no difference in the number of revertant worms between the control worms and *vbh-1* mutants (Table 3). If VBH-1 were responsible for DNA damage repair, we would expect to see a higher number of *unc*-58 revertant worms in *vbh-1* mutants than in wild type worms. However, no change was observed, indicating that VBH-1 is likely not important in DNA damage repair.

Strain	N2	Ne4400 (null)	Ne4406 (null)
Revertants	2	1	2

Table 3. *unc-58* mutator assay results. After 1 month, 40 plates were scored per strain. Each plate was scored based on the presence of worms that showed a revertant phenotype; specifically, the number of plates with crawling, rather than twitching, worms were counted and totaled.

VBH-1 is not important for CSR-1 localization

CSR-1 normally localizes to the P-granules (Figure 7, left), structures made of mRNAs and RNA binding proteins. To localize to the P-granules, CSR-1 may be utilizing VBH-1, an RNA binding protein, to tether itself to the P-granules. Without proper VBH-1 function, we may expect to see an inability of CSR-1 to bind to the mRNAs of the P-granules, and as a result a mis-localization of CSR-1. To determine if VBH-1 function is important for CSR-1 localization, a *vbh-1* DQAD mutant was made in a *gfp::csr-1* background strain. This strain already has a gfp-tagged CSR-1 protein, allowing for the imaging of CSR-1 localization under UV fluorescence. In the DQAD mutant, this localization was not disrupted, as CSR-1 still localized to the P-granules (Figure 7, right).

Originally, the DQAD mutant was chosen over the null mutant for this experiment because it had the most severe sterility (Figure 6, left). Because sterility was more severe in DQAD mutants, it may follow that function is greater disrupted in DQAD mutants than null mutants. However, experiments performed in other DEAD-box helicases have shown that a DQAD

mutation results in a loss of ATP hydrolase activity by the helicase (Turner et al., 2007), and that this loss results in the helicases inability to release from the target mRNA. If CSR-1 recruits VBH-1 as the helicase allowing it to localize to the P-granules through the binding to mRNAs within the P-granules, it may follow that the dominant nature of the DQAD mutation may not result in mis-localization of CSR-1. To fully understand if VBH-1 is required for CSR-1 localization, this experiment should be performed with the *vbh-1* null mutant.

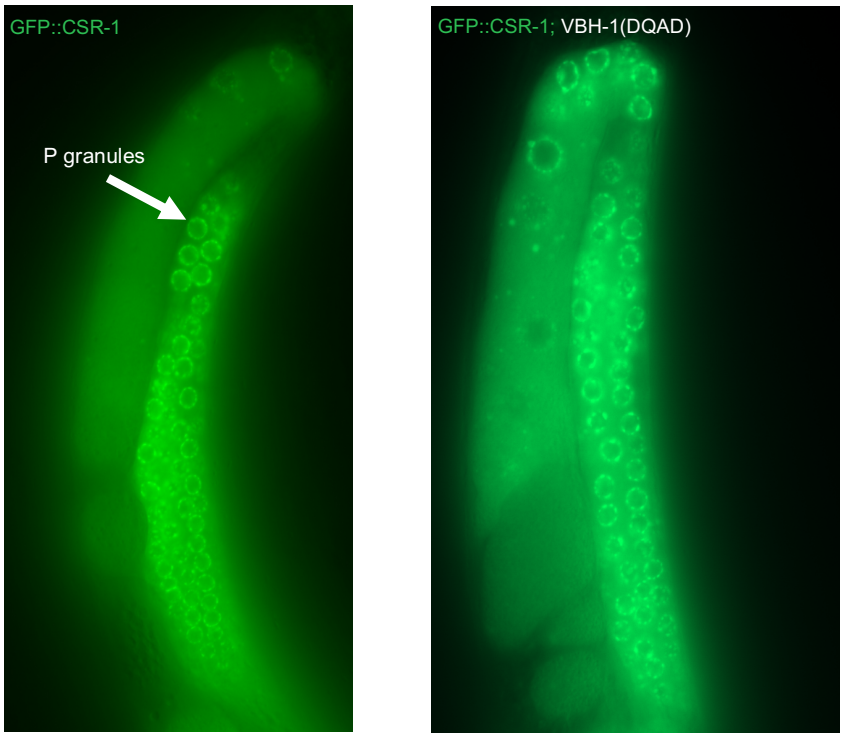

Figure 7. CSR-1 localization in *vbh-1* DQAD mutants. CSR-1 localizes to the P granules in wildtype worms (left, taken at 63x). In the *vbh-1* DQAD mutant (right, ne4635, taken at 40x) this localization is not disrupted.

VBH-1 is not important for CSR-1 mediated RNAa

To determine if VBH-1 is important for CSR-1 mediated RNAa, CRISPR was used to knockout VBH-1 in an RNAa/RNAe strain (A/E strain). This strain contains two transgenes, *gfp::cdk-1* and *gfp::oma-1*. In a properly functioning worm, the *gfp::oma-1* is actively expressed

(on), resulting in a uniform fluorescence in the distal end of the gonad. In contrast, the *gfp::cdk-1* transgene is silenced (off) by PIWI-mediated RNAe (Table 4). Though the precise reason for this silencing is not known, it has been suggested that the presence of the foreign GFP sequence triggers PIWI-mediated silencing. For an unknown reason, *gfp::oma-1* escapes this silencing, resulting in the on state seen for this transgene. Though it is unknown why *gfp::oma-1* escapes this silencing, it has been shown that CSR-1 is required for the activation of this transgene, in a process termed RNAa. By incurring mutations to VBH-1 in the A/E background, information can be gained regarding the importance of VBH-1 in both CSR-1 RNAa, and PIWI RNAe. In this scenario, the turning on of *gfp::cdk-1* (normally turned off by PIWI RNAe) after mutating would indicate a dysfunction in RNAe, implicating VBH-1 in RNAe. Alternatively, the turning off of *gfp::oma-1* (normally kept on by CSR-1 RNAa) in the *vbh-1* mutant would implicate its role in CSR-1 RNAa.

However, after creating a *vbh-1* null mutant in the A/E strain, no changes were observed in the *gfp::oma-1* activation and *gfp::cdk-1* silencing (data not shown), indicating that VBH-1 likely does not play a significant role in either process.

	OMA-1::GFP Expression	GFP::CDK-1 Expression
Wild Type	ON	OFF
RNAa defective	OFF	OFF
RNAe defective	ON	ON
vbh-1 null	ON	OFF

Table 4. Expression of transgenes. In wild-type conditions, the OMA-1::GFP transgene is not silenced, while GFP::CDK-1 is silenced. In RNAa/RNAe defective worms, the expression of these transgenes differs.

VBH-1 may be important for the proper expression of CSR-1 targets

CSR-1 is an argonaute protein likely targeting all endogenous germline transcripts. CSR-1 has been shown to be important in RNAa, protecting transcripts from PIWI-mediated silencing (RNAe). As a result, if CSR-1 mediated RNAa is compromised, protein levels of CSR-1 targets are expected to decrease as a result of uninhibited RNAe.

To determine if VBH-1 is important for the translation of CSR-1 targets, western blot analysis was carried out to check the protein levels of CSR-1 targets in worms grown on *vbh-1* RNAi expressing bacteria. Using an antibody targeted to GFP, western blotting was performed. Two proteins were tagged with gfp, KLP-7, and CSR-1, because these proteins are among CSR-1's top targets (Gerson-Gurwitz et al., 2016). As shown in Figure 8, there is a slight decrease in CSR-1, and a significant decrease of KLP-7 in worms grown on *vbh-1* RNAi plates. This data was obtained at 25°C, and at 20°C this change was not observed (data not shown). The loss of protein of CSR-1 targets may suggest that under temperature stressed conditions VBH-1 is important for proper CSR-1 mediated RNAa of germline-expressing genes.

One thing to note regarding the western shown in Figure 8 is that there is no proper loading control to verify that similar amounts of protein was loaded into the gel. This is due to errors made performing the western blot. However, this result is currently being repeated to verify the results shown.

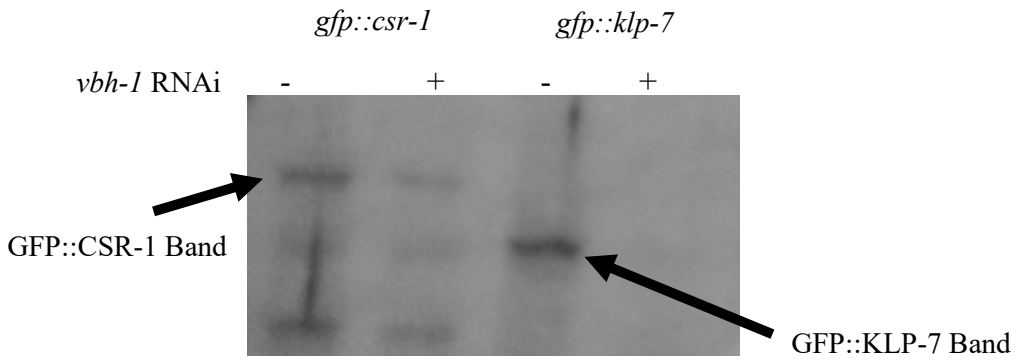

Figure 8. Protein expression of CSR-1 targets in worms grown on *vbh-1* RNAi. To test the importance of VBH-1 in the expression of CSR-1 targets, worms were grown at 25°C on *vbh-1* RNAi plates. For both CSR-1 and KLP-7, levels decreased in worms grown on *vbh-1* RNAi.

Conclusions and Future Work

Genetic regulation plays a key role in the development of organisms. Throughout the life-cycle of an organism, protein production levels are carefully tuned to prevent over-expression and under-expression. RNAe and RNAa act in counteracting pathways to silence and protect germline mRNA transcripts, respectively. In RNAa, the argonaute protein CSR-1 is responsible for binding to target mRNAs, in turn preventing PRG-1 mediated RNAe. However, it remains a mystery how CSR-1 can bind target mRNAs in their native state. Studies focusing on argonaute proteins in other organisms have identified the use of RNA helicases by argonautes. Co-IP experiments of CSR-1 have found many reads for the RNA helicase VBH-1, a helicase unique to the germline which has been found to localize to the P-granules (Salinas et al., 2008). This may suggest that VBH-1 is the RNA helicase recruited by CSR-1 to unwind target mRNAs.

To identify if VBH-1 interacts with CSR-1 in RNAa activities, several experiments were conducted. Firstly, the phenotype of *vbh-1* mutants was characterized. *Vbh-1* null and DQAD mutants exhibit a high sterility at 20°C and are completely sterile at 25°C. This corresponds to the sterile phenotype observed in *csr-1* mutants, suggesting a possible functional overlap between the two proteins. However, CSR-1 targets all endogenous transcripts at some rate, so loss of CSR-1 function may lead to sterility for a variety of reasons. To further assess the role of VBH-1 in CSR-1 function, we looked to see if CSR-1 localization was disrupted in *vbh-1* mutants. In the DQAD mutants, CSR-1 localization was not disrupted. However, RNA helicases with DQAD mutations have been shown to bind tighter to target mRNAs. If CSR-1 recruits VBH-1 to bind to P-granules (consisting heavily of mRNA), and the DQAD mutant can still bind to target mRNAs, it may

follow that the DQAD mutation is not enough to disrupt localization. To properly assess if VBH-1 is required for CSR-1 localization, this experiment is being repeated with a *vbh-1* null mutant.

Because *csr-1* mutants are RNAa defective, we looked to see if *vbh-1* mutants share this defect. *Vbh-1* null mutants were made in the A/E background to observe if the transgene OMA-1::GFP was expressed. Fluorescence in the gonad caused by expression of OMA-1::GFP indicates the presence of a functioning RNAa system. No fluorescence would indicate an RNAa defect, as seen in *csr-1* mutants. However, this was not the case in *vbh-1* mutants, as OMA-1::GFP was shown to be on. This indicates that VBH-1 is not important for the expression of a foreign transgene in the RNAa context. However, this does not indicate that VBH-1 does not function with CSR-1.

CSR-1 targets the entire germline transcriptome at some rate and is thought to protect these transcripts from PRG-1 mediated silencing through RNAe. If CSR-1 function is disrupted, it would follow that its targets would now be silenced at a higher rate, resulting in lower protein production of CSR-1 targets. This has been shown in previous experiments (Gerson-Gurwitz et al., 2016). If VBH-1 is required for CSR-1 function, it would follow that protein levels of CSR-1 targets would decrease in *vbh-1* mutants. When grown in temperature stressed conditions (25℃) on plates with bacteria expressing *vbh-1* RNAi, we found that worms produced a smaller protein amount for CSR-1 targets. This suggests that *vbh-1* has a role in protein production of CSR-1 targets, though the reason remains unknown based on the data generated. To truly understand the importance of VBH-1 in protein production, this experiment must be redone in *vbh-1* null mutants. It is also important to observe protein levels of proteins not targeted by CSR-1, as VBH-1 may be important for the expression of all proteins, not solely CSR-1 targets.

One explanation exists regarding the temperature-sensitive loss of protein in *vbh-1* knockdown worms. DED-1 is a homolog of VBH-1 in yeast which has been found to be important for ribosomal positioning (Guenther et al., 2018). Under temperature-stressed conditions, it was found that ribosomes in *ded-1* mutants get stuck in the 5' UTR of some mRNA transcripts. As a result, protein levels of these transcripts decrease. This protein decrease only occurs in temperature-stressed conditions, similarly to the protein decrease observed in *vbh-1* mutants. To fully assess the ribosomal function in relation to VBH-1, we are developing a ribosome profiling protocol in *C. elegans* to determine if ribosomes position properly in *vbh-1* mutants. This may explain the decrease of protein levels in *vbh-1* mutants.

Several experiments are being conducted to further assess the role of VBH-1 in *C. elegans,* as well as in relation to CSR-1. Currently, a tagged VBH-1 protein is being used to conduct a Co-IP experiment. By pulling down VBH-1, we can analyze any and all proteins that Co-IP with VBH-1, to determine its interactors. Though it has been shown to pull-down with CSR-1 in a CSR-1 IP, it would be nice to verify this result through the corollary – a pulldown of VBH-1. This may also identify other proteins it interacts with, to further elucidate a possible role of VBH-1. Another experiment aims to identify the target mRNAs of VBH-1. This will utilize a Cross-linking IP experiment of VBH-1, in which mRNAs bound to VBH-1 will be chemically linked to VBH-1 before pulling it down. RNA sequencing of the RNAs pulled down with VBH-1 will identify mRNAs targeted by VBH-1, as well as where on the mRNA VBH-1 binds. If this data corresponds with the binding profile of CSR-1, it will be a clear connection between VBH-1 and CSR-1.

Currently, there is no significant data to suggest a clear role of VBH-1 in CSR-1 function. Experiments must be conducted to clearly implicate it in CSR-1 function. However, the data presented identifies a clear role of VBH-1 in germline function, specifically fertility. Furthermore,

VBH-1 has been shown to be important for protein expression under temperature stressed conditions. However, the reasoning of this change in expression is yet to be determined. Future experiments aim to further explore the role of VBH-1 in *C. elegans* function.

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