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Identification of a Novel Endogenous Small RNA Pathway Specifically Targeting the 3'UTRs of mRNAs in C. elegans

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by

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Dedication

I would like to dedicate this work to my mother, Colleen Maloney, and my brother, Paul Luis Weston Randolph. Without their love and support this and many more achievements would have not been possible. Through the good and the very bad, they were always willing to listen when needed and give advice when asked. I could not have made it this far without them. I love you both.

To my friends and all of their glorious distractions. You all made what little down time I had all the more enjoyable, making the grind of lab work and the tough times all the better with something to look forward to.

ABSTRACT OF THE THESIS

Identification of a Novel Endogenous Small RNA Pathway Specifically Targeting the 3' UTRs of mRNAs

by

James W.J. Randolph

Master of Science, Graduate Program in Genetics, Genomics and Bioinformatics
University of California, Riverside, June 2019
Dr. Weifeng Gu, Chairperson

We are reporting a novel WAGO small RNA pathway which specifically targets the 3' UTR of hundreds of functionally important genes. In *C. elegans*, endogenous small RNAs, 22G-RNAs, bind Argonautes to regulate almost all germline genes. There are two major 22G-RNA-mediated pathways in *C. elegans* germline cells. One is mediated by Argonaute CSR-1 and plays important roles in chromosome segregation and embryonic development; the other is mediated by multiple Argonautes, WAGOs, and play critical roles in silencing transposons, pseudogenes, viruses, and some functional genes. In all these small RNA pathways, 22G-RNAs are generated by RNA-dependent RNA polymerases (RdRPs) using mRNAs and other RNAs as templates. Usually these 22G-RNAs are generated from both coding regions and UTRs of RNAs. Here we are reporting a novel small RNA pathway which specifically targets the 3' UTRs of hundreds of genes, many of which have been well studied and play important roles in germline and embryonic development. Our preliminary results indicated that these genes are targeted both by CSR-1 and WAGO Argonautes. However, CSR-1 targets the 5' UTRs,

coding regions and 3' UTRs, while the WAGO Argonautes only target the 3’ UTRs. Interestingly, the WAGO-22Gs are not dependent on *rde-3,* which is usually required for generating 22Gs in other WAGO-dependent pathways including exogenous RNAi pathways. Our RNA-seq results suggest that the CSR-1 22G-RNAs may be involved in silencing the target RNAs. We are currently using genetics, high-throughput sequencing and ribosome profiling to investigate why WAGO 22Gs are only generated from the 3’ UTR regions and if these small RNAs are involved in regulating mRNA and/or translation. We are also analyzing if these 22G-RNAs affects miRNA-mediated gene regulation at the 3’UTR of RNAs, In all, we are reporting a novel WAGO-mediated 22G pathway which specifically targeting the 3’UTR of hundreds of functional genes and this pathway is different from the canonical WAGO pathway since RDE-3 is not required for the 22G biogenesis and this pathway regulates CSR-1 targets.

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List of Acronyms

Ribonucleic acids (RNA)
RNA interference (RNAi)
RNA-dependent RNA polymerases (RdRPs)
Caenorhabditis elegans (C. elegans)
Double stranded RNA structure (dsRNA)
Short interfering RNA (siRNA)
Argonaute (AGO)
Worm-specific argonautes (WAGO)
Chromosome-Segregation and RNAi deficient (CSR)
Coding regions (CDS)
Untranslated regions (UTR)
Nematode growth media (NGM)
Terrific broth (TB)
Lysogeny broth (LB)
Optical density (OD)
Ribosomal RNA (rRNA)
Polysome extraction buffer (PEB)
Messenger RNA (mRNA)
Dimethylsulfoxide (DMSO)
Polyethylene glycol 8000 (PEG-8000)
Polyvinylidene Fluoride (PVDF)
Uncoordinated (*unc*)
Larval stage 1 (L1)
Larval stage 4 (L4)
Deoxyribonucleic acid (DNA)
Reverse transcriptase (RT)
Polymerase chain reaction (PCR)
Micro RNA (miRNA)
Piwi-Interacting RNA (piRNA)
Alkaline Phosphatase, Calf Intestinal (CIP)
Ribonuclease H (RNase H)
Ethylenediaminetetraacetic acid (EDTA)
Sodium Acetate (NaAc)
Monohydrogen dioxide, water (H2O)
Polyacrylamide gel electrophoresis (PAGE)
Altered avermectin sensitivity (*avr*)
Glutamate-gated Chloride channel (*glc*)
Chromatin immunoprecipitation (ChIP)
Argonaute (plant)-Like Gene (ALG)
Svendval Unit (S)
RNAi Defective (*rde)*

Heritable RNAi deficient (hrde-1)
Wild-Type (WT)
Phosphatase Interacting with RNA/RNP (Pir-1)
P-GranuLe abnormality (Pgl-1)
Dicer Related Helicase (Drh-1)
Enhancer of Glp-One (glp-1) (Ego-1)
Kinesin-Like Protein (Klp-7)
Cyclin-Dependent Kinase (Cdk-1)
Sodium Chloride (NaCl)
Deionized Monohydrogen dioxide, water (di-H2O)
Potassium dihydrogen phosphate (KH2PO4)
Disodium hydrogen phosphate (Na2HPO4)
Magnesium sulfate heptahydrate (MgSO4-7H2O)
Calcium Chloride (CaCl2)
Magnesium Sulfate (MgSO4)
Potassium Phosphate (KPO4)
Dibasic potassium phosphate (K2HPO4)
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid potassium salt, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) potassium salt (HEPES-KOH)
Potassium Acetate(K-Acetate)
Dithiothreitol (DTT)
phenylmethylsulfonyl fluoride (PMSF)
Potassium Chloride (KCl)
RNA Ligase 2 (RNL2)
RNA Ligase 1(Lig1)
SuperScript II(SSII)
Tetramethylammonium chloride (TMAC)
deoxyribonucleotide triphosphate (dNTP)
Molar (M)
Micro Molar(uM)
Micro Liter (uL)
Liter (L)
Micro Molar(uM)
Micro Liter (mL)
Micro Molar (mM)

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Introduction

siRNA and RNAi

The biogenesis of various small ribonucleic acids (RNAs) and their use within RNA interference (RNAi)-related mechanisms has been identified in a wide variety of organisms, ranging from plants to *Caenorhabditis elegans* (*C. elegans*) (1, 2, 4, 9, 15). RNAi is the name given to the cellular process that utilizes these small RNAs in a dose dependent manner to silence gene expression at transcriptional and/or posttranscriptional levels (Figure 1). This process could be a temporary effect and has been seen to affect cells outside of the original silencing event (4). A number of factors contribute to the identification of the various small RNA classes, ranging from the method of biogenesis, the argonaute (AGO) protein that the small RNA binds to, and the way the small RNA regulates or functions within the cells of the organism (2). These small RNAs range from sizes between 21-26 nt in length (15). The shorter of these small RNAs, 21-22nt, have been shown to participate in mRNA degradation (4, 9). While the larger, 24-26nt, contribute to the silencing and methylation of homologous DNA (deoxyribonucleic acid) within the organism in question (9). These short interfering RNAs (siRNAs) are long enough to induce messenger RNA (mRNA) degradation in mammalian cells but short enough to avoid triggering an interferon response due to their double stranded RNA structure (dsRNA) (4). The origin of the target genetic material used in the biogenesis of

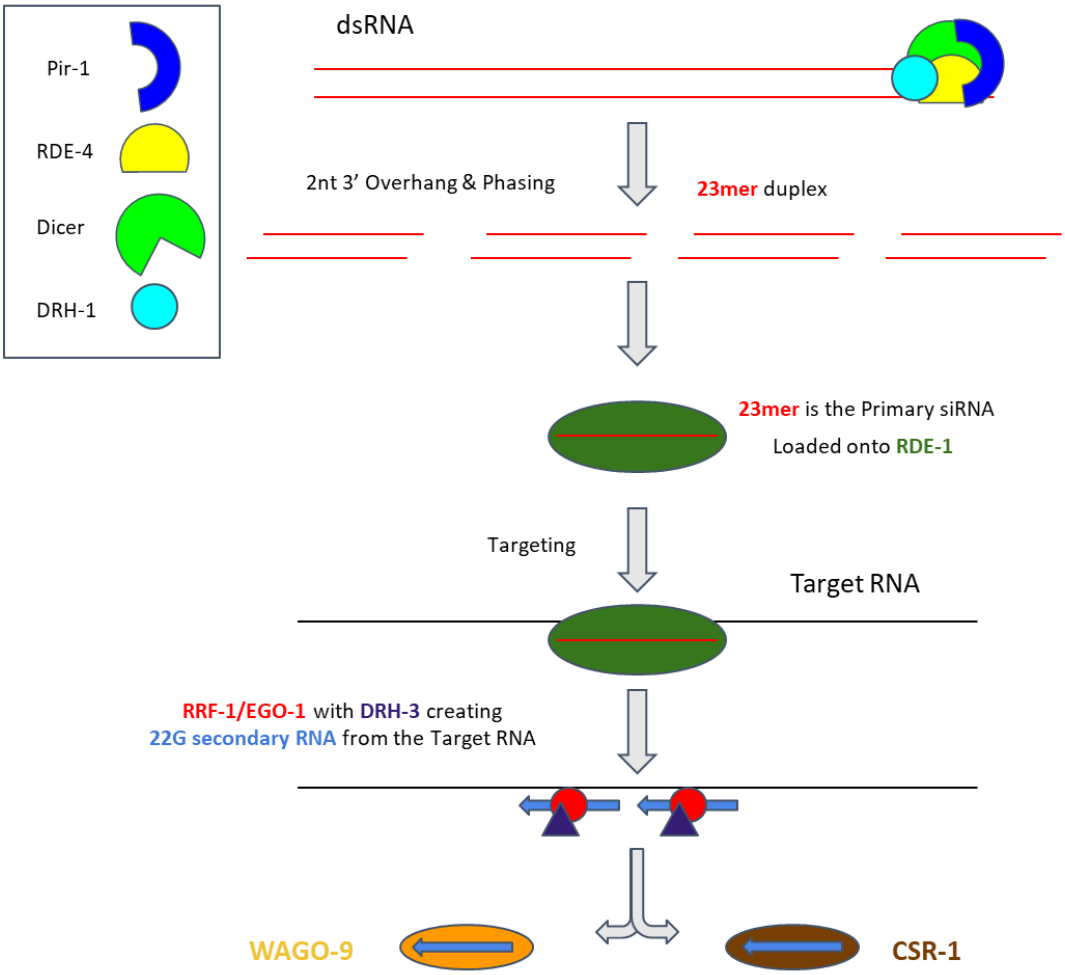

Figure 1: Simplified representation of the biogenesis of 22G siRNA within the germline of C. elegans.

these small RNAs can either be exogenous, coming from outside of the biological system, or endogenous, or coming from within the biological system. In C. elegans, small RNA products of RNA dependent RNA polymerase (RdRP) are referred to as 22G due to their prevalence of being 22 nucleotides in length with a 5' guanosine (1,2). Originating from coding genes, transposons, pseudogenes, and non-annotated loci; these 22G RNAs

are found predominantly within the germline and target 50% of the currently annotated genome of C. elegans (2) (Figure 1).

siRNA Roles in Human Diseases

A group of 22nt small RNAs, siRNAs, have anti-viral properties in many non-mammalian species using a process called RNA interference. Within the past couple of decades, these siRNAs and the RNAi pathway, have shown great promise in the realm of various therapeutic and anti-viral applications for humans. Viruses such as Hepatitis B, Influenza, and Human Papillomavirus (HPV) to name a few, have all been shown to be interrupted by the introduction of siRNA treatment (9). Targeting the viral gene expression of oncoprotein E6/7 or nucleocapsid protein (NP) using siRNA could lead to effective therapies for viruses such as HPV and influenza respectively. While targeting the chemokine receptor CXCR4 and CCR5 within a patient's own B cells could block the Human Immunodeficiency Virus (HIV) from ever entering the hosts B cells and effectively preventing its replication (9). Now siRNAs are not limited to antiviral uses, they also have the promise to change the face of cancer therapy by completely revamping chemotherapy. The MDR1 (multiple drug resistance) gene on the cancer cells, which is responsible for the overexpression of P-glycoproteins, is used by the cancer cells to remove the various chemotherapeutic drugs out of the cells. Targeting this gene could lead to a drastic improvement to the overall effectiveness to chemotherapy (9). Decreasing or silencing the expression of certain oncogenes may also be possible. For instance, inhibiting the expression of PLK1 or u-PA would prevent the cancer cells to

block the apoptosis signaling and reduce the movement and proliferation of tumors respectively (9). Neurodegenerative diseases also could benefit from siRNAs and RNAi. There are eight known neurodegenerative diseases that are caused by the trinucleotide CAG repeat, Huntington's disease and spinobulbar muscular atrophy are some common examples of these diseases. Using RNAi these diseases can be managed by targeting the gene that is being expressed with siRNA to decrease of inhibit the expression of the mutant allele (9). So, by using endogenous or exogenous targets for the creation of these siRNAs, various new strategies can be developed to combat viruses, genetic disorders, and cancer in ways that a decade ago would have not seemed possible. The study and discovery of new pathways and families of siRNAs could very well lead to the next big medical therapy.

Argonaute

As stated previously, the small RNA in the RNAi process is bound to a protein called argonaute. This protein is a core effector in all RNAi processes (2). Argonaute proteins are found in a variety of organisms and within those organisms the argonautes vary in location, function and targets (2, 6, 7, 18). Using the siRNAs as a guide, the argonaute proteins can target a specific complementary sequence within the cells to perform a specific action, which varies depending upon the argonaute being bound (2). In C. elegans there are currently twenty-seven known argonaute proteins found in either the germline or somatic cells (11). These argonautes bind to 22G siRNAs that are antisense to their intended targets, and perform various tasks dependent upon the argonaute they

are bound too. For the purposes of this paper two argonautes were focused on, CSR-1 and WAGO family.

WAGO Family

Worm-specific argonautes (WAGO) are a group of argonautes found within C. elegans. This family of argonautes is found in both the somatic and at least one role within germline cells, with multiple WAGO-dependent pathways existing (1, 2). WAGO 22G RNAs come from 2911 non-functional genes and bind to the entire WAGO family, WAGO 1 through 12 (data not shown). The WAGO family is known to silence transposons, pseudogenes, cryptic loci and certain genes in both the cytoplasm and the nucleus (1, 2). As of now there are twelve identified members of the WAGO family (2). Though this may not be the entirety of the WAGO family it is believed that not all of these other possible argonautes are functional, possibly due to them being pseudogenes that have lost their functions over time (2). These twelve WAGO proteins are needed for the production of the germline 22G siRNA's in C. elegans. It was found that in a deletion mutant strain targeting all twelve WAGO proteins lead to undetectable levels of germline WAGO 22G RNA's (2).

CSR-1

There are two distinct argonaute based pathways within the germline of C. elegans, one WAGO based and the other based in CSR-1 (Chromosome-Segregation and RNAi deficient). Unlike the WAGO family argonautes that are found within both the

somatic and germline cells, the CSR-1 argonaute has so far only been found in the germline cells of C. elegans (1, 2). Where the WAGO family argonautes has some function in the biogenesis of the 22G siRNA in the germline, the CSR-1 argonaute binds to the majority, if not all, the endogenous germline-expressed genes (1). These 22G RNAs originate from 3659 functional genes and do not require RDE-3 or MUT-7 for biogenesis (Data not shown). When bound to siRNA the CSR-1 argonaute does not seem to silence their intended target (2). Instead of silencing targets CSR-1 may actually prevent the silencing pathways of PRG-1 and WAGO family argonautes. CSR-1 is needed to promote the expression of male specific germline genes, males become infertile over the course of multiple generations of not paternal CSR-1 activity. These CSR-1 argonautes small RNAs also seem to act as a memory of previous germline expression to protect the endogenous genes from piRNAs (piwi-interacting RNA), which indirectly helps with the scanning of foreign sequences of dsRNA (1). Alongside its role with 22G siRNA CSR-1 may also have a role with regulating the transcription of its own target genes. When mRNA reads were analyzed from a homozygous *csr-1* deletion mutant it was found that a group of over 100 mRNA transcripts were upregulated (Figure 2). This data suggested the role of CSR-1 in the mRNA regulation of its own gene targets.

C. elegan Model Organism

Nematodes have been used in science in some capacity since the 1890's, originally using the parasitic members. The switch to the free-living nematodes in 1900's

lead to the organism becoming much easier to use (13). This meant that instead of needing a host to allow the nematode to grow, they instead could grow on a enriched media plate. The initial draw to this organism was not for the study of molecular biology but for its reproduction and development. Sydney Brenner is credited with starting the popularity of using free-living nematodes, beginning in 1974, for use within molecular biology (13). The success of Brenner's work, the ability to store strains indefinitely, the ease of growing these nematodes, and more lead to the rapid adoption of this model organism in the years following. C. elegans has gone on to be used to research into molecular, cellular, physiological, and behavior (13). Since 2002, six Nobel Prizes have been awarded to labs that used C. elegans as the model organism, which just goes to show the power and success this organism has within the field of science (16). This study is done within the C. elegans model organism due to its power when studying RNAi pathways. Mello and Fire discovered RNAi within C. elegans and was awarded the Nobel prize for it in 2006 and it is their work that helped to set the foundation of this study (16).

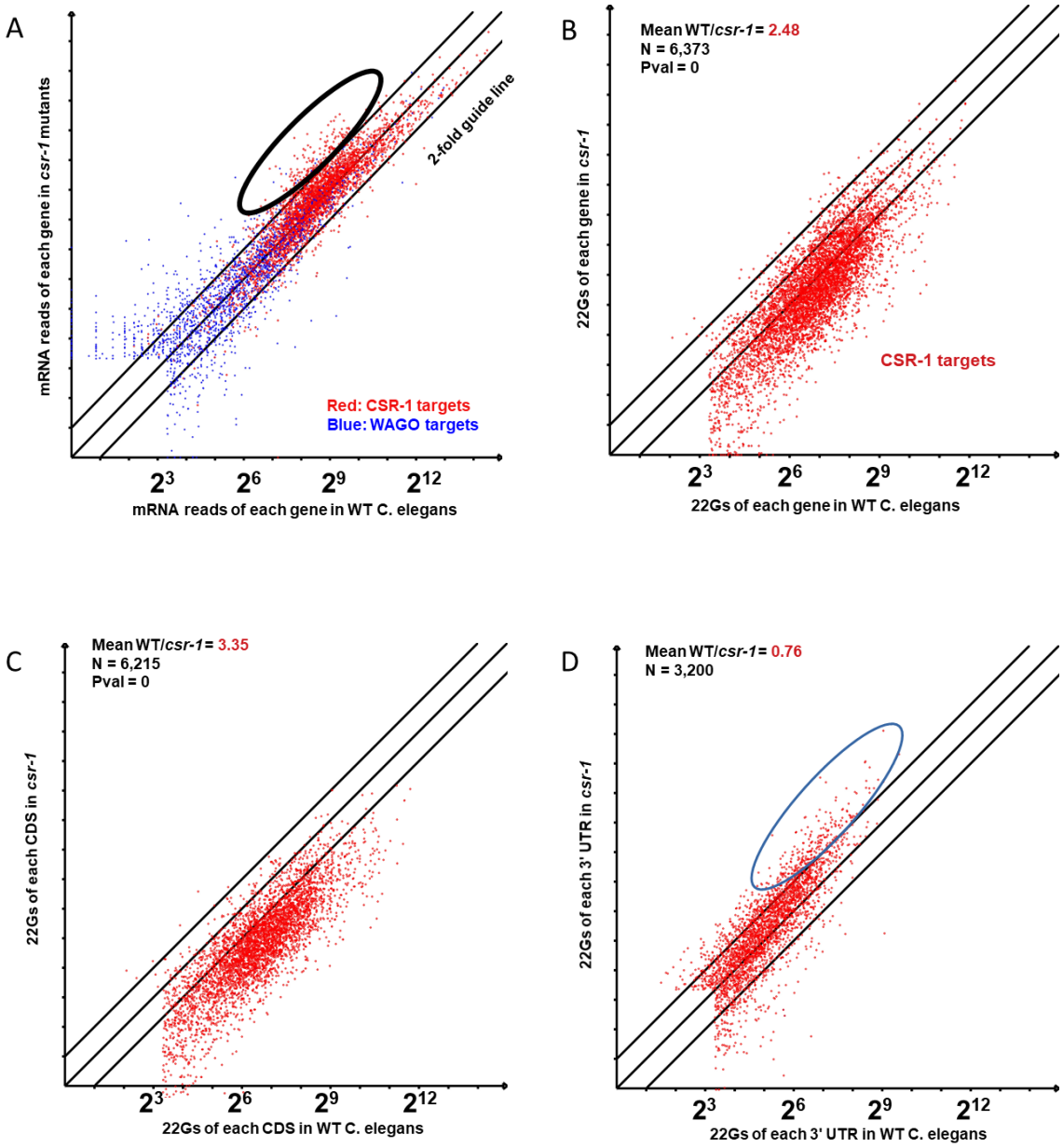

Figure 2: Each dot on the plots represents a single gene. A) Plot representing the mRNA reads in a csr-1 mutant. The circled portion are mRNA reads that have been upregulated in the csr-1 mutant. B) Plot of the 22G reads between a WT and csr-1 mutant, showing the decrease in overall 22G small RNA reads in the csr-1 mutant. C) Plot of the 22G small RNA reads in the CDS of csr-1 mutant compared to that of WT, obvious downregulation occurring. D) Plot of the 22G small RNA reads between the csr-1 mutant and WT in the 3' UTR showing an upregulation in the csr-1 mutant.

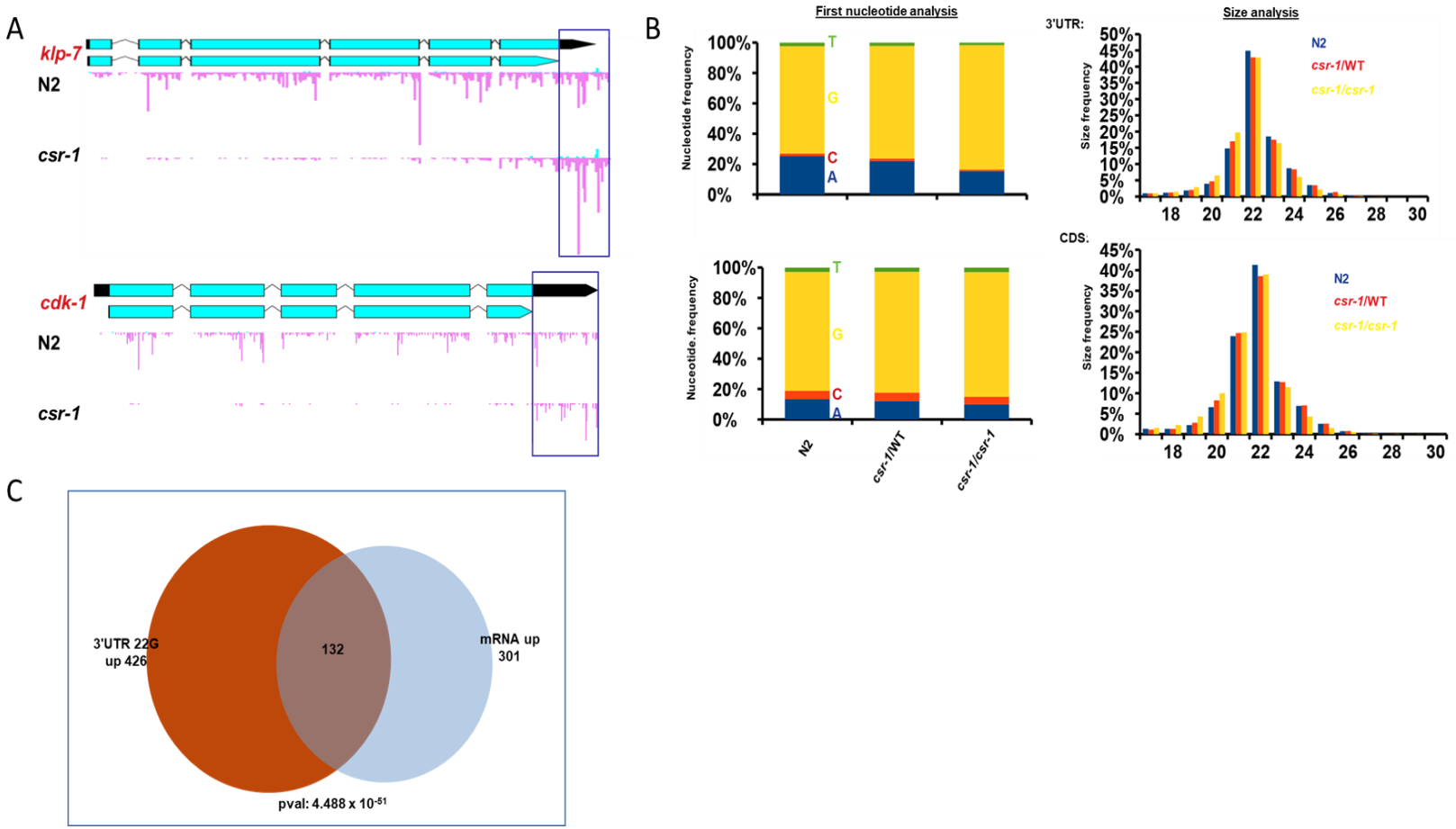

Figure 3: A) Representation of 22G small RNA reads from a genome browser on known CSR-1 dependent genes. The reads are compared between a csr-1 mutant and a N2 WT strain. The boxed area is the 3' UTR of the genes and shows a significant increase in reads in the csr-1 mutant over that of the N2 WT. B) Graphical representation of the starting nucleotide and the size of the reads from the genome browser results. Indicating that the small RNA in question is 22 nt. long and starts with a guanosine. C) Ven diagram showing the overlap between the genes that contain this 3' UTR 22G small RNA and the genes that contain upregulated mRNA in the absence of functional CSR-1 argonaute.

In previous work done by Dr. Weifeng Gu's lab within the model organism C. elegans, a novel small RNA pathway has been identified through the presence of unpredicted 22G small RNA reads being located in the 3' UTR. When the 22G small RNAs were mapped to CSR-1 target genes the downregulation is noticeable. However, when this data for the CSR-1 gene targets is split between the coding regions (CDS) and the 3' untranslated regions (UTR) a new pattern is able to be seen. The CDS matches the observed pattern from the combined graph of both the CDS and 3' UTR (Figure 2C). Where the pattern changes in when the 3' UTR is seen isolated, many of the reads now appear to be upregulated in the absence of the assumed argonaute that would regulate and load these 22G small RNA (Figure 2D). All of this data was found using high throughput sequencing of small RNA. In this previous research it was found that it was 22G small RNA reads in the 3' UTR of CSR-1 dependent genes, and these reads were not decreased in the *csr-1* mutant background (Figure 3A & B). As it was assumed that the entirety of the CSR-1 dependent genes would have reads decreased this raised many new questions about these 22G small RNAs. First, if CSR-1 is not the argonaute that is loading these 22G small RNAs, then what another germline based argonaute is? And second, since the function of the 22G small RNA is heavily dependent upon the argonaute that it is bound to, what is the function and target(s) of these 3' UTR 22G small RNAs? These are the two primary questions the following research hopes to uncover.

Methods

Growth of C. elegans

A

Nematode Growth Media (NGM)		
NaCl	3 g	
Agar	17 g	
Peptone	2.5 g	
H2O up to	975 mL	
Post Autoclave Additions	**Stock**	**per 1L of NGM**
CaCl2	1 M	1 mL
Cholesterol in Ethanol	5 mg/ml	1 mL
MgSO4	1 M	1 mL
KPO4 (for NGM)	1 M	25 mL

B

KPO4 Buffer for NGM Plates	
KH2PO4	108.3 g
K2HPO4	35.6 g
H2O up to	1 L

C

M9 Buffer	
KH2PO4	3 g
Na2HPO4	6 g
NaCl	5 g
MgSO4-7H2O	0.246 g
H2O up to	1 L

Table 1: A) Nematode growth media is the basis of all growth media used. The creation is split onto two major steps, the first being the creation of the media and then autoclaving for 45 minutes on a liquid cycle. Second involves the addition of (need to find out what each does again). B) The potassium phosphate buffer used in the NGM media post-autoclaving. C) M9 buffer that is primarily used for washing worms and resuspending the OP50 bacterial food. This buffer is autoclaved after being distributed into 500 ml Pyrex bottles.

The general maintenance and growth of C. elegan nematodes is described in detail on WormBase (3). Below are the methods, including the various tables that contain the recipes for various buffers and media, used given the tools available within the lab using the information on WormBase as a foundation.

Creation of Nematode Growth Plates

Nematode growth media (NGM) is the base for the rest of the growth media used in the study (Table 1A). This media is generally made in batches of 1L, needing to autoclaved before pouring into the desired plate. Ensure that there is a large stir bar placed within the media before autoclaving, as after the autoclaving is completed the flask containing the NGM media is placed on an Excella E24 incubator shaker and set to medium high spin settling and allowed to cool down to lukewarm temperature. Once the media is lukewarm plates can be poured. If the plates that are going to be made are not simple NGM plates (i.e. Ivermectin plates, RNAi plates, etc.) then the additional components to those plates are added right before pouring onto the plates. A single liter of NGM media can produce; ~250 35mm plates, ~25 10 cm plates, ~13 150mm. Plates are to be stored in labeled bags or boxes at 4oC. These are then used for plating the C. elegans and growing at various temperatures.

Growth of OP50 Food

A	TB Media	
	Tryptone	12 g
	Yeast Extract	24 g
	Glycerol	5 g
	H2O up to	900 ml
	Post Autoclaving	
	KPO4 Buffer (for TB)	100 mL

B	KPO4 Buffer for TB Media	
	KH2PO4	23.1 g
	K2HPO4	125.4 g
	H2O up to	1 L

Table 2: Terrific Broth (TB) media is use to grow the OP50 bacteria food for the nematodes. The creation of this broth has a pre- and post-autoclaving step with the latter requiring the addition of 100 mL potassium phosphate buffer to bring the broth up to roughly 1 L total volume.

As a means of long-term storage and ease of creating large batches of OP50 bacteria food, OP50 is frozen down as pellets and stored at -80oC. To accomplish this OP50 is inoculated onto a 10 cm LB (Lysogeny broth) agar plate, near an open flame of a Bunsen burner, and incubated at 37oC overnight to allow for proper single colonies to develop (Table 3B). A single colony from this plate is then used to inoculate 100-500 ul of TB (Terrific broth) media in the appropriately sized flask, and near an open flame to maintain sterility (Table 2A). The incubation of the flasks is done within the Excella E24 incubator shaker set to 200 rpm at 37oC until an OD (optical density) of 0.4-0.6 is achieved. Once this OD has been reached the bacterial is aliquoted into 1.7 ml microcentrifuge tubes at 500 ul per tube. These tubes are then frozen in with dry ice before they are placed into the -80oC freezer for long term storage. Each tube of 500 ul OP50 can be used to inoculate 1 L of TB media.

A

LB Media	
Tryptone	10 g
Yeast Extract	5 g
NaCl (Solid)	10 g
H2O	990 mL

B

LB Agar Plates	
Tryptone	10 g
Yeast Extract	5 g
Agar	15 g
NaCl (Solid)	10 g
H2O	990 mL

Table 3: LB media is used for growing bacteria other than OP50 food. Both require autoclaving before use A) Base LB media in a liquid state mainly used for growing bacteria overnight in a shaker. B) For growing bacteria on plates, the same media mixture is used except with the addition of agar to allow for the media to solidify in the desired plates.

Near an open flame of a Bunsen burner, pour the contents of a thawed seed tube of OP50 into the 1 L of TB media (Table 2A). Setting the Excella E24 incubator shaker to 200 rpm at 37oC, place the now inoculated TB media into the shaker overnight. Target OD of the food should be between 0.4 to 0.6 OD, and should reach it after around 16 hours of growth in the shaker. When the target OD has been reached there are two options to collect. The first option is when the OP50 is needed immediately. This will entail, near an open flame from a Bunsen burner, to pour the media into centrifuge bottles and spinning it down with a swing bucket rotor at 4500 rpm at 4oC for 10 minutes. After the spin down, the supernatant should be removed and disposed of in the biohazard waste, and the centrifugation step repeated until all the OP50 containing TB media has been pelleted into the centrifuge bottles. Using M9 buffer, and over an open flame, resuspend the pelleted OP50 and place into a 50ml conical tube (Table 1C). Once all of the resuspended pellet has been transferred to a 50ml conical tube, it is spun down using a fixed angle rotor at 4500 rpm for 10 minutes at 4oC. Remove the supernatant from the 50 ml tubes and refill with clean M9 buffer. This will clean the remaining TB media from the OP50. Store at 4oC for less than a month. The other method to collect OP50 is used if the food is not needed immediately. For this method, the OP50 containing TB media is placed in a 4oC fridge for at least 24 hours to allow the bacteria to settle to the bottom of

the 2L flask. When the OP50 bacteria have settled to the bottom of the flask, near an open flame, remove the TB solution by vacuum. TB media is removed until roughly less than 50 ml of TB media and bacteria remain in the flask; being careful not to remove too much of the precipitated bacteria in the process. The steps then mirror that of the previous collection method, what is left the OP50/TB mixture is then collected into one 50ml conical tube per 2L flask and spun down at 4500 rpm for 10 minutes at 4oC. Remove the supernatant and refill to 45 ml mark with fresh M9 buffer. Storage at 4oC for less than a month. Checking periodically to see if contaminated before use by dropping variable dilutions of the OP50 onto an empty 10cm plate let stay at room temperature overnight and then check for contamination growth using a microscope.

Immunoprecipitation

The sample for the immunoprecipitation consists of between 100,000 to 1,000,000 gravid adult worms. These worms need to be cleaned with M9 buffer (Table 1C). This is done as many times as needed to remove the bacterial debris and other possible contaminants. Once all debris has been removed from the tube containing the worms, lysis buffer (Table 4B) is added at the same volume as the worm pellet. This usually falls around 1-2 ml. Using a tissue tearor, sheer the worms at speed eight for between seven to ten minutes. Check to see if the worms have been properly sheared by placing 1 ul drop on the lid of a plate and check with a microscope for cuticles of all the worms are twisted

A

Wash Buffer		
	Stock	Final
H2O		
HEPES-KOH	1000 mM	40 mM
K-Acetate	3 M	0.15 M
MgCl2	1000 mM	2 mM
DTT	1000 mM	1 mM
Triton X-100	10 %	0.5 %

B

Lysis Buffer		
	Stock	Final
PMSF	100 mM	1 mM
Pefabloc sc	40 mM	1 mM
Add Wash Buffer to bring up to 4 mL		

Table 4: Buffer recipes used in immunoprecipitation. A) IP wash buffer. Roughly 10 to 15 ml is needed per sample. B) IP lysis buffer. The worm pellet is resuspended with equal volumes of lysis buffer. Generally, this is between 1-2 mL of lysis buffer per sample. But this recipe defaults for 4ml, so about 2 samples.

or in pieces. Centrifuge the teared samples at 200000 xg for fifteen minutes at 4oC. The supernatant is collected into a fresh tube avoiding the pellet as that contains the sheared cuticles.

A PVDF (Polyvinylidene Fluoride) low binding filter is used to clean the sample further. Ensure that the syringe is firmly attached to the filter to prevent the likelihood of the sample from leaking. A Bradford assay can be performed to quantify the protein, however the average yield for one million adult gravid worms is 20 mg of protein. For the remainder of the protocol, one to two mg is required.

Clean 40 ul of anti-FLAG M2 magnetic beads, from Sigma, by washing them with the wash buffer three times (Table 4A). When the beads have been cleaned the protein is added to them and allowed to rock at 4oC for an hour and a half in parafilmed tubes. The beads are then resuspended in 100 ul of water and 1.5 ul of 20 g/l glycogen. Add 100 ul of neutral phenol to each of the sample tubes and vortex those tubes for two minutes. Taking 1.7 ml microcentrifuge tubes centrifuge grease is added to the tubes using a 200 ul pipette tip; doing this for as many tubes as needed. Spinning down these tubes on burst in a centrifuge to allow the grease to settle on the bottoms of the tubes. The

supernatant of the tubes containing the beads is added to the tubes containing the grease. Tubes are then spun down at 12000 rpm for four minutes at room temperature. There will be three phases when the spin is complete, the top phase of the sample is collected into a new 1.7 ml tube. Samples are then precipitated with a 1:10 3M NaAc and 1 - 1.2 volumes of isopropanol. Ensure that the tubes are mixed well and have them place them in -20oC freezer for a minimum of thirty minutes. Samples are then spun down in a 4oC fridge for fifteen minutes at 13000 rpm. The pellet is cleaned with 75% ethanol and then resuspended in 10mM Tris-HCl. The samples can then be used in downstream experiments.

Creation of Mutant Strains

Days before the actual cross begins a male stock should be created. Collecting the OP50 bacterial food from a small 35mm nematode NGM plate and placing it in the center of a new 35mm plate that previously did not have OP50 bacteria food. Using an unused 35mm plate, that has also been previously been dropped with OP50 food, decreases the chance of the worms migrating away from the center and instead near the streaks of OP50 that will form due to the collection of OP50 to the center. Taking the initial parent strain that is meant to be used as males in the first cross, take as many male worms as can be found on the initial growth plates and re-plated onto the new NGM plate with the food placed in the center. From the same strain that the males were collected from, plate 1.2x as many young adult hermaphroditic worms onto the same plate. When the hermaphrodites left alone the rate of males produced is close to 1:1000 in a lab setting,

but in the environment containing this higher population of males the rate is closer to 1:2. This plate is considered a male stock and will help in collecting enough male worms for the beginning of the cross.

The two strains that will be used in the cross are to have been grown onto between ten to twenty small 35mm NGM plates before use in the cross. Though more worms can be grown on larger plates, like 150mm and 10cm plates, these 35mm plates make it significantly easier to search for the male worms and properly aged hermaphroditic worms. To begin the cross, another 35mm plate has its OP50 bacterial food collected towards the center of the plate just as is done for the male stock plates. From the male stock, created in previous days, add as many males as can be found on the plate with the centrally placed OP50. Following the addition of the male worms, 1.2x young adult hermaphroditic worms are added. The hermaphroditic worms should not be from the same strain as the males, instead should be the other strain in the cross. Any strain that contains a mutation that impairs movement (i.e. *unc, dpy*, etc.) should be used as the hermaphrodites. If those strains are used as the males, which would be required to move to reproduce with the plated hermaphrodites, the chance of a successful cross will be drastically reduced. This process of plating males and then 1.2x the number of hermaphrodites continues until the target strain is believed to be reached.

Brood Size

Grow the selected worm strains until they are at larval stage 4 (L4). This would mean that the hermaphroditic worms are almost at the adult stage before they have a fully developed vulva and have yet to begin laying eggs. Single ~25 of the L4 stage hermaphrodites onto numbered 35mm plates containing the appropriate growth media. Allow the worms to stay on the plates for a full 24 hours. When the 24 hours is up, re-plate the adult hermaphroditic worms onto new numbered 35mm plates. Save the plates from the previous day along with the new plates, and wait another 24 hours. The next day, record the amount of hatched larval stage 1 (L1) hatched worms and the amount of unhatched eggs on the original numbered plates the hermaphrodites were singled onto. Just like the previous day, re-single the hermaphroditic worms onto another set of new numbered 35mm plates while keeping the previous plates for counting, wait for another 24 hours. When the 24 hours are up, once more count and record the number of hatched L1 worms and unhatched eggs from each numbered plate. The singled hermaphroditic worms are also moved to another set of new numbered 35mm plates, with the previous numbered plates being saved for counting the next day. Wait for another 24 hours, after this wait the worms should have stopped laying eggs all together so the plates containing the worms now should only contain the hermaphrodites after the 2 hours. When the last 24-hour wait is finished, count the amount of L1 worms and unhatched eggs are on the numbered plates. Also, the plates containing the singled hermaphroditic worms should be checked for any eggs, if there are then the process should be continued for another day.

Ribosomal Profiling

The protocol for the ribosomal profiling was provided by Dr. Yonghui Zhao, a post-doctoral researcher in Dr. Xuemei Chen's lab. Slight modifications have been made to accommodate for the use of C. elegans instead of Arabidopsis (10). Mainly in the area of sample preparation and analysis of the produced graph. Below goes through the protocol detail as done within Dr. Weifeng Gu'slab.

Sucrose Gradients

% Sucrose	2M Sucrose	10X Sucrose Salt	H2O	Cycloheximide	Chloramphenicol	Volume/Gradient
60.00%	8.8 ml	1 ml	0.2 ml	1 ul	1 ul	0.75 ml
45.00%	9.9 ml	1.5 ml	3.6 ml	1.5 ul	1.5 ul	1.5 ml
30.00%	6.6 ml	1.5 ml	6.9 ml	1.5 ul	1.5 ul	1.5 ml
15.00%	2.2 ml	1 ml	6.8 ml	1 ul	1 ul	0.75 ml

Table 5: Recipe for the different layers of the sucrose gradients. This makes nine discontinuous sucrose gradients that need to be stored at -80oC.

Nine sucrose gradients are created after each run of the protocol. These sucrose gradients are considered discontinuous gradients due to the concentration of sucrose being nonconsecutive (i.e. 15%, 30% to 45% instead of 10%, 11%, and 12%). Due to their thinner walls, polypropylene centrifuge tubes are used and pre-cooled in a -80oC freezer. The bottom 60% sucrose layer is created in a 50ml conical tube while on ice and is vortexed to ensure that it is thoroughly mixed (Table 5). Using serological pipettes, add the solution slowly to the centrifuge tubes. If the solution is added too quickly bubbles will form in the sucrose layer which will cause the fraction collector to register "spikes" on the graph created through the Brendel software. Placing a sheet of parafilm over the

Polysome Extraction buffer (PEB)

A

	stock	final	8 ml/Rx
Tris-Hcl, pH 7.5	1 M	0.1 M	0.8 ml
Kcl	3 M	0.2 M	0.533333 ml
EGTA, pH 8.0	0.5 M	0.025 M	0.4 ml
MgCl2	1 M	0.035 M	0.28 ml
Detergent Mix	20 %	1 %	0.4 ml
c0mplete Cocktail	25 X	1 X	0.32 ml
DTT	1 M	0.005 M	0.04 ml
Cycloheximide	50 mg/ml	0.05 mg/ml	0.008 ml
Chloramphenicol	25 ug/ul	0.05 ug/ul	0.016 ml
ddH2O			5.202667 ml

Sucrose Cushion Solution

B

	stock	final	8 ml/Rx
Tris-Hcl, pH 9.0	1 M	0.4 M	3.2 ml
Kcl	3 M	0.2 M	0.533333 ml
EGTA, pH 8.0	0.5 M	0.005 M	0.08 ml
MgCl2	1 M	0.035 M	0.28 ml
Sucrose		1.75 M	4.8 g
DTT	1 M	0.005 M	0.04 ml
Cycloheximide	50 mg/ml	0.05 mg/ml	0.008 ml
Chloramphenicol	25 ug/ul	0.05 ug/ul	0.016 ml
ddH2O		Add up to	8 ml

Resuspending Buffer

C

	stock	final	2 ml/Rx
Tris-Hcl, pH 9.0	1 M	0.2 M	0.4 ml
Kcl	3 M	0.2 M	0.133333 ml
EGTA, pH 8.0	0.5 M	0.025 M	0.1 ml
MgCl2	1 M	0.035 M	0.07 ml
DTT	1 M	0.005 M	0.01 ml
Cycloheximide	50 mg/ml	0.05 mg/ml	0.002 ml
Chloramphenicol	25 ug/ul	0.05 ug/ul	0.004 ml
ddH2O			1.280667 ml

Table 6: Recipes for the buffers needed through the ribosomal profiling protocol. These are all to be made the day before the protocol. DTT, c0mplete cocktail, cycloheximide, and chloramphenicol are added right before the use of the buffer in the protocol.

openings of each of the centrifuge tubes, place them back into the -80oC freezer for an hour. During this hour the next layer is created, 45% sucrose, and is left on ice until the hour is finished (Table 5). Once again using a serological pipette, slowly add the 45% sucrose solution to the centrifuge tubes that should now have a frozen bottom layer. Cover the centrifuge tubes again with parafilm and place into the -80oC for another hour. The creation of the 30% sucrose layer is then created in this time as was done with the 45% sucrose solution (Table 5). The same process is repeated until the final 15% sucrose solution is created and poured (Table 5). The sucrose gradients can be stored indefinitely in the -80oC freezer. Due to the preparation time of these gradients, they are prepared at least a day ahead of the actual ribosomal profiling protocol.

Sample Preparation and Fraction Collection

Preparation for the ribosomal profiling begins with the growth of the mutant strain. Ensuring that the proper growth media is being used for the specific strain, grow 650,000 or more young adult stage worms. The stage is critical, at this point in their development the worms have yet to start producing eggs which means that the rRNA (ribosomal RNA) collected at the end of the experiment is collected solely from the adult worms and not the next generation. These young adult worms are then cleaned by spinning them down in a 15 ml conical tube at 4000 rpm for 30 seconds to a minute until the solution is clear containing only the worms. If this is not enough than the worms can be precipitated once again, using M9, until the solution is clear (Table 1C). To do this, add M9 to the 10 ml mark on the 15 ml conical tube and vortex. Allow the worms to settle to the bottom of the tube before carefully vacuuming out the M9 buffer, ensuring not to take up any of the worms. Repeat this process until the solution is clear. As with the importance of the development state of the worms, cleaning the worms ensures that no bacterial contamination will be present during the collection of the rRNA.

After collecting and cleaning the worms the next step is to lyse the worms. Remove as much of the M9 buffer from the 15 ml tube as possible and replace it with 8ml of the polysome extraction buffer (PEB)(Table 6A). Ensuring that the tube stays enclosed in ice, tear the worms for seven minutes on speed eight. Check under a microscope to see if the cuticle of the worms has been complete sheared, if not continue to tear until the worms are sheared. If this is not done, then the yield of rRNA will be too

low to proceed with the final steps of the protocol. To remove the cuticle and other unwanted particulate from the solution centrifuge the 15ml tube at 16000 xg for 10 minutes. Transferring the supernatant to a new 15ml tube, centrifuge again at 16000 xg for 10 minutes. During each of these steps a pellet should form, do not disturb this pellet as it contains the cuticles and other debris.

While on ice, add 8ml of the sucrose cushion to 2" polypropylene ultracentrifuge tubes (Table 6B). This solution is very viscous, add this solution slowly to ensure that no bubbles form on the cushion. Take the cleaned worm lysate and add it to the tubes contain the sucrose cushion. Using the remaining PEB, balance the tubes. Load the tubes into a Type 70TI fix angle rotor making sure that there is a clear mark on the lids of the tubes pointing towards the outside of the rotor to indicate which side of the tube will contain the pellet, and then load into the Beckman L8-70M ultracentrifuge. Setting it for 50000 rpm at 4oC for three hours. During this time, place the discontinuous sucrose gradients from the -80oC storage into a 37oC incubator for 45 minutes to an hour, this is meant to thaw them for use in the next steps. When the gradients are thawed, making sure to not disturb the gradients, place them into a 4oC fridge until they are needed.

When the first round of ultracentrifugation is completed, remove the tubes from the rotor and make sure that the mark on the lid is still facing towards the outside of the rotor. Check the side of the tube matching the mark on the lid for the pellet. The pellet is likely to be translucent and faint but should be slightly visible under direct light, circle

the pellet clearly on the tube. Vacuum the remaining PEB and sucrose cushion out of the tubes making sure to avoid the pellet. Take 1ml of di-water using a micropipette to clean the sides of the tubes and around the pellet. To wash around the pellet, hold the plunger still while trapping water between the pipette tip and the inside wall of the tube, slowly move the trapped water around the perimeter of the pellet. Do not release any water over the actual pellet or it will begin to dissolve and the sample will be lost. After completing this cleaning procedure two to three times, use the resuspension buffer to dissolve the pellet (Table 6C). The starting number of worms dictates the volume of buffer used in this step. If the number of worms is on the lower end of the scale use 400ul of buffer, if on the higher end then use 1 ml. This will ensure the OD260 will be in a reasonable range for loading onto the sucrose gradient. To calculate the OD260 for the sample take 1ul of the sample for use in a nanodrop, DeNovix DS-11 Spectrophotometer, and record the value of A260 that it provides. Take 5000OD and divide it by the OD260 that the nanodrop provided to get the volume of your sample you need to load onto the sucrose gradient. If this volume is greater than 400 ul than too much resuspension buffer was used. Slowly add the proper volume of the sample to the labeled sucrose gradient to ensure once again that there are no bubbles. Balance these gradients so there is no difference between any of them. Load the gradients into a SW55TI swing bucket rotor and then into the ultracentrifuge. Spin the gradients at 50000 rpm for 1.5 hours at 4oC.

After the ultracentrifugation of the sucrose gradient they should be immediately placed on ice while still within the swing buckets holders for the tubes ensuring that the

samples do not spill into the ice. The sucrose gradients are then loaded into a Brandel sucrose fraction collector. It will push fluorinert FC-40 oil through the bottom of the sucrose gradient containing the samples through the machine which will then use an attached UV spectrophotometer to detect the A260 which is then displayed in graphical form using software provided by Brendel. While this is happening, the fraction collector is distributing the sample in a set volume into 1.5ml microcentrifuge tubes dropwise. Collect these numerous tubes and place them on ice. Store them temporarily at -20oC, but for long term storage -80oC. The tubes containing the polysome are then pooled by taking 1/3rd of each tube into a new tube and proceeding to precipitate the sample using isopropanol. The resuspended samples are then able to be used in downstream protocols.

Small RNA and mRNA Cloning

Starting with the total RNA for small RNA and pelleted RNA for the mRNA (messenger RNA), the cloning protocols begin with 3' ligation. Just like with starting material, the common mixture for the 3' ligation changes dependent upon whether small RNA or mRNA is being cloned. Small RNA differs through the use of PEG-8000 (Polyethylene glycol 8000) instead of DMSO (Dimethylsulfoxide), which is used in mRNA cloning, and the addition of a catalytically dead pir-1 protein (Table 7A). The mixture in both cases needs to be mixed well but especially in the case of the small RNA due to the viscous PEG-8000, the small RNA samples need to be watched while mixing to ensure that they are all mixed completely. DMSO and PEG-8000 is used to slow down

A

	stock	final	10 ul
H2O			1.91003 ul
Buffer noATP	10 X	1 X	1 ul
PEG-8000	50%	25%	5 ul
Purified 165*	20 uM	0.25 uM	0.125 ul
RNL2	20 uM	0.25 uM	0.125 ul
pir-1	20 uM	0.25 uM	0.125 ul

B

	stock	final	20 ul
ATP	20 mM	0.5 mM	0.5 ul
Purified RNA164	20 uM	0.2 uM	0.2 ul
Lig 1	20 uM	0.125 uM	0.125 ul
H2O			8.675 ul

C

	stock	final	20 ul
dNTP	10 mM	0.5 mM	1 ul
RT dilution buffer	10 X	1 X	2 ul
DTT	100 mM	5 mM	1 ul
SSII	20 uM	0.125 uM	0.125 ul

D

	stock	final	50 ul/Rxs
H2O			37.25 ul
PFU buffer	10 X	1 X	5 ul
TMAC	1000 mM	15 mM	0.75 ul
dNTP	10 mM	0.1 mM	0.5 ul
wg239	10 uM	0.1 uM	0.5 ul
wgnnn	10 uM	0.1 uM	0.5 ul
cDNA			5 ul
pfu	100 X	1 X	0.5 ul

Table 7: Recipes for the various steps of the small RNA cloning A) 3' ligation, step 1 of the cloning procedure. B) 5' ligation, step 2 C) Reverse Transcription (RT), step 3 D) PCR amplification of mRNA, step 4.

the movement of the molecules in the solution and help the RNA ligase bind to its target and increase the reactions efficiency. When the samples are as homogenized as possible allow them to sit at room temperature for between two to three hours. During this incubation the activated linker is bound to the 3' end of the target RNA. This linker is considered activated due to the adenosine phosphate group located at its 5' end, this allows for a phosphate anhydride bond between the two pieces of genetic material to form. The use of RNA Ligase II (Rnl2) prevents the formation of self-ligation of the primers and higher efficiency over that of RNA ligase I.

This incubation at room temperature is followed by another incubation at 65oC for ten minutes. After this the addition of 0.5 ul of a DNA oligo meant to bind to the remained of the free-floating activated oligo from the 3' ligation step. This will prevent the T4 RNA ligase 1 from using the activated linker from the previous steps. The concentration of this oligo is different between the two versions of cloning, 5 uM for small RNA and 10 uM mRNA. Another incubation at 65oC for five minutes follows the

addition of the oligo. Cool the samples back down to room temperature by 0.1oC per second. These incubation steps are completed using a thermocycler to allow for precise control for cooling down.

When the samples have been cooled back down to room temperature the next common mixture is made on ice and is meant for the 5' ligation step (Table 7B). This common mixture is the same for both the small RNA and mRNA cloning protocols. ATP is added to this reaction for the addition of the adenosine phosphate to the 5' end of the target. This adenosine phosphate is then the target of the RNA oligo creating another phosphate anhydride bond. An RNA oligo is used over the DNA oligo for the use in the next step of reverse transcription. When the common mixture is finished 9.5ul of this mixture is added to each of the samples. As with the 3' ligation, mix as close to homozygous as the solution allows. This is then left out for two to three hours at room temperature.

Next the samples are to be converted to DNA. This is accomplished through reverse transcriptase, otherwise simply known as RT. Using the RNA oligo that was recently bonded to the 5' end of the target material the superscript II enzyme will use as a starting point to transcribe the newly formed DNA product. The common mixture is made the same way between the two versions of the RNA cloning (Table 7C). Adding 4.125 ul of the common mixture into each of the samples and incubating them in a

thermocycler for 30 minutes at 42oC followed by 85oC for five minutes and finally a holding step at 4oC.

The final step is polymerase chain reaction (PCR). Due to the identical ligated ends the chance for the formation of botched products is higher in this PCR reaction than in a normal PCR reaction. To work around this 0.1 uM of oligos (these oligos are represented as wg239 and wgnnn in Table 7) is used during the first sixteen cycles of the reaction. Using less oligos at the beginning of the reaction decreases the formation of botched products and still allows for the actual target to gain a higher concentration in the sample(s). An additional 0.5 uM of oligos is added to the reaction(s) for the final two cycles. Given the higher concentration of the target at this point in the reaction means that the added oligos will have a higher chance to bind to the target and amplify them over the botched products. Important to this step is the DNA oligo that is used as a barcode for the individual sample to help identify the reads later on during high throughput sequencing. Each oligo is a unique nucleotide sequence that binds to the end of the products, and one of these is used for each sample ensuring there is no overlap between samples. Each sample may be run multiple times through this step to help amplify the sample to a level that can be used downstream in throughput sequencing (Table 7D). To check for this, each sample is run on an 8% native PAGE gel to compare their relative intensities to that of the other samples in that specific set. Based on the intensities one or more PCR runs of a given sample may be done to get them all to have roughly the equivalent amount of genetic material.

mRNA Sample Preparations:

Ribo Zero

Starting concentration of total RNA should be around 0.1 g/l, and will go through two initial protocols before mRNA cloning. The initial preparation step is RiboZero, also known as Ribominus. This process is designed to cleave the different subunits of rRNA into smaller, ~50 nt, pieces to help mitigate their possible interference with the mRNA cloning downstream.

A

	stock	final	20 ul/Rx
ce rRNA oligo	1 g/l	0.06 g/l	1.2 ul
ce rRNA oligo 2	1 g/l	0.06 g/l	1.1 ul
Annealing buff.	10 X	1 X	2 ul
H2O	g/l	g/l	12.9 ul
N2 inp (Total)	2.45 g/l	0.1 g/l	0.82 ul

B

	Stock	final	20 ul/Rx
H2O			3 ul
10X P1 buffer	10 X	1 X	2 ul
RNA	1 X	0.75 X	15 ul
P1	0.02 U/ul	0.000625 U/ul	0.625 ul

Table 8: A) Reagents needed for the completion of the Ribozero protocol. The ce rRNA oligo 1 and 2 are DNA oligo sets that have been designed to target the 26S, 18S, 5S and 5.8S ribosomal subunits. 10X annealing buffer is made with 500 mM Na3Citrate, pH 7.0, 100 mM MgCl2 B) Reagents needed for the creation of the reaction solution for the P1 partial digest. 10X P1 buffer is made with 0.5M Na3 citrate pH7, MgCl2 0.1M.

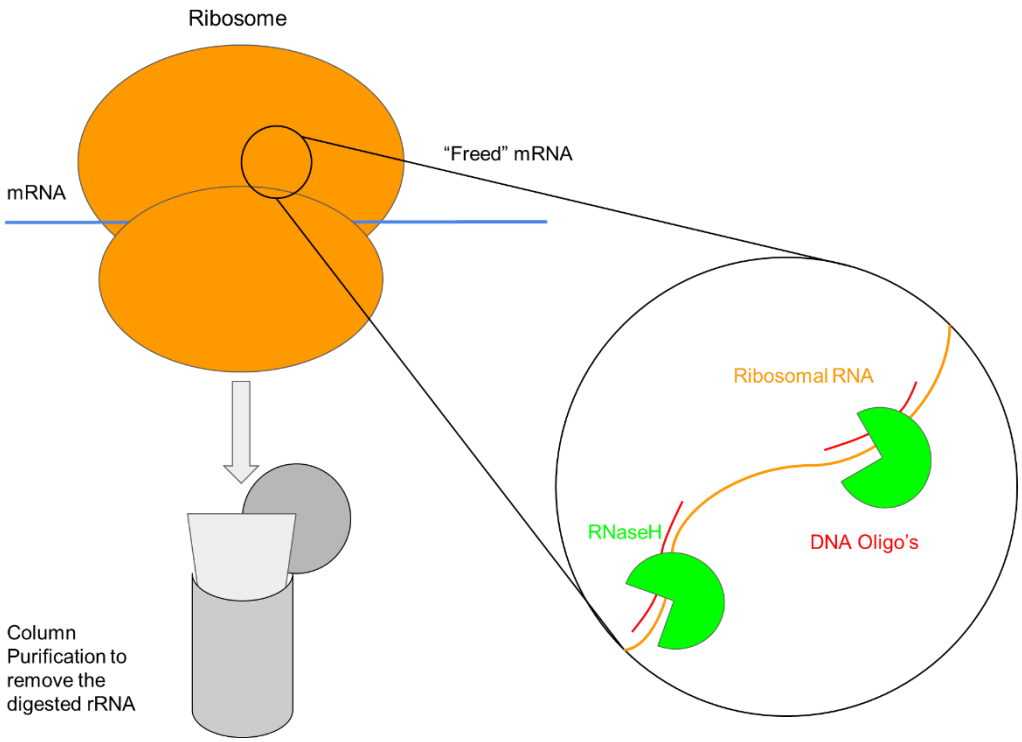

Figure 4: Animated simplified representation of the mRNA cloning sample preparation step, Ribozero. The polysome from samples collected post polysome profiling go through this process to remove the bound ribosome and free the mRNA they are bound too. The column purification portion leads to the final sample preparation step, P1 partial digest.

MirVana lysis/binding buffer, parts of an industry phenol-based miRNA (micro RNA) isolation kit, should be placed at room temperature before beginning the protocol. On ice, prepare the sample by following the recipes in Table 8A. The ce rRNA oligos 1 and 2 are premixed DNA oligo sets that have been designed to target the 26S, 18S, 5S and 5.8S ribosomal subunits. Once this solution has been mixed thoroughly, heat it to 95oC for 2 minutes and then cooling the sample down to 30oC by 0.1oC every second using a thermocycler. This should help to degrade the rRNA enough to allow the oligos enough space to bind to their intended targets. When the sample has been cooled to 30oC add 2uM of RNase H (Ribonuclease H) to each tube, samples should now be placed at

room temperature for the remainder of the protocol. RNase H is an enzyme that targets the hybrid molecule of RNA binding to DNA. This is important, as at this point the DNA oligo should be bound to the rRNA subunit at 50 nt intervals, so only these molecules should be cut and not the mRNA still found in the samples. Incubate the samples at 37oC for 50 minutes and add 1 ul of 10 U/ul CIP (Alkaline Phosphatase, Calf Intestinal) followed by another 20 minutes at 37oC, however the addition of CIP does not seem to affect the overall process so may be skipped and simple incubate for a full hour with only RNase H (data not shown). When the incubation is complete immediately move onto the next step, which entails column purification. Following the incubation, 100 ul of the mirVana lysis/binding buffer is added to each of the samples, mixing well. Allow the samples to then stay at room temperature for five minutes. This is then followed by the addition of 12 ul of 3M NaAc (Sodium Acetate) pH 5.2 and 34 ul of 100% ethanol and mixed well without the use of a pipette. Using a pipette may lead to the shearing of the mRNA to a degree that makes it useless in downstream applications. Transfer the samples onto RNA tini columns, ensuring not to disturb the filter at the bottom. These columns will help to isolate the RNA from other possible contaminants in the original sample. Spin the samples in a centrifuge at 10000 rpm for 1 minute and dump the flow through. Using 500 ul of 80% ethanol, wash the samples twice spinning them down in centrifuge at 10000 rpm for a minute for each wash. After removing the flow through from the last wash, centrifuge the now liquid free columns at 10000 rpm for two minutes to dry the columns. Replace the collection tubes from under the columns with fresh 1.7ml centrifuge tubes. Adding 18 ul of 95oC 2.5 mM Tris pH 8, gently to each of the columns

to ensure that the column is not disturbed. Wait for one minute at room temperature before spinning the samples at 10000 rpm for two minutes. Placing the samples on ice, the next preparation step is prepared.

P1 Partial Digestion

For this protocol the entirety of what was collected from the columns in the previous protocol will be used. Now due to volume loss in the column the collected total volume is actually closer to 15 ul instead of the expected 18ul, so that will be the volume used to do the calculations for this protocol. Add the mixture indicated in Table 8B and allow to incubate at 60oC for 10 minutes in a thermocycler. The addition of P1 is meant to digest the mRNA in the sample to slightly smaller length, but not to completely digest them. The size of the mRNA directly affects the efficiency of the mRNA cloning protocol, the protocol favors the smaller pieces over larger. So partially digesting the mRNA helps to increase the efficiency of the mRNA cloning protocol. Immediately the samples should be placed onto ice and 1ul of 0.5 M EDTA (Ethylenediaminetetraacetic acid) is added. Next is a phenol chloroform extraction. Using a pipette tip, place a pea size amount of centrifuge vacuum grease into a 1.7ml centrifuge tube, this is done once per sample and is referred to as a phase lock column. Add 80 ul of H2O and 2ul glycogen to the sample(s), mixing well before the addition of 100 ul acidic phenol/chloroform. This mixture is than added to the phase lock column(s). Spin the columns at 12000 rpm for 4 minutes at room temperature. After this spin down three phases should be visible. The top phase is a liquid that will be collected to continue to isopropanol precipitation. In

the middle should be the vacuum grease, and the final phase should be the phenol and other unwanted materials from the sample. The top phase should be collected into a fresh 1.7ml centrifuge tube and have 10ul of 3M pH 5.2 NaAc and 80ul isopropanol added. Mix the solution well by inverting the tube multiple times and gently finger vortexing. Place he sample(s) into a -20oC freezer for at least 30 minutes. When the samples have been in the -20oC for the minimum 39 minutes, they are centrifuged for 15 minutes at 12000 rpm at 4oC. A white pellet should form at one of the bottom sides of the tubes. The supernatant is then removed and the pellet is washed with 1mL of 75% ethanol. The pellet is resuspended with 10mM pH 7.5 Tris buffer between 10-15ul. This volume is dependent of the size of the pellet, the larger the pellet the larger the volume to resuspend. Next the samples are gel purified to attempt to remove as much of the rRNA from the samples as possible. Load the samples unto a 10% Urea PAGE gel using a two-dye loading dye, xylene cyanol and bromophenol blue. Loading alongside the samples is a lab made total RNA ladder. Two pieces are cut out of the gel for each sample, one between 75 bp and 165 bp and the other between 165bp and 300bp. Both pieces, per sample, are gently dried on a Kimwipe and placed into a 1.7ml centrifuge tube. Spin down the tubes on burst in a centrifuge for a few seconds to get the gel pieces to the bottom. Using a 1 ml micropipette tip, grind the gel pieces against the walls of the centrifuge tube. When the gel pieces have become very fine, 750 ul of TE buffer is then added to each sample and the lid wrapped with parafilm and allowed to shake at room temperature overnight. The following day the sample(s) are spun down on burst in a centrifuge to have the pieces of the gel reach the bottom of the tubes. The supernatant is

then collected into a new 1.7 ml tube. Ensuring that all liquid is collected, while rotating the pipette so that the tip is against the wall of the tube, gently lift off the plunger to collect the liquid that is still with the gel pieces at the bottom of the tube. Depending upon the volume collected from the previous tube, ~75 ul of 3M NaAc, 1.5 ul of 20 ug glycogen and ~800 ul isopropanol is added to the samples. The tubes should be just about filled at this point so it is essential to work slowly as to not spin any of the mixture. Mix by inverting multiple times and finger vortexing. Allow the sample(s) to stay in a -20oC freezer for a minimum of 30 minutes followed by being centrifuged for 15 minutes at 12000 rpm at 4oC. A pellet should form at the bottom side of the tubes. The supernatant is removed and the pellet washed with 75% ethanol. This wash step is followed by the resuspension of the pellet with ~10 l of the 3' ligation mixture from the beginning of the mRNA cloning protocol.

Results

Identifying the Argonaute Loading the 3' UTR 22G Small RNA Using Immunoprecipitation and High Throughput Sequencing.

There are twenty-seven argonautes in C. elegans but only a handful have been discovered to work within the germline, of that handful CSR-1 and the WAGO family are known to be there (11). Even given the large number of genes these two argonautes target

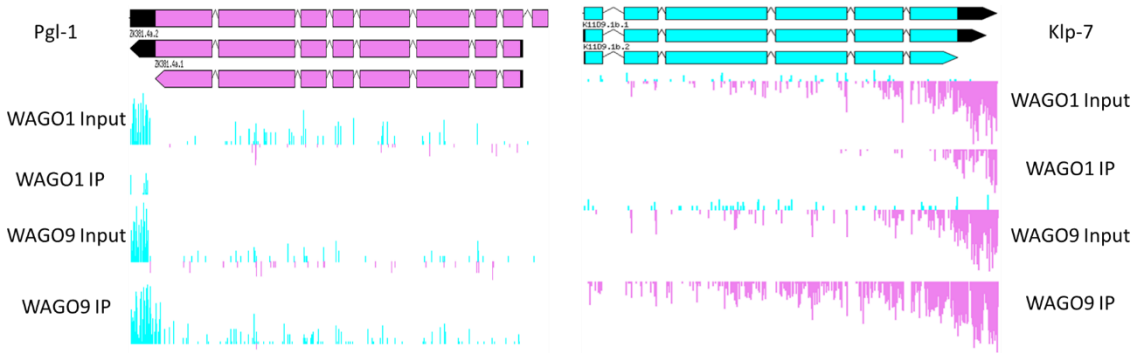

Figure 5: These two images represent the reads of CSR-1 associated gene targets. The light blue represents the Watson strand, and the pink represents the Crick strand. Both Pgl-1 and Klp-7 are CSR-1 dependent genes. The reads presented are associated with the results of the immunoprecipitation of WAGO-9 and WAGO-1 transgeneic worm strains.

the overlap between the two is significantly smaller than the total pool, with only 32 genes overlaping (data not shown). Though the WAGO family has twelve argonautes, only two were focused on, WAGO-1 and WAGO-9. WAGO-1 even has a known function within the germline localizing P-granules and gene silencing (2). WAGO-9 is also known as hrde-1 (heritable RNAi deficient), is seemingly found only within the germline of C. elegans, and abundantly expressed within these cell's nucleus (8). This argonaugte has been found to play a critical role in epigenetic silencing within the germline genome and the inheritance of these modifications through to the next generation (8, 12). As apparent with the above statements, these two WAGO argonautes are well researched and documented within the WAGO family. It is because of this that they were chosen to be used in the immunoprecipitation first before any others within the family were attempted.

Transgenic worm strains containing one FLAG-tagged transgene for either one of two chosen members of the WAGO argonaute family, WAGO 1 and WAGO9, were

grown for use in this immunoprecipitation (IP) protocol. The transgenic strains are within a *csr-1* mutant background. Meaning that these strains also do not have a functioning CSR-1 argonaute when homozygous. Lysates to these transgenic strains were collected and then had a portion of each collected lysate set aside to be used as input samples for each strain. The remainder of the lysate for each sample proceeded through the IP protocol. The 22G small RNAs that were collected from these pulled down loaded argonautes should reveal which of the two, if any, load these 22G small RNAs when in the absence of csr-1 argonaute.

Using known CSR-1 dependent genes, Klp-7 and Pgl-1, the 22G RNA reads were analyzed through a genome browser (Figure 5). It was noticed that the coding regions are void of 22G small RNA reads, which is consistent with the csr-1 homozygous mutant background. CSR-1 argonaute loads 22G small RNAs that was created using the CDS of its dependent genes. This lack of CDS reads is consistent between both the WAGO-1 and WAGO-9 transgenic samples. However, the difference between the two samples, WAGO-9 and WAGO-1, is noticed not in the CDS but in the 3' UTR. Comparing the input to the experimental WAGO-1 sample shows no amplification to the 3' UTR (Figure 5). The WAGO-9 input looks like the WAGO-1 input but the changes show in the experimental sample. Reads in the 3' UTR of the WAGO-9 sample are upregulated at least two-fold over that of the input sample.

The outcome of this IP answered the first of many questions regarding this new 22G small RNA family, what argonaute is the 22G small RNAs being loaded onto? Taking the analysis all together shows that WAGO-9, not WAGO-1, likely loads these new 22G RNAs. This is apparent due to the noticeable enrichment seen in the 3' UTR regions from the experimental reads over that of the input reads for WAGO-9 sample from within these CSR-1 dependent genes (Figure 5). So as WAGO-9 was being pulled down it was loaded with members of this novel 22G small RNA family.

C. elegan *csr-1 wago-9* and *csr-1 alg-5(ram2)* Mutant Strain Crosses

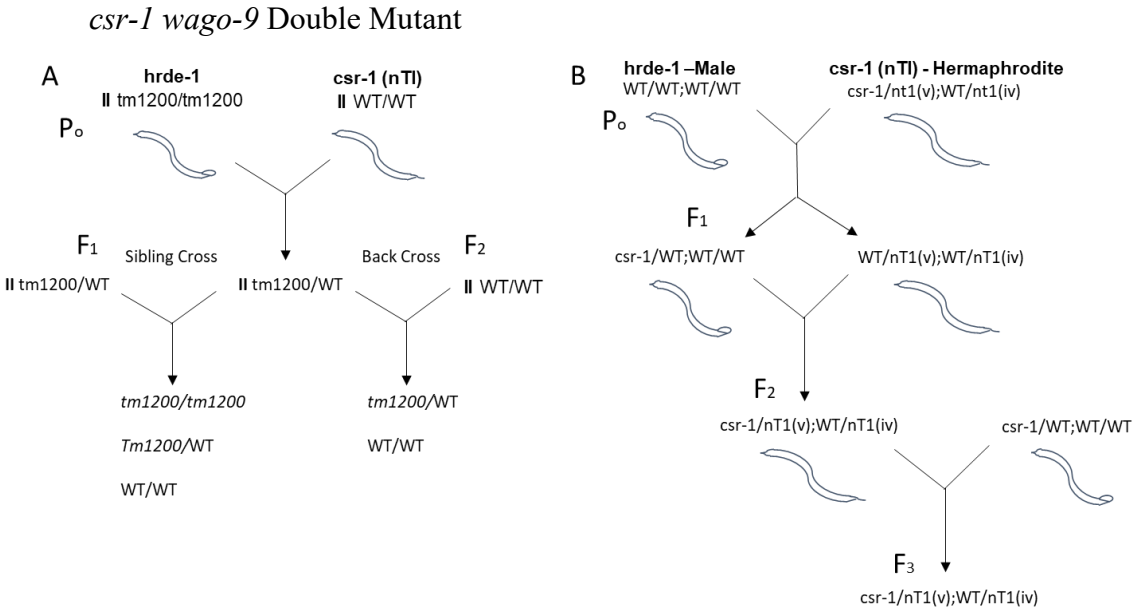

Figure 6: Simplified figure representing the creation of the csr-1 hrde-1 (wago-9) double mutant. A) The cross from the viewpoint of the second chromosome, where the hrde-1 mutant loci are located. B) The cross from the viewpoint of the csr-1 mutation which involves both the fourth and fifth chromosome due to the needed nT1 balancer.

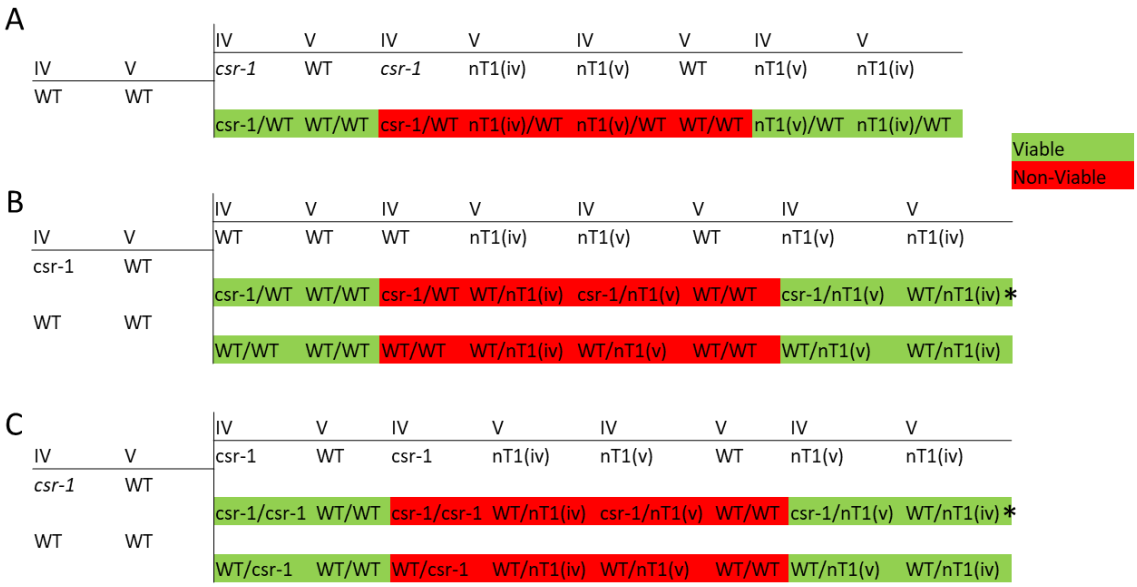

Table 9: Progeny marked with the asterisk (*) is what is used in the next crossing step. A) The progeny of the initial cross between the csr-1 balanced and hrde-1 (or wago-9) mutant worms. B) Progeny results of a sibling cross between the two viable progenies of the initial cross. C) Progeny results of the backcross using the selected strain from the previous sibling cross.

Seeing as the CSR-1 argonaute was found to not be loading the 3' UTR originating 22G small RNAs the argonaute that actually loads these small RNAs needed to be found. Previous immunoprecipitation work had found that it was in fact the WAGO-9 argonaute that loads these 3' UTR 22G small RNAs (Figure 5). This result lead to the decision to use a WAGO-9 mutant in this cross over a WAGO-1 mutant. This double mutant containing both *csr-1* and *wago-9* deletions will be used to collect and then sequence the mRNA and small RNA, and then observe the strain for any newly developed phenotypes.

This cross begins with a *hrde-1* (*wago-9*), which is represented as tm1200 in Figure 6, homozygous mutant and a *csr-1* nT1 balanced heterozygous mutant. Viewing

from the second chromosome, the initial cross leads to the progeny being heterozygous for the *wago-9* deletion mutation. The cross then continues with a sibling cross, followed by a back cross. With three fourths of the progeny containing at least one copy of the tm1200 mutation in the sibling cross and half of the progeny containing at least one copy after the back cross. Taking the same cross and viewing it from the fourth and fifth chromosome shifts the focus from the *wago-9* (tm1200) deletion mutation to the *csr-1* balanced mutation. The first cross between the *wago-9* homozygous mutant and the heterozygous *csr-1* mutant results in only 50% of the progeny being viable (Table 9A). This is due to the balancer with in the *csr-1* mutant strain containing a WT (wild-type) copy of either a portion of the fourth chromosome, located on the balancer on the fifth chromosome, or a portion of the WT fifth chromosome, located on the balancer on the fourth chromosome. This extra copy of either chromosome when in conjunction with a strain that already has WT copies of these chromosomes causes a trisomy to form and a non-viable worm. The two viable progenies are then crossed together in a sibling cross. The progeny that is not exhibiting the *unc* phenotype is used as the male for this cross and contains a copy of the *csr-1* deletion mutation (Figure 6). While the *unc* exhibiting progeny contains the balancer and is used as the hermaphrodite (Figure 6). The resulting progeny of this cross also suffer from 50% viability due to the balancer. Of the remaining progeny 50% contain the *unc* phenotype, and only half of those *unc* worms contain the wanted genotype (Table 9B). However, checking for the genotype at this stage is impractical as it would consume the worm. So, all the hermaphroditic worms exhibiting

the *unc* phenotype were collected and backcrossed with a male from the initial cross (Figure 6). This backcross helps to clear any background mutations that may have formed during the cross and "cleaning" up the strain. The resulting *unc* expressing progeny are singled onto new 35mm NGM plates and subjected to further screening using PCR (Table 9C).

Through the use of various genotyping protocols for the *csr-1* and *wago-9* mutant alleles, the possible newly created mutant strains were tested. Mutant deletion allele tm1200 for the WAGO-9 argonaute is not essential for the development of the worms so its existence as a homozygous mutant is tested. The *wago-9* homozygous mutant seemed to grow and propagate at a higher rate than that of the N2 WT worm strain, though this has only been observed anecdotally not tested as of yet. The *csr-1* deletion mutant on the other hand creates non-viable offspring when it is homozygous in the current generation. Due to this, the *csr-1* mutant should exist in the new strain in a heterozygous form paired with a balancer. As with the previous *wago-9* deletion allele, the genotype is determined through a PCR protocol. Though unlike the previous deletion allele, the *csr-1* deletion allele is expected to be heterozygous. All of these expected double mutant strains are created within a background of ivermectin resistance. Ivermectin resistance is made up of three homozygous deletion mutations that must all exist within the strain for it to have the resistance. The three genes that are to be deleted are *avr-14, glc-1* and *avr-15*. To test for the presence of these three mutants the strains that passed the two genotyping experiments were plated onto small 35mm ivermectin plates and allowed to grow. These

plates were checked daily for the worm's survival. This ivermectin screening process usually only lasts between 24-48 hours. When the screening of the possible new mutant strains was completed, four were discovered to be the correct genotype. These four new strains had phenotypes similar to that of heterozygous *csr-1* mutants, slower growth rates. Those four that manage to survive contained the necessary three mutations for ivermectin resistance, alongside those of the *wago-9* homozygous and *csr-1* heterozygous balanced mutations. These four new strains were then stored in -80oC. One of these four strains collected after this process is then to be used in the downstream polysome profiling and cloning protocols

When this new strain is on ivermectin, which leads to the C. elegan to possess both *wago-9* and *csr-1* in a homozygous state, a new phenotypic trait was observed. The hermaphroditic worms in this strain seemingly lacked the ability to develop any eggs within their germline. This phenotype may shine new light on a new role for WAGO-9 in the germline if this new phenotype persists throughout the reproductive lifespan of the worms. The male worms of this mutant strain seem to possess no noticeable, unexpected, phenotypes. Data collected from the downstream sequencing should bring to light the possible genes being affected to allow this phenotype to become expressed.

csr-1 alg-5(ram2) Double Mutant

A

IV	V	IV	V	IV	V	IV	V
csr-1	WT	*csr-1*	nT1(iv)	WT	nT1(v)	nT1(v)	nT1(iv)

IV	V
WT	WT

csr-1/WT	WT/WT *	csr-1 /WT	nT1(iv)/WT	WT/WT	nT1(iv)/WT	nT1(iv)/WT	nT1(iv)/WT

Viable
Non-Viable

B

IV	V	IV	V	IV	V	IV	V
csr-1	nTI1iv)	csr-1	avr-15 glc-1	nT1(v)	avr-15 glc-1	nT1(v)	nT1(iv)

IV	V
csr-1	WT
csr-1	avr-15 glc-1
WT	WT
WT	avr-15 glc-1

csr-1/csr-1	WT/nT1(iv)	csr-1/csr-1	WT/avr-15 glc-1	csr-1/nT1(v)	WT/avr-15 glc-1	csr-1/nT1(iv)	WT/nT1(iv) *
csr-1/csr-1	avr-15 glc-1/nT1(iv)	csr-1/csr-1	avr-15 glc-1/avr-15 glc-1	csr-1/nT1(v)	avr-15 glc-1/avr-15 glc-1	csr-1/nT1(v)	avr-15 glc-1/nT1(iv) *
WT/csr-1	WT/nT1(iv)	WT/csr-1	WT/avr-15 glc-1	WT/nT1(v)	WT/avr-15 glc-1	WT/nT1(v)	WT/nT1(iv)
WT/csr-1	avr-15 glc-1/nT1(iv)	WT/csr-1	avr-15 glc-1/avr-15 glc-1	WT/nT1(v)	avr-15 glc-1/avr-15 glc-1	WT/nT1(v)	avr-15 glc-1/nT1(iv)

Table 10: Progeny marked with the asterisk (*) were what is meant to be used in downstream crosses. A) Table representing the progeny from the initial cross between the csr-1 balanced mutant strain and the alg-5(ram2) mutant strain. Only the first part of the table is what is used in the next step due to it containing the csr-1 mutant allele. B) This table represents the offspring from a backcross with the progeny from the previous cross and the original csr-1 mutant strain.

Due to ChIP (chromatin immunoprecipitation) sequencing data previously completed by Dr. Weifeng Gu, it was discovered that the mir-58 family miRNA shares the same target loci of the CSR-1 dependent gene 3' UTR that this novel small RNA pathway also targets (Data not shown). The ALG-5 gene encodes a necessary protein found in the biogenesis of many miRNA families within C. elegans germline, including the miR-58 family (14). The goal was then to introduce a deletion mutant allele of the ALG-5 gene in a *csr-1* deletion and ivermectin resistant background. The *alg-5(ram2)* strain for this cross was provided by Dr. R.M & B.E Montgomery's lab, while the *csr-1* mutant strain was the same lab strain used for the previous *csr-1 wago-9* cross (14). A strain containing this double mutation will bring to light any possible role overlap or regulation that the miR-58 family may have with the CSR-1 argonaute.

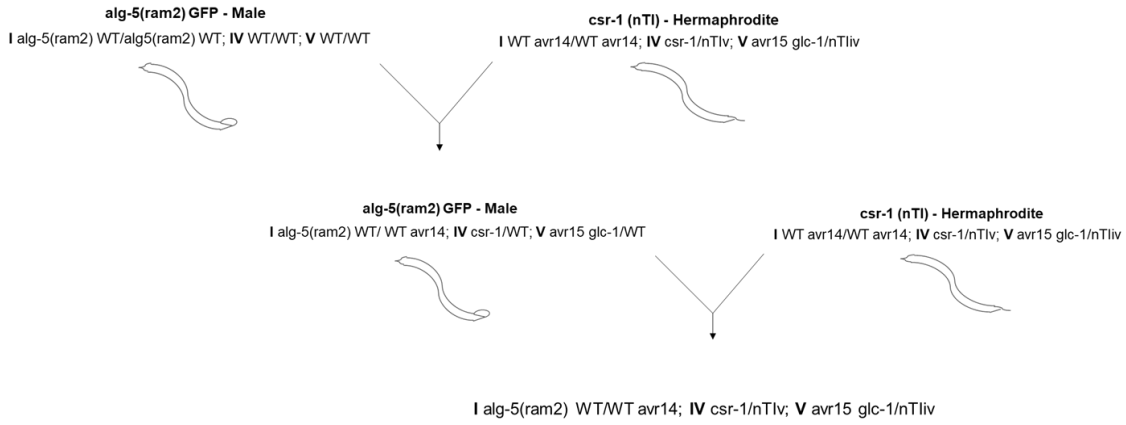

Figure 7: Simplified road map to the creation of the csr-1 balanced alg-5(ram2) homozygous double mutant strain. The F2 progeny will be self-crossed and the offspring plated on ivermectin plates to identify strains homozygous for avr-14 and possibly alg-5(ram2)

The *csr-1* deletion mutant strain was used as the hermaphrodite and the *alg-5(ram2)* deletion mutant was used as the male for the cross (Figure 7). This was decided due to the *unc* mutation present on the *csr-1* mutant balancer. As mentioned previously in the cross between the *csr-1* and *wago-9* mutants, this *unc* mutation makes the movement of the worms to be severely hindered, so it was decided to have the *alg-5(ram2)* mutant take the position of the male as they do not have hampered movements. The results of this cross should have progeny containing four different genotypes. However, due to *csr-1* having to exist with the nT1 balancer, only 50% of the progeny would be viable (Table 10A). This is for the same reason as described before in the *csr-1 wago-9* cross. Of the remaining 50% of progeny that actually are viable only one is what is needed for the next part of the cross. The one that is used is the only one that contains the *csr-1* mutant and not the balancer containing the *unc* mutation. Because of the lack of the *unc* mutation, distinction between the two viable progeny is significantly easier, as the worms moving normally are the males to be used for the next cross. The next cross, a back cross

with the same *csr-1* balanced strain as the initial cross, also creates offspring with a 50% viability (Figure 7). Half of the viable offspring will be noticeably *unc* and the other half will not, unlike the previous cross the progeny to collect this time are those that are *unc* mutants (Table 10B). Of those worms that are *unc* only one combination is what is wanted. At this stage however, it is not possible to determine which is which. So, all *unc* worms are collected. Hermaphroditic *unc* progeny from this backcross are then singled onto 35mm NGM plates and allowed to self-cross. The progeny of this self-cross is plated onto ivermectin 35mm plates and observed for 24-48 hours. Plates that contain progeny that survive indicate that the mother of those offspring contains the desired genotype, as the mother would have to have the three needed mutations for ivermectin resistance. To double check that this is not a strain containing WT CSR-1, as there is another possible progeny that could be ivermectin resistant but not contain the *csr-1* mutation, a PCR protocol designed to target the CSR-1 gene is run of a few of the progeny per plate that survive to make sure that the correct strain is found.

From the now identified correct hermaphroditic mothers of the back cross, more of their self-cross hermaphroditic progeny is singled onto as many 35mm NGM plates as possible. When these singled hermaphroditic worms start to lay eggs, they are collected and run through various PCR protocol to help screen for the correct genotype. These PCR protocols tested the homozygosity of the *alg-5(ram2), avr-14, glc-1* mutant alleles and the heterozygosity of the *csr-1* mutant. Four strains were identified as having homozygous *alg-5(ram2)* mutation alongside the *csr-1* balanced and ivermectin resistant

backgrounds. One of these strains is used in the following ribosomal profiling and cloning protocols.

Unlike the previous cross, no noticeable phenotype was noticed with any of the completed mutant strains. Though it is disappointing that a new phenotype wasn't observed, that doesn't mean that the changes this mutant incurred could not still be significant. These *csr-1 alg-5(ram2)* strains will hopefully identify the role the miR-58 family has with the CSR-1 dependent germline genes. Data that should be completed once the mRNA and small RNA sequencing is complete.

Brood Size Observations of *csr-1* and *wago9* Mutants

After recording the presence of a new phenotype in the *csr-1 wago-9* double mutant, a test was devised to track and prove the consistency of this new phenotype. This brood size experiment was used to track the egg laying and hatching of the new strain (*csr-1 wago-9* homozygous deletion), the two original strains from the cross (*csr-1* and *wago-9* both homozygous deletion), and a control (*avr 3x*). All of these strains, including the control strain, exist within an ivermectin resistant background and as such were singled and grown on ivermectin plates. Recording these other strains, alongside that of the *csr-1 wago-9* homozygous double mutant, allows for reasonable comparisons for the severity of the new phenotype.

This ivermectin background is needed due to the *csr-1* mutant and the need for the existence of a balancer in the strain. As mentioned in the design of the cross, to create the double mutant this brood size experiment is meant to test, the *csr-1* mutant cannot exist as a homozygous mutant and successfully sustain a second generation. So, this ivermectin resistance is placed to ensure when it is needed only the homozygous version of this strain is what is being tested.

A

		avr3x	*csr-1*	*wago9*	*csr-1 wago9*
Worms	Day 1	869	0	1514	0
	Day 2	1674	0	793	0
	Day 3	569	0	648	0
	Day 4	90	0	276	0
Eggs	Day 1	2	238	165	0
	Day 2	79	11	2	0
	Day 3	389	2	3	0
	Day 4	336	1	97	0

B

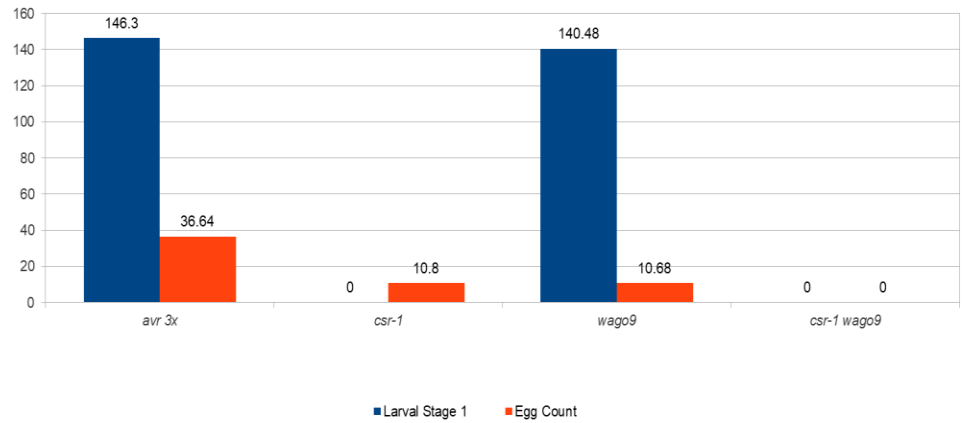

Figure 8: Data representing the number of L1/2 worms and eggs recorded over the course of four days. A) Tables with the day-to-day counts for the L1/2 worms and eggs for the various mutant strains. B) This graph represents the average combined totals for the L1/2 and eggs counted over a four-day period for the various mutant worm strains. Strain *avr 3x* represents the control strain. Homozygous *csr-1* strain follows its expected phenotype by not producing viable offspring and very few eggs compared to the *avr 3x* control. The *wago9* homozygous mutant has similar numbers of L1/2 offspring as the *avr 3x* control and similar number of remaining unhatched eggs as the homozygous *csr-1* mutant. Double homozygous *csr-1 wago9* mutant had neither L1/2 or eggs on the plates the entire four days.

Observations of the first day shows that mutants containing the homozygous *csr-1* deletion had no L1 worms present on the any of the 25 plates. This continues for the remaining three days as well (Figure 8A). Maintaining the expected phenotype of this deletion mutant, as it would seem these hermaphroditic worms have problems laying viable eggs. As it is seen in the *csr-1* homozygous mutant, though no L1 worms were seen, these plates had the highest concentration of eggs still present on the plates. However, the homozygous *csr-1 wago-9* double mutant had neither the eggs or the L1 worms (Figure 8A). The control worms, *avr 3x*, contained almost a thousand L1 worms and almost no remaining eggs on the plates. Showing that the vast majority of the eggs laid before the first day hatched (Figure 8A). The *wago-9* homozygous deletion had more eggs laid on average than the control strain on the first day. Having almost 600 more L1 worms and 160 more unhatched eggs than the *avr 3x* control (Figure 8A). At the end of the first day of counting, the *csr-1* mutant strains produced no living offspring and maintained a multitude of unhatched eggs, for the homozygous *csr-1* mutant, and the homozygous *wago-9* mutant appears to produce more offspring than that of the *avr 3x* control (Figure 8A).

The following days the homozygous *wago-9* strain begins a steady decline of the amount of L1 worms hatched from the laid eggs and the amount of remaining eggs unhatched decreased as well. An opposite pattern appears with unhatched eggs within the control strain, as the days went on the amount of eggs left unhatched increased. After a peak in L1 worm hatchings on day two of the *avr 3x* control and then followed a decline.

When taken all together the averages of these hatched L1's between the *avr 3x* control and the homozygous *wago-9* mutant strains are nearly identical (Figure 8A). Though the unhatched eggs are far fewer in the *wago-9* mutant strain than in the control strain. Mutant homozygous *csr-1* and *csr-1 wago-9* double mutant strains continued to show the lack the living hatched L1 offspring. Unhatched eggs for the *csr-1* homozygous mutant drops off dramatically by the second day and then again by the third and fourth day. Seen with the initial creation of the *csr-1 wago-9* mutant strain during the cross experiments, this strain does not produce any eggs throughout the entire four days (Figure 8A & B). With the addition of the *wago-9* homozygous mutation the previous *csr-1* homozygous mutation phenotype seems to become exacerbated, having the worms go from producing no seemingly viable eggs to absolutely no eggs produced at all. These results indicate that the WAGO-9 argonaute may have a yet undiscovered process within the germline of C. elegans. This role may be discovered with downstream experiments into the double mutant strain.

The results of the brood size tracking confirm what was previously seen during the creation of the *csr-1 wago-9* double mutant strain. That being the lack of any eggs being laid or any being produced in the hermaphroditic worm's germline in the homozygous mutant. Comparing the *csr-1 wago-9* homozygous double mutant strain with the *csr-1* homozygous strain shows that both do not seem to produce any viable offspring, but unlike the *csr-1 wago-9* mutant, the *csr-1* strain still lays eggs (Figure 8B). This phenotype has persisted through this tracking experiment and ever since, leading to

the acceptance of this phenotype being a result of the addition of a *wago-9* mutation within the *csr-1* mutant background. Alongside the confirmation of the *csr-1 wago-9* double mutant strain phenotype, the *wago-9* mutant strain seemed to exhibit no noticeable change between it and the *avr 3x* control strains progeny count but did have less eggs laid on the plates when counting was done (Figure 8B). A closer look at the day to day progeny counts between the *avr 3x* and *wago-9* strains reveals that the *wago-9* produces a lot more progeny early on compared to that of the *avr 3x* control strain (Figure 8A). This initial burst of progeny is eventually balanced out to that of the *avr 3x* control over the last three days of counting. Previous observations of the homozygous *wago-9* strain have shown that it seems to outpace the WT control in progeny development and this may be the first bit of evidence to back up that claim. Though it balances out in the long term, in the short term the *wago-9* strain seems to produce progeny faster than a control strain. Of course, more detailed work will have to be done before this is certain. There is also a decrease in eggs remaining on the plates compared to the *avr 3x* control strain. This may be a lesser form of the phenotype exhibited within the *csr-1 wago-9* double mutant strain but this is not known for sure, and it still does not seem to hamper the overall production of progeny within the *wago-9* strain.

Polysome Profiles of C. elegan Mutant Strains

Ribosomal profiling was an approach decided upon due to the location of the newly identified 22G small RNA reads, the 3' UTR. Their position on the 3' UTR raised questions about their function and how to study it. In a biological system there is only

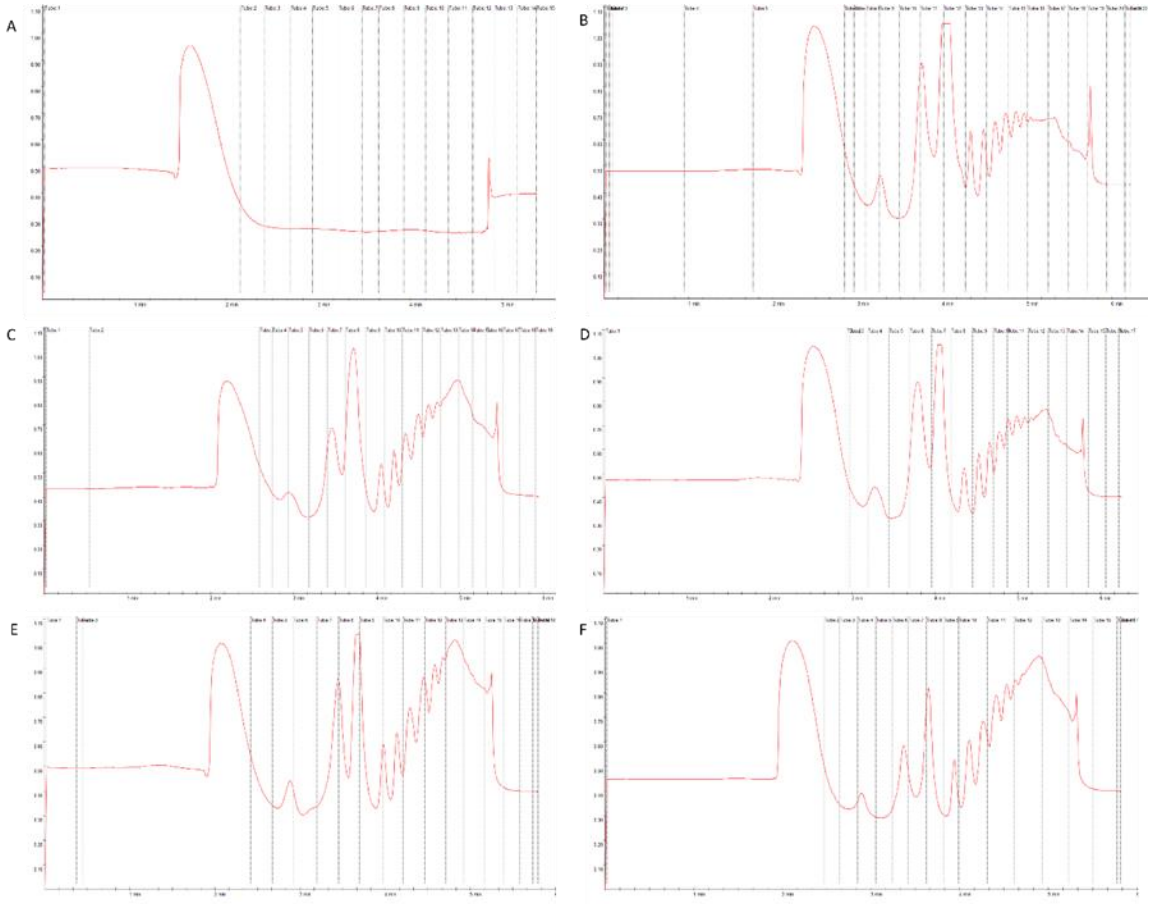

Figure 9: The above graphs were generated using the software provided by Brendel, and are generated during the sucrose fraction collection. A) Control run of the protocol, with only the sucrose gradient and buffer. The initial curve would appear to be caused by the PEB buffer or the sucrose gradient its self. B) Avr3x mutant strain C) Homozygous csr-1 mutant strain D) Homozygous wago9 mutant strain E) Homozygous csr-1 wago9 double mutant F) Homozygous csr-1 alg-5(ram2) double mutant

one currently known way that the 3' UTR is read and/or utilized, and that is during the process of translation (5). Translation being the biological function that creates the proteins used in the system by converting the genetic code read out on mRNA into various amino acids in a chain like series (17). The process of translation uses ribosomes to translate the mRNA to the specified protein (17). Ribosomal Profiling is a procedure in which the actively translating ribosome is stalled on the mRNA template, and then this currently translated mRNA is isolated from the rest of the RNA and used in downstream

experiments. Knowing about the location of the new 22G small RNAs being on the 3' UTR, and translation being the biological process recognizing this loci, ribosomal profiling would help to shed light on exactly what this 22G small RNA family does to the genes they are associated with.

Once the isolation of the ribosomes is complete, the samples are analyzed through a sucrose fraction collector and a proprietary program from Brendel. There are six major peaks or sections of the graphs produced from the Brendel program (Figure 9). The initial peak is present in all runs of the samples, even in the control run. This peaks presence in both the control and the sample runs indicates that this specific peak is likely due to a reagent found within the resuspension buffer. The next peak witnessed is associated with the 40 Svendval Unit (S) subunit of the ribosomes followed by the 60S peak and then the 80S peak. So, the two peaks following the first represent the two individual subunits of the whole ribosome; while the next is the complete ribosomal unit. At the bottom of the right-hand side of the 80S peak is where the polysome peaks begin. The polysome peaks are seen as multiple peaks of increasing height, and the greater the area under these multiple peaks' correlates to the possible final concentration of the combined polysome, a greater area meaning a higher possible concentration and a lower peak a lower possible concentration (Figure 9B-F). Following immediately after the multiple peaks of the polysome, there is a sharp peak followed by a sharp drop. This represents the oil, fluorinert FC-40, being introduced to the tubes and the end of the sample being run through the fraction collector (Figure 9A-F). Once the various strains have been

processed through the Brendel sucrose fraction collector they are quantified using a nanodrop. This is to verify the positions of the peaks correspond to the correct tube numbers, and to pool the tubes together that are correctly associated with the polysome. This process is done for each sample to allow for the use of the samples downstream. Generally, each sample is off by one or two tubes compared to the graphs.

The amount of worms used through the rest of the runs varied based on the mutations the strains contain, some of these actually directly link to difficulty in propagation of the strain. The control strain, *avr 3x*, and the *wago-9* homozygous mutant showed no signs of problems with propagation which allowed for the highest collection of worms for this protocol, 1,000,000 and 1,500,000 worms respectively. Due to the *csr-1* mutations effects on the propagation and growth of the worms containing this mutant, the worms collected for these strains was less than that of the previous mentioned mutant and control strains. Double homozygous mutant *csr-1 wago-9* had 800,000 worms able to be used for the protocol, a theory as to why the heterozygous *csr-1* version of this double mutant strain doesn't struggle to propagate as much as the single heterozygous *csr-1* mutant may be attributed to the presence of the homozygous *wago-9* mutant. As mentioned before, the *wago-9* homozygous mutant seems to grow and propagate at a much higher rate than the WT strain. The homozygous *csr-1* mutant strain had only 650,000 worms for its run shown (Figure 9C). Though there were previous runs containing < 500,000 worms. These runs lead to polysome profiles containing significantly smaller peaks, especially in the polysome portions (Data not shown). So,

with this data it was found that the lowest total worms needed for a successful run of the protocol is at least 650,000 worms.

Starting with the control (Figure 9A) it can be seen that the initial peak is all that is shared between the various samples. The missing peaks are only found in samples that have been properly process through the ribosomal profiling protocol. A preliminary estimation to the amount of polysome that can be recovered from a specific strain can be found by checking the area under the polysome peaks. A cursory glance over the graphs show that three of the five experimental sample graphs have significantly larger areas under the polysome peaks (Figure 9C, E & F). Given that the two strains that have lower polysome peaks do not contain a *csr-1* mutation, *avr 3x* control and *wago-9* homozygous deletion mutant, may show that the CSR-1 argonaute may have a role in mRNA regulation. This is not the first time that this argonaute has been noted to regulate mRNA, and this may add to the evidence of its possible involvement within the biological process of translation. The collected samples from the sucrose fraction collector are checked with the nanodrop to ensure that the proper tubes are collected. All tubes associated with the areas of the graph that contain multiple peaks in close proximity, which indicate the position of the polysome, are pooled together and purified through isopropanol precipitation and is then able to be used in the cloning protocols.

Taken alone the charts produced through this protocol do not show much in terms of differentiation between the various strains. The graphs have helped in the preparation

of each strains samples for used downstream, to obtain data that can be properly analyzed. Though this is not to say that there wasn't a trend that occurred in the graphs. It was noticed that samples containing strains that did not contain the *csr-1* homozygous mutation had smaller polysome peaks compared to samples that contained strains with the *csr-1* homozygous mutation. As seen in Figure 2, it is possible that CSR-1 argonaute has a role in mRNA regulation. So, these peaks may contain further proof that the CSR-1 argonaute performs some sort of mRNA regulation on its own gene targets and possibly others since these results come from a total lysate of the nematode not just of the germline CSR-1 specific targets.

Small RNA and mRNA Cloning of Polysome and Input Samples of C. elegan Mutant Strains

From the isolated and pooled polysome and input samples, mRNA and small RNA cloning is performed. These cloning protocols will further isolate a specific RNA species from the samples and then amplify that specific species. This further isolation is needed due to the nature of how abundant certain RNA species are over other species of RNA, referring mainly to rRNA. Ribosomal RNA (rRNA) is the most abundant RNA found within a living system, ~80%, and for the samples in their current state is now considered an abundant contaminant (19). So, the pooled post-ribosomal profile and input samples are further process to remove the abundant rRNA and allow the originally minor

species (either mRNA or small RNA in these cases) to be amplified. All this is to allow for clean sequence reads downstream.

Small RNA cloning was performed on just the input samples of the *csr-1*, *avr 3x*, *wago-9*, *csr-1 wago-9* and *csr-1 alg-5(ram2)* mutant strains. The polysome samples from the ribosomal profiling experiments are excluded due to the purification of the samples removing most of the small RNAs and leaving mainly the mRNAs and rRNAs. Comparisons between the *csr-1*, *wago-9* and *csr-1 wago-9* reads should bring light to the CSR-1 dependent genes that 3' UTRs that are dependent as well on the WAGO-9 argonaute. Finding genes where the 22G small RNA reads are down-regulated in the *csr-1 wago-9* mutant over that of the *csr-1* mutant will accomplish this.

The mRNA cloning involves both the input samples and the pooled polysome samples. Polysome fractions are included since they contain the actively translated mRNAs located in the germline of the *csr-1*, *wago-9*, *avr 3x*, *csr-1 wago-9* and *csr-1 alg-5(ram2)*. This is important due to the location of the newly discovered 22G small RNAs, the 3'UTR. The only known biological process that identifies this region of the transcribed mRNA is the act of translation. Since the polysome is a collection of 80S ribosomes bound to an mRNA, the collection of the polysome is actually collection of the currently translated mRNA in the specified strain. These samples will be sent to be sequenced through high throughput sequencing.

Sadly, though the cloning is finished, the results of the high-throughput sequencing has yet to be completed. This doesn't mean that there is not speculation over how to compare the samples. The *csr-1* homozygous mutants reads compared to the *csr-1 wago-9* homozygous double mutant strain should provide insight into the possible functional regulation these new 22G small RNAs are involved in. Comparing the reads from the *csr-1* mutant to that of the *avr 3x* control may also add more evidence to the ability of CSR-1 regulating its own dependent genes (2). Looking at the reads for the *csr-1 alg-5(ram2)* mutant over that of the *csr-1* mutant will bring insight into the possible roles of miRNA in this new 22G small RNA family. Due to ALG-5 being involved in the biogenesis of miRNA which in turn is involved in many roles within the germline of C. elegans, and the removal of it leads to many miRNA families to not be properly created. The germline reads from the *csr-1 alg-5 (ram2)* mutant over that of the *csr-1* mutant could show how, if at all, the lack of miR-58 effects the 22G small RNA reads (14). This could then lead to a better understanding of regulation of these 22G small RNAs in this novel small RNA pathway.

Discussion

During the analysis of reads in a *csr-1* homozygous strain of C. elegans a new family of 22G small RNAs was observed. This new family resided within the 3' UTR region of many of the CSR-1 dependent genes and so is assumed to be expressed within the germline (Figure 2). As these were CSR-1 dependent genes, they are known to be

used in the transcription of 22G small RNAs that load onto the CSR-1 argonaute. The continued presence of 22G small RNA reads within the 3' UTR with the depletion of CSR-1 in the system raised the question as to what argonaute these 22G small RNAs were loading onto and what function do they have within the germline of the C. elegan organism.

Two transgenic worm strains in a *csr-1* mutant background, containing either WAGO-1 or WAGO-9, were used to help identify which argonaute loads these newly identified 22G RNAs. Through the use of immunoprecipitation and high throughput sequencing the 22G RNA reads from the input and IP samples were analyzed. Using the lab genome browser genes known for being CSR-1 dependent, two specifically were used as examples Klp-7 and Pgl-1, were examined and a pattern emerged for the WAGO-9 argonaute. This WAGO argonaute had at least two-fold more 22G small RNA read within the 3' UTR over its input sample, which indicated that it was indeed the argonaute loading this newly identified 22G RNA family (Figure 5).

Seeing as the argonaute that loads the 22G small RNA, WAGO-9, is now known and then discovering miR-58 family has overlapping targets to the 3' UTRs of the same CSR-1 dependent genes (data not shown), mutant strains needed to be made. These mutant strains were crossed within a *csr-1* mutant and ivermectin resistance background. The *wago-9* deletion exists as a homozygous null mutant alongside a balanced *csr-1* mutant allele. While the miR-58 family is removed from the worm through the deletion

of the ALG-5 miRNA associated protein. This protein is known to me required for the biogenesis of many germline-based miRNA, including miR-58 family (14). These mutant strains are used downstream for the eventual analysis of small RNA and mRNA reads post high throughput sequencing.

The newly created mutant strains, alongside the *csr-1* mutant and *avr 3x* mutant control, were put through the ribosomal profiling protocol to isolate the mRNA currently being translated. It was noticed through the graphs created through this process that the strains that possessed the *csr-1* mutant contained significantly higher peaks than those strains that did not contain this mutation (Figure 9B-F). This discovered pattern added more evidence to the CSR-1 argonaute possibly having a role in mRNA regulation, as the smaller peaks indicate that there is possibly less mRNA available for the 80S ribosomes to bind to for translation. This would in turn lead to less mRNA translation, and thus smaller polysome peaks. The mRNA transcripts and the input samples are collected and run through the mRNA and small RNA cloning protocols. The results of this are to be sent out for sequencing once they have been properly pooled together. High throughput sequencing will then be performed. From the reads of the small RNA, the role of WAGO-9 as the loading argonaute will be proven through the decrease in reads at the 3' UTR and the effect of miR-58 family on the 22G small RNA in the 3' UTR will be seen. Using the resulting reads from the mRNA sequencing a possible target for what these 22G small RNAs regulate can be deciphered through which genes have upregulated reads in the *csr-1 wago-9* double homozygous mutant over that of the *csr-1* homozygous

mutant. The reads from the mRNA cloning will also provide insight into whether CSR-1 argonaute can actually regulates its target dependent genes and/or other genes.

Though it would be expected that small RNAs created from loci associated with a certain argonaute would be loaded onto said argonaute, this newly identified 22G small RNA breaks that mold. This new 22G small RNA family is created from the 3' UTR of a CSR-1 dependent gene, but loads onto the WAGO-9 argonaute. WAGO-9 being one of two of the known argonauts in the WAGO family to exist in the germline. The origin of these 22G small RNAs also raised a few questions about their possible roles within the germline of the C. elegan, originating from the 3' UTR instead of the CDS. Through the future analysis of the reads from the various cloning procedures mentioned previously, the reasons to this family's origin location of biogenesis and its role(s) within the germline should hopefully be answered.

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