Analysis of RNA Interference Pathways in *C.elegans*

"Investigating Argonaute Protein Interactions using a
Yeast Two-Hybrid Screening System"

A Major Qualifying Project

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Abstract

RNA interference (RNAi) is a sequence-specific silencing phenomenon that is conserved in animals, plants and some bacteria (Fire and Mello, 1998; Meister and Tuschl, 2004). At least 4 different AGO pathways are thought to function in the germline of *C. elegans*. In an effort to understand how these pathways remain independent from one another, we are using yeast two-hybrid to identify proteins that interact with and potentially regulate AGOs. The results showed colonies which grew on secondary selection plates indicated potential interacting proteins for the argonautes of interest.

Acknowledgements

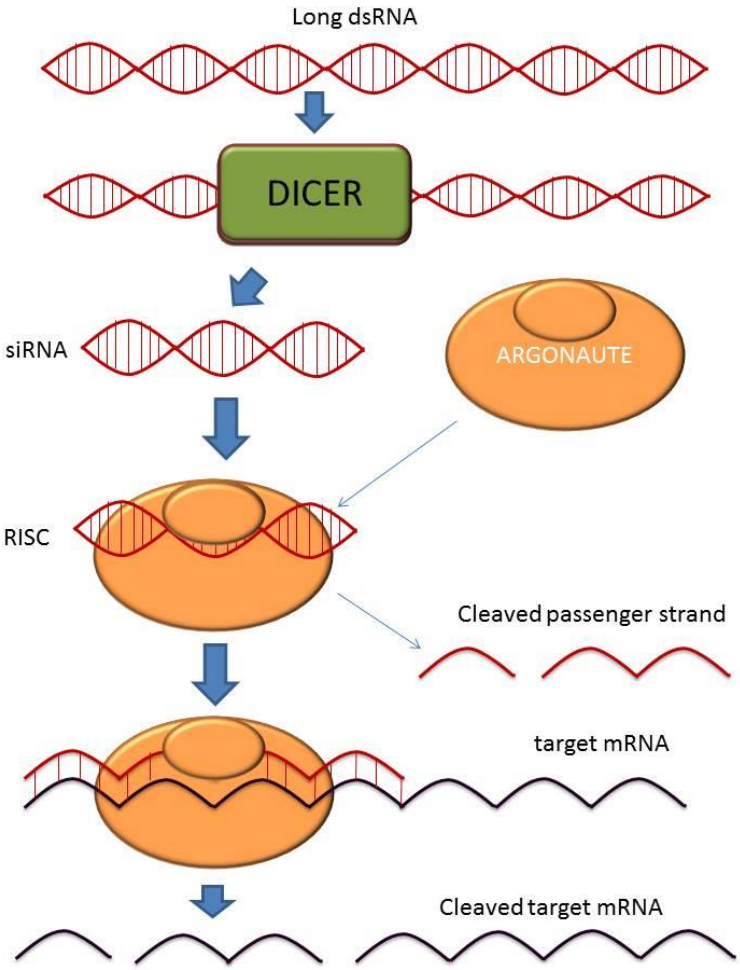
I would like to thank Dr. Craig Mello and UMass Medical School for giving me this opportunity to collaborate with them on this project. I would also like to thank my advisor Dr. Destin Heilman for overseeing the progress and final outcome of my project. In addition, I would like to thank Dr. Darryl Conte and William Stanney III for all the help and guidance they have given me in developing and executing this project, I could not have done this without all the support and guidance that I have been awarded.

Introduction

Argonaute proteins are specific to the process of RNA interference. These proteins have the ability to bind to single stranded RNAs which are about 22-23 base pairs long and are homologous to the mRNA targeted for silencing (Miyoshi et al, 2005). Some of the primary argonautes identified in RNAi are WAGO-1, PRG-1, CSR-1, and ERGO – 1. Although there has been evidence to indicate the general processes argonautes facilitate inside C. elegans, there is still much that is not understood. Therefore investigations of the primary argonautes and in particular WAGO – 1 are necessary to better understand the RNAi process.

Gene Silencing by RNA Interference

First discovered in plants, the process of RNA interference (RNAi) is a very intricate and involved form of post transcriptional silencing. RNAi has been attributed to to suppressing gene function and coding regions that were not well understood previously (Fire and Mello, 1998). Since its identification and discovery, RNAi has been used as a therapy both in vivo and in vitro to regulate gene expression. Figure 1 below is an illustration of the process of RNA interference within the organism C.elegan.



Adapted from RNAiweb (Fire et al, 1996)

Figure 1: Illustration of the Process of RNA Interference

RNA interference is initiated by double stranded RNA which is either made within the body or introduced by laboratory procedure. The double stranded RNA has a homologous region that corresponds to the mRNA targeted for silencing. The DICER protein then cuts the double stranded RNA into miRNA which are later transformed into small interfering RNA recognized by the appropriate RISC complex. The RISC complex is comprised of Argonaute and Helicase which has a specific domain that binds to the siRNA. Although not much is known by which the RISC complex binds to its designated

siRNA, the completed complex silences genes by either cleaving the target mRNA which occurs at the +10 and +11 nt of the guide siRNA or binding to it in order to stop it from undergoing translation by ribosomes.

Argonaute Specificity: AGO and Piwi protein Argonautes

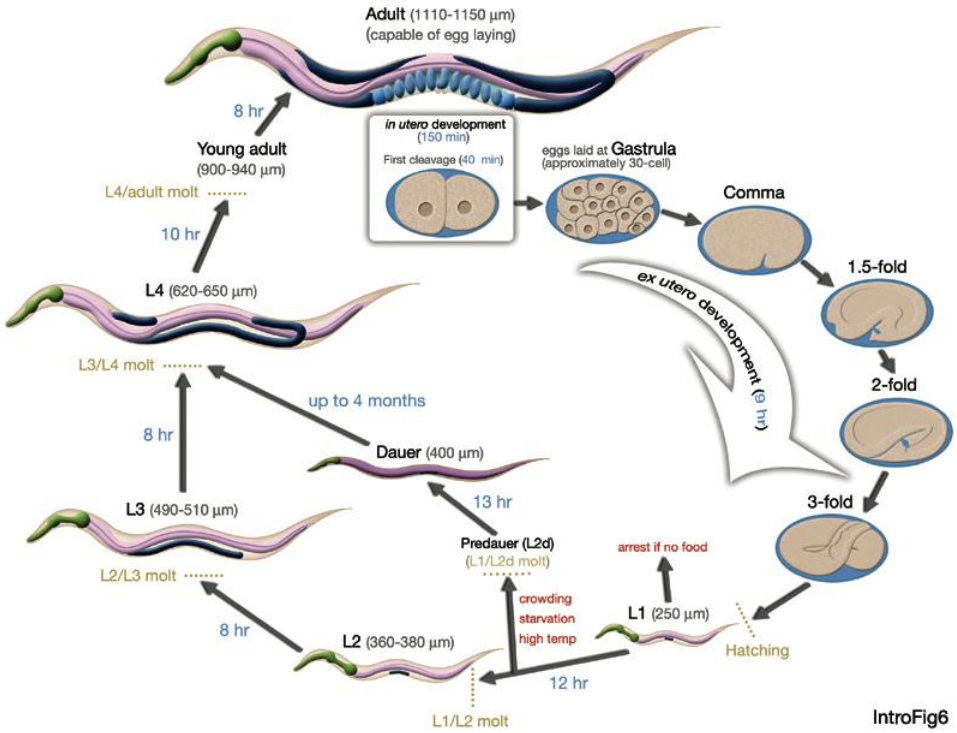
Argonautes are the key effectors of the RNAi process (Lee et al, 2012). Argonautes are members of different subfamilies and are very distinctive in their function. Argonaute proteins can be divided into two subgroups known as the Argonaute proteins and the PIWI proteins. These two subgroups differentiated and identified by the presence of PAZ or Piwi domains found on specific miRNA of which they bind. They are also specified by their location in the germ line. AGO proteins are distinguished by their association with the miRNA pathway and their ability to perform posttranscriptional silencing in many different areas of the organism, while Piwi proteins interact specifically with piRNA. piRNA and their respected pathways differ from miRNA and traditional RNAi by size and sequence conservation. In addition the piRNA pathway is specific to germline generation.

The PAZ domain in AGO proteins is distinguished by the binding of the 3’ end of the siRNA. When the target RNA begins to anneal to the siRNA the PAZ domain falls off and this allows the target RNA to form a full duplex complex with the siRNA before actual cleavage occurs. The PIWI domain, one of the most common domains within argonautes, has an RNaseH like structure and the endonucleolytic activities are also conserved in the argonautes (Miyoshi et al, 2005). The Argonaute clade can be divided into subgroups based on sequence similarities, and the PIWI clade is found directly in ciliaties, slime molds, and animals (Das et al, 2008). However in Drosophila and other

non-vertebrates, PIWI proteins also act on somatic cells that are associated with the germ line. For example PIWI proteins are required in Drosophila for oogenesis and stem cell maintenance while also having the responsibility of for transposon silencing within the germline.

Argonaute Proteins in C. elegans

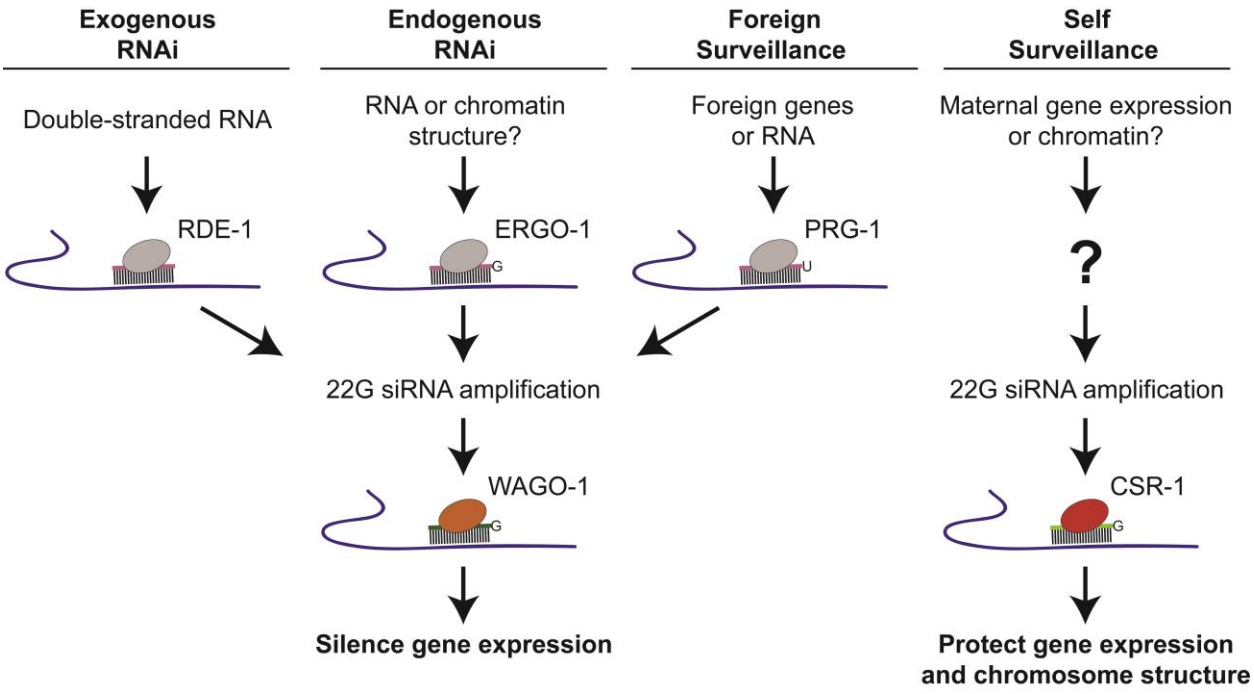
Caenorhabditis elegans are eukaryotic, multicellular organisms which undergo embryogenesis, morphogenesis, and have the ability to grow into mature adults (Rutgers University, 2007). C. elegans have been used as a model organism for study because of their simple composition, small genome, and quick reproduction rates. Figure 2 below is a depiction of the life cycle of a male C.elegan.



Adapted from Worm Atlas (Altun & Hall, 2009)

Figure 2: Picture of male Caenorhabditis elegans life cycle

C. elegans also contain RNAi pathways and proteins that are similar to mammals in their function and composition (Meister and Tuschl, 2004). Although the main function of argonautes has been identified for RNAi, there has been indication that certain argonautes are needed for other functions and processes. For example, the argonaute ERGO-1 has been identified in the accumulation and biogenesis of small RNA in C.elegans (Vasale et al, 2009) and CSR-1 has been identified in proper holocentric chromosome segregation (Claycomb et al, 2009). It has also been proposed that argonaute pathways and mechanisms interact and overlap on many occasions. The argonaute WAGO-1 appears to be a part of pathways that contain ERGO -1, CSR-1, and PRG-1 (Claycomb et al, 2009. Gu et al, 2009. Lee et al, 2009). It is generally understood that PIWI proteins are needed for correct germ line development in C. elegans and are also responsible for silencing transposons in the worm's genome. Previous research has allowed for scientist to generate ideas of how the primary argonautes interact, with one such depiction shown below.



RNAi and Primary Argonaute Pathways in C.elegans

With the complex processes attributed to argonautes that are coming to light, more needs to be understood about each argonaute individually and the roles they play within different pathways.

Identifying Protein Interactions

Greater understanding of the primary argonautes and associated proteins may aid in pinpointing mutations and defects that can affect the worm development. One way to accomplish this is by finding specific proteins that interact directly with each argonaute. Identifying proteins that interact directly with our proteins of interest can give insight to where they are located both in the pathway and organism as a whole. In addition, identification of other interacting proteins can be studied to see what their specific functions are. Protein domains and specificity may also play a part in argonautes activity

making it useful to identify if certain domains are responsible for interactions. Protein identification be accomplished by utilizing a sensitive protein-protein interaction screening process such as the yeast-two hybrid mechanism depicted below.

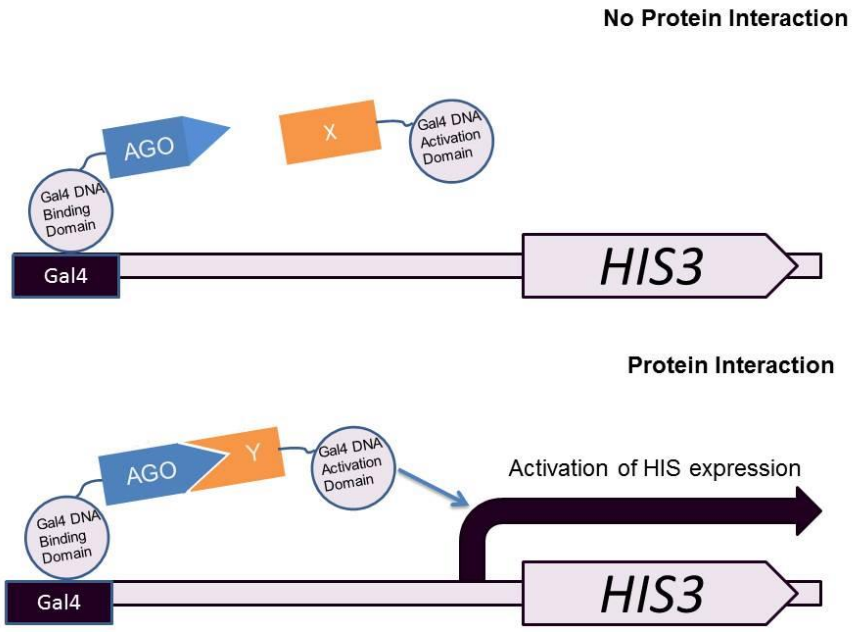


Figure 2: Yeast Two hybrid mechanism

This project will focused on building the constructs for the primary argonautes of ERGO – 1, WAGO – 1, PRG-1, CSR – 1, and RDE- 1 needed to run a yeast two hybrid screen. In addition WAGO-1 was the protein of interest screened against a cDNA library to see which, if any colonies would emerge indication positive reactions.

Methods and Materials

The WAGO - 1 Yeast Two-Hybrid Screen assay was conducted using the Invitrogen Yeast Two Hybrid system according to the companies presented protocol.

RT-PCR of WAGO -1 Gene and Other Primary Argonautes

Obtaining RNA extraction of WAGO -1 from C.elegans

Total RNA containing the genome of C. elegans was obtained from stock previously available to the laboratory of Dr. Craig Mello. WAGO -1 oligo primers were obtained through independent design that was sent for construction to the Integrated DNA Technologies company. The WAGO -1 primers used for the reverse transcription reaction are the same as the WAGO -1 primer sequences used for PCR. The sequences are displayed below.

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTCTCCTCATCCACAACCTCAATCAGAATGCATAG
GGGGACCACTTTGTACAAGAAAGCTGGGCTATGCATTGACGCGCTTCTGATTGA

The same process that was used to obtain the primer sequences for WAGO-1 was utilized to obtain the primers for the other argonautes of PRG-1, CSR-1, ERGO – 1, and RDE – 1. Below are the sequences for each of the primary argonautes of interest.

ERGO - 1

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAGCTATAACAACCGGCGGCGGCTCGAAGGAATGACATTTGTCTAA
GGGGACCACTTTGTACAAGAAAGCTGGGTTAGACAAATGTCATTCCTTCGAGC

RDE -1

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTCCTCGAATTTTCCCGAATTGGCCCTGGAATGTCGTTCGCATAA
GGGGACCACTTTGTACAAGAAAGCTGGGTTATGCGAACGACATTCCAGGG

PRG-1

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCATCTGGAAGTGGTC
GGGGACCACTTTGTACAAGAAAGCTGGGTTACAAGAAGAACAGCTTGTCACGAA

The primers for CSR – 1 were ordered previously for other experiments in the lab and therefor did not need to be constructed.

Extraction and Purification of mRNA from Total RNA

Total RNA was used to perform a RT-PCR of the WAGO -1 gene but the results were less than desirable when amplifying the gene. To eradicate this problem, mRNA was purified from the available total RNA using the mRNA extraction protocol with the utilization of Dynabeads by Invitrogen. First 75 µl of Total RNA was added to 100 µl of distilled water and heated to 65 degrees Celsius and then put on ice. Afterward 200 µl of re-suspended Dynabeads were placed in a centrifudge tube which was then placed on a magnet wall for 30 seconds. The supernatant was removed leaving only the beads attracted to the magnet. Next 100 µl of binding buffer was added to the beads. The supernatant was removed and then the step was repeated leaving the buffer in the solution. Afterward the Total RNA was added to the binding Buffer and the Dynabead solution. This solution was mixed with a roller for 5 minutes at room temperature. The tubes were then placed on the magnet again and when the solution segregated completely the supernatant was removed. Lastly the final solution was washed with Washing Buffer (200 µl, 2X). The supernatant that contains the mRNA was stored in the -4 C freezer.

Reverse Transcriptase of Purified mRNA into cDNA

cDNA was synthesized using the purified mRNA and the SuperScript III First Strand Synthesis System kit along with the protocol provided by Invitrogen life Sciences technologies. The following components were added in a 70 µl tube and then vortexed for complete mixture. 2.8 µl of purified mRNA, 2 µl of designated primers, 1 µl of 50 ng/µl random hexamers, 1µl of 10mM dNTP mix, and 5.2 µl if distilled water. The mixture was then allowed to incubate in a thermal control machine at 65 degrees for 5 minutes. After the incubation period the mixture was then placed on ice for 1 minute. Afterward the following cDNA synthesis mix was prepared: 12 µl of 10X RT buffer, 3 µl of 0.1 M DTT, 3 µl of RNaseOut (40 U/µl), 2 µl of SuperScript III RT (200 U/µl), and 9 µl of distilled water. 10 µl of the cDNA synthesis mix was added to the RNA primer mixture and mixed gently by pipetting a small volume up and down the tube. Next the entire solution was allowed to incubate at 50 degrees for 50 minutes and then the temperature was moved to 25 degrees for 10 minutes followed by 50 degrees for another 50 minutes. The reaction was then terminated by allowing the solution to incubate at 85 degrees and then being removed to chill on ice. Lastly, 1 µl of RNase H was added to the mixture which was then allowed to sit at 37 degrees for 20 minutes.

PCR of WAGO -1 cDNA and Other Argonautes

The newly synthesized cDNA was then amplified by Polymerase Chain Reaction (PCR) utilizing the appropriate WAGO -1 Gateway primers, along with the iProof High-Fidelity DNA Polymerase kit created by Bio-Rad Laboratories. The reaction performed was 50 µl in volume and was done in a 70 µl tube. The components consisted of 10µl of 5X

iProof HF buffer with a concentration of 1X, 1 µl of 10 dNTP mix with a concentration of 200 µM, 2.5 µl of the designated primers, 2 µl of DNA template, 5 µl of Proof DNA polymerase, and 31.5 µl of sterile water. After the solution was created it was then placed into a thermal cycling process under the following conditions: 98 C for 30 seconds (1X), 98 C for 5-10 seconds, then 72 C for 30 seconds, 72 for 15 seconds (34X), and then at 72 C for 10 min (1X).The final step included leaving the solution at 4 degrees Celsius. The solution was then placed and stored in a negative 20 degrees Celsius freezer. After the PCR was completed the reaction was tested by gel electrophoresis using a 1KB marker to examine if the gene were not only amplified but also the correct size.

BP Cloning of WAGO -1 Gene into Vector pDONR 207

In order to prepare the amplified product to enter the necessary domains for the yeast two hybrid assay the WAGO -1 gene was placed in the pDONR 207 vector, an entry clone, by BP reaction using the Gateway BP Clonase II Enzyme Mix kit produced by Invitrogen.

The pDONR 207 Vector

The pDONR 207 vector was constructed to have selective properties such as lacZ promoter gene and kanamycin resistance. This particular plasmid contains a pUC origin and replicates less frequently, leading to a smaller DNA yield. This donor vector also contains two transcription termination sequences, which prevents transcription genes that could be toxic from being expressed.

Cloning of the Amplification Product into the pDONR Vector

The procedure provided by the manufacturer was as follows: First the following components were mixed together in a 70 µl tube and spun down for complete integration. 6 µl of PCR product, 1 µl of Donor Vector, 1µl of BP Clonase II Enzyme, and 2 µl of TE Buffer pH 8. Afterward the product was allowed to incubate at 25 degrees Celsius in order to initiate the reaction, and then was allowed to stay for one hour letting the reaction run to completion. Afterward the final product was taken from the incubator and allowed to sit at room temperature.

Transformation of BP Reaction into E.Coli Bacteria

After the reaction was completed the BP solution was then transformed into One Shot Top 10 Competent E.coli cells and were later cultured and mini-preped isolating only the vector and WAGO -1 gene. The correct protocol was also provided by the Gateway BP Clonase II Enzyme Mix kit produced by Invitrogen. The TOP 10 competent cells were obtained from a -80 degree freezer and allowed to thaw on ice. When completely thawed 2 µl of the BP cloned solution was added to the vial containing the bacteria and was then placed on ice for another 30 minutes. Afterward the cells were heat shocked at 40 degrees Celsius for 30 seconds and were then immediately placed back on ice. Next 250 µl of S.O.C solution was added to the vial and was then allowed to incubate at 37 degrees with light shaking for 1 hour. At the end of the incubation period the cells were taken out of the vial and plated on ampicillin coated plates for selection and allowed to stay overnight in a 37 degree incubator. The cells were then cultured and mini-preped isolating the vector and WAGO -1 gene. The product was then taken and examined by gel electrophoresis to determine whether there was an insert present in

the vectors, those samples that appeared to match the desired weight and size of the vector were then selected and sent for ECONO sequencing. When returned the sequences were check against the accepted sequences provided by the National Center for Biotechnology Information. Sequences that were correct were then stored in a 4 C refrigerator.

LR Reaction of WAGO -1 into Destination Vector pDEST32 (Binding Domain)

The WAGO -1 gene was put into the pDEST32 destination vector which serves as the binding domain within the yeast two-hybrid assay. This was performed by using the Gateway LR Clonase II Enzyme Mix Kit produced by Invitrogen.

The pDEST 32 Vector

The pDEST 32 vector is also known as the bait vector and will contain the main gene of interest, WAGO -1. This vector contains ampicillin resistance and also the leucine gene which will allow for the mutated yeast to produce that particular amino acid. The LR reaction procedure followed was provided by the manufacturer and the following components were mixed together in a 70 µl tube. Six µl of the Entry Clone with PCR product, 1 µl of the Destination Vector, 2 µl of LR Clonase II, and 1 µl of TE Buffer with pH 8.The components were then vortexed and centrifuged in order to have complete integration and were allowed to incubate at 25 degrees for I hour. Transformations of the vector into E. Coli bacteria and sequence conformation was also performed in accordance with the procedures stated previously in relation to the BP cloning.

LR Reaction of WAGO -1 and Other Argonautes into Destination Vector pDEST22 (Activation Domain)

The WAGO -1 gene was put into the pDEST22 destination vector which serves as the activation domain within the yeast two-hybrid assay. This was performed by using the Gateway LR Clonase II Enzyme Mix Kit produced by Invitrogen.

The pDEST 22 Vector

The pDEST 22 vector is also known as the prey vector and will contain the genes meant for interactions with the gene of interest WAGO -1. Similar to the pDEST 32 vector, this vector also contains ampicillin resistance and the gene for the amino acid tryptophan, which is essential for the survival of the mutated yeast. The protocol for the LR reaction of WAGO -1into the pDEST 22 vector was provided by the manufacturer. The following components were mixed together in a 70µl and allowed to incubate at 25 degrees Celsius for 1 hour: Six µl of the Entry Clone with PCR product, 1 µl of the Destination Vector, 2 µl of LR Clonase II, and 1 µl of TE Buffer with pH 8.Transformations of the vector into E. Coli bacteria and sequence conformation was also performed in accordance with the procedures stated previously in relation to the BP reaction and LR reaction of the pDEST32 vector.

LR Reaction of cDNA Library into Destination Vector pDEST22 (Activation Domain)

A premade cDNA library was inserted into the pPC86 vector which is available through the ProQuest Two Hybrid System. The pPC86 is a cloning vector that has the GAL4 Activation Domain but does not contain the recombination sites. The same LR reaction

was used in order to have the cDNA inserted into the vector containing the activation domain. The following components were added to a 70 µl tube and were mixed completely. Six µl of the Entry Clone with PCR product, 1 µl of the Destination Vector, 2 µl of LR Clonase II, and 1 µl of TE Buffer with pH 8. The solution was then allowed to remain at 25 C for one hour and then moved to the 4 C refrigerator. The library mix although created in this fashion was obtained from another lab which had previously made the mixture for other experimentation.

Yeast Strain MaV203

The yeast strain used was MaV203 which contained single copies of the reporter gens HIS3, URA3, and lacZ. These reporter genes are stably integrated at different loci in the yeast genome. The promoter regions for each of individual genes are unrelated. The yeast also contains the features of having a non-reverting set of auxotrophic mutations of leu1 and trp1 to allow for selection of the bait and prey fusion vectors.

Transformation of LR Vectors into Yeast Strain MaV203

The PEG/LiAc solution was thawed in a beaker containing room temperature water and was mixed. The competent yeast cells were also thawed in a 30 degree water bath for 90 seconds. The tubes containing the cells were inverted several time and then allowed to sit at room temperature. Afterward 250 µl of the cell solution was transferred to two 15 ml polypropylene tubes. Afterward 25 µl of the cells were placed in a 1.5 ml tube and underwent a control assay to assess the quality of the competent yeast cells. For the rest of the cells 10 µg of the DB-WAGO -1 and 10 µg of the AD-library were added to the solution and mixed completely. Next 1.5 ml of the PEG/LiAc solution was added to the cells and mixed together by swirling. The solution was incubated for 30 minutes in a

30 degree water bath and was mixed by swirling occasionally for re-suspension of the cells. Afterward 88 µl of DMSO was added and mixed well. The cells were then heat shocked for 20 minutes in a 42 degree water bath and was again mixed occasionally. The cells were centrifuged for five minutes at 640 g and the supernatant was removed carefully. The pellet was then suspended in 8 ml of autoclaved saline and the final solution was then prepared and plated on SC-leu-trp plates.

Pre – Screening Controls and Parameters

Control test were administered to determine if interactions occurred between designated proteins and also the strength of such interactions. PIE – 1 (BD)/MEP – 1, WAGO -1 (BD) / F58.11 (AD), WAGO – 1/ EMPTY (AD), and WAGO – 1 (BD)/WAGO -1 (AD).

WAGO -1/ cDNA Library Screen

Preparation of Yeast for Library Transformation

The protocol for a library scale screen was obtained from the Invitrogen Proquest two hybrid System. In the experiment, 1/10 of the amount called for in the procedure was used in order to save the cDNA library. First 20 ml of the yeast cells containing the bait vector was cultured overnight. Next the yeast were taken and grown in 150 ml cell culture to an OD of .8. When the yeast has reached the desired OD they were transferred into two 50 ml conical tubes and centrifuged at 1500 rmp for 5 minutes at room temperature. The supernatant was then removed and the cells were re-suspended in 30 ml if sterile water. The yeast were then centrifuged again at 1500 rpm for 5 minutes at room temperature, the supernatant removed, and then re-suspended in 1 ml

of 1M LiAc/TE. The cells were then ready for transformation and the experiment proceeded directly to the next step.

Transformation of cDNA library Prey Vector into Yeast

First 1 µl of the prey vector containing the cDNA library was added to 5 µl of 50 µg/µl salmon sperm DNA and placed into a 1.7ml micro centrifuge tube. Ten tubes were made in total using 10 µl of prey vector and 50 µl of salmon sperm. The components were spun down and then 25 µl of the yeast cells that were prepared in the previous step was added to the solution. Afterward 300 µl of PEG 3550/LiAc/TE was added to the solution and the tubes inverted to thoroughly mix everything. The tubes were then left to incubate for 30 minutes at room temperature. Afterward the tubes were heat shocked at 42 C for 15 minutes and then removed quickly. Next 10 µl from one of the newly transformed cells tubes were placed in a new 1.7 m tube along with 990 µl of autoclaved saline, making a 1:100 dilution. The step was then repeated with 1 µl of transformed cells and 999 µl of autoclaved saline, making a 1:1000 dilution. These dilutions were plated on designated –leu/-trp 12 cm plates and allowed to grow at room temperature for 24 hours and then moved to the 30 C incubator to grow. Next the contents of each tube were split in half and plated on 15 cm –leu/-trp plates, making 20 transformed plates in total. The plates were then allowed to sit at room temperature for 24 hours and then were moved to the 30 C incubator to grow.

Replica Plating of Yeast from Primary Selection Plates to Secondary Plates

The yeast plated on the –leu/-trp plates were allowed to grow for 72 hours at 30 C producing visible large colonies. To replica plate them onto the secondary selection

plates of –leu/-trp/-his + 3AT, velvet was placed on a replica plating block and secured tightly around. Next the primary plates with growing yeast were placed on the velvet and gently pressed down to transfer the cells. Next a clean secondary plate containing 20 mm of 3AT was pressed gently onto the velvet transferring yeast cells unto the plate. The same step was repeated with plates containing 40 mm of 3AT and 60 mm of 3AT. All plates were then allowed to stay at room temperature for colonies to emerge.

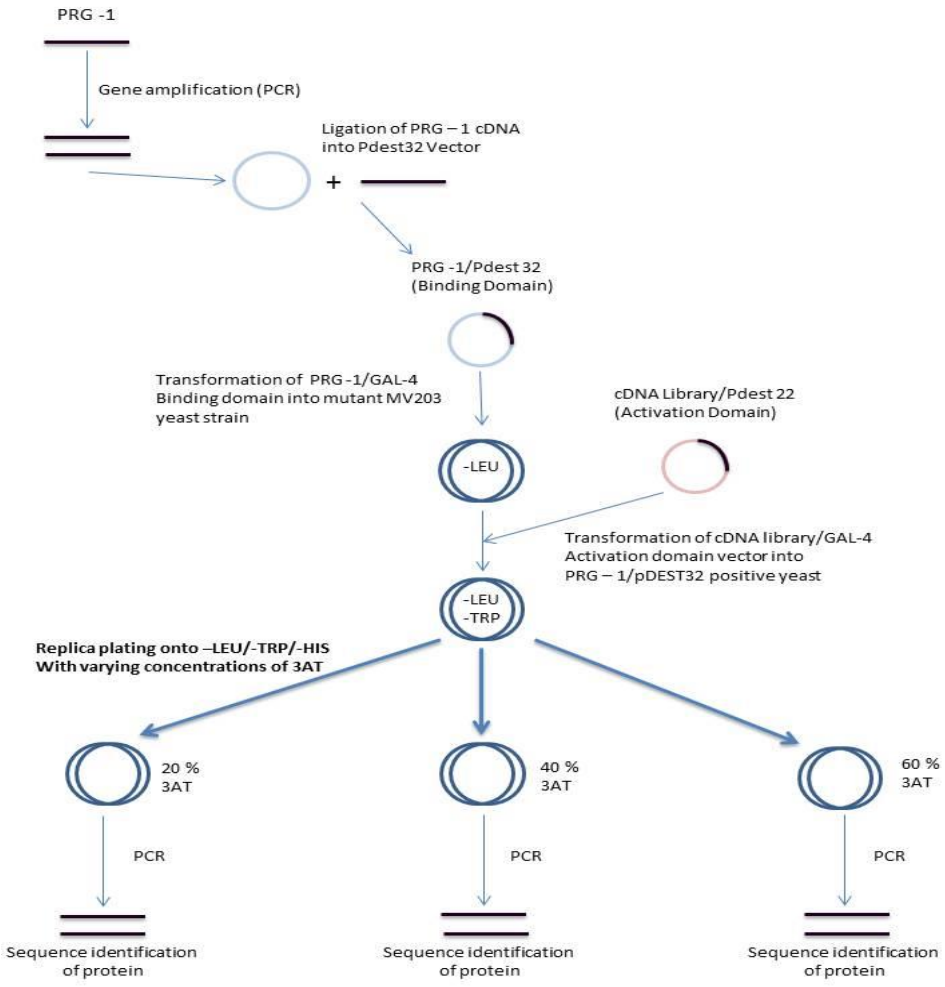


Figure 2: Yeast screening process for reporter genes obtained from the Proquest Yeast Two-hybrid Screen Protocol Manual pg 21.

Results

Argonautes have been implicated as very important protein in not only their own specific RNAi pathway, but as a regulators and secondary proteins of different processes in C.elegans. Because WAGO - 1 has been implicated in many RNAi and argonaute pathways, investigation of its interactions will help give us a more clear understanding of the specific roles and responsibilities it has in substantiating and maintaining processes in C. elegans. Investigation of potential protein interactions was done by utilizing a yeast two-hybrid system screening the WAGO - 1 protein against a cDNA library.

Construction of Vectors and Control Screens

The initial step to running the yeast – two hybrid system involved extracting the genes of interest and amplifying them to be used for a large scale screens. This was accomplished by first isolating and purifying mRNA of the PRG- 1, CSR -1, WAGO – 1, RDE – 1, and ERGO – 1 genes, and performing a reverse transcription using designated primers to create cDNA. They were then amplified by PCR and grown inside E.coli to increase the amount for experimentation. Because the yeast two hybrid screen works by expressing a reporter gene which occurs from the close proximity of the bait and prey vectors, the WAGO - 1 gene and cDNA library needed to be incorporated into the domain vectors, this would allow them to express the reporter gene of histidine if there were a potential interaction. Therefore WAGO - 1 was ligated into the pDEST32 vector which contains the binding domain for the yeast two – hybrid system, and the cDNA library obtained from a L4 developed male worm was ligated into the pDEST22

vector which contains the activation domain of the system (**Figure 1**). The results would ideally show that if there is an interaction between WAGO - 1 and another protein then the reporting gene of histidine will be expressed inside the yeast.

The advantage of using a yeast two-hybrid system is to observe protein interactions in vivo. In vivo experimentation helps to create an environment which is closely related to what may occur naturally, although the experimental organism may by different from the original. Because yeast are eukaryotic organisms which have the ability to grow and multiply quickly, they are readily chosen for experimentation when looking at in vivo applications. Yeast which are chosen for the two – hybrid screen usually have their genes altered in order to detect the reporter gene when it is activated by interactions. Yeast used for the screen was the mutant strain MV203 which lacked the ability to produce leucine, tryptophan, and histidine. The WAGO - 1/pDEST32 vector, which contains the gene to express leucine, was then transformed into MV203 yeast and were plated on leucine deficient medium plates. This was done to ensure that yeast which did not take up the plasmid did not have access to leucine and would subsequently die due to the lack of the essential amino acid. Yeast that took up and kept the WAGO - 1/pDEST32 expressed the leucine gene and therefore grew on the – leu selection plates. Colonies were then taken from the leucine selective plate and cultured to grow and multiply the yeast. To test the quality of the vectors, and also to recognize the outcome of a positive reaction, a control test was done by adding a F58.11/pDEST22 activation domain vector and transformed into the cultured yeast. The yeast that grew on these plates indicated that they had both the activation domain which

contained the genes to express tryptophan, and the binding domain which contained the genes to express leucine.

False positives in context of the yeast-two hybrid system are when colonies emerge on selection plates, but genetically do not have the desired components that produce a protein – protein interaction. False positives have the ability to emerge at any time during the screening process and therefore measures must be taken regularly throughout the screen to eliminate them. To ensure the integrity of our results several initial controls were run to set parameters for colony morphology and numbers. The combination of proteins PIE -1 (BD)/MEP -1 (AD) was used as a control to determine a successful screen basis because of the known interaction between both genes inside C.elegans. WAGO - 1(BD)/F58.11(AD) were used as a control to determine the integrity of the WAGO-1 binding domain construct because of the specific known interaction of WAGO-1 and F58.11. WAGO-1 and the empty activation domain were used as a control to investigate self-activation, and the WAGO-1 (BD)/WAGO – 1 (AD) control was used to investigate self-interactions. The results showed that PIE-1 and MEP- 1 yeast hybrid screen produced colonies on the initial selection plate of –try/-leu and also on the secondary selection plates of –his/-leu/-trp which contained 3AT at the different concentrations of 20, 40, 60 percent. The WAGO-1 and F58.11 reaction grew on both the initial and secondary selection plates on all concentrations of 3AT as well, indicating that there was a very strong reaction between both proteins. In comparison the WAGO-1 and empty activation domain produced colonies on the initial selection plate of –trp/leu, but did not produce any colonies when replicated onto the secondary selection plate of –his/-leu/trp. This indicates that the reporter gene histidine was not activated

supporting the assumption that WAGO-1 is not self-activating within the yeast two-hybrid system. The same result was met when the control parameter of WAGO -1 in both the binding domain and activation domain was tested. This strongly suggests the WAGO - 1is also not self – interacting.

Screening of WAGO – 1 Argonaute protein against cDNA library

The success of the yeast-two hybrid system is dependent on the ability of the yeast to take up the designated constructs and their overall transformation efficiency. This allows for larger designated protein interactions to potentially occur. For the WAGO-1 yeast screen dilutions of 1:100 and 1:1000 of the transformation reaction were made for only the binding domain transformations, and then for both plasmid transformations. They were then plated on small –leu plates and -leu/trp plates. On the –leu selection plate with only the binding domain 357 colonies emerged and 262 colonies were found on the –leu/-trp dilution plate. Using the results generated on the dilution plates we were able to calculate the transformation efficiency of the library screen.

$$Number\ of\ Colonies\ on\ (-leu, -trp) \div Number\ of\ Colonies\ on\ (-leu)$$
$$= Number\ of\ Transformants$$

According to the overall calculations 2.2 x 10^5 colonies were transformed on each 15 cm plate. There were 20 plates in all and therefore the total amount of 4.4 x 10^6 colonies for the screen. According to the Gateway protocol the number of expected

transformants with a cDNA library is 4.3 x 10^6 colonies and so it can be concluded that the screen transformation was successful **(Figure 2, Figure 3a).**

After cleaning, the replicated yeast were allowed to grow on –leu/-trp/-his plates with varying concentrations of 3AT **(Figure 3b).** From the replicated plates 247 colonies emerged on the 20mm 3AT concentration, 2 colonies emerged on the 40mm 3AT concentration, and there were no colonies that grew on the 60mm 3AT concentration plates. Of the colonies that did emerge they were relatively small and had only the distinct feature of very small bumps which indicated that there could be some growth, but nothing definitive. The lack of characteristic large colony growth could indicate that interactions may have occurred, but were very weak hence why colonies of interest only grew on the lower concentrations of 3AT.

Discussion

The yeast-two hybrid system helped to investigate protein interactions with the primary argonautes found in C.elegans. These argonautes are responsible for RNAi and other processes that happen inside C.elegans. The screen produced over 249 potential interactors with the argonaute WAGO-1, and more interacting proteins may be recognized with the other screens that are yet to be run. The next steps will involve ensuring positive interactions are occurring and any weak or false reactions are eliminated. This is usually accomplished by streaking the colonies from secondary selection plates, in this case –leu/-trp/-his plates with 3AT, onto new secondary selection plates with the same parameters as before. This ensures that any colonies or yeast that were on the screening plate which managed to live even without the specific selection factors are eliminated. Colonies that then emerge on the streaked plates are very good indicators of positive interactions and also have the ability to grow larger with more space and resources provided by the new plate. After the colonies have been streaked and regrown on the new selection plates, the next step is to positively identify the proteins that are interacting with each argonaute of interest. It should be considered that many of the interactions that have occurred could already have been positively identified from previous research, such as F58.11. Identification of the interacting proteins can be accomplished using different biochemical analysis. Using the method of PCR to amplify the interacting gene and then sequence it has been favored when dealing with unknown proteins, and should be applied to this situation once the yeast have been selected, opened, and the DNA has been extracted.

Following identification of the unknown proteins the next step that would be most beneficial is to establish what role the proteins play inside C.elegans. If there were previous research that has been conducted on the protein which yields this information, it can be readily verified and applied to the results obtained by the screen. If information is limited, knocking out the known gene inside the actual worm and see what effects occur can be done as well. It is important to take into consideration that removing an interacting protein from a pathway will potentially have adverse effects. If the knockout worms die it could be a very good indicator that the gene is needed in very important processes of survival. Previous research supports this with the knockout mutation of the primary argonautes. PRG-1 is needed for transposon maintenance and when knocked out of the worm is proven lethal. The same can be said for knockout mutations of CSR-1 which is needed for proper chromosome segregation, although there are no immediate repercussions to the organism; without this important argonaute the offspring of the worm cannot develop correctly and leads to developmental arrest. Another investigation tactic that could be used is knocking out the argonaute itself and seeing what effects occur on the interacting protein and rest of the pathway. A key factor to investigate is if the identified protein interacts with more than one of the primary argonautes, such as if the argonautes are able to share more than one helicase protein. Helicase proteins such as F58.11 are needed in the formation of the RISC complex for the WAGO-1 RNAi pathway.

Once there has been conformation of interaction proteins, understanding the specific areas and process in which these interactions occur are also very important to the overall understanding of RNAi and other processes in C.elegans. Another screen

should be performed using only parts of each protein in the bait vector and then running it against the cDNA library to find potential interactions. The binding domain of the protein to the interactor is arguably the most important aspect within the investigation. Putting in only the N terminal or C terminal of a protein and finding if the interaction still occurs can pinpoint the specific spot and sequence that is of importance. Manipulation of these proteins can determine whether or not just the binding domain of the protein is important or the entire protein is needed for binding. In terms of protein complex formation understanding of the specific binding areas is crucial. Mutations in terms of the binding domain of a protein can lead to potentially severe problems functionally for the organism. Understanding relationships between proteins is important to gaining an overall better picture of pathways and processes inside organisms. If there are interactions between the primary argonautes it could indicate that the pathways are more interdependent and closely related than previously depicted. Integrated pathways can also be identified by proteins that interact with more than one of the primary argonautes.

Figures

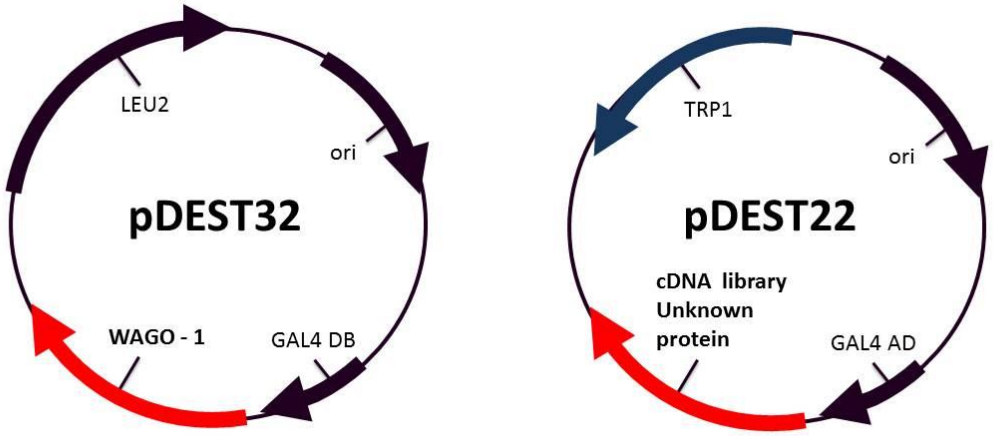


Figure 1 Bait and Prey Constructs for WAGO – 1 Y2H Screen: The pDEST32 bait vector made in accordance with the Invitrogen Gateway Yeast Two – Hybrid System. Important components are the WAGO-1 gene inserted through LR reaction, open reading frame for replication inside the yeast. Positive leucine gene and GAL4 Binding Domain sequence. Important components of the pDEST22 vector were the inserted cDNA library, Positive tryptophan gene, open reading frame, and GAL4 activation domain.

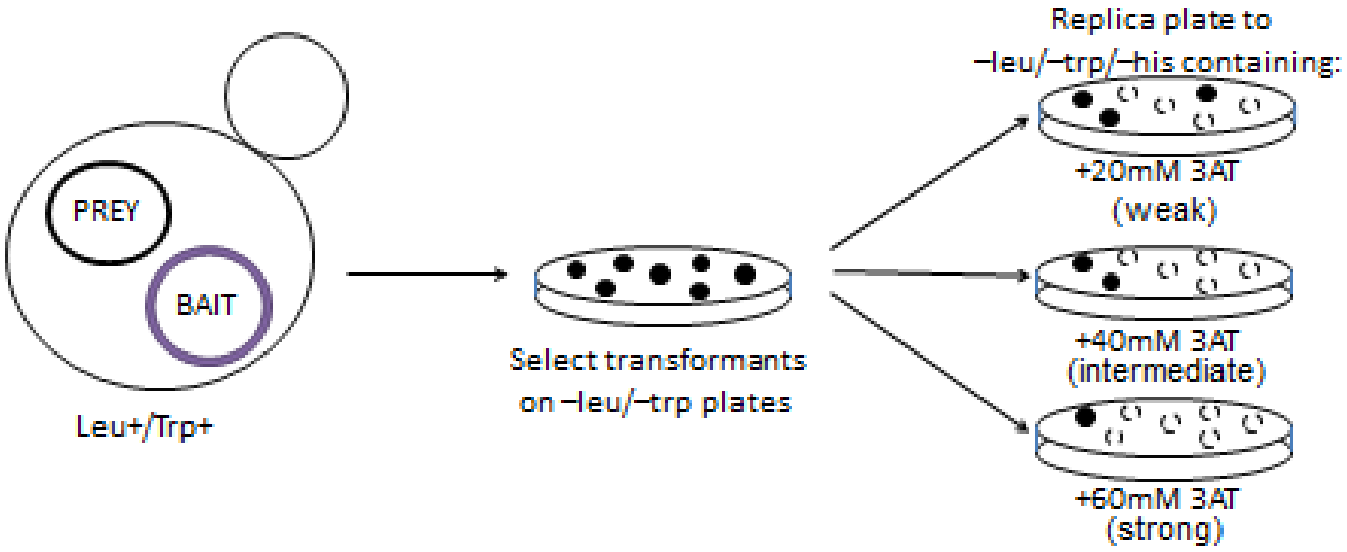


Figure 2 Mechanism of Performing the Yeast – Two Hybrid Screen: Both the bait and prey plasmid are inserted into yeast which begin to expressed the genes incorporated to ensure selection. The yeast are plated on primary selection plates in order to confirm the yeast which possess both plasmids. The yeast were then transferred to secondary selection plated to test for interactions by expression of the reporter gene. Inhibitors allow for analysis of the strength of interactions by increasing the difficulty of the reporter gene to undergo its metabolic processes.

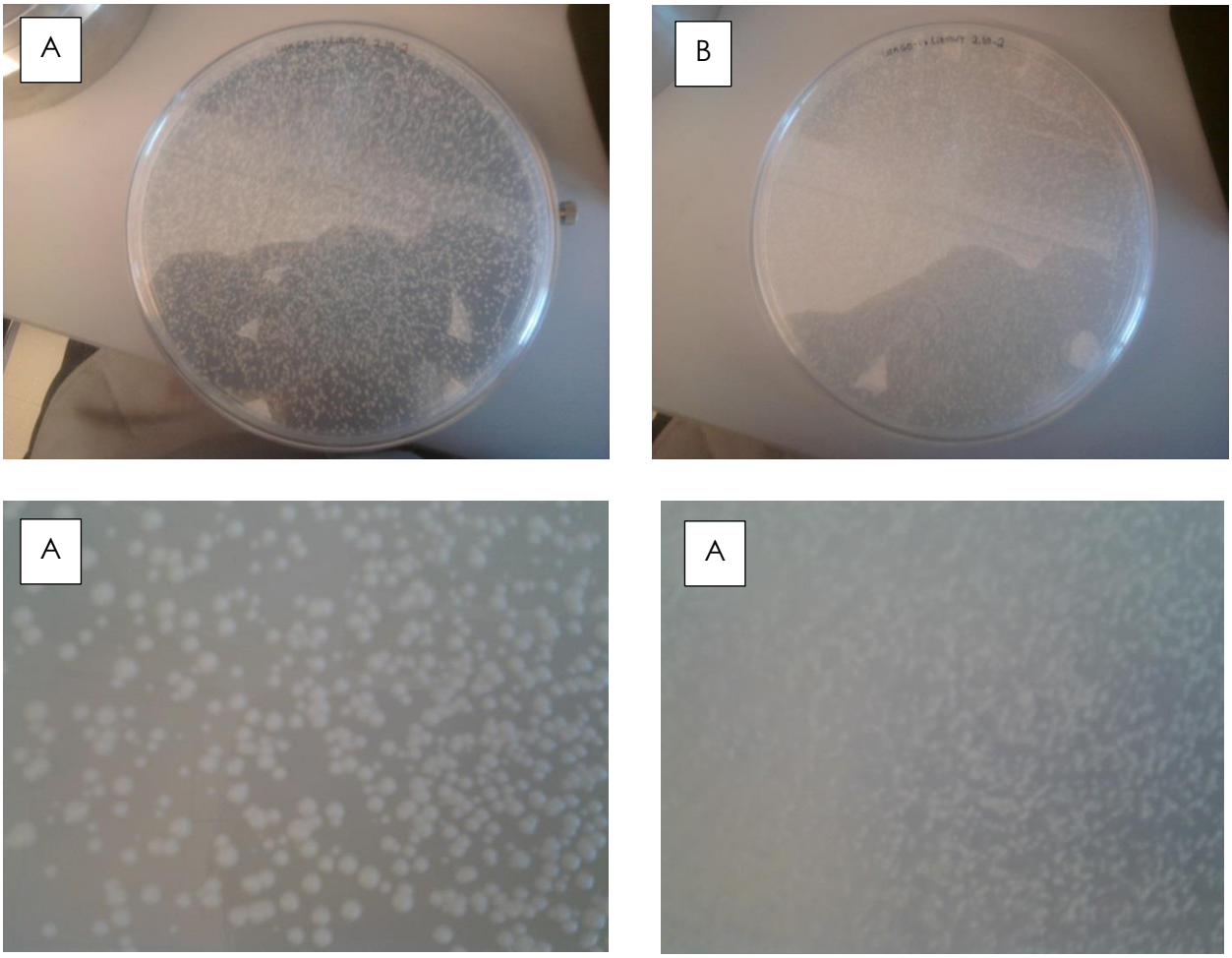


Figure 3 WAGO – 1 Library Screen: A) WAGO – 1 library screening plate pre-replication B) WAGO-1 library screening plate post replication, allowed to grow at room temperature for 72 hours.

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