2015

Genome Engineering to Create Dominant Alleles in Caenorhabditis elegans Using CRISPR-CAS9 Technology

Abrar Sulaimani
Rowan University

GENOME ENGINEERING TO CREATE DOMINANT ALLELES IN *CAENORHABDITIS ELEGANS* USING CRISPR-CAS9 TECHNOLOGY

Abrar Sulaimani, B.S

A Dissertation submitted to the Graduate School of Biomedical Sciences, Rowan University, in partial fulfillment of the requirements for the M.S. Degree.

Stratford, New Jersey 08084

July 2015

Table of Content:

Acknowledgments

I would like to thank all of the people who made this dissertation possible, starting with my mentor Dr. Eric Moss. He has provided all the support I needed to earn my Master's degree. Also, I would like to thank my committee, Dr. Ronald Ellis and Dr. Susan Muller-Weeks, for being in my thesis committee and guiding me with their comments. Special thanks to the GSBS administration office and the Rowan international center for providing instructions to finish my program.

I would like to thank my husband Ahmad and my parents for their support from the beginning of my scholarship. I want to thank my friend Jennifer Tsialikas for everything. She was mentoring and motivating me. Thanks to Angels Nguyen, and Tasneem Khokhon for being there for me.

Finally, I would like to thank the Saudi Cultural Mission (SACM) advisors for making my scholarship a success and getting my Master degree. I thank King Abdul-Allah Scholarship program supervisors for giving me a great opportunity to study in the USA.

Abstract

Many investigators have being using CRISPR-Cas9 as a method of genome engineering because it is easy, accurate and fast. This technique has been used to modify the genomes of a wide variety of organisms, including the nematode *Caenorhabditis elegans (C. elegans)*. The short life cycle and ease of introducing exogenous plasmids make *C. elegans* an ideal system for advancing this technique. My thesis had two aims that focused on developing methods to create dominant alleles in *C. elegans*. Genetic modifications like precise deletion and insertions into a locus of chromosome are technically challenging. Additionally, although there are several ways of generating transgenic models that are useful for developmental studies, the efficiency of getting stable transgenic lines can be an issue. To address these problems, my first aim was using CRISPR-Cas9 specifically to generate *ztf-16* mutants by deleting *lin-4* and *let-7* microRNAs regulatory regions in its 3' UTR. I used microinjection to introduce the CRISPR plasmids into the gonad of a hermaphrodite *C. elegans*. In addition, I employed a co-CRISPR strategy to enhance the modification events and to visualize the morphology of the transgenic animals in the F1 progeny. PCR based screenings confirmed that two candidates have the *ztf-16* 3'UTR deletion in the F2 progeny. My second aim was to set up a method that allows locus-specific insertion of any desired sequences using a Cre/loxP system. The first step was to use the CRISPR-Cas9 method to insert mutant loxP (lox66) into chromosome IV and then inject a second loxP (lox71) to cause the single-copy insertion.

Introduction

Targeting precise modifications in eukaryotic genomes like that of *Caenorhabditis elegans* genome has been fast, easy and accurate. Manipulating the *C. elegans* genome by using genome-engineering technologies is valuable for studying genes, especially those that are conserved from nematode to human. [1] In recent years, programmable nucleases have become a powerful method for successful genome editing. These nuclease enzymes are site-specific and modify DNA by double-stranded breaks (DSBs). Examples include the transcription activator-like effectors (TALEs) from *Xanthomas* bacteria or the Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) associated endonuclease Cas9 from type II bacterial adaptive immune system CRISPR. Basically, TALE proteins recognize and edit one nucleotide in a DNA target directly by protein-DNA interaction. [2]

CRISPR technology has been adapted to yield many desired modifications. [2] CRISPR is more common in basic biotechnology research and medical technologies that utilize genome editing. In 1987, CRISPR was a mystery sequence of 29 nucleotides found by researchers in the *E. coli* genome. Since this discovery, CRISPR has rapidly evolved and almost all details about this system are known now. After 25 years of research, we know now how CRISPR is working and facilitating genome editing studies. The CRISPR-Cas9 has been adopted in wide experiments in agriculture, biotechnology, nutrition, and drug development due to its flexibility to accomplish genome editing. This accelerated technology in 2013 was successfully used in mammalian cells and since then studies continue to develop rapidly. [2]

The CRISPR-Cas9 system is derived originally from a bacterial defense mechanism against viral infections. [2] Together, the Cas9 endonuclease and small guide RNAs (sgRNA) protect the bacterial cells from viral invasion because the Cas9-sgRNA complex identifies the viral DNA in specific sites and prevent its's replication. In 2011, researchers showed that they could design synthetic sgRNAs that have a 20 nucleotide sequence complementary to almost any desired DNA target. [1] CRISPR-Cas9 technique requires three elements to work: Cas9 protein, its sgRNA and protospacer adjacent motif (PAM) in the target DNA. This system is based on DSBs by the Cas9 enzyme at defined site so the sgRNA distinguishes the target and matches to it. Then Cas9 cleaves both strands of targeted DNA and there are two possible ways to repair the cleavage: by non-homologues end joining (NHEJ) that causes indels — small insertions, deletions, or both or homology-directed repair (HDR) which requires a donor or template DNA to repair the double cleavage. [3], (Figure 1)

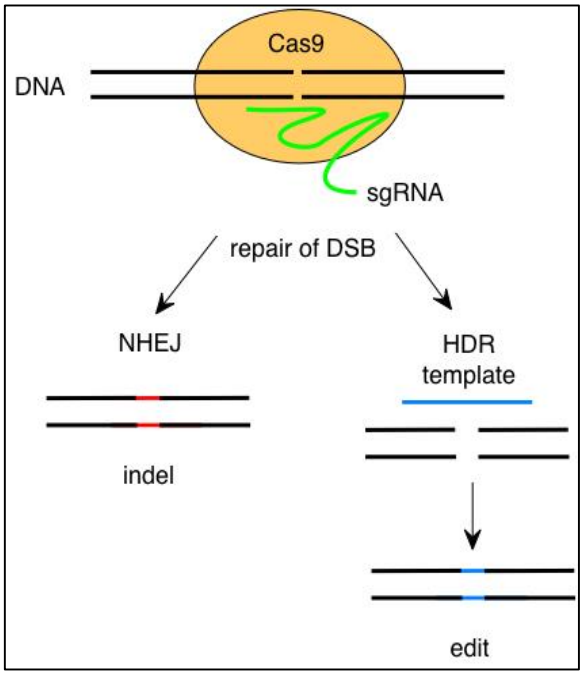

Figure 1: Genomic editing by double-stranded break (DSB) using CRISPR-Cas9 technology. Cas9 cuts at specific sites and there are two possible repair methods: non-homologues end joining (NHEJ) which causes random insertion and deletions (indel) or homology-directed repair (HDR) which requires a template DNA (in blue) and results in precise gene editing.

Researchers are improving the CRISPR method to ensure the success of genome editing and developing new protocols that are used to get highly efficient genome modification. For example, a co-CRISPR strategy has been shown to optimize homologous recombination events and yield highly efficient gene editing. In this strategy, co-injecting an experimental CRISPR with a verified CRISPR allows direct identification of genome-editing events. [1] Researchers have found that mutants displaying the verified CRISPR's phenotype were enriched for the second, experimental modification. For both aims in this study, I used a *dpy-5* co-CRISPR that was previously developed in the lab. Mutations in *dpy-5* cause a short and fat phenotype, which is easily screened for under the dissecting microscope. Moreover, there is a recent study that focused on the enhancement of Cas9 action *in vivo*. This study suggested designing sgRNAs that have an extra GG motif at the 3' end of their target-specific sequences in order to increase mutation frequency. [4] (Figure 2) In this research, I applied the recent updated CRISPR techniques in order to design CRISPR plasmids that were used to generate new *C. elegans* mutants that will be used for understanding more about genes regulation by microRNAs in heterochronic pathways.

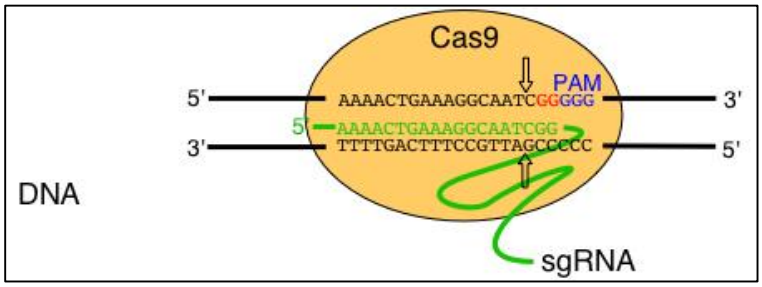

Figure 2: The sgRNA targets Cas9 enzyme to cleave double stranded DNA. The extra GG motif in red next to the PAM in blue. The sgRNAs shows in green, Cas9 enzyme in orange and the arrows

In *C.elegans*, the heterochronic pathway regulates succession of the cell fates so that they occur at the correct time. If there is a mutation, the cells will divide differently: dividing in advance or delaying the division. This concept is referred to as heterochrony, in other words, is the result of the developmental events occurring at alternative times. [5] Even though *C. elegans* is a simple model, the cellular and genetic characteristics are complex. [7] Heterochronic mutations cause two main characteristic phenotypes: precocious or reiterative developments. [5] Precocious development of specific cell lineages causes the stage-specific events to occur early because one or two development stage is skipped. On the other hand, reiterative development causes these events to occur later due to the repetition of one or two stages. [8] Studies done on heterochronic genes using genetic analysis found that multiple genes incorporated in this pathway act as central regulators and a number of these regulators encode microRNAs. Mainly, microRNAs regulate most genes that act during postembryonic development of *C. elegans*. [6] Moreover, the genes are regulated by microRNAs that have binding sites in their 3' UTRs to cause an inhibition. (Figure 3)

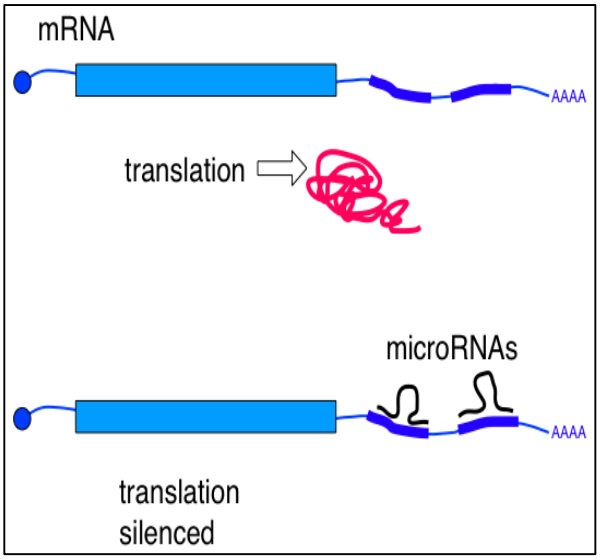

Figure 3: Gene regulation by microRNAs. The messenger RNA (mRNA) of a gene has binding sites in its 3'UTR (thick blue lines). These microRNAs (black loop shape) cause an inhibition activity and no protein produced (red).

Studies suggest that microRNAs control their targets at 3'UTR binding sites. When these sites are deleted, which has occurred in some natural mutants like *lin-14*, a strong phenotype occurs. Based on genetic analysis, it was discovered that the microRNA *lin-4* negatively regulates the transcription factor *lin-14*. This genetic interaction was identified by heterochronic phenotypes of *C. elegans* mutants. [9] The dysregulation of a microRNA, or its target, results in two possible developmental phenotypes as shown in Figure 4. Importantly, the 3'UTR region of the target gene that has its microRNAs binding sites disrupted, causes reiterative development, the same phenotype as when the microRNA gene is mutated. (Figure 4), [10]

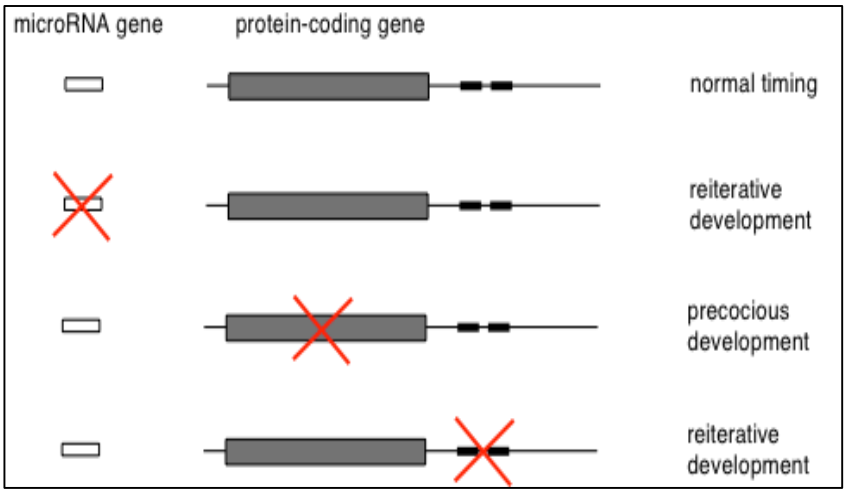

Figure 4: The regulation of heterochronic gene encodes microRNA in developmental timing. Mutation (red X shape) in microRNA gene (white box) causes reiterative development while a mutation in the target gene (gray box) causes precocious development. A mutation in microRNA binding sites (two black boxes) in 3'UTR of the target gene results reiterative development.

In this study, my first aim focuses on creating new mutants of *ztf-16* by using the CRISPR method to alter the microRNA regulatory regions of *ztf-16's* 3'UTR. Gene *ztf-16* is an Ikarose family member and encodes zinc-finger transcription factor. In *C. elegans*, the ZTF-16 protein has role in remodeling of glial cells during dauer larval development

via controlling the expression of *ver-1* which encodes a receptor tyrosine kinase. [11] The gene *ztf-16* was recently identified as a loss-of-function suppressor of reiterative development in our lab. The project was done to determine a potential partner for the hunchback-like-1 (*hbl-1)* gene, which is also an Ikarose family member and encodes a zinc-finger transcription factor.

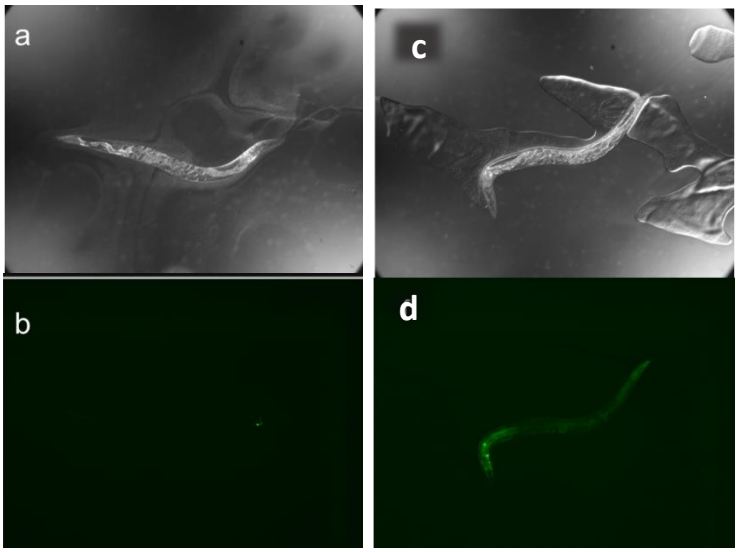

Figure 5: Comparison showing expression of *col-19::gfp* in adult worms: a) *hbl-1* DIC b) *hbl-1GFP* filter applied. c) *ztf-16* DIC d) *ztf-16* GFP filter applied. From b panel, *col-19::gfp* is not expressed in *hbl-1* retarded mutant while in 5d panel, shows expression of *col-19::gfp* after *ztf-16* RNAi feeding procedure was used.

Mutation in *hbl-1* causes a retarded phenotype where larva stage 2 (L2) is repeated and the adult animal is not formed. The RNA interference method was performed to find a partner and *col-19::GFP* was used as marker for the adult cuticle to determine which RNAi suppressed the retarded phenotype of mutated *hbl-1*. The results showed that *ztf-16* RNAi suppresses *hbl-1* retarded development. As shown in Figure 5b, *col-19::gfp* is not expressed in *hbl-1* retarded mutant. Conversely, Figure 5d, shows expression of *col-19::gfp* after *ztf-16* RNAi feeding procedure was used. That demonstrates that *ztf-16* is a possible partner for *hbl-1* gene and could have an undiscovered role in developmental timing. The question was then raised: what is the role of *ztf-16* in developmental timing?

Genetically, it is a candidate target for microRNAs because its 3'UTR contains *let-7*and *lin-4* microRNAs binding sites as shown in Figure 6. I deleted most of its 3'UTR including the region of potential binding sites using CRISPR-Cas9 modification method. My CRISPR plasmids targeted at each end to cause large deletion. In wildtype *ztf-16,* PCR primers (small arraows) will detect a 900 bp product, while in the mutants, primers will detect about a 300bp PCR product. (Figure 6)

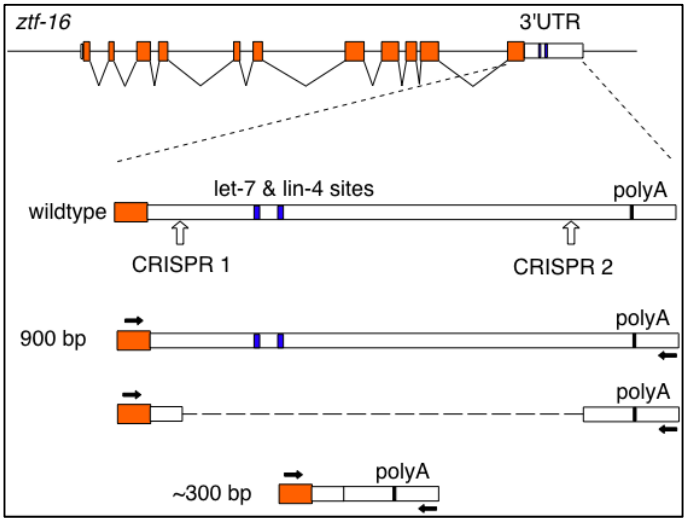

Figure 6: Deletion of the microRNA regulatory regions of *ztf-16* by using two CRISPR plasmids 1 and 2 are in white arrows. PCR primers (small black arrow) detect a 900 bp product in wildtype but a 300 bp product in the mutants.

For my second aim, I want to design a method to make an insertion of any desired sequence into a targeted locus using genome engineering technologies. This will allow the comparison of the functions of a series of related heterologous sequences as stable, single-copy insertions in identical chromosomal locations. There are several technologies used to make transgenic animal such as germline transformation by injection, irradiation and micro-particle bombardment techniques. These methods are based on introducing exogenous DNA into the animal. [12] Making transgenes has been challenging due to several features: extrachromosomal array formation, variation in copy number, variation in stability, mosaicism, and some transgenes are inconvenient for comparing subtle differences between transgenes. [13][14] To overcome some of these

issues, I used CRISPR and Cre\loxP methods to facilitate locus-specific insertion of any desired sequences. The Cre-dependent loxP excision system is a site specific recombination (SSR) technique that is used to cut or invert DNA between two short sequences called loxP sites which are short (only 34bp). The orientation of the loxP sites has to be known because whether excision or inversion occurs is dependent on this orientation. [15] Recent studies show that SSR using Cre/loxP is a good option in genome modification for obtaining a desired genotype and phenotype. [16] I used one CRISPR plasmid to insert a loxP site into the genome [17] Then, a second loxP site will be integrated into a plasmid by researchers the site-directed mutagenesis method. When the plasmid is introduced into the animal along with the Cre recombinase, it will integrate into that single site in the gene. Furthermore, to ensure that plasmid can integrate but not be deleted by the same Cre, I used loxP mutants (lox66, lox71). [18] (Figure 7)

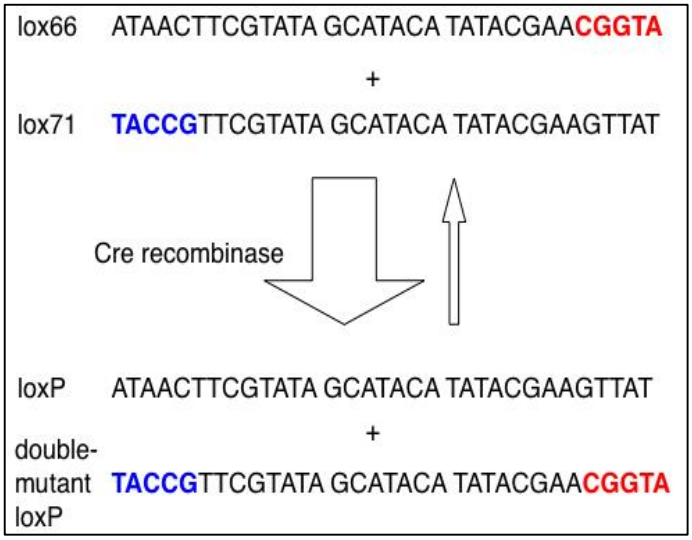

Figure 7: The loxP mutants (lox66 and lox71) drive the reaction in one direction in the presence of Cre recombinase. The thinner arrow indicates insufficient reverse reaction. The products of this reactions: normal loxP and double- mutant loxP.

Rational:

The goal of aim one is to make a large deletion of regulatory elements in the 3' UTR of *ztf-16*. The purpose is to understand if *ztf-16* is a novel target for microRNAs, and also discover if the deletion of regulatory regions causes developmental timing phenotypes. Bioinformatics, indicates at least 3 binding sites for microRNAs in *ztf-16* 3' UTR, all of which were deleted. Moreover, the gene *ztf-16* was recently identified as a suppressor of *hbl-1* reiterative development. Here the *ztf-16* mutant strains will be characterized and the *ztf-16* mutant will be crossed with other developmental timing mutants to measure suppression and enhancement of precocious and reiterative phenotypes.

My second aim is to establish a method of locus-specific insertion to overcome some problems that arise from making *C. elegans* transgenes. In research, there are many uses for plasmid integration: gene specific reporters as plasmids, microRNA sponges and RNA circles to assay microRNA activity. Injected plasmids do not normally lead to integration into the genome, but rather, they form extrachromosomal arrays. These arrays are not stable and are lost at high frequency in later generations; additionally, there is no drug-based selection used in *C. elegans*. Therefore, populations that carry an array are heterogeneous making some kinds of analysis difficult for example, genome expression studies on populations may be compromised by heterogeneity of genotypes. Various methods of integrating plasmids have been tried. Some, involved radiation, others required elaborates transposon systems, but all were labor-intensive and inefficient. The advantages of CRISPR technology, makes it a simple solution to this problem.

Material and Methods

Worm Strains:

Nematode strains were maintained at 20˚C. RG733 was used for this study as the wild type.

Plasmids preparation:

The Q5 site-directed mutagenesis kit protocol (E0554) was used, to insert the targeting sequence of the sgRNAs into the CRISPR plasmid (pDD162 made by Goldstein B. [19]). This plasmid has the Cas9 coding region for the worms. (Figure 8) The sgRNAs were designed with the extra GG motif. CRISPR plasmids that has sgRNAs were used in worm microinjection. [4]

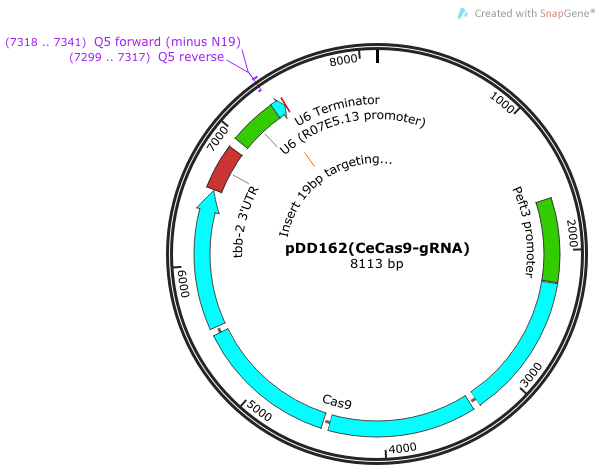

Figure 8: CRISPR-Cas9 plasmid (pDD162) for *C. elegans* contains Cas9 sequence and a site for inserting the sgRNA sequence. This site is used to insert sgRNA.

The *ztf-16* sgRNAS sequences are:

dn1175: AAAACTGAAAGGCAATCGGGGTTTTAGAGCTAGAAATAGCAAGT.

dn1176: AAAGAAATGTTTGGGATGGGGTTTTAGAGCTAGAAATAGCAAGT.

The lox66 insertion sgRNA sequence is dn1184:

TAATAATCGTAACTTAAAGGGTTTTAGAGCTAGAAATAGCAAGT

This is a locus on LGIV that has been shown to permit a good expression of integrated gene. [17]

Germline Transformation by Microinjection:

All the plasmids for microinjection were purified using the Midi Plasmid Purification kit. The method of microinjection is derived from Singson *et al*. (2009) protocol. [20] Microinjection mixtures were prepared in 50µl. Injected animal were incubated on fresh plates at 25°C. For both experiments, 30 worms were injected. For the deletion *ztf-16* 3'UTR, the DNA microinjection was: the *dpy-5* co-CRISPR which is pJT.8 (100ng\µl), pAS.18 (200ng\µl) and pAS.19 (200ng\µl) are *ztf-16* CRISPRs. For the locus-specific insertion experiment: pJT.8 (100ng\µl), pAS.20 (200ng\µl), and dn1185 (500uM) were used.

Screening mutants by PCR:

Injected animals were allowed to self-fertilize for 3-4 days. The dissecting microscope was used to screen F1 dumpy animals and then cloned them. Next, lysates from F2 dumpy animals were screened by PCR to identify the mutants. Primers used for PCR were complementary to the *ztf-16* 3'UTR outside the CRISPR sites. Further PCR and DNA sequencing analysis was done on F3 progeny to confirm they had a deletion in 3'UTR.

Experimental Results:

CRISPR plasmids with target sequences generation

Using direct-site mutagenesis, CRISPR plasmids (pDD162 with sgRNAs) were generated *in vitro.* After bacterial transformation and DNA purification, the sequences of CRISPRS plasmids were confirmed by sequencing. The sgRNAs have the extra GG at 3' of the target-specific sequences.

Deletion of microRNAs binding sites in *ztf-16* 3'UTR

The two *ztf-16* CRISPR plasmids have both the Cas9 and sgRNA sequences on a single plasmid and each one was designed to target a site in the *ztf-16* 3'UTR. Transgenic lines were made by injecting a DNA mixture that included, *dpy-5* co-CRISPR and the two *ztf-16* CRISPR plasmids that target regulatory regions into 30 young adult *C. elegans*. Next, 40 F1 progeny with the dumpy phenotype were cloned. Out of 40 F1 clones, only 9 produced F2 dumpy animals. These 9 dumpy F2 were selected to screen based on PCR. Two new alleles were found and confirmed by sequencing. (Table 1) The first candidate, T2 (T2 in Figure 9), showed a band about 300bp, indicating a deletion when compare to the wild type band of 900bp. This band was expected size to see if the sequence in between the two CRISPR targets was deleted. The other candidate was T5 (T5 in Figure 9), had a band less than 500bp. This suggested there was a deletion, but it was not the expected size. Subsequent sequencing confirmed that T2 contained the desired deletion between the CRISPR target sites, removing the microRNA binding sites from *ztf-16* 3'UTR. In contrast T5 had the deletion, but also contained additional sequence of

unknown origin inserted into the locus. This sequence was searched against the BLAST database but was not found to match any known sequences.

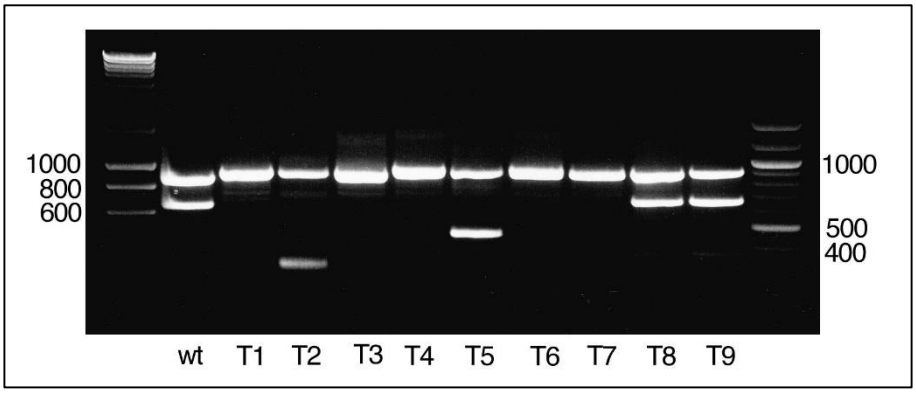

Figure 9: PCR products of *ztf-16* 3'UTR. This gel shows the two new mutants T2 and T5 that have about a 300bp and a 400bp band, respectively.

Microinjection mixture components	Description	injected worms	Dumpy F1	Dumpy F1 that gave F2 Dumpy	mutants
pJT.8 (100ng\µl) pAS.18 (200ng\µl) pAS.19 (200ng\µl)	*dpy-5* (Co-CRISPR) *ztf-16* CRISPR at 3' *ztf-16* CRISPR at 5'	30	40	9	2

Table 1: DNA microinjection mixture includes two CRISPR plasmids for targeting *ztf-16* 3'UTR and co-CRISPR plasmid to ensure Cas9 is active. Number of injected worms is 30 and produced 40 dumpy F1 progeny. Out of the 40 F1 9 gave dumpy F2 progeny that were screened for mutation. Only 2 mutants showed the deletion.

Locus-specific insertion of any desired sequences using CRISPR-Cas9

I used one CRISPR plasmid to insert the first loxP (lox66) site into the genome on chromosome IV or linkage group IV (LGIV). This is because by *Mos1*-mediated single copy transgene insertions (MosSCI), chromosome IV is a site in *C. elegans* that allows the inserted sequence to express very well. [17] The injection mixture includes CRISPR plasmids: CRISPR targets LGIV, *dpy-5* co-CRISPR and an oligonucleotide containing lox66 flanked by homology arms were injected into 30 young adult animals. Dumpy F2 progeny were screened for mutations by PCR and out of 28 candidates only one showed a possible insertion. (Table 2) The PCR screening determined that number 2 had shifted up a little as shown in Figure 10. This result indicated CRISPR plasmids cleaved and there is possibly a lox66 insertion. Following sequencing confirmed that sequence is not the lox66 site.

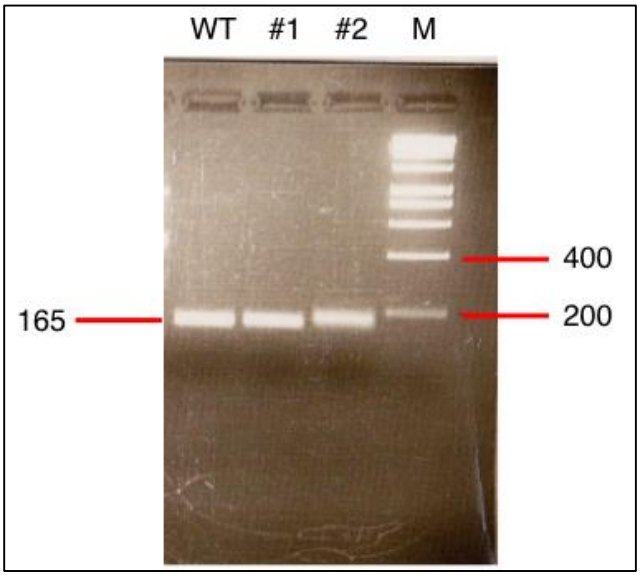

Figure 10: The 3% agarose gel indicates possible candidate #2 that has the lox66 site because it was shifted up.

Microinjection mixture components	Description	injected Worms	Dumpy F1	Dumpy F1 that gave F2 dumpy	mutants
pJT.8 (100ng\µl) pAS.20 (200ng\µl) dn1185 (500uM)	*dpy-5* (Co-CRISPR) LGIV CRISPR Single strand oligonucleotide	30	70	28	1

Table 2: Summary of the DNA microinjections to insert the lox66 into chromosome IV. The mixture includes a CRISPR plasmids targets the chromosome IV, co-CRISPR plasmid to ensure Cas9 activity, and single strand oligonucleotide which is the lox66 site. Number of injected worms is 30 and produced 70 dumpy F1. Out of this 70 dumpy F1 gave 28 dumpy F2. After screening for mutation, only 1 candidate showed possible insertion.

Discussion:

In the nematode *C. elegans*, exploring the function of conserved genes is the main focus of many studies, however, a great number of these gene's activities have not been discovered yet. [21] The method of genome engineering using CRISPR-Cas9 system will facilitate on understanding the genes regulation of many genetic pathways. Researchers, who modify a specific gene in a developmental pathway can determine whether the gene acts as a suppressor or an enhancer of the phenotype of other genes. For example, in *C. elegans*, genes that produce microRNAs control their targets at the 3'UTR in *C. elegans*. Gene regulation by microRNAs is essential for succession through development. In this study, the gene *ztf-16* was chosen because it has microRNAs binding sites in its 3'UTR, and *ztf-16* RNAi was used previously in the lab to successfully suppress a retarded development. Using the CRISPR-Cas9 method, I was able to generate a deletion in the 3'UTR and the microRNAs binding sites for *lin-4* and *let-7* microRNAs were eliminated. (Figure 11) Future work will discover if this mutant line has a phenotype when crossed with other related mutants.

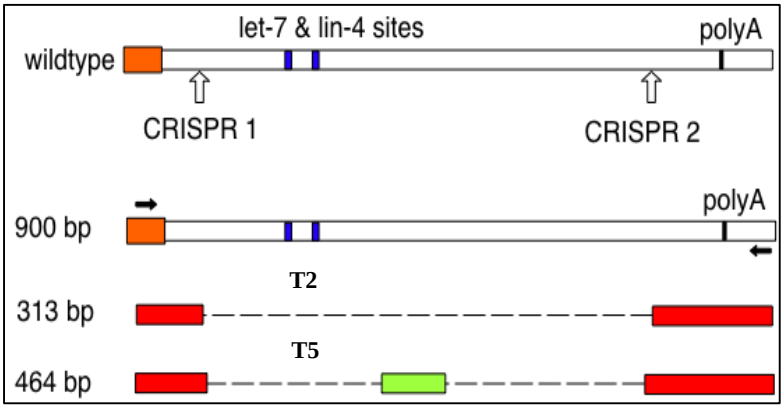

Figure 11: Two new alleles of *ztf-16* are missing the microRNAs binding sites. T2 is a candidate that has the deletion and results 313bP PCR product. T5 is the candidate which has a deletion and unknown DNA got inserted and results 464bp PCR product.

Building up a strategy that improved genomic engineering in *C. elegans* was the second aim of this project. I sought to solve one problem that happens often in getting mutants for research. The main problem with the transgenic line made in *C. elegans* after the germline transfection is the formation of extrachromosomal arrays. I used the Cre\loxP system method to make a single-copy insertion site at chromosome IV in *C. elegans*. CRISPR was used to utilize the first loxP insertion into chromosome IV. Special lox sites were selected to ensure the direction of the reaction moves one way to insert the desired sequences into the genome. (Figure 12) This method is promising because CRISPR cleaved and I have made a candidate line that had an insertion but the sequence is not belong to lox66.

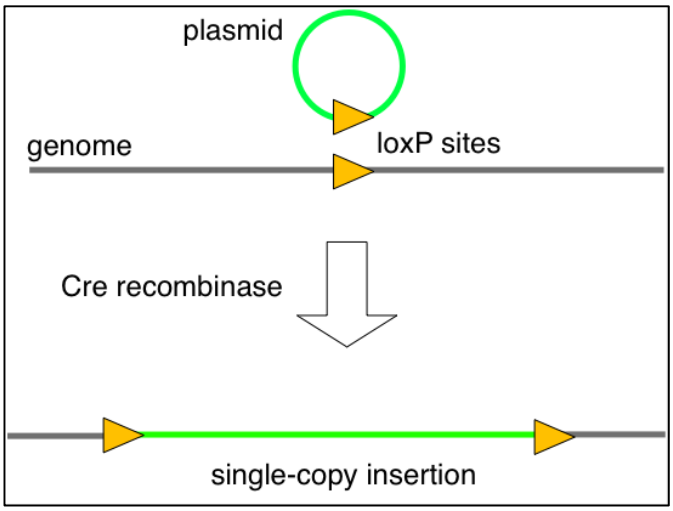

Figure 12: Method of locus-specific insertion of any desired sequences using Cre/loxP system. Cre recombines tow loxP sites to insert the plasmid which has the desired sequences.

Summary and conclusions

CRISPR-Cas9 technology was used to target two sites in the *ztf-16* 3' UTR to eliminate the microRNAs binding sites. I generated a novel viable deletion of the *ztf-16* 3' UTR and I am in the process of obtaining homozygous strains. The *ztf-16* mutants will be characterized genetically with other developmental timing mutants. For future directions, these mutants of *C. elegans* will be crossed with other related mutants for further investigations, like measuring suppression and enhancement of precocious and reiterative phenotypes.

Developing a method of locus-specific insertion of any desired sequences is in progress. I have one candidate which was not lox66 site that got inserted in in chromosome IV. The goal was after I get the lox66 inserted, I will proceed to insert lox71 into a plasmid. Next, I will inject the plasmids into the young adult worms and screen them. For future directions, this method will be used to insert desired sequences like a microRNA sponge into a specific location to obtain gain-of function mutants and use that for further studies.

References

[1] Kim H, Ishidate T, Ghanta KS, Seth M, Conte D Jr, Shirayama M, Mello CC. A co-CRISPR strategy for efficient genome editing in Caenorhabditis elegans. Genetics. 2014 Aug; 197(4):1069-80. doi: 10.1534/genetics.114.166389. Epub 2014. May 30. PubMed PMID: 24879462; PubMed Central PMCID: PMC4125384.

[2] Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014 Jun 5; 157(6):1262-78. doi: 10.1016/j.cell.2014.05.010. Review. PubMed PMID: 24906146; PubMed Central PMCID: PMC4343198.

[3] Arribere JA, Bell RT, Fu BX, Artiles KL, Hartman PS, Fire AZ. Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. Genetics. 2014 Nov; 198(3):837-46. doi: 10.1534/genetics.114.169730. Epub 2014 Aug 26. PubMed PMID: 25161212; PubMed Central PMCID: PMC4224173.

[4] Farboud B, Meyer BJ. Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. Genetics. 2015 Apr; 199(4):959-71. doi:10.1534/genetics.115.175166. Epub 2015 Feb 18. PubMed PMID: 25695951; PubMed Central PMCID: PMC4391549.

[5] Ambros V, Horvitz HR. Heterochronic mutants of the nematode Caenorhabditis elegans. Science. 1984 Oct 26; 226(4673):409-16. PubMed PMID: 6494891.

[6] Moss EG, Lee RC, Ambros V. The Cold Shock Domain Protein LIN-28 Controls Developmental Timing in C. elegans and Is Regulated by the lin-4 RNA. Cell. 1997 Mar7; 88(5):637-46. PubMed PMID: 9054503.

[7] Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell. 1989 Apr 7; 57(1):49-57. PubMed PMID: 2702689.

[8] "Introduction to C. Elegans". C. Elegans as a model organism. Rutgers University. Retrieved from http://avery.rutgers.edu/WSSP/StudentScholars/project/introduction/worms.html

[9] Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell. 1989 Apr 7; 57(1):49-57. PubMed PMID: 2702689.

[10] Moss EG. Heterochronic genes and the nature of developmental time. Current Biology. 2007 Jun 5; 17(11):R425-34. Review. PubMed PMID: 17550772.

[11] Procko C, Lu Y, Shaham S. Sensory organ remodeling in Caenorhabditis elegans requires the zinc-finger protein ZTF-16. Genetics. 2012 Apr; 190(4):1405-15. doi: 10.1534/genetics.111.137786. Epub 2012 Jan 31. PubMed PMID: 22298710; PubMed Central PMCID: PMC3316652.

[12] Kadandale P, Chatterjee I, Singson A. Germline transformation of Caenorhabditis elegans by injection. Methods Mol Biol. 2009; 518: 123-33. doi: 10.1007/978-1-59745-202-1_10. PubMed PMID: 19085141; PubMed Central PMCID: PMC2796118.

[13] Falgowski KA, Kmiec EB. Gene editing activity on extrachromosomal arrays in C.elegans transgenics. Gene. 2011 Apr 15; 475(2):87-93. doi: 10.1016/j.gene.2010.12.014. Epub 2011 Jan 15. PubMed PMID: 21241788.

[14] Mello C, Fire A. DNA transformation. Methods Cell Biol. 1995; 48:451-82. Review. PubMed PMID: 8531738.

[15] Hubbard EJ. FLP/FRT and Cre/lox recombination technology in C. elegans. Methods. 2014 Aug 1; 68(3):417-24. doi: 10.1016/j.ymeth.2014.05.007. Epub 2014 May 27. PubMed PMID: 24874786; PubMed Central PMCID: PMC4210360.

[16] Kage-Nakadai E, Imae R, Suehiro Y, Yoshina S, Hori S, Mitani S. A conditional knockout toolkit for Caenorhabditis elegans based on the Cre/loxP recombination. PLoS One. 2014 Dec 4; 9(12):e114680. doi: 10.1371/journal.pone.0114680. eCollection 2014. PubMed PMID: 25474529; PubMed Central PMCID: PMC4256423.

[17] Frøkjær-Jensen C, Davis MW, Ailion M, Jorgensen EM. Improved Mos1-mediated transgenesis in C. elegans. Nat Methods. 2012 Jan 30; 9(2):117-8. doi: 10.1038/nmeth.1865. PubMed PMID: 22290181; PubMed Central PMCID: PMC3725292.

[18] Liu WY, Wang Y, Qin Y, Wang YP, Zhu ZY. Site-directed gene integration in transgenic zebrafish mediated by cre recombinase using a combination of mutant lox sites. Mar Biotechnology (NY). 2007 Jul-Aug; 9(4):420-8. Epub 2007 May 15. PubMed PMID: 17503154.

[19] Dickinson DJ, Ward JD, Reiner DJ, Goldstein B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nat Methods. 2013 Oct; 10 (10):1028-34. doi: 10.1038/nmeth.2641. Epub 2013 Sep 1. PubMed PMID: 23995389; PubMed Central PMCID: PMC3905680.

[20] Kadandale P, Chatterjee I, Singson A. Germline transformation of Caenorhabditis elegans by injection. Methods Molecular Biology. 2009; 518:123-33. doi: 10.1007/978-1-59745-202-1_10. PubMed PMID: 19085141; PubMed Central PMCID: PMC2796118.

[21] Petersen C, Dirksen P, Schulenburg H. Why we need more ecology for genetic models such as C. elegans. Trends Genet. 2015 Mar; 31(3):120-7. doi: 10.1016/j.tig.2014.12.001. Epub 2015 Jan 8. Review. PubMed PMID: 25577479.

Attributes

Figure 1- Moss, Eric Ph.D. created schematic of the CRISPR-Cas9 double-stranded break and methods of the repair.

Figure 2- Moss, Eric Ph.D. created schematic figure for targeting Cas9 to the target sequence.

Figure 3- Moss, Eric Ph.D. created schematic of the miRNAs action site.

Figure 4- Moss, Eric Ph.D. created schematic of the role of miRNAs in developmental timing.

Figure 5- Chelsea Karacz M.S. created all the constructs and performed the experiment and imaging.

Figure 6- Moss, Eric Ph.D. created the schematic of eliminate the microRNA regulatory region of *ztf-1*.

Figure 7- Moss, Eric Ph.D. created the schematic of loxP mutants that drive the reaction one way.

Figure 8- the schematic diagram of CRISPR plasmid Goldstein lab, UNC created the construct. Figure from reference [19].

Figure 9- Sulaimani, Abrar created the all constructs and worms strains and performed PCR but Moss, Eric Ph.D. designed the PCR primers.

Figure 10- Sulaimani, Abrar created the all constructs and worms strains and performed PCR but Moss, Eric Ph.D. designed the PCR primers.

Figure 11- Moss, Eric Ph.D. created the schematic diagram of new *ztf-16* mutants but Sulaimani, Abrar performed the experiments.

Figure 12- Moss, Eric Ph.D. created the schematic diagram of a method of locus-specific insertion of any desired sequences using Cre/loxP system.

Table 1: Sulaimani, Abrar performed all the experiments listed.

Table 2: Sulaimani, Abrar performed all the experiments listed.