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Nikita Tapiawala
Southern Methodist University, ntapiawala@mail.smu.edu

Combinatorial Genetics of RNA Binding Proteins for Lifespan Regulation in C. *elegans*

Nikita Tapiawala *

ntapiawala@mail.smu.edu

ABSTRACT

RNA binding proteins control post-transcriptional aspects of gene regulation. While transcriptional control over the aging process has been well-established, less is known about post-transcriptional control over the aging process. We have used CRISPR/Cas9 genetics to study combinations of RNA binding proteins in C. *elegan*, a transparent nematode or roundworm one millimeter in length, and their role in aging. The manner in which the research was conducted was through experimental laboratory work in which the lifespan of the nematodes was monitored until their death.

The three mutations affecting RNA binding proteins that assays were performed with are exc-7, fox-1, and mbl-1. We found that double mutants have stronger effects on lifespan than single mutants. Thus, RNA binding protein mutations, although separate, appeared to be functionally connected. These observations, though being considered in C. *elegans*, could have a substantive impact on the study of gene regulation in humans and in turn, provide insight into the human aging processes.

1. INTRODUCTION/ MOTIVATION

Through genetic studies in various model organisms, it has been concluded that the aging process is not just passive depreciation, but in the hands of a variety of signaling pathways[1]. With this in mind, the research we conducted focused on the nature of RNA binding proteins in C. *elegans*. RNA binding proteins, although quite essential for the intermediary process of gene regulation, have not been as well studied as other gene regulators that appear in the early (transcription) and late (translation) stages[1]. The intermediate stage of gene regulation could have been overlooked due to the fact that there is redundancy or combinatorial aspect associated with it[1]. Thus, the purpose of the research lays in the fact that these previously under-appreciated RNA binding proteins, although being studied in something as small as C. *elegans*, is of great value to human development and survival because humans share several of the same RNA binding proteins as C. *elegans*. With this in mind, we honed in on what would happen if certain RNA binding proteins were lost in single or double mutant combinations in regards to the nematode's lifespan. To study this, we focused on RNA binding proteins which the lab had identified as potentially playing a role in lifespan regulation. These proteins were deleted using CRISPR/Cas9 technology; the CRISPR/ Cas9 technology involves specialized forms of DNA that are able to cut other strands of DNA that need to be modified[1]. For this instance, deleted means that the RNA binding proteins lost their ability to properly function[1]. The three RNA binding proteins that were isolated using this method are exc-7, mbl-1, and fox-1[1]. With these particular proteins, along with wild-type worms (N2), three separate lifespan assays were conducted. By performing these lifespan assays, we were hoping to further our understanding on the cellular and molecular mechanisms by which RNA binding proteins play a role[1].

The chief hypothesis for this research was that although a single mutation may have minor effects on the worm's survival, a double mutant with any combination of the single mutations mentioned above will have a greater effect in the manner in which the worm ages because losing two of the three genes would be more detrimental than just losing one. This hints at the notion that the proteins could be working together and that losing one will not harm the worm as much because the other two proteins will be a protective factor against the single mutation's effects.

2. DESCRIPTION/METHODOLOGY

Creation of Strains

The strains were created prior to the start of this assay. As stated earlier, all single mutant strains were made using the CRISPR/ Cas9 technology. Through this method, specialized DNA strands were able to go in and mutate the particular RNA proteins being analyzed: exc-7, mbl-1, and fox-1. To make the various double mutants, the single mutants were used to perform a simple cross. With this, the exc-7; mbl-1, mbl-1; fox-1, and exc-7; fox-1 double mutants were created.

Strains Analyzed

The first assay we analyzed to measure lifespan was between exc-7 and fox-1. In this assay, we compared the phenotypes of the exc-7 single mutant, fox-1 single mutant, exc-7; fox-1 double mutant, and N2. The second assay was between fox-1 and mbl-1. This assay was used to study the relationship between the fox-1 single mutant, mbl-1 single mutant, fox-1; mbl-1 double mutant, and N2. The third and final assay was between exc-7 and mbl-1. In this we viewed the nature of the exc-7 single mutant, mbl-1 single mutant, exc-7; mbl-1 double mutant, and N2.

* Mentor: Dr. Adam Norris, Professor of Biology

Preparation of Worm Strains

To begin the process, worms were chunked from plates of the four different types of strains (depending on which assay was being worked on at the time) and put onto fresh plates to ensure that there was a sufficient supply of non-starved worms to work with. To "chunk" simply means to place a portion of an old plate onto a fresh plate with ample bacteria for food. Proper measures such as using ethanol and a flame on the tool used to chunk were employed to make sure there was no contamination. After a few days of allowing the fresh worms to populate the new plates, a bleaching procedure was exercised. The worms were placed in 100 microliters of bleach solution and then washed 3-4 times with M9 (a simple saline solution) to ensure no bleach was left behind. The bleach solution contained 2.75 mL water, 1.25 mL NaOH 1M, and 1 mL of bleach. The bleaching allowed the worms to be staged so that all of them were at the same period in life when their lifespans began being tracked. Bleach kills the adult worms but can't permeate the shells in which the eggs are encased. Therefore, all the worms that are adults or in any other stage in life disintegrate and only the eggs survive. With this in mind, once the worms had been bleached, the eggs hatched overnight in a test tube filled with M9 and plated the next day onto a normal seeded NGM plate.

Creation of FUDR Plates

On the same day L1s (stage right after the worms hatch) were plated, an FUDR plate was prepared using 100-150 microliters of 1mg/1 mol H_2O FUDR solution. FUDR, Floxuridine, is a drug that works in a similar manner to chemotherapy by inhibiting cell division; the newly hatched hermaphrodite nematodes would be unable to have offspring of their own as a result of it. This ensures that the worms' lifespans being tracked are not confused with other worms entering the plates. The worms were allowed to grow up on the normal plate for a day and then were picked from each respective phenotype onto their respective FUDR plates.

Picking Worms for Aging Assays

Once again, the nematodes were staged so that they were all L4s (right before adults) when they were picked to guarantee there was consistency. To ensure that the results were statistically significant, each plate that was picked onto consisted of 100 C. *elegans*. Moreover, wild-type worms (N2) were included into each of the three assays conducted to provide a basis for comparison. All of the three assays conducted consisted of four plates: each of the single mutants, the double mutant, and an N2. All of the single mutants, double mutants, and N2s were observed under identical conditions and time frame. To pick the worms from a normal seeded NGM plate onto the seeded FUDR plate and progressively review the worms, a small metal pick and microscope were utilized.

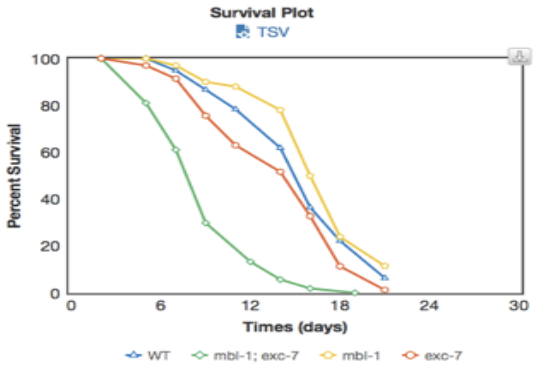
Statistical Analysis

The entire process of preparing the worms took about 5-6 days and subsequently the nematodes on each FUDR plate were counted every 2-3 days until all the worms from each plate died. To graph our results and test for statistical significance, the Oasis software was used[3]; this enabled us to determine if the assay was of value by using a log-rank test to determine if Bonferroni-corrected $p<0.05$.

3. RESULTS

This research has shed light on three separate but connected RNA binding proteins. The three assays included exc-7 vs. mbl-1, fox-1 vs. mbl-1, and exc-7 vs. fox-1.

The exc-7 vs. mbl-1 assay, shown in figure one, appears to be the most consistent with the hypothesis because the double mutant did significantly worse than the other phenotypes. This data also appeared to be statistically significant as the p-value between N2 vs. exc-7, N2 vs. exc-7; mbl-1, and exc-7 vs. exc-7; mbl-1 was less than 0.05. Therefore, we fail to reject the null hypothesis. Additionally, 50% of the mbl-1 single mutant lived about the same amount as 50% of the N2. Along with the significant data, notably, any plate that consisted of exc-7 appeared to have visibly more bacteria growth than plates that that exc-7 did not inhabit.

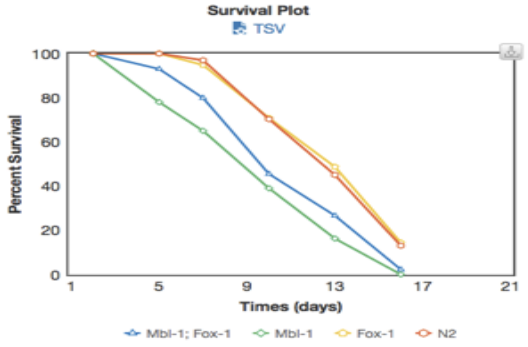


Condition	Statistics		
	χ^2	P-value	Bonferroni P-value
WT v.s. mbl-1; exc-7	81.3	0	0
WT v.s. mbl-1	3.36	0.0669	0.2006
WT v.s. exc-7	4.21	0.0401	0.1204
mbl-1; exc-7 v.s. WT	81.3	0	0
mbl-1; exc-7 v.s. mbl-1	113.53	0	0
mbl-1; exc-7 v.s. exc-7	53.44	0	0
mbl-1 v.s. WT	3.36	0.0669	0.2006
mbl-1 v.s. mbl-1; exc-7	113.53	0	0
mbl-1 v.s. exc-7	14.85	0.0001	0.0003
exc-7 v.s. WT	4.21	0.0401	0.1204
exc-7 v.s. mbl-1; exc-7	53.44	0	0
exc-7 v.s. mbl-1	14.85	0.0001	0.0003

Figure 1: Exc-7 single mutant (red), Mbl-1 single mutant (yellow), Mbl-1; Exc-7 double mutant (green), and N2 (blue) survival plot. P-values between Mbl-1; Exc-7 double mutant and N2, Mbl-1; Exc-7 double mutant and Mbl-1 single mutant, Mbl-1; Exc-7 double mutant and Exc-7 single mutant, and Mbl-1 single mutant and Exc-7 single mutant appear to be statistically significant.

The fox-1 vs. mbl-1 assay, shown in figure two, appears to mostly coincide with the hypothesis. The double mutant had a statistically significant p-value of less than 0.05 in regards to the N2 and fox-1 mutant. The fox-1 mutant behaved in a similar fashion to the N2, performing only

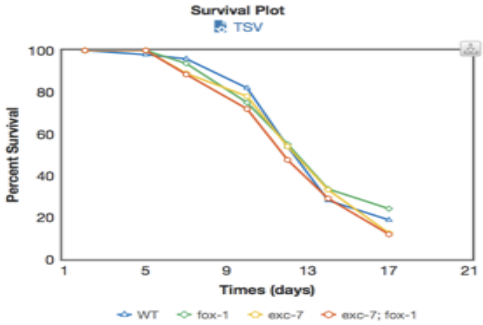
slightly better. The data differed, however, because the mbl-1 single mutant appeared to do significantly worse than not only the N2 and fox-1, but also the fox-1; mbl-1 double mutant. This, although cannot be validated unless attempted again, could have been due to human error—bleach solution could have been left in the tube housing the mbl-1 mutants causing 50% of them to live to only about 8 days.



Condition	Statistics		
	χ^2	P-value	Bonferroni P-value
Mbl-1; Fox-1 v.s. Mbl-1	5.46	0.0195	0.0584
Mbl-1; Fox-1 v.s. Fox-1	18.16	0.00002	0.0001
Mbl-1; Fox-1 v.s. N2	16.26	0.0001	0.0002
Mbl-1 v.s. Mbl-1; Fox-1	5.46	0.0195	0.0584
Mbl-1 v.s. Fox-1	35.01	3.3e-9	1e-8
Mbl-1 v.s. N2	33.19	8.4e-9	2.5e-8
Fox-1 v.s. Mbl-1; Fox-1	18.16	0.00002	0.0001
Fox-1 v.s. Mbl-1	35.01	3.3e-9	1e-8
Fox-1 v.s. N2	0.11	0.7411	1
N2 v.s. Mbl-1; Fox-1	16.26	0.0001	0.0002
N2 v.s. Mbl-1	33.19	8.4e-9	2.5e-8
N2 v.s. Fox-1	0.11	0.7411	1

Figure 2: Fox-1 single mutant (yellow), Mbl-1 single mutant (green), Fox-1; Mbl-1 double mutant (blue) and N2 (red) survival plot. P-values between Mbl-1; Fox-1 double mutant and Fox-1 single mutant, Mbl-1; Fox-1 double mutant and N2, Mbl-1 single mutant and N2, and Mbl-1 single mutant and Fox-1 single mutant appear to be statistically significant.

The exc-7 vs. fox-1 assay, shown in figure three, appears to greatly differ from the hypothesis as all the worms appeared to live the same amount with 50% of the four phenotypes dying at around 11-12 days. This data not only differs from the hypothesis, but also appears unusual as typical N2 worms live to around 12-18 days[4]. As a result of this, the data was not statistically significant and appeared to have another factor affecting it. It was inferred that the bleach solution used in preparing the worms for the assay may have been carried over and thus, affected the lifespan of all the worms, making them appear similar. Additionally, as mentioned above, any plate that exc-7 was present on consisted of more bacteria growth than the other plates.



Condition	Statistics		
	χ^2	P-value	Bonferroni P-value
WT v.s. fox-1	0.11	0.7376	1
WT v.s. exc-7	0.27	0.6059	1
WT v.s. exc-7; fox-1	1.55	0.2137	0.641
fox-1 v.s. WT	0.11	0.7376	1
fox-1 v.s. exc-7	0.81	0.3679	1
fox-1 v.s. exc-7; fox-1	2.45	0.1176	0.3528
exc-7 v.s. WT	0.27	0.6059	1
exc-7 v.s. fox-1	0.81	0.3679	1
exc-7 v.s. exc-7; fox-1	0.47	0.4926	1
exc-7; fox-1 v.s. WT	1.55	0.2137	0.641
exc-7; fox-1 v.s. fox-1	2.45	0.1176	0.3528
exc-7; fox-1 v.s. exc-7	0.47	0.4926	1

Figure 3: Exc-7 single mutant (yellow), Fox-1 single mutant (green), Exc-7; Fox-1 double mutant (red), and N2 (blue) survival plot. No p-values appear to be statistically significant.

4. DISCUSSION

The preliminary findings through the research conducted indicate that there could be important interactions between RNA binding proteins that affect the lifespans of the nematodes. Through observations, we surmise that the exc-7 mutation could be more noteworthy in altering the nematode's lifespan as it did significantly worse than the N2 in the first assay discussed whereas the mbl-1 and fox-1 performed about the same as the N2 with no implications of significance. As previously mentioned, in addition to the data, any plate with the exc-7 mutation (whether single or double) appeared to have more bacteria growth than the other plates. This agrees with the idea that exc-7 could be a more detrimental mutation than mbl-1 or fox-1 because the bacteria growth was consistent and coincides with the data. Together, these results strengthen our hypothesis that certain RNA binding proteins interact to regulate lifespan in C. *elegans*. We hope to further our understanding of RNA binding proteins by performing these particular assays again and possibly even working with a triple mutant of mbl-1, exc-7, and fox-1 to see if that would result in a stronger decline of lifespan.

5. REFERENCES

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