INVESTIGATING MODIFIERS OF ALS TOXICITY THROUGH A GENOME WIDE RNAI SCREEN OF C. ELEGANS

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INTRODUCTION
Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s Disease, afflicts over 400,000 people worldwide, making it the most common neuromuscular disease [1]. It is an age-dependent disease that usually onsets around age 50, though symptoms develop differently in each individual [1]. ALS is characterized by a deterioration of motor neurons that leads to paralysis and eventual loss of vital functions such as swallowing and breathing. This neurodegeneration results in death, usually within three years of diagnosis. Currently, there is no cure for ALS, and no highly effective therapies.

Clinical diagnoses of ALS can be classified into 2 groups: Familial (fALS; 10%) and Sporadic (sALS; 90%) cases. Most known genetic causes of this disease are single gene mutations, and the familial form is more commonly studied due to a more predictable pattern of inheritance. Sporadic cases usually contain similar genetic mutations to fALS cases, allowing sALS to be studied using fALS findings. Previous findings, such as mutations in TDP-43, SOD1, and FUS have been linked to up to ~15% of fALS cases, as well as ~5% of sALS cases [2]. Recent discoveries, however, identify mutations of the C9orf72 gene as the most common cause of ALS, accounting for ~50% of familial forms of the disease and ~20% of sporadic cases [3]. Mutations in C9orf72 result in the expansion of a ‘GGGGCC’ sequence (G4C2 repeats). Affected patients carry 100s -1000s of G4C2 repeats, while unaffected individuals carry <20. Interestingly, this repeat is contained within a C9orf72 intron. Evidence from multiple systems, shows that the G4C2 repeats are actually translated in both sense and antisense directions in all possible reading frames to generate five distinct dipeptide proteins. Two of these dipeptides, PR and GR, are highly toxic in animal models. Evidence shows proteins aggregation in cases of mutation, potentially disrupting the function of various pathways [4]. Little is known regarding the mechanism(s) of PR toxicity in any system, which could potentially reveal new ALS disease mechanisms, biomarkers, and therapeutic drug targets.

RNAi interference, a popular technique for knocking down genes in large quantities, can be used to identify how diseases, such as ALS, progress. RNAi in C. elegans works through inducing an RNA sequence that, when ingested by the worm, binds to a complementary mRNA, leading to its destruction. The protein can thus not be translated, resulting in the loss of that gene’s function.

OBJECTIVE
Based on previous experiments, PR dipeptide expression in C. elegans is an accurate model of ALS-like age-dependent paralysis. The objective of this experiment was to determine which genes are involved in modifying PR toxicity in C. elegans to determine relevant pathways involved. This was done by using RNA interference to knockdown each C. elegans gene individually and examining resultant worm phenotypes to identify disease suppressors.

HYPOTHESIS/SUCCESS CRITERIA
Based on previous literature that utilized large-scale RNA interference screens to identify genetic suppressors of ALS in model organisms such as yeast, further investigation of genetic modifiers of ALS in C. elegans needed to be performed. It was hypothesized that in a C. elegans PR dipeptide model of ALS, knocking down a gene that plays a role in modifying PR toxicity will result in a modified phenotype of the organism. If a gene is knocked down resulting in healthier mobility phenotypes, the gene can be characterized as a suppressor. The criteria for a verified suppressor is that it screens as a healthy phenotype 4 out of 5 times [5].

METHODS
The worm strain used was drIs34, which consists of a transgene containing 50 repeats of the Proline-Arginine (PR) dipeptide protein. Both the PR dipeptide and a Red Fluorescent protein were attached to a muscle promoter so that expression of the transgene could be detected in the body muscle under fluorescence. A Green Fluorescent Protein sequence was directly linked to the PR sequence so that the PR dipeptide would be knocked down when the GFP is knocked down, eliminating the toxic effects of PR.

Bacterial stocks containing RNA interference clones were grown up for each individual gene. 20 μL of culture were spotted onto 24 well plates, each well containing a clone that would knock down a specific gene. Eggs were harvested from drIs34 adults, and ~25 eggs were seeded onto each well and grown at 20°C for 6 days. Phenotypic traits, such as motility and proper development, of adults and first generation progeny were then observed. These wells were given a score of -3 to 3 indicating the strength of the PR40 phenotype suppression (healthy; scored 1,2,3) or enhancement (paralyzed; scored -1, -2, -3). Worms feeding on bacteria carrying the empty vector were used as a negative control and served as the basis for a score of ‘0’. Worms feeding on GFP RNAi bacteria were used as positive controls, and served as the basis
of comparison for a score of ‘3’. Each well was scored blinded to which gene was knocked down in that particular well.

Each positive hit scored above a ‘2’ was rescreened four times to test consistency and meet the success criteria. RNAi clone bacteria of both hit wells and controls were streaked out from stock plates and grown overnight. A single colony was then grown in for 24 hours, and 20 μL of culture were spotted onto 24 well plates, 4 wells per hit. Wells were screened as stated above, and analyzed under fluorescence to determine if positive hits still showed proper transgene expression. ‘Overall’ suppressor scores were calculated by adding up the score of the 5 wells, subtracting transgene suppression scores (where wells appeared less fluorescent), and divided by 5 to find the average.

A miniprep of the bacterial pellets was performed on the clones that rescreened as positive hits. DNA was sent in for sequencing and compared to the genomic sequence of C. elegans using the BLAST program to ensure the RNAi clones were accurate for the genes.

RESULTS

8543 Genes were screened, and 93 genes came up preliminarily as potential hits. The 93 potential hits represent 1.1% of all genes screened. These wells typically exhibited characteristics such as healthy larval motility and a substantial amount of offspring that existed in various stages of development. Shown below in Figure 1 is an image of a well scored a ‘non-hit’ and a well identified as a ‘hit’:

![Image A](image.png)  ![Image B](image.png)

Figure 1. Wells of genes during preliminary screening. Image A shows a non-suppressor (scored 0/3), exhibiting visible paralysis and limited development. Image B shows a suppressor (scored 3/3) with high concentration of developed animals and no paralysis observed.

Of the initial 93, 3 genes rescreened as verified ‘hits’, scoring positive all 4 times on the rescreen for a total of 5/5 hit rate, satisfying the success criteria. Clone ZK20.5 (rpn-12) received a final suppressor score of 1, with all but 1 well showing normal transgene expression. Clone T06D8.8 (rpn-9) received a final suppressor score of 1.4 with all but 1 well showing proper transgene expression. Clone T05H10.5 (ufd-2) received a final suppressor score of 2, with all wells showing proper expression of the transgene.

DISCUSSION

The results of the screen confirmed the hypothesis that knocking down certain genes will result in a modified phenotype of an ALS disease model in C. elegans. Along with the three consistent hits, significant transgene expression was observed, demonstrating that the healthy phenotypes were a result of the proper gene knockdown, not a function of external factors. Two of the hits, rpn-9 and rpn-12, have regulatory proteasome function, which is key in modification and degradation of proteins. rpn-9 and rpn-12 both have related human orthologues (PSMD13, PSMD8, respectively). The third suppressor hit, ufd-2, has the human orthologue UBE4B and is involved in Ubiquitin chain assembly. Additionally, this gene is expressed alongside rpn-10, potentially indicating its relationship with rpn-9 and rpn-12. A primary function of this ubiquitin chain pathway is the degradation of abnormal proteins. All three of the observed suppressors are involved in the recognition or degradation of proteins, which makes sense in the context of the C9orf72 dipeptide toxicity model. These results suggest that when targeting the proteasome regulatory subunits or the ubiquitination pathways, the foreign dipeptides will potentially be degraded at a higher rate, leading to less toxicity and a healthier phenotype.

While the three genes identified as suppressors have not themselves been found in previous studies, similar genes in related pathways have been identified to modify ALS phenotypes. A recent study shows that impaired degradation contributes to disease onset and progression of C9 ALS and the associated disease FrontoTemporal Dementia [6]. Another second study identified 4 UBE genes involved in ubiquitination are affected in C9orf72 ALS, providing evidence that ufd-2, with its human UBE4B orthologue, could be a primary genetic target in future C. elegans studies on C9orf72 [7].

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