Creating A *Drosophila* SOD1 Mutant Through Homologous Recombination

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Acknowledgement

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Chapter 1- Introduction

Amyotrophic Lateral Sclerosis

Neurodegenerative diseases are among the most common diseases affecting aging population including 50 million Americans targeted each year (Brown 2005). Neurodegenerative diseases are characterized by death of neuronal cells. Neurons are post-mitotic, hence once the cells die, there is no replenish of the lost neurons (Friedman 2011). There are many factors contributing to the onset of neurodegenerative diseases. However, most common factors in neurodegenerative diseases include 1) protein aggregation and 2) oxidative stress, which is associated with mitochondria dysfunction (Sheikh 2012).

First discovered in 1869 by French neurologist Jean Martin Charcot, amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting both upper and lower motor neurons leading to paralysis and eventually death due to diaphragm failure (Redler 2012). Upper motor neurons extend from the motor cortex in the brain and lower motor neurons extend from the spinal cord to innervate skeletal muscles that allows for voluntary movement. Interestingly, ALS does not affect cognitive ability, sensation, and autonomic function, such as breathing and digestive process. It is currently the most common neurodegenerative disease affecting 1-2 person per 100,000 people worldwide (Redler 2012). Death usually occurs 2 to 5 years after disease onset. In recent studies, the U.S ALS mortality rate is 1.84 per 100,000 persons.

There are two onset forms of ALS: either spinal or bulbar (Wijesekera 2009). Spinal ALS onset is characterized by muscle weakness in upper and lower limbs. It also includes focal muscle atrophy of upper and lower limbs along with fasciculations (spontaneous contraction) and spasticity (pull). On the other hand, bulbar ALS onset is characterized by difficulty in speech,

swallowing and develop excessive drooling. Bulbar onset also express fasciculations and spasticity of the tongue. As ALS progresses, patients experience both spinal and bulbar traits (Wijesekera 2009). Roughly 82% of ALS cases are sporadic showing no trace of hereditability and about 5-10% cases are familial ALS (fALS) and autosomal dominant. Of the fALS cases, 20% are associated with mutations in superoxide dismutase 1 (SOD1). SOD1 is an enzyme that scavenges toxic superoxide radicals to produce hydrogen peroxide (H_2O_2). To date, over 100 mutations in SOD1 have been identified as causative for fALS.

Protein aggregation

In neurodegenerative diseases, protein aggregates are found in the brain tissues and these misfolded proteins can cause dysfunctions in neurons. It has been discovered that protein aggregates in neurodegenerative diseases are usually in beta-sheet structures, which increase protein rigidity (Takalo 2013). There are different proteins aggregates found in cases of ALS. Aggregates of TAR binding DNA protein 43 (TDP-43), a gene regulator protein, are found in neuronal and glial cells and localized to the cytoplasm from the nucleus of spinal cord motor neurons in both sporadic and familial cases of ALS (Blokhuis 2013). Another mutated protein associated with ALS is fused in sarcoma/translocated in liposarcoma (FUS), a nuclear protein. FUS aggregates were initially discovered in fALS cases but then found in 1% of sALS cases. These protein aggregates tend to cause dysfunction of the lower motor neurons (Blokhuis 2013). Mutation in a protein with unknown function is characterized to be a major cause of fALS and is called C9orf72 (chromosome 9 open reading frame 72) (Blitterswijk 2014). Normally, there are hexanuceleotide GGGGCC repeats in this protein but it becomes toxic when there are hundreds to a thousand repeats present, causing a gain-of-function in RNA (Blitterswijk 2014).

Protein aggregates produce cell death because they can cause cellular important proteins to misfold and aggregate to the growing protein aggregation causing those proteins to lose their function and consequently cell survival might be affected. Cells have protective mechanisms against protein aggregation called the protein quality control (PQC), which include chaperones and ubiquitin-proteasome system (UPS) (Takalo 2013). Chaperone proteins help prevent protein misfolding, disaggregate protein complexes, and cooperate with proteases to degrade proteins with the aid of ATP hydrolysis (Lindberg 2015). Heat shock proteins are the largest chaperone family and are upregulated under conditions of cellular stress including high temperature and oxidative stress. The UPS targets proteins for degradation. Within the UPS, ligases (E1, E2, E3) first recognize and tag misfolded proteins with a chain of four to five ubiquitin to lysine residues. This tag is then recognized by 26R proteasome complex, a protease which degrades misfolded proteins. The UPS is usually accompanied by chaperones to recruit the ligases and so both chaperones and UPS work hand- in- hand to clear misfolded proteins. It has been hypothesized that deficiencies in PQC system could also initiate protein accumulation and mutations in proteins that play a role in aiding PQC (Takalo 2013).

Oxidative stress

Cellular oxidative stress is characterized by the formation of superoxide radicals, reactive species which produce cellular toxicity through adduct formation, protein misfolding and lipid peroxidation (Uttara 2009) which possess an extra unshared electron. Because the brain consumes about 20-30% oxygen, making it the tissue utilizing the most oxygen in our body, reactive oxygen species (ROS) are more prevalent and produce greater levels of toxicity than in other tissues (Sultana 2013). Under normal conditions 1-2% of the oxygen is converted to ROSs (Uttara 2009). In neurons the major producers of ROSs are NADPH oxidase and xanathine

oxidase present in the cytosol and monoamine oxidase, complex I and complex III in the mitochondria (Gandhi 2012). NADPH oxidase is an enzyme that donates an electron from NADPH to oxygen to form superoxide (O_2^-); xanathine oxidase is an enzyme that transfers electron to oxygen in order to form uric acid, superoxide, and hydrogen peroxide (Gandhi 2012). Monoamine oxidase converts amine to aldehyde and hydrogen peroxide (Holschneider 2000). Complex 1 and complex III are enzymes that play a role in oxidative phosphorylation on the inner mitochondria membrane transferring electron from NADH to the final electron acceptor oxygen (Gandhi 2012). Complex I releases superoxide to mitochondria matrix and complex III releases superoxide to the inner mitochondria membrane (Hroudova 2014). Hence, dysfunction of mitochondria can lead to oxidative stress and has been a characteristic in neurodegenerative diseases (Gandhi 2012).

ROS are problematic because they are highly reactive due to their electron deficiency and consequently participate in many redox reactions with macromolecules leading to a cascade of redox reactions (Sultana 2013). ROS can cause carbonylation of proteins, which, in turn, produce reactive ketones and aldehydes that react with other molecules and so on. Furthermore, ROS can cause DNA breaks and DNA mutations by reacting with DNA backbone and individual nucleic acid bases. ROS react with brain's high concentration of polyunsaturated fatty acids (PUFA), which are fatty acids containing more than one double bond in its structure, in neuronal membranes (Sultana 2013). PUFA are important for signal transduction (Bazinet 2014) and ROS breaks it down producing malondialdehyde which in turn disrupts proteins required for DNA stability. (Gemma 2007). When there are too many ROSs for cells to overcome, cell toxicity reaches its maximum and the cell dies (Fulda 2010).

SOD1

Cu/Zn Superoxide dismutase 1 (SOD1) is an antioxidant enzyme that coverts superoxide (O_2^-) to dioxide (O_2) and (H_2O_2), which can then detoxify to water by oxidase (Banci 2008). It is conserved throughout many organisms, including humans (Figure 1). SOD1 is a homodimer with each SOD1 containing copper and zinc in its structure. Copper plays a catalytic role while zinc plays a structural role (Rahkit 2006). The dimer also consists of a disulfide bond which holds the two SOD1 units together (Sea 2015) and prevents it from aggregation if metal is deficient (Franco 2013). It is noted that the metals promote the disulfide bonds (Redler 2012). When SOD1 interacts with superoxide (O_2^-), it donates an electron from Cu^{2+} to oxide making molecular oxygen (O_2) and copper reduces to Cu^+, which becomes Cu^{2+} again when it comes in contact with another O_2^- and 2H to produce hydrogen peroxide (H_2O_2) (Franco 2013).

Mutations in SOD1 affect both protein aggregation along with oxidative stress. There are roughly over 100 missense mutations in SOD1 that are associated with ALS. Interestingly, the function of SOD1 is retained in the presence of a mutation. Moreover, transgenic mice with SOD1 knockout did not develop ALS, leading to the idea that the function of SOD1 is not required for onset of the disease (Redler 2012). Many experiments have discovered that there is a correlation between increased mutant SOD1 aggregation and decreased survival time (Redler 2012). In human SOD1, there is a cysteine on the 111^{th} amino acid sequence but a serine is present in *Drosophila* SOD1 at the corresponding position. In previous study by Cozzolino et al., a mouse motorneuron cell line NSC-34 was transfected with human fALS associated mutant SOD1. Mutants of human SOD1, C6F and G93A, formed insoluble aggregates in the cells. However, in double mutants containing C111S, solubility of aggregates was increased and toxicity decreased. Moreover, the presence of $SOD1^{C111S}$ increased solubility of triple mutant

$SOD1^{C6F/C57S/C146R}$ and inhibited aggregate formation in $SOD1^{G93A/C111S}$ and $SOD1^{C6F/C111S}$. Furthermore, it was shown the wildtype SOD1 became insoluble in the presence of mutant $SOD1^{G93A}$ but with co-transfection of $SOD1^{G93A/C111S}$, insoluble $SOD1^{wt}$ recovered to it normal, soluble state. These results led to the hypothesis that the 111Cysteine residue is essential to aggregate formation and toxicity. To test this idea further, we modified the endogenous *Drosophila* SOD1 gene to create $SOD1^{S111C}$ and assessed general toxicity through lethality studies.

***Drosophila melanogaster* as a model of ALS**

Drosophila melanogaster proves to be an excellent model organism to study disease mechanisms *in vivo* for many reasons including a quick life cycle (10-14 days), a large number of progeny, a small and fully sequenced genome with high conservation with mammals cheap and easy maintenance, and most importantly, it contains many genes that have been conserved among of mammalian (McGurk 2015). *D. melanogaster* has homolog of about 75% of human genes making *D. melanogaster* a very valuable model organism to study genetic diseases (Jackson 2008). Furthermore, *D. melanogaster* and human neurons share common cellular components, like cell signaling and trafficking, which makes *D. melanogaster* even more valuable to study neurodegenerative diseases (Sheikh 2013).

One key feature that makes *D. melanogaster* an important model organism is its ability to undergo homologous recombination (Rong 2002). Homologous recombination is the process by which *D. melanogaster* can exchange a part of its genomic DNA with an injected donor plasmid DNA. This process leads to the manipulation of *D. melanogaster* genomic DNA and allows researchers to study mutations and even study human genes (Rong 2002).

In this thesis, we are testing the idea that human-like SOD1 will promote protein aggregation since *D. melanogaster*-like SOD1 abolished protein aggregation. Additionally, we are testing the eclosion ratio of *D. melanogaster* with $SOD1^{S111C}$ in the presence of another mutation $SOD1^{G85R}$ to test the effect, if any, of $SOD1^{S111C}$.

Chapter 2- Materials and Methods

Materials

Drosophila Stocks with Bloomington Stock Numbers

5905 w^{1118}

6934 y[1] w[*]; P{ry[+t7.2]=70FLP}11 P{v[+t1.8]=70I-SceI}2B sna[Sco]/CyO, S[2]

2475 w[*]; T(2;3)ap[Xa], ap[Xa]/CyO; TM3, Sb[1]

851 y[1] w[67c23] P{y[+mDint2]=Crey}1b; D[*]/TM3, Sb[1]

33821 w[1118]; wg[Sp-1]/CyO; sens[Ly-1]/TM6B, Tb[1]

5580 y[d2] w[1118] P{ry[+t7.2]=ey-FLP.N}2

Mutagenesis Primers

sod S111C 1 CGTCCGATGATGCAGTCGGCACCGAAG

sod S111C 2 CTTCGGTGCCGACTGCATCATCGGACG

Sequencing Primers

A2-S-F1	TTCCAAGGAATGCAACAGC
A2-S-F2	CTAGCGAAACGGAAGGAG
A2-S-F3	TTCGTCGAGCAACAAGTG
A2-S-F4	TACGGATTGAAGTGCGGTC
A2-S-R1	GATCTGGGCAACATTGAGG
A2-S-R2	ATCAAGATACTCGCCACATG
A2-S-R3	TCAGGCTGCTAAACGAAG
A2-S-R4	GACCAGAATAAAGGGCAGC

Methods

Fly Care

D. melanogaster were obtained from Bloomington Drosophila Stock Center and kept at 23^{o}C fly incubator with a 12h/12h light/dark cycle and raised on standard Gelbart food.

Mutagenesis

Mutagenesis was performed on pW25.2sod^{wt} vector that contained sod^{wt} and ampR gene (Figure 2) using QuikChange II Xl Site-Directed Mutagenesis Kit and then digested with EcoR1 enzyme. Five products of mutagenesis (1, 3, 6, 12, 13) were digested with Acc65i and Not1 to isolate mutated arm-2. Arm-2 from clone 6 was cut-out from low melting agarose gel and ligated with pW25.2SOD1^{wt} without arm-2 using standard protocols. After isolating DNA by Qiagen Mini Prep Kit purification, samples were sent to University of Rhode Island for Sanger sequencing.

Injection and Homologous Recombination

pW25.2 vector containing SOD1^{S111C} was sent to Genetics Service Inc. for injection into the *D. melanogaster* embryos' germ line cells.

Homologous recombination was performed based on protocols adopted from Staber et al (2011). After receiving injected flies, the first step was to mate the injected flies with w^{1118} flies and then cross the progeny with red eyes to Drosophila stock line 2475. we crossed the flies with flpI-sceI/CYO and heat shocked the embryos for one hour to activate the enzyme flippase that will excise the p-element out of the genomic DNA and the flies will then undergo homologous recombination with the endogenous sod^{+} on the third chromosome. The final step was to excise

the mini white gene and this was done by crossing the flies with fly stock containing Cre recombinase.

Genomic DNA of *D. melanogaster*

I isolated heterozygous *D. melanogaster* [sod^{S111C}/ sod^{+}] DNA before crossing the flies with Cre recombinase stock. I used GeneJet Genomic DNA Purification Kit; however, I eluted in 100 µl elution buffer instead of 200 µl to obtain a greater yield of DNA. After isolating genomic DNA, I amplified the *sod*1 gene from the genomic DNA via PCR using PfuII polymerase (Agilent). Cycling parameters consisted of 55 C annealing (30s), 72 C extension (2 min), 92 C denaturing for 40 cycles.

Following Cre recombination to remove the mini-white gene, genomic DNA was isolated and PCR was performed as described above. Then the samples were sent to University of Rhode Island for sequencing with primer A2-S-R1.

Eclosion Rate

I crossed virgin females that contain pW25.2sod^{S111C}/CYO on the second chromosome with males that contained sod^{G85R}/Tm3-Ser on the third chromosome. The cross resulted in four different genotype progenies: [pW25.2sod^{S111C}/+; sod^{G85R}/+], [pW25.2sod^{S111C}/+; TM3-Ser/+], [CYO/+; sod^{G85R}/+], [CYO/+; TM3-Ser/+]. I scored the number of progeny of each genotype (Table 1).

I also scored progeny from three additional crosses to test the eclosion rate in the presence of sod^{S111C}. All the sod mutants were located on the third chromosome and had undergone homologous recombination.

♀ [sod^{S111C}/sod^{S111C}] X [sod^{H71Y}/TM3-Ser-GFP] ♂

♀ [sod^{S111C}/sod^{S111C}] X [sod^{loxp}/sod^{loxp}] ♂

♀ [sod^{S111C}/sod^{S111C}] X [sod^{G85R}/TM3-Ser-GFP] ♂

I scored the progeny on CO_2 pad and performed a chi-square test (Table 2).

Chapter 3- Results

Sod gene is highly conserved among eukaryotic organisms (Figure 1). If we focus just on the 111th amino acid position, we can see that position to be highly conserved in most organisms as well. However, in human sod there exists a cysteine in 111th position but a serine in *Drosophila* sod. Previous work by Cozzolino et al. showed that human sod^{C111S} abolished protein aggregation in a mouse motor neuron cell line when this mutant protein was expressed in combination with ALS-causing sod mutations (such as sodA4V). To test whether a cysteine is associated with toxicity, our experimental approach was to mutate *Drosophila* sod^{wt} to sod^{S111C} and assess phenotypes..

Mutagenesis

In order to model sod^{S111C} in *Drosophila* we first had to create the mutation in a vector that will be injected in *Drosophila* embryonic cells. We used pW25.2sod^{wt} vector (Figure 2) to introduce sod^{S111C} via site-directed mutagenesis at the appropriate location. Our mutation of interest is located in arm-2 within the sod gene. The ampR gene, coding for ampicillin resistance, allowed us to select transformed bacterial cells containing the plasmid. The pW25.2 vector serves as a construct for homologous recombination in Drosophila. Embedded between arm-1 and arm-2 is the presence of the mini-white marker gene and loxp that allowed us to trace our mutation in *Drosophila* by the expression of red-eyes. The FRT sites are where flippase will excise the P-element out of the genomic DNA to initiate homologous recombination.

We performed site-directed mutagenesis to introduce S111C into the Drosophila sod gene. The process of mutagenesis can cause the vector to rearrange and to check for any rearrangements in our vector, we digested our mutagenesis product with EcoR1 restriction

enzyme. Following mutagenesis, the *sod1* gene was cloned into the *Drosophila* transformation vector and diagnostic digests were performed to verify the integrity of the clone. There are five total EcoR1 sites within the plasmid clone with predicted sizes of 295 bp, 662 bp, 696 bp, 5350 Kb, 6570 Kb. After transformation, 18 ampicillin resistant colonies were selected at random, and DNA was isolated from grown colonies. EcoRI restriction digests were performed to determine the integrity of the clones. Among clones tested, 5 different banding patterns were observed (Figure 3). None of the clones tested showed expected DNA fragments and we inferred pW25.2SOD1^{S111C} to have rearranged during mutagenesis.

To determine if the relevant portion of genomic sequence for *sod1* was intact, we performed Not1 and Acc65i digests because these restriction sites are located on both ends of sod arm-2. After Not1 and Acc65i digest of samples we expected arm-2 to drop out producing 2 bands on a gel: 10 Kb band and 2.5 Kb band (Figure 4). Sample 6 was the only sample among the five that gave us the expected 2 bands. Sample 6 was sequenced to confirm that we had the S111C mutation in *sod1* gene (Figure 5). We confirmed the presence our mutation because there exists thymine peak in the proper location. Since the remaining vector of sample 6 was rearranged, we ligated arm-2 of sample 6 to the properly arranged pW25.2SOD^{wt} vector without arm-2. Now that we had a vector that contained S111C mutation, we wanted to amplify the amount of the properly mutated vector by transforming it into competent *E. coli* cells, isolate and sequence the DNA, and finally inject it into *Drosophila* embryonic cells.

Genomic DNA

In order to introduce sod^{S111C} in *Drosophila*, the injected sod1^{S111C} had to replace a copy of the endogenous dsod1^{wt} on the third chromosome and this was accomplished through homologous recombination (Figure 6). Briefly, the sod^{S111C} construct was injected into the

Drosophila embryonic cells and introduced stably into the *Drosophila* genome by P-element transposition. Stable insertion of the construct was determined by a dominant selectable red eye marker. To determine heritability of the construct, we crossed the injected flies with w^{1118} stock and scored for progeny with red-eyes. Once verified, the stable insertions were balanced to prevent recombination and loss of the transposable element. Flies containing the stable insertion were crossed to *Drosophila* stock line (2475) which contain balancer chromosomes (CyO for the 2^{nd} chromosome and TM3Sb for the 3^{rd} chromosome). To determine the location of the P-element, flies that now expressed CyO and TM3Sb were crossed with *Drosophila* stock line (2475) for a second time. Progenies that expressed red-eyes also expressed TM3Sb, but not CyO showed that the location of our P-element was on the second chromosome. To excise the P-element from the second chromosome so it can undergo homologous recombination with $dsod^{wt}$ on the third chromosome, the flies were crossed with flpI-sceI/CYO and the eggs were heat shocked for one hour. Heat shock activates the flippase enzyme and excise the P-element out of the second chromosome at FRT sites and then undergo homologous recombination with the wildtype sod gene, thus replacing endogenous sod^{wt} with sod^{S111C}. The product of homologous recombination was determined by an eye color change and we screened 5 lines and identified 5 independent lines. In order to remove any disruptions in the sod^{S111C} gene that might affect expression, we removed the mini-white gene from the sod^{S111C} intron. This was accomplished by crossing the flies with *Drosophila* stock (line 851), expressing the Cre recombinase enzyme. leaving behind sod^{S111C} with only loxp embedded in the gene. Hence, the final product is *Drosophila* in which sod^{S111C} was knocked into the genome within the endogenous sod gene on the third chromosome.

Mid-way through the process of homologous recombination, we wanted to confirm that the flies still contained our P-element in their genome. We isolated genomic DNA and amplified only a portion of the sod gene that contained our S111C mutation (500 bp). The electrophoresis of the amplified genomic heterozygous showed one clean band at 500 bp, as expected (Figure 7). Sequencing of the heterozygous genomic DNA confirmed that the flies did have the mutated sod^{S111C} in their genome. The sequencing results (Figure 8) showed a large thymine band and a small adenine band right underneath it. Since the genomic DNA was derived from heterozygous flies, it contained one copy of the wild-type endogenous sod and one copy of the mutated sod^{S111C}, hence the reason why a thymine and an adenine bands are both present. Additionally, to determine the presence of sod^{S111C} in homozygous flies we isolated genomic DNA, amplified the portion of the gene that contained our mutation and sequencing results showed us one clean adenine peak at the expected location (Figure 9).

Analyzing Progeny Eclosion Rate in the Presence of sod^{S111C}

To determine if the presence of sod^{S111C} on the second chromosome will have any effects in flies heterozygous for sod^{G85R}, a mutation which causes ALS in humans and produces adult lethal phenotypes in flies, we crossed virgin females [pW25.2sod^{S111C}/CYO; +/+] with [+/+; sod^{G85R}/Tm3-Ser] males and that resulted in four different progeny genotypes, including a double mutant [pW25,2sod^{S111C}/+; sod^{G85R}/+] (Figure 10). After scoring progeny eclosion rate, we found that the eclosion percent of expected for all four progeny classes were 100% (Table 1). Hence, all progeny types eclosed in Mendelian ratios. To take one step further, we also analyzed the effects of sod^{S111C} in the presence of other ALS-associated sod mutants on the third chromosome to test if sod^{S111C} will rescue flies containing other sod mutations. We crossed homozygous sod^{S111C} flies with heterozygous sod^{H71Y} and also crossed homozygous sod^{S111C}

with heterozygous sod^{G85R}. Additionally, we also crossed homozygous sod^{S111C} with homozygous sod^{loxp} as a control. The eclosion percent of expected for sod^{S111C}/sod^{H71Y} and sod^{S111C}/loxp were 100%, whereas the eclosion percent of expected of sod^{S111C}/sod^{G85R} was 150%, with a p-value < .001 (Table 2).

Chapter 5-Conclusions

This works describes the creation and partial phenotypic analysis of a point mutation within the endogenous *Drosophila* sod gene. The sod^{S111C} allele was generated through a process of mutagenesis and homologous recombination *in vivo*. Sod^{S111C} stocks were created to analyze the mutation's influence on protein aggregation, if any. We were able to test the effects of this mutant by assessing the function of the protein using genetic assays. To examine the effect of sod^{S111C} on the second chromosome in the presence of sod^{G85R} on the third chromosome appropriate genetic crosses were performed and it was determined that there was no significant difference among the eclosion rates of progenies. All four genotypes of progenies eclosed in Mendelian ratios. This could be explained by the fact that though one class of progeny genotype contained a double mutant sod, there was still the presence of the endogenous sod^{wt} that could have masked for the double mutant.

We also assessed the eclosion rate of flies containing sod^{S111C} in the presence of ALS-associated sod mutations to determine if S111C mutation rescue those sod mutations. There was no effect in eclosion rate with the presence of sod^{S111C} with sod^{H71Y} and sod^{S111C} with sod^{loxp}. However, there was a significant increase in the eclosion rate of sod^{S111C}/sod^{G85R}. This showed that sod^{S111C} rescued sod^{G85R} because the presence of sod^{S111C} and sod^{G85R} lead to more fly eclosions. Hence, it is safe to say that there was no effect on protein aggregation with one copy of sod^{G85R} and we can further conclude that sod^{S111C}/sod^{G85R} acts in a similar manner as sod^{wt}/sod^{G85R} in *Drosophila*. In the presence of sod^{S111C}, flies containing sod^{G85R} seem to eclosed in higher numbers than progenies that did not contain the sod^{G85R} mutation. If sod^{S111C} did not function in a similar manner to the wildtype sod gene, then the eclosion rate of sod^{S111C}/sod^{G85R}

would be lower than sod^{S111C}/TM3Ser. The fact that our results were the opposite, showed that the presence of sod^{S111C} could have masked sod^{G85R}, like the endogenous $dsod^{wt}$ in [sod^{S111C}/+; sod^{G85R}/$dsod^{wt}$]. Thus, we can conclude that sod^{S111C} behaves in a similar manner as the endogenous $dsod^{wt}$.

In future studies, longevity experiments will be performed to assess the ability of S111C to rescue pathogenic mutations over time as flies age.

Figures

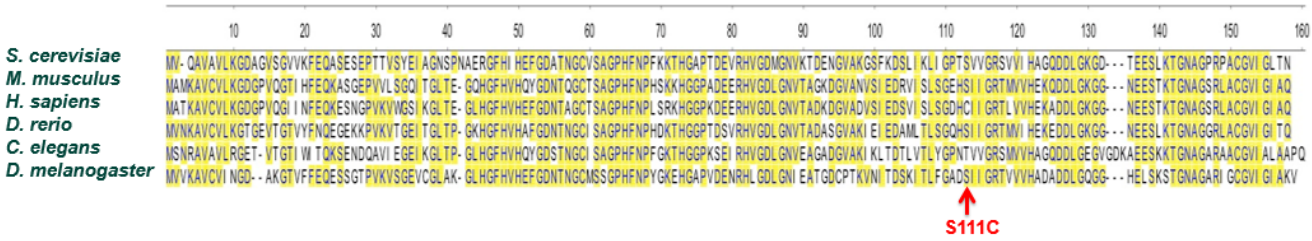

Figure 1. Clustal W alignment for SOD1 in selected model organisms. SOD1 protein shows a high degree of evolutionary conservation. Highlighted areas represent areas of conservations and arrow represents the site of S111C mutation.

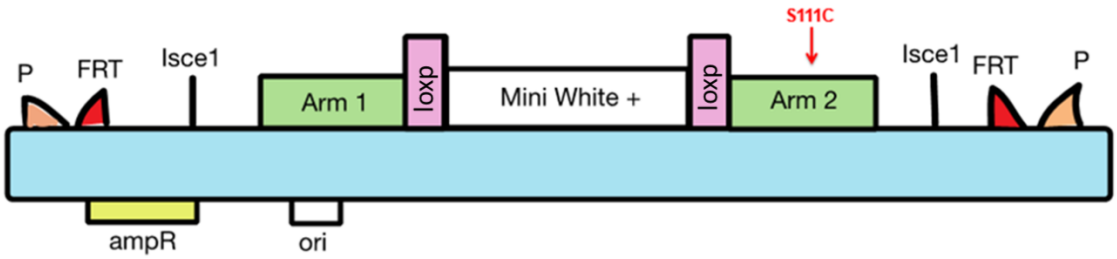

Figure 2. **Cartoon depiction of pW25.2SOD1^{wt} vector with ampicillin resistance gene.** The vector was obtained from Reenan lab at Brown University (Staber et al 2011). Arm-1 and arm-2 make up SOD1^{wt} gene which is disrupted by mini white marker gene and loxp that allows us to follow our vector in *D. melanogaster*. The FRT sites are where flippase is going to cut and excise the vector out of *D. melanogaster* genome.

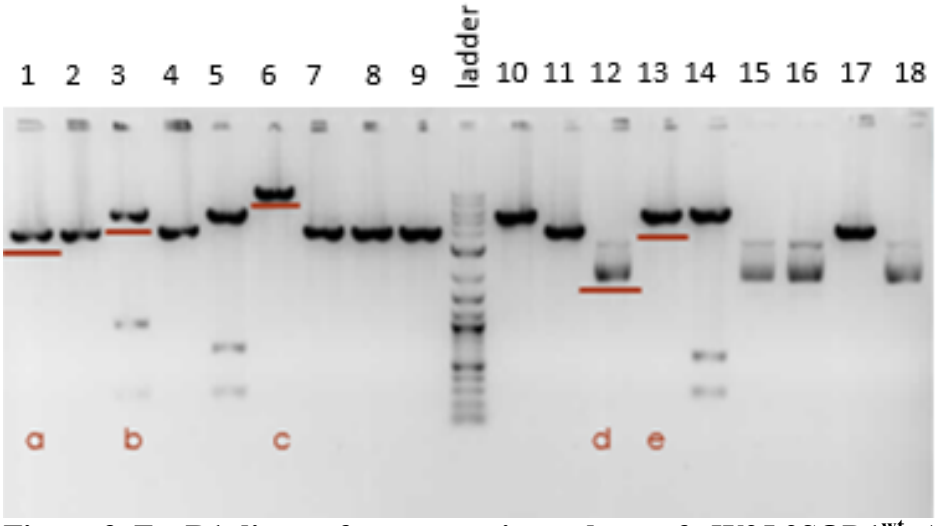

Figure 3. EcoR1 digest of mutagenesis products of pW25.2SOD1^{wt}. After mutagenesis, 2 µl of products were digested with EcoR1 at 37^{o}C for 2 hours and then ran on 1% agarose gel with 2 log ladder. Expected 3 band patterns but the gel showed five distinct patterns (a-e).

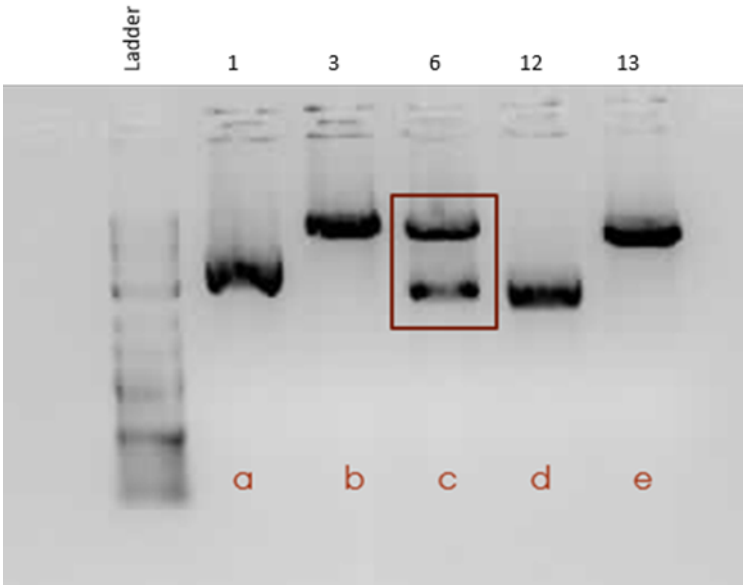

Figure 4. Not1 and Acc65i digest of mutagenesis samples 1, 3, 6,12,13 (see Figure 3). Not1 and Acc65i isolate arm-2 from pW25.2 vector. Sample were digested for 2 hours at 37^{o}C and then ran on 1% agarose gel with 2 log ladder. Expected bands are arm-2 (2.5 Kb) and the remaining vector (10 Kb). Lane 6 is the only sample that showed the expected bands.

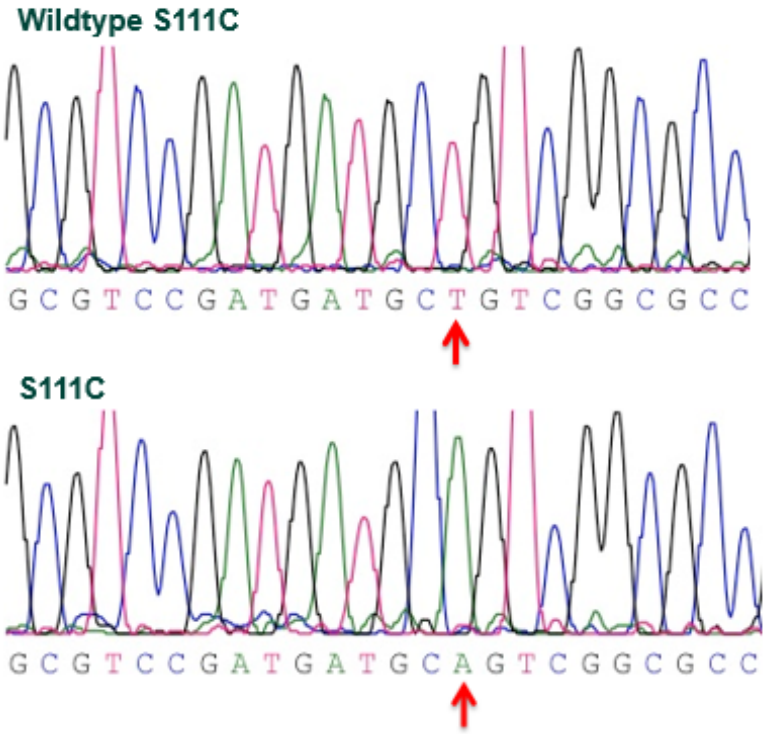

Figure 5. Sanger sequencing results of pW25.2SOD1^{S111C} after the process of site-directed mutagenesis. Vector was sent to University of Rhode Island. In wild-type SOD1^{wt} (top), there exist a thymine but after mutagenesis that thymine is mutated to an adenine (bottom).

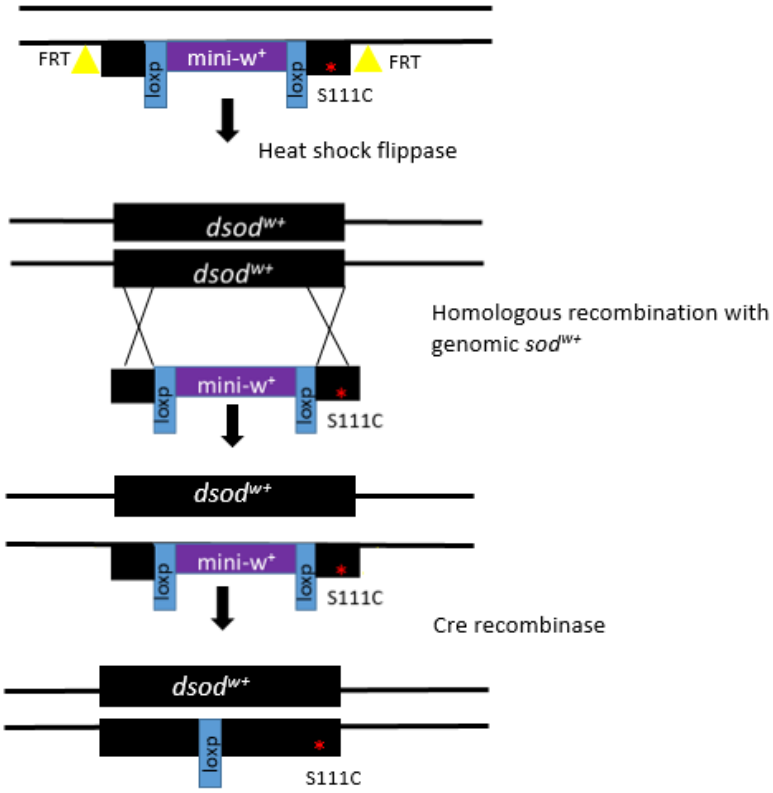

Figure 6. Process of homologous recombination in *D. melanogaster* with injected pW25.2SOD1^{S111C}. After injection, only the p-element incorporates into the genomic DNA. Flippase excises the vector at FRT sites and then undergoes homologous recombination with *D. melanogaster's* third chromosome that contains the endogenous SOD1^{wt}. Cre recombinase removes the mini white gene between arm-1 and arm-2. In the end we have one copy on the endogenous SOD1^{wt} and one copy of SOD1^{S111C} on the third chromosome.

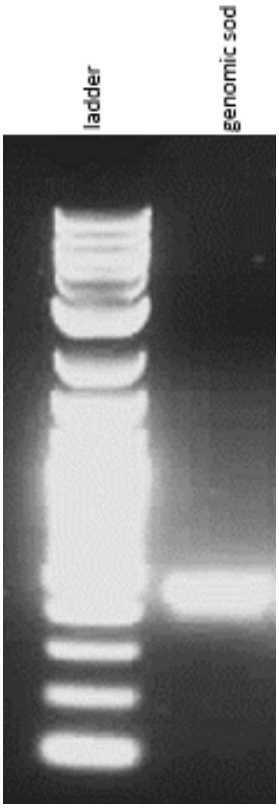

Figure 7. PCR amplification of sod from genomic DNA. Genomic DNA of heterozygous sod^{+} /sod^{S111C} flies were isolated and amplified with primer set [F_{3A}, R_{1A}] on 1% agarose gel with 2 log ladder. The expected band is 500 bp.

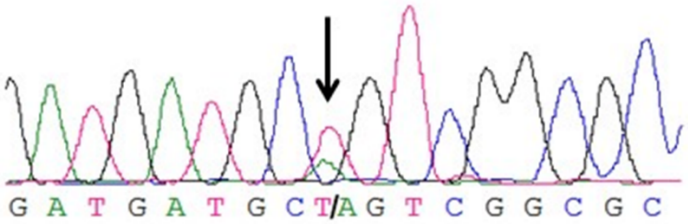

Figure 8. Sanger sequencing result of genomic *D. melanogaster* DNA. Heterozygous sod^{+}/sod^{S111C} flies' genomic DNA was isolated after homologous recombination and amplified with primer A2-S-R_1. Sample was sent to University of Rhode Island for sequencing. Arrow shows thymine (T) from endogenous sod^{+} and adenine(A) from mutated sod^{S111C}.

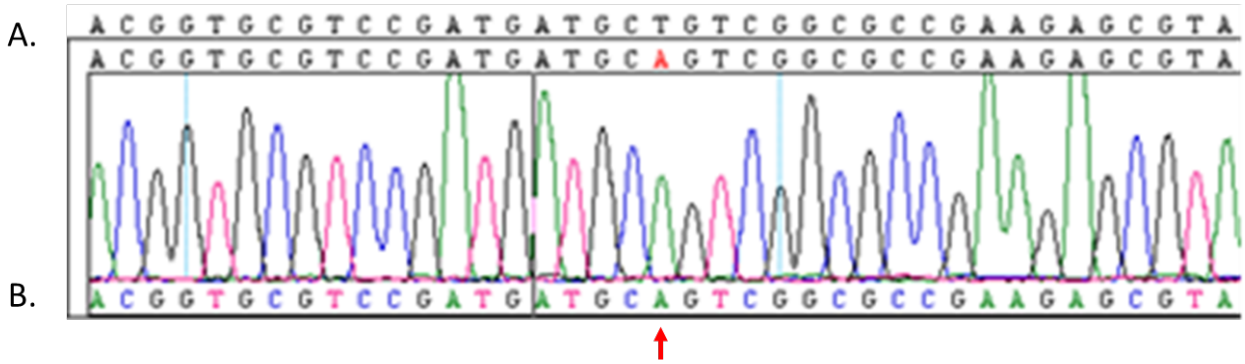

Figure 9. Sanger sequencing result of genomic *D. melanogaster* DNA. Homozygous sod^{S111C}/sod^{S111C} flies' genomic DNA was isolated after homologous recombination and amplified with primer A2-S-R1. Sample was sent to University of Rhode Island for sequencing. (A) Top alignment shows the sequence of wildtype sod and the bottom alignment shows S111C mutated sod sequence, with the red A denoting the mutation from thymine to adenine. (B) Genomic DNA sequence of sod^{S11C} peaks. Arrow shows the clean adenine peak, instead of thymine, at the site of mutation.

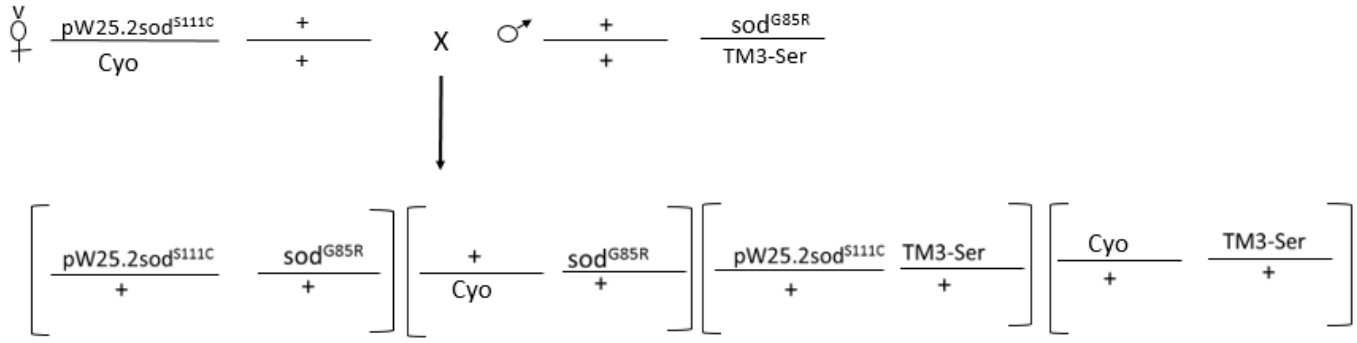

Figure 10. Crossing scheme of eclosion rate experiment. The diagram depicts only second and third chromosome of *D. melanogaster*, where the pW25.2sod^{S111C} vector and sod^{G85R} are located along with marker genes. The cross results in four different progeny types.

Genotype	Eclosion % of Expected	Sample size (n)
pW25.2sod^{S111C}/+; sod^{G85R}/+	100%	182
pW25.2sod^{S111C}/+; TM3-Ser/+	100%	160
CYO/+; sod^{G85R}/+	100%	144
CYO/+; TM3-Ser/+	100%	181

Table 1. Eclosion data of progeny from a cross involving heterozygotes of sod^{S111C} and sod^{G85R}. Adult flies of genotypes pW25.2sod^{S111C}/Cyo; +/+ and +/+; sod^{G85R}/TM3-Ser were crossed and eclosion numbers of the resulting F1 progeny were scored every other day. Expected percentages were based on predicted Mendelian ratios.

Genotype	Eclosion % of Expected	Sample Size (n)
S111C/H71Y	100%	688
S111C/loxp	100%	505
S111C/G85R	150% *	81

Table 2. Eclosion data of progeny from three crosses consisting of sod^{S111C}. Progeny were scored on CO_2 pad every or every other day. Eclosion percent of expected was calculated as (observed/sample size divided by expected) *P-value< .0001

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