GENETICALLY ACCURATE MODELS OF SOD1-BASED ALS IN DROSOPHILA: VALIDATION AND CHARACTERIZATION

by

Ceren İskender

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GENETICALLY ACCURATE MODELS OF SOD1-BASED ALS IN DROSOPHILA: VALIDATION AND CHARACTERIZATION

APPROVED BY:

Prof. A. Nazlı Başak (Thesis Supervisor)	……
Assoc. Prof. Arzu Çelik	……
Prof. Uğur Özbek	……

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To my beloved and distinguished grandfather,

Prof. Yalçın Sanalan, who is also

a neurodegenerative disease patient

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ABSTRACT

GENETICALLY ACCURATE MODELS OF SOD1-BASED ALS IN DROSOPHILA: VALIDATION AND CHARACTERIZATION

SOD1 has been a unique target of ALS research as the first identified gene of the disease; since 20 years, SOD1 has not been related to any other disease unlike other ALS-causing genes. There are more than 160 mutations seen in ALS patients scattered all around the small protein that cause the mutant protein to gain a toxic function. Up to date, more than 20 SOD1-based animal models have been developed that helped us gain insights to the mechanisms in which mutant SOD1 is involved, e.g. oxidative stress, misfolded protein aggregation, endoplasmic reticulum stress and glutamate excitotoxicity. Even so, all these models had their own drawbacks as being either knock-out or over-expression studies. In this respect we aimed to develop an accurate model of SOD1-based ALS using ends-out homologous recombination (HR) in *Drosophila.* In this study, four previously defined SOD1 missense mutations (G37R, H48R, H71Y and G85R) were introduced into the endogenous locus of *Drosophila* SOD1 (*dSOD).* For the characterization of mutants, life span and larval motility assays were used. In addition, genotypic ratios of the mutants were observed for genotype-phenotype correlations and expression levels of several candidate genes, possibly involved in ALS pathogenesis, were compared among mutants using quantitative RT-PCR. This is the first study, which successfully implements homologous recombination, a new and powerful approach to ALS. We hope that, genetically accurate models of SOD1-based ALS, generated via homologous recombination, will further help us gain insights into the complex mechanisms involved in neurodegenerative processes.

ÖZET

SOD1-TEMELLİ ALS'NİN GENETİK AÇIDAN HASSAS DROZOFİLA MODELLERİ: DOĞRULAMA VE KARAKTERİZASYON

SOD1 geni, ALS'ye yol açtığı belirlenen ilk gendir. 1993 yılında ilk defa tanımlanmasından beri SOD1'deki mutasyonlar, ALS'ye neden olan diğer genlerin aksine, başka hiçbir hastalık ile ilişkilendirilmediğinden, SOD1, ALS alanında süregelen araştırmaların, en önemli odak noktası olmuştur. Sadece 153 amino asitten oluşan SOD1 proteinin her tarafına eşit olarak dağılmış olan 160'tan fazla mutasyon bulunmaktadır ve bu mutasyonlar proteine toksik bir işlev kazandırmaktadır. Bugüne dek 20'den fazla SOD1-temelli ALS hayvan modeli geliştirilmiş ve bu modeller mutasyona uğramış SOD1'in de rol aldığı, oksidatif stres, yanlış katlanmış protein birikimi, endoplazmik retikulum stresi ve glutamat eksitotoksisitesi gibi hastalık mekanizmalarının anlaşılmasında son derece faydalı olmuştur. Buna rağmen, tüm bu modeller, *knock-out* veya *over-expression* çalışması olmanın olumsuzluklarını taşımaktadırlar. Bu tez çerçevesinde, SOD1-temelli ALS'nin, *Drozofila*'da genetik açıdan hassas bir modelinin yapılması hedeflenmiş ve dört SOD1 mutasyonu (G37R, H48R, H71Y ve G85R), *Drozofila* SOD1 geni içerisine homolog rekombinasyon yöntemi kullanılarak yerleştirilmiştir. Mutantların karakterizasyonu için yaşam süresi ve larva hareketliliği analizleri uygulanmıştır. Bunun yanında, yaşamsal bütünlük gibi, genotip-fenotip ilişkilendirmeleri için, mutantların genotip oranları incelenmiştir. Ayrıca, ALS patogenezinde olası rol oynayan aday genlerin anlatım düzeylerini ölçmek için RT-PCR metodu uygulanmıştır. Bu çalışma, yeni ve güçlü bir yöntem olan homolog rekombinasyonu, ALS alanında uygulayan ilk çalışmadır. Çalışma kapsamında oluşturulan genetik açıdan hassas *Drozofila* modellerinin ALS ve nörodejeneratif süreçlere yol açan mekanizmaları daha iyi anlamamıza yardımcı olacağını umuyoruz.

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*	Asterix
%	Percentage
^{o}C	Centigrade degree
cq	Cycle Value
g	Gram
g	G-force
kb	Kilobase
µg	Microgram
µl	Microliter
µM	Micromolar
mM	Milimolar
mg	Miligram
ml	Mililiter
M	Molar
ng	Nanogram
V	Volt
β	Beta
Δ	Delta

LIST OF ACRONYMS/ABBREVIATIONS

AD	Alzheimer's disease
ADAR	Adenosine Deaminase Acting on RNA 2
ALS	Amyotrophic Lateral Sclerosis
ALS2	Alsin
ALS-FTD	Amyotrophic Lateral Sclerosis-Frontotemporal Dementia
ALSoD	Amyotrophic Lateral Sclerosis Online Genetics Database
AMPA	L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANG	Angiogenin
AO	Age of Onset
AR	Autosomal Recessive
ATXN2	Ataxin 2
C	Cysteine
C9orf72	Chromosome 9 Open Reading Frame 72
CHMP2B	Charged Multivesicular Body Protein 2B
Ca^{2+}	Calcium Ion
CCS	Copper Chaperone for Superoxide Dismutase
CO_2	Carbondioxide
Cre	Causes recombination
Cu	Copper
DAO	D-amino-acid oxidase
DCTN1	Dynactin 1
DNA	Deoxyribonucleic Acid
dADAR	Adenosine Deaminase Acting on RNA

dNTP	Deoxyribonucleotide Triphosphate
dSOD	Drosophila Superoxide Dismutase
EAAT2	Excitatory Amino Acid Neurotransmitter 2
EDTA	Ethylenediaminetetraacetic Acid
EMS	Ethyl Methane Sulphonate
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
EtOH	Ethanol
fALS	Familial Amyotrophic Lateral Sclerosis
FIG4	Polyphosphoinositide phosphatase
FLP	Flippase
FRT	Flippase Recognition Target
FTD	Frontotemporal Dementia
FUS	Fused in Sarcoma
G	Glycine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GluA2	Glutamate Receptor A2
GOF	Gain of Function
H	Histidine
H_2O	Water
H_2O_2	Hydrogen Peroxide
HR	Homologous Recombination
KO	Knock-out
L3	Larval Stage 3
LMN	Lower Motor Neuron
LOF	Loss of Function
LoxP	Locus of X-over P1

MBR	Metal-Binding-Region
MND	Motor Neuron Disease
mRNA	Messenger Ribonucleic Acid
mt	Mutant
NEFH	Neurofilament, Heavy Polypeptide
ND	Neurodegenerative diseases
NMJ	Neuromuscular Junction
O_2^-	Superoxide
O_2	Oxygen
OPTN	Optineurin
OH^-	Hydroxyl radicals
PBS	Phosphate Buffered Saline
PD	Parkinson's disease
PCR	Polymerase Chain Reaction
PFN1	Profilin 1
PRPH	Peripherin
PXX	Proline-Unknown-Unknown
Q	Glutamine
qPCR	Quantitative PCR
R	Arginine
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
sALS	Sporadic Amyotrophic Lateral Sclerosis
SETX	Senataxin
SIGMAR1	Sigma Non-Opioid Intracellular Receptor 1
siRNA	Small Interfering RNA
SMA	Spinal Muscular Atrophy

SOD1	Superoxide Dismutase1
SPG11	Spatacsin
TAF15	TATA-binding protein-associated factor 2N
TARDBP/ TDP-43	TAR DNA binding protein
TBE	Tris/Borate/EDTA
TM3	Third Multiple Three
UAS	Upstream Activation Sequence
UBQLN2	Ubiquilin 2
UMN	Upper Motor Neurons
UPR	Unfolded Protein Response
UV	Ultraviolet
VABP	Vesicle-associated Membrane Protein-associated Protein B
VCP	Valosin-Containing Protein
WB	Western Blot
white+	Red-eye Marker
wt	Wild-Type
wt-SOD1	Wild-Type Superoxide Dismutase1
WST	Water Soluble Tetrazolium Salt
WTL	Wild-Type-Like
Y	Tyrosine
Zn	Zinc

1. INTRODUCTION

1.1. Introduction to ALS

Neurodegenerative diseases (NDs) are a heterogeneous group of progressive disorders that affect specific populations of cells in the central and peripheral nervous systems. Most of the patients are sporadic, however familial cases are also seen enabling identification of disease causing genes. Clinical symptoms are typically adult onset and include memory loss, cognitive impairment and loss of movement or loss of control over movement (Ambegaokar *et al.*, 2010). Although several features are shared by NDs, different regions of the brain, distinct neuronal types or glial cells, are affected in each disease. For instance in the case of Alzheimer's disease (AD), the temporal lobe of the brain is affected and neurofibrillary tangles or plaques are accumulated, whereas in Parkinson's disease (PD) loss of dopaminergic neurons in the nigrostriatal system is observed with the formation of Lewy bodies (Gama Sosa *et al.*, 2012).

Amyotrophic Lateral Sclerosis (ALS), also known as motor neuron disease (MND), is also a late-onset neurodegenerative disease that leads to the progressive death of the upper motor neurons (UMN) in the cortex and the lower motor neurons (LMN) in the brainstem and spinal cord, followed by muscle weakness and paralysis (Pasinelli and Brown, 2006). The French physician Jean-Martin Charcot first described ALS, in 1869, as a non-hereditary disease, making a clear distinction between Spinal Muscular Atrophy (SMA) and ALS. However, almost a century later, with the identification of genes contributing to ALS, brought out the concept of both familial (fALS) and sporadic (sALS) forms of the disease. Today, it is accepted that 5-10% of the disease is seen as fALS with multiple affected family members, whereas the remaining portion is considered as sALS (Andersen and Al-Chalabi, 2011). Phenotypically it is almost impossible to distinguish between both forms of the disease, but there is evidence that the age of onset is lower in fALS, being around 47 as opposed to 57 in sALS (Kiernan *et al.*, 2011). Also, considering the pathological similarities of fALS and sALS, it can be said

that this discrimination is only applicable in genetic terms (Andersen and Al-Chalabi, 2011).

Symptoms and disease progression vary between ALS patients depending on the location of motor neuron degeneration. The death of upper motor neurons in the motor cortex of the brain leads to spasticity, hyperexcitability of reflexes and slow speech; whereas the death of lower motor neurons in the brain stem and the spinal cord leads to atrophy, fasciculation and weakening of the muscles controlling voluntary movement. At the later stages of the disease the patients become dependent on long-term mechanical ventilation assistance, due to the denervation of diaphragm muscles (Pasinelli and Brown, 2006). The limb type of onset with the involvement of both upper and lower motor neurons is more common (70%) than the bulbar onset (25%) that starts with speech and swallowing difficulties and continues with limb features. In lower percentages, primary lateral sclerosis with pure LMN involvement and progressive muscular atrophy with pure UMN involvement can also be seen (Kiernan *et al.*, 2011).

ALS is the most common adult-onset motor neuron disease: annually, 2-3 per 100,000 individuals develop ALS, with a slightly higher incidence in men (Kiernan *et al.*, 2011, Vande Velde *et al.*, 2011). The prevalence on the other hand, being 4-6 in 100,000 individuals, is similar to the incidence of the disease (Pasinelli and Brown, 2006). This similarity may be explained by the fast disease progression, which in a way balances the rate of incidence and the rate of death due to the progressive disease. For the increased incidence of the disease throughout the years, several suggestions have been made like improved diagnosis, better awareness and the aging population (Sathasivam, 2010).

Survival in ALS is dependent on clinical phenotype, progressiveness of the disease, early occurrence of respiratory defects and life style of the patient including nutrition (Kiernan *et al.*, 2011). However, usually, by the time the disease's first signs appear, 80% of the motor neurons are already dead and the majority of the patients survive for only two to five years, whereas only about 20% of the cases survive for five to ten years with good patient care (Kiernan *et al.*, 2011). There is no cure for ALS yet and the current treatment allows extending the life span for only two months with

Riluzole, a palliative glutamate-release inhibitor (Bensimon *et al.*, 1994). Availability of new treatment approaches will only be possible, as the pathogenesis of this complex disease is unraveled in more detail.

1.2. Genetic Basis of ALS

So far, via linkage analyses and next generation sequencing techniques, many genes have been reported to be disease-causing in fALS (Table 1.1). The full list of ALS associated genes can be found in the ALSoD database (http://alsod.iop.kcl.ac.uk/als/). Mutations in genes identified so far were only able to clarify 25-30% of fALS cases (Andersen and Al-Chalabi, 2011). Some of these genes also contribute to sALS, however in a much lower frequency. Familial ALS appears with different modes of inheritance, autosomal-dominant with complete penetrance, autosomal-dominant with incomplete penetrance and autosomal-recessive, being the most common (Andersen and Al-Chalabi, 2011, Robberecht and Philips, 2013). Exceptionally, an X-linked dominant form of inheritance is observed in individuals with mutations in the *UBQLN2* gene, recently discovered as ALS-causing (Deng *et al.*, 2011).

Table 1.1. ALS-associated genes (adapted from (Al-Chalabi *et al.*, 2012b)).

Gene	ALS #	Locus	Affected Motor Neuron	Mean AO	Mode of inheritance
SOD1	ALS1	21q22.11	UMN, LMN	47	AD, AR, de novo
ALS2	ALS2	2q33.2	UMN, LMN	1	AR
SETX	ALS4	9q34.13	UMN, LMN	18	AD
SPG11	ALS5	15q21.1	UMN, LMN	16	AR
FUS	ALS6	16p11.2	UMN, LMN	46	AD, AR, de novo
VAPB	ALS8	20q13.33	UMN, LMN	44	AD
ANG	ALS9	14q11.1	UMN, LMN	55	AD
TARDBP	ALS10	1p36.22	UMN, LMN	55	AD, AR
FIG4	ALS11	6q21	-	55	AD
OPTN	ALS12	10p13	LMN, UMN	51	AD, AR
AD: autosomal dominant, AR: autosomal recessive, LMN: lower motor neuron, UMN: upper motor neuron					

Table 1.1. ALS-associated genes (adapted from (Al-Chalabi *et al.*, 2012b)(cont.).

Gene	ALS #	Locus	Affected Motor Neuron	Mean AO	Mode of inheritance
ATXN2	ALS13	12q23-q24.1	LMN, UMN	57	AD
VCP	ALS14	9p13	UMN, LMN	49	AD
UBQLN2	ALS15	Xp11.21	UMN, LMN	41	SD
SIGMAR1	ALS16	9p13	LMN, UMN	1	AD
PFN1	ALS18	17p13.3		45	AD
C9orf72	ALS-FTD2	9p21.2	UMN, LMN	57	AD
CHMP2B	ALS-FTD3	3p12.1	UMN, LMN	-	AD
NEFH	-	22q12.1-q13.1	UMN, LMN	60	AD
DCTN1	-	2p13	UMN, LMN	55	-
PRPH	-	12q12	LMN	-	-
DAO	-	12q24	UMN, LMN	44	-
TAF15	-	17q11.1-q11.2	LMN, UMN	50	AD, AR
AD: autosomal dominant, AR: autosomal recessive, LMN: lower motor neuron, UMN: upper motor neuron					

1.2.1. Cu/Zn Superoxide Dismutase 1 (*SOD1*) Gene

Cu/Zn superoxide dismutase 1 (*SOD1*) was first related to ALS in 1993 and identification of mutations in this gene became a milestone in understanding the genetic basis of ALS (Rosen, 1993). Mutations in *SOD1* explain almost 20% of fALS and 2-3% of sALS (Andersen, 2006). So far, there are 166 disease-associated mutations distributed on the 9.3kb human gene with 5 exons, encoding for a 153 amino acid-long protein. Among these, 147 are missense and the remaining 19 mutations are nonsense mutations, small insertions and deletions. SOD1 mutations cause autosomal-dominant disease with the exception of D90A, which can be seen both in autosomal-dominant and recessive forms. 23 SOD1 mutations show incomplete penetrance (Andersen and Al-Chalabi, 2011).

SOD1 is a cytoplasmic metalloenzyme that catalyzes the conversion of superoxide radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) in order to protect cells from oxidative damage caused by reactive oxygen species (ROS) (McCord

and Fridovich, 1969). The enzyme is functional as a homodimer, each subunit consisting of eight β-barrels and seven β-loops (Figure 1.1). Binding of one copper ion and one zinc ion per subunit is required for the activity of the enzyme and is represented as green and red in Figure 1.1, indicating copper and zinc binding residues, respectively. There is also a conserved intrasubunit disulfide bond between two cysteines (C) at positions 57 and 146. SOD1 mutants can either referred to as metal-binding-region (MBR) or wild-type-like (WTL) mutants according to the mutated residue (Valentine *et al.*, 2005).

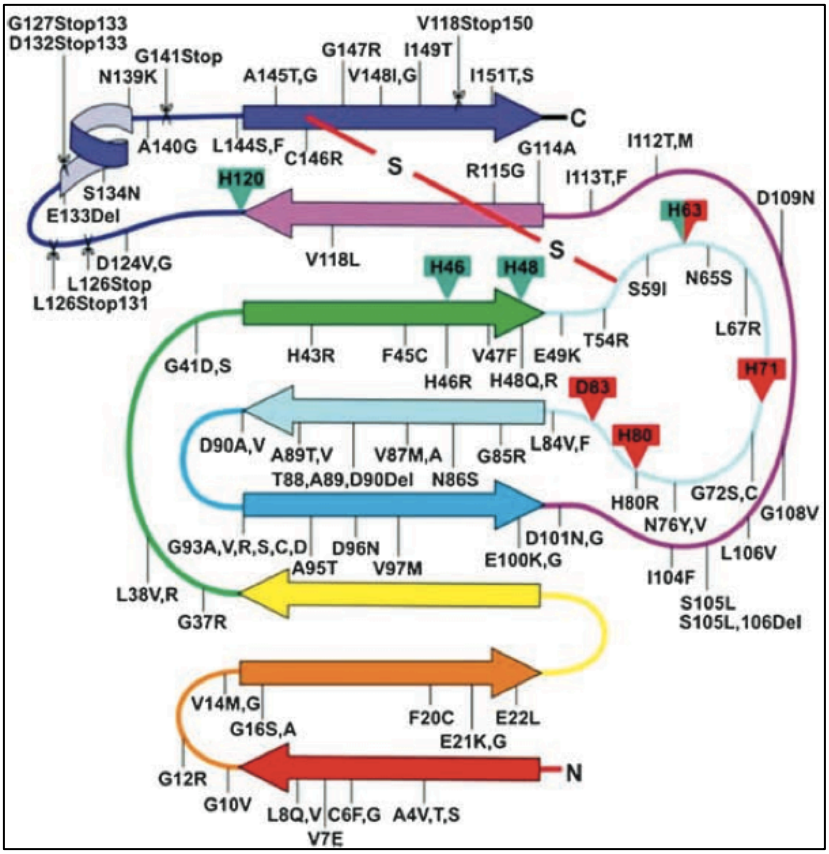

Figure 1.1. Distribution of ALS causing mutations on the SOD1 protein (Valentine *et al.*, 2005).

There is no certain genotype-phenotype correlation for mutations of SOD1, however there are observations regarding the age of disease onset and survival time. The age of onset ranges from six years (I104F) to 94 years (D90A), and a reduced mean age of onset is observed in G37R, L38V and G114A. The range for the survival time of patients with SOD1 mutations is reported to be changing from a few months (C6G) to 44

years (L144F). In general, A4V, G41S, G93A and R115G are related with short survival time, while longer survival is seen in patients carrying the G41D, G93C and H46A mutations and the D90A mutation in homozygous form. Survival times have loose borders for some mutations like G37R (ranging from 2-36 years), I104F (ranging from 3-38 years) and I113T (ranging from 2-20 years) (Andersen and Al-Chalabi, 2011, Andersen *et al.*, 1995, Cudkowicz *et al.*, 1997, Valentine *et al.*, 2005).

Extra motor features are also observed specifically for some genotypes; mild cerebellar ataxia (D90A, E100K), bladder disturbance (G41S, D90A, V118L), heat sensation (D90A), sensory neuropathy (A89V, D90A) and severe neuralgic pain (A4V, G12R, G37R, D90A, E100G, G127R) are some examples (Andersen and Al-Chalabi, 2011).

1.2.2. Other ALS Genes

After the identification of the association between SOD1 mutations and ALS, consecutive studies reported several genes as ALS-causing, supporting the complex genetic nature of the disease. Most of these mutations are missense mutations, however with some exceptions, including frameshift and nonsense mutations leading to truncation of the encoded proteins.

Mutations in two DNA/RNA binding proteins, TAR DNA-binding protein (*TARDBP*/TDP-43) and Fused in sarcoma (FUS) were also reported to be causing typical ALS (Gitcho *et al.*, 2008, Kabashi *et al.*, 2008, Kwiatkowski *et al.*, 2009, Sreedharan *et al.*, 2008, Vance *et al.*, 2009). C-termini of both proteins are hotspots for these autosomal-dominantly inherited mutations (Andersen and Al-Chalabi, 2011). Both TDP-43 and FUS resemble each other being involved in different steps of RNA processing. Contributions of *TARDBP* and *FUS* to ALS pathogenesis are also similar being around 4-6% for fALS and 0-2% for sALS worldwide (Andersen and Al-Chalabi, 2011, Robberecht and Philips, 2013).

The recent discovery regarding the pathogenicity of the hexanucleotide (GGGGCC) repeat expansions in the non-coding region of *C9orf72 (Chromosome 9 open reading frame 72)* in ALS, have made a clear change in understanding ALS genetics (DeJesus-Hernandez *et al.*, 2011, Renton *et al.*, 2011). Identification of these expansions in the chromosome 9p21 locus, which was previously linked to ALS, explained almost 30% of fALS cases and became the new leading causative factor for ALS, switching places with *SOD1* (Hosler *et al.*, 2000, Majounie *et al.*, 2012, Morita *et al.*, 2006, Turner *et al.*, 2013).

UBQLN2, encoding the ubiquitin-like protein ubiquilin 2, causes chromosome X-linked ALS, with a dominant inheritance pattern (Deng *et al.*, 2011). Several fALS-linked mutations were identified, which are predominantly located inside the PXX domain of the protein. Mutations in this protein explain almost 2% of fALS cases in varying populations (Gellera *et al.*, 2013, Millecamps *et al.*, 2012). Juvenile patients with UBQLN2 mutations are also seen (Deng *et al.*, 2011). In patients with UBQLN2 mutations the male to female ratio of disease onset is significantly low, when compared to ALS patients with different mutations. This is explained by the hemizygous nature of the mutation in males (Andersen and Al-Chalabi, 2011).

Mutations in other fALS genes, like *alsin (ALS2)*, *senataxin (SETX)*, *spatacsin (SPG11)*, *angiogenin (ANG)*, *optineurin (OPTN)*, *dynactin 1 (DCTN1)*, *VAMP-associated protein B (VAPB)*, *valosin-containing protein (VCP)* and *profilin 1 (PFN1)* are considered as rare causes of ALS (Al-Chalabi *et al.*, 2012a). Autosomal-recessive mutations in *ALS2*, *SETX* and *SPG11* were found to be associated with juvenile-onset ALS with slow disease progression (Chen *et al.*, 2004, Hadano *et al.*, 2001, Orlacchio *et al.*, 2010). Almost all of the above mentioned genes have contributions also to sporadic ALS, however *C9orf72* expansions play a significant role in sALS genetics.

There are cases where features of ALS and frontotemporal lobe dementia (FTD) are seen interchangeably in both diseases. Genes responsible for this dual phenotype are not totally explored, however the recently identified *C9Orf72* locus helped us gain insights about this issue and marked the *C9Orf72* locus as the ALS-FTD locus (DeJesus-

Hernandez *et al.*, 2011, Renton *et al.*, 2011). ALS-FTD also has been related to the mutations in genes *TARDBP*, *FUS*, *ANG* and *UBQLN2* (Benajiba *et al.*, 2009, Borroni *et al.*, 2009, Ticozzi *et al.*, 2009, Yan *et al.*, 2010). In addition, dementia has been reported in ALS patients with *VCP* mutations (Johnson *et al.*, 2010). Altogether, this inevitable clinical heterogeneity of ALS, with the newly identified causative genes, directs scientists to work on a more sophisticated taxonomy for the disease (Turner *et al.*, 2013).

1.3. Mechanisms of SOD1-based ALS

A significant subset of ALS cases is caused by mutations in the *SOD1* gene, which gains a toxic function, making this small protein an attraction point for scientists. Interconnected pathological processes involved in disease progression are discussed below.

1.3.1. Oxidative Stress

An imbalance between the generation and removal of reactive oxygen species (ROS) in the cell results in oxidative stress, which in turn may lead to structural damage in proteins, lipids, DNA and even some RNA species. Evidence from free radical damage markers and postmortem tissue suggests that ALS patients with *SOD1* mutations are exposed to oxidative damage. Surprisingly, this exposure is due to other mechanisms than its catalytic activity, since no change in enzymatic activity of SOD1 is detected in the *SOD1*^{G93A} mice, and also overexpression of wt-SOD1 does not rescue the disease phenotype (Barber and Shaw, 2010, Bruijn *et al.*, 1998). In addition, neurodegeneration is not observed in *SOD1*-null mice, supporting the gain of function hypothesis (Ho *et al.*, 1998, Phillips *et al.*, 1989, Reaume *et al.*, 1996). Rather, prolonged activation of the superoxide production in microglia and also dysregulation of antioxidant production pathway in neurons are probable causes of oxidative damage leading to ALS pathogenesis. It is also thought that oxidative damage triggers other mechanisms that eventually play a role in the death of motor neurons, like protein aggregation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, glutamate excitotoxicity,

also resulting in changes in neighboring cell pathologies (Figure 1.2) (Ferraiuolo *et al.*, 2011, Redler and Dokholyan, 2012, Robberecht and Philips, 2013).

1.3.2. Protein Misfolding and Aggregation

The tendency of the mutant protein to misfold and form aggregates due to loss of stability of the native SOD1 homodimer and the stabilizing posttranslational modifications has been accepted as the major cause leading to disease pathogenesis (Redler and Dokholyan, 2012). SOD1-immunoreactive proteinaceous aggregates seen in motor neurons of patients with sALS designate oligomerization as a common concept for the disease (Gruzman *et al.*, 2007). In addition to SOD1 inclusions, detection of TDP-43-positive cytoplasmic inclusions is discussed between early pathogenic events for ALS patients. Altogether, the observation of cytoplasmic inclusions containing additionally FUS protein in *FUS*-related ALS cases and involvement of other ALS-causing genes, like *VCP* and *UBQLN2* in proteosomal degradation pathways, indicates the importance of protein aggregation in disease pathogenesis.

1.3.3. Other Subcellular Pathways Triggered by Oxidative Stress

Another subcellular event playing a role in ALS pathogenesis is glutamate excitotoxicity, which results from insufficient removal of glutamate (main excitatory neurotransmitter) from the synaptic cleft by glutamate reuptake transporters. Glutamate excitotoxicity is driven by oxidative stress and caspase-3-mediated proteolysis. Selective loss of astrocytic glutamate transporter, excitatory amino acid transporter 2 (EAAT2), results in increased levels of glutamate, thereby affecting neuronal energy homeostasis (Ferraiuolo *et al.*, 2011). Several serial events start with reduced activity of EAAT2, like increased Ca^{2+} uptake by the Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) receptor of motor neurons, further leading to increase in ROS production, to disruption in protein homeostasis, and eventually to apoptosis (Redler and Dokholyan, 2012).

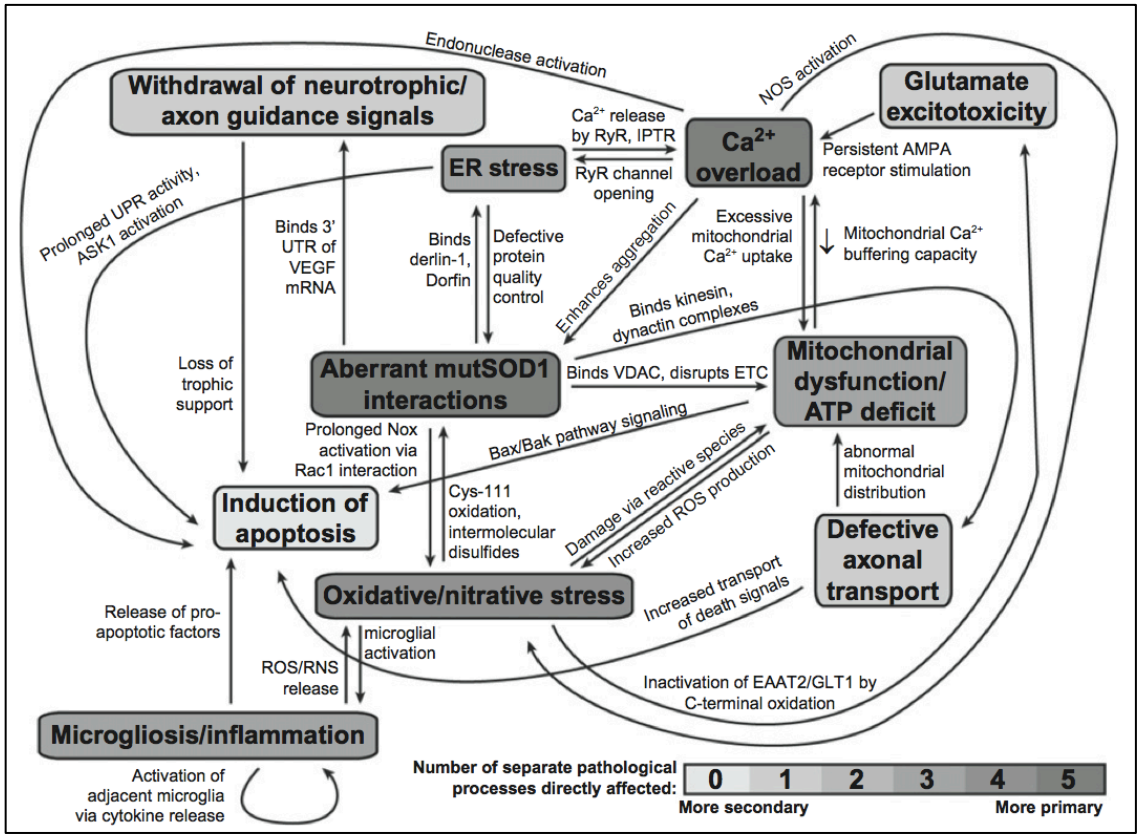

Figure 1.2. Interconnected pathways underlying SOD1-based ALS (Redler and Dokholyan, 2012).

Mitochondrial dysfunction and ER stress are other proposed mechanisms of the disease that are affected by excess Ca^{2+} influx, due to mutant SOD1 (mtSOD1) aggregation, resulting from disrupted Ca^{2+} homeostasis. Lowered electron transport chain activity, low membrane potential and increase in mitochondrial DNA damage are some of the features indicating malfunctioning of mitochondria in samples of ALS patients and in *SOD1* mouse models (Barber and Shaw, 2010, Martin *et al.*, 2007, Wong *et al.*, 1995). As described above, protein misfolding and accumulation are of high importance in ALS pathogenesis, bringing ER-associated protein degradation (ERAD) and unfolded protein response (UPR) pathways into the scene. Regardless of the disease, these mechanisms are responsible for the recognition, repair and removal of unfolded or misfolded proteins in the cell. However, hyperactivation of initially protective mechanisms may trigger apoptotic signaling in case of ALS (Ferraiuolo *et al.*, 2011). Increased levels of ER stress

markers have been detected in spinal cord of sporadic ALS patients and in *SOD1*^{G93A} mice (Atkin *et al.*, 2008, Kikuchi *et al.*, 2006).

1.3.4. Axonal Transport Defects and Involvement of non-Neuronal Cells

Motor neurons have specialized characteristics, including very long axons (up to 1m), in order to deliver components like RNA, proteins and organelles from the cell body through the axon to the synaptic structures at the neuromuscular junction (NMJ) (Redler and Dokholyan, 2012). The transport towards the axon is mediated by microtubule-dependent kinesin and called anterograde transport, whereas the transport in the reverse direction that is from the NMJ to the cell body is called retrograde transport and depends on cytoplasmic dynein molecular motors. In case of *SOD1* mutations, both anterograde and retrograde transports are affected, however the defects do not have to be bidirectional for a specific cargo. As discussed in *Ferraiuolo et al*., most of the mechanisms leading to ALS pathogenesis are interconnected, defects in axonal transport may also be the result of the impairment of mitochondrial transport due to its dysfunction (Ferraiuolo *et al.*, 2011). Because of the lack of energy in the axonal compartment of the affected neuron, subsequent defects may follow in the transportation of other cargoe. Also it has been shown that excess glutamate reduces axonal transport of neurofilaments, which are responsible for maintaining axonal integrity by activating protein kinases. Hyperphosporylation of neurofilaments results in disrupted axonal transport by their detachment from motor complexes (Ackerley *et al.*, 2003).

In addition to all pathophysiology within the motor neurons, the literature on ALS implies the role of non-neuronal cells in ALS pathology as undeniable. It is shown that chimeric mice expressing mtSOD1 in glial cells, but not in motor neurons, developed signs of ALS pathology; also it is reported that survival of motor neurons expressing mtSOD1 is increased when they are surrounded by normal non-neuronal cells (Clement *et al.*, 2003, Swarup and Julien, 2011). Impairment of the astrocytic glutamate transporter EAAT2 results in excess glutamate in the synaptic cleft, thereby increasing excitotoxicity and inducing oxidative stress in motor neurons, an example indicating the importance of non-neuronal cells. Oxidative stress can further activate glia resulting in release of

reactive nitrogen species and proinflammatory cytokines and thus can be neurotoxic for motor neurons (Barber and Shaw, 2010).

Altogether, as can be seen in Figure 1.2, the underlying mechanisms leading to ALS pathogenesis are highly complex, and it is not clear whether an event is the primary cause of the disease or a secondary consequence. However, as studied so far, oxidative stress and protein aggregation, both as causes or consequences, play crucial roles in the pathology of *SOD1*-based ALS, therefore become of high importance for therapeutic targeting (Barber and Shaw, 2010, Ferraiuolo *et al.*, 2011, Redler and Dokholyan, 2012).

1.4. Animal Models of SOD1-based ALS and *Drosophila melanogaster* as a Model Organism

Although animal models do not perfectly resemble humans, they are the most effective tools to study human diseases. The mechanisms underlying disease processes can be mimicked in animals, to better understand the cellular events leading to disease pathogenesis from initiation to maturation. As a major subgroup of human diseases, NDs are progressive disorders of the nervous system and are mostly incurable. Fortunately, innovations and new technologies in human genetics allowed identification of disease-associated genes, and it became possible to understand disease mechanisms at molecular level and target these mechanisms therapeutically, via generation of animal models. Generating animal models of ALS became possible with the identification of *SOD1* mutations as ALS-causing and the recent discovery of causative genes like *TARDBP*, *FUS*, *OPTN* and *VCP*.

The ubiquitously expressed SOD1 is responsible for the superoxide dismutase activity within the cell, thus at first it was suspected that the loss of this activity caused the disease. However, *SOD1* knock-out mice and *SOD1*-null *Drosophila* did not show motor neuron degeneration, only reduced fertility and life span due to oxidative stress were observed, supporting the gain of toxic function hypothesis for the disease (Ho *et al.*,

1998, Reaume *et al.*, 1996). So far, several animal models are generated to understand the mechanism behind this toxic gain of function (Table 1.2).

1.4.1. Rodent Models

The first animal model of ALS was generated by overexpressing the human *SOD1* gene, carrying the G93A mutation, in mice using the human *SOD1* promoter (Gurney, 1994). *SOD1*^{G93A} mice resembled human ALS phenotypes with weaknesses in hind limbs leading to paralysis. This model was followed by overexpression of other mutations including G37R, A4V and G85R. However in all cases the observed phenotypes were dependent on the dosage of the expressed mutant protein. Transgenic *SOD1*^{G93A} and the *SOD1*^{A4V} mice expressing the mutated human gene in lower copy numbers either did not develop disease symptoms or developed them at later stages (Joyce *et al.*, 2011, Swarup and Julien, 2011, Turner and Talbot, 2008). Interestingly, it was also reported that wild-type SOD1 (wtSOD1), when overexpressed, caused neuronal defects and crossing *SOD1*^{A4V} mice with mice that overexpress wtSOD1, accelerated disease progression (Joyce *et al.*, 2011). This phenomenon additionally supports the toxic function gained by the SOD1 protein.

Another study further rules out the loss of function hypothesis by showing that there was no change in disease progression of the *SOD1*^{G93A} mice, lacking the copper chaperone for superoxide dismutase (CCS), although reduction in copper-loaded SOD1 amount was observed (Subramaniam *et al.*, 2002). The cytoplasmic inclusions that became a common hallmark for both familial and sporadic ALS were also inspected in SOD1 mice models. Several groups reported the presence of these proteinaceous inclusions in *SOD1*^{G93A}, *SOD1*^{A4V} and *SOD1*^{G85R} mice (Bruijn *et al.*, 1998, Gurney, 1994, Watanabe *et al.*, 2001).

In addition to human *SOD1* mutations, artificially induced *SOD1* mutations that lead to inhibition of copper binding (H46R/H48R and H46R/H48Q/H63G/H120G) or

truncation of the protein (T116X) were also expressed in mice, resulting also in ALS-like phenotypes (Joyce *et al.*, 2011).

Table 1.2. Animal Models of Sod1-based ALS (adapted from (Joyce *et al.*, 2011)).

Species	**Mutation**	**Protein expression (fold)**	**Activity (fold)**	**Symptom Onset (weeks)**	**Survival (weeks)**
Mouse	A4Va	-	-	35	48
	G37R	-	14	15-17	25-29
	H46R	-	-	20	24
	H46R/H48Q	-	0	17-26	-
	H46R/H48Q/H63G/H120G	-	0	35-52	-
	L84V	-	-	21-26	26-30
	G85R	1	0	35-43	37-45
	G85R	1.5	-	39.5-48	46-54
	G86Rb	-	0	13-17	17
	D90A	20	6-8	52	61
	G93A	17	13	13-17	17-26
	G93Adl	8	-	24-26	40-50
	I113T	-	-	52	60
	T116X	-	-	41	43
	L126X	0-0.5	-	28-36	-
	L126X	0-1	-	44	47
	L126delTT	2	0	17	18
	G127X	0.5-1	0	35	36
Rat	H46R	6	0.2	20	24
	G93A	2.5	3	16	17
	G93A	8-16	-	16	17
Dog	E40K/E40Kc	1	1	after 5 years	6-19 months duration
Zebrafish	G93Rd	3	-	48	72-108
Drosophila	WT	3-7	-	3	normal
	A4V	3-5	-	4	normal
	G85R	1-2	-	2	normal
C.elegans	G85R	-	-	1	reduced
	H46R/H48Q	-	-	1	-
	G85R, G93A, G127X	<1	-	-	-
a: double transgenic with wtSOD; b: mouse transgene; c: spontaneous mutation in endogenous gene; d: zebrafish transgene; dl: G1 del-low copy					

Although mouse models take the lead, rat models of *SOD1*-based ALS are also generated and considered as advantageous due to their bigger size, which makes them more suitable for drug trials. H46R and G93A mutations have been the target in rat models. Animals represented both UMN and LMN signs, and the severity of the disease

was observed as directly proportional to the onset site (forelimb or hind limb) (Joyce *et al.*, 2011).

1.4.2. Zebrafish and C. *elegans* Models

To model ALS in zebrafish, several commonly studied human SOD1 mRNAs (wt, G93A, G37R and A4V) were injected to the fish by *Lemmens et al.*, and axonal defects in the animals were observed (Lemmens *et al.*, 2007). In addition to human mutations, the endogenous *SOD1* gene, carrying the transgenic G93R mutation, was expressed in zebrafish. Threefold expression of the transgene led to loss of motor neurons and reduction in survival (Ramesh *et al.*, 2010).

Introduction of the human G85R mutation and the wtSOD1 to C. *elegans* (expression is restricted to neurons) resulted in reduced forward crawling in the G85R mutant as compared to the wild-type. Also aggregates were observed in the cytosol of ventral nerve cord cell bodies of the G85R-YFP animals. The double transgenic strain H46R/H48Q-YFP, showed much less locomotion defects compared to the G85R-YFP strain (Wang *et al.*, 2009).

1.4.3. *Drosophila melanogaster* as an Animal Model

Flies and humans are similar in terms of several cellular processes including regulation of gene expression, subcellular trafficking, synaptic transmission and cell death. Important pathways like Wnt, ERK, and Toll-like signaling are identified in flies and are conserved also in humans. Flies with only four pairs of chromosomes as opposed to 23 in humans, 12,000 genes as compared to 20,000 in humans have simpler genetics. They have a simple nervous system harboring around 200,000 neurons instead of over 100 billion in humans, that is however, capable of complex motor behaviors like walking, climbing and flying. Flies can also be trained to test learning and memory (Ambegaokar *et al.*, 2010). Due to these properties successful results can be obtained from behavioral assays, especially when studying NDs (Chen and Crowther, 2012). In addition,

Drosophila is advantageous with a short reproductive cycle, where an embryo can grow into a reproductively mature adult in 10 to 14 days. The maintenance of flies is easy and inexpensive, since hundreds of lines can be kept in a small area in separated vials. Flies are also advantageous for drug screening, since the pharmacological agent can easily be mixed with the fly food. To study neurodegenerative diseases, another important aspect related to drug screening is, that it is easy for the agent to enter the nervous system of the organism; since no blood-brain barrier exists in flies (Lu and Vogel, 2009). Altogether, these properties make flies good models to study diseases and NDs in particular.

In addition to all these facts, *Drosophila* is suitable for genetic manipulations with the use of the endogenous transposable P element. P elements insert themselves randomly into the genome, however promoter regions are usually hotspots. When P elements insert into the promoter region of a gene, they can disrupt the transcription. Thus, this approach is used to create animals with null mutations, which enable researchers to study the function of the disrupted gene. P elements are also being genetically engineered in order to carry a gene and insert that gene into the genome, while exerting its own transposase activity. It is also possible to use this tool in a tissue-specific manner, by introducing a tissue/cell-specific promoter in addition to the gene (Ambegaokar *et al.*, 2010, Chen and Crowther, 2012).

Another tool is the GAL4/UAS system, which is widely used in *Drosophila*. In this system, the yeast-derived transcription factor GAL4 that binds to the Upstream Activating Sequence (UAS) enhancer element, drives the expression of the gene that is downstream of the UAS. In this manner a tissue-specific enhancer can control GAL4, which will drive the expression of the gene of interest downstream of the UAS. As the transcription with this system can be tissue-specific, it can also be conditional and can be used to introduce siRNA for the use of knocking down a gene.

1.4.3.1. *Drosophila melanogaster* and ALS. An ALS model was generated in *Drosophila* ALS using the GAL4/UAS system by expressing $hSOD^{G85R}$ and $hSOD^{A4V}$ under neuron-specific promoters and observed ALS-like phenotypes were observed including climbing deficits, ubiquitinated SOD1 aggregates and stress responses in glia (Watson *et al.*,

2008). Another model of ALS in *Drosophila* was a transgenic model, where human wtSOD and five fALS mutations were expressed in a *dSOD* (*Drosophila SOD1*) null background. In this study it was observed that fALS-SOD mutations caused oxidative stress and physiological defects in adults, however in a recessive manner, and thus were not relevant to the gain of function nature of the human disease (Mockett *et al.*, 2003).

1.5. Genetically Accurate Animal Models: Homologous Recombination as a Useful Approach

Generating transgenic animals, as summarized in the previous part, is so far the most common approach to study ALS, where mostly human disease-causing alleles are expressed exogenously in the model organism in its wild-type background. In current models of the disease, phenotypes observed are directly proportional to the amount of mutant protein expressed, thus the reliability of these phenotypes can be suspected. Furthermore, observation of similar phenotypes even when the wild-type protein is overexpressed strengthens this thought. The GAL4/UAS system used in *Drosophila* is especially very much prone to this outcome, where high expression of the gene of interest is a significant feature.

In order to obtain trustable results, while generating animal models of human diseases, it is important to select the suitable organism and advantageous to protect the organism's endogenous environment. In this perspective, using targeted gene mutagenesis is a favorable approach, to protect the genomic structure and to keep the protein amount at a constant level in all mutants. Gene targeting based on homologous recombination (HR) uses the advantage of abolishing background wild-type protein expression by the excision of the endogenous gene and the introduction of the donor sequence including the transgene with the desired mutation. The transgene can than be traced by a marker that is located in the donor sequence in order to end up with the actual mutants. HR, previously was used in mouse embryonic stem cells and human somatic cells, however in *Drosophila* it was first described in 2000s (Rong and Golic, 2000).

Altogether, *Drosophila,* with a short reproduction cycle, cheap maintenance, highly conserved genes and useful genetic tools, is a good candidate to perform targeted gene mutagenesis in order to make a ‘knock-in’ model to understand the pathology behind a disease.

2. PURPOSE

ALS is the most common adult-onset motor neuron disease with a highly progressive nature, leading to death in five to ten years. Although most of the research is based on the inherited form of the disease, similarities between the familial and sporadic forms is expected to enable the availability of any treatment for the benefit of all patients.

Recent research indicates, however, that even the familial forms of this fatal disease is more complex than anticipated. Availability of new treatments for ALS will only be possible, as the pathogenesis of this complex disease is studied in detail. For this purpose, animal models are the most effective tools. Up to date, more than 20 SOD1-based animal models have been generated that helped us gain insights into the mechanisms of SOD1-based ALS. Even so, as being either knock-out or over-expression studies, resemblance and the accuracy of the observed phenotypes may be speculated. To overcome these drawbacks and generate a 'knock-in' model of ALS based on a targeted gene mutagenesis approach, a structured collaboration between Robert Reenan Laboratory at Brown University and NDAL was set-up aiming to build-up a *Drosophila* model of SOD1-based ALS, using ends-out homologous recombination.

In this respect, this study aims to;

- validate the proper integration of four previously defined ALS-causing missense mutations (G37R, H48R, H71Y and G85R) into the endogenous locus of *Drosophila* SOD1 (*dSOD).*
- characterize the mutants in terms of phenotype severity and other phenotypic properties.
- perform molecular analyses on mutants to understand the cellular events behind disease pathogenesis.

3. MATERIALS

The fly stocks, buffers and solutions, PCR and sequencing reagents, kits and primers used in this thesis are compiled in Tables 3.1-3.7, respectively.

3.1. Fly Stocks and Maintenance

The fly stocks used in this thesis are obtained from Bloomington Drosophila Stock Center at Indiana University, USA (Table 3.1).

Table 3.1. Fly stocks.

Genotype and Stock Description
Canton S.
wi; TM3GFP/Ser

Table 3.2. Fly maintenance materials.

Fly Food Mix	Fisher Scientific, USA
Fly Morgue	70% Ethanol

3.2. Buffers and Solutions

Buffers and solutions used for RNA isolation, gel electrophoresis and validation PCR are listed in Tables 3.3, 3.4 and 3.5.

3.2.1. RNA isolation

Table 3.3. RNA isolation materials.

RNaseZap ®	Sigma-Aldrich, USA
TRI Reagent ®	Sigma, USA
Chloroform	Merck KGaA, Germany
2-propanol	Merck KGaA, Germany

3.2.2. Gel Electrophoresis

Table 3.4. Gel electrophoresis materials.

0.5X TBE buffer (pH 8.3)	0.89 M Tris-Base
	0.89 M Boric Acid
	20 mM Na_2 EDTA
6X DNA Loading Dye	Fermentas, Lithuania
Agarose	Prona, Poland
DNA ladder, 1kb	New England Biolabs, USA
	Fermentas, Lithuania
Ethidium Bromide (EtBr)	10 mg/ml

3.2.3. Validation PCR

Table 3.5. Reagents for validation PCR.

10 mM dNTP	New England Biolabs, USA
5X Phusion HF Reaction Buffer	New England Biolabs, USA
Phusion High-Fidelity *Taq* Polymerase (2000 units/ml)	New England Biolabs, USA

3.2.4. Sequencing

- Magnetic beads: Agencourt Clean Seq, Beckman Coulter Genomics, USA
- 80% Ethanol

3.3. Kits

All kits used in this thesis are listed below.

- Maxwell 16 Tissue DNA kit, Promega, USA
- Wizard® SV Gel and PCR Clean-Up System, Promega, USA
- BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA
- Transcriptor High Fidelity cDNA Synthesis Kit, Roche, Germany
- FastStart Essential DNA Green Master, Roche Germany

3.4. Primers

Primers used for validation PCR and qPCR are stated in Tables 3.6 and 3.7.

Table 3.6. Primer sequences for validation PCR.

SODseq3.5.1	5'-GCTCGTTATCATAATCAGTGCTTCTGC-3'
SODseq5rev	5'-GATGAGACACCGCTCATAGAACTAA-3'

Table 3.7. Primer sequences for qPCR.

dGAPDH1-QF	5'-GAAATCAAGGCTAAGGTCGAGGAG-3'
dGAPDH1-QR	5'-GGAGTAACCGAACTCGTTGTCGTA-3'
dADAR-QF	5'-CTGTCCTAAATGATTCCCATGCTG-3'
dADAR-QR	5'-GGGTATTGCCCATCAGTATTCCTC-3'
dSOD-QF	5'-CTAAGCTGCTCTGCTACGGTCACA-3'
dSOD-QR	5'-CAGACAGCTTTAACCACCATTTCG-3'

3.5. Equipment

The equipment used in this study is shown in Table 3.8.

Table 3.8. Equipment used.

Equipment	**Model/ Company**
Balance	TE612, Sartorius, Germany
Centrifuge	Centrifuge 2-16K, Sigma, USA
CO_2 Tank	Genç Karbon, Turkey
Culture Plate	Bacteral culture plate, Corning Incorporated Costar, USA
Deep Freezer	2021D (-20 °C), Arçelik, Turkey
	HT5786-A (-86 °C), Hettich, Germany
DNA extraction	Maxwell® 16 System, Promega, USA
Documentation System	GelDoc Documentation System, BIO-RAD, USA
Electrophoretic Equipment	EC250-90 Compact Power Supply, Thermo Scientific, USA
	Mini Sub Cell GT, BIO-RAD, USA
Falcon Tubes	EasyOpen 50-ml Centrifuge Tubes, JETBIOFIL, USA
Fly Pad	Fly Stuff, USA
Fly vials and closures	Fly Stuff, USA
Foot Valve	Fly Stuff, USA
Heat Block	Grant, UK

Table 3.8. Equipment used (cont.).

Equipment	**Model/ Company**
Hood	IP44/I, Wesemann, Germany
	Biosafety Cabinet Class II, Tezsan, Turkey
Incubator (18 °C)	WTW TS 606-G/4-i, Carl Stuart Limited, Ireland
Light Source	S2030, Pripor Scientifc Instruments, England, UK
Magnetic Plate	Agencourt SPRIPlate 96R ring magnetic plate, Beckman Coulter Genomics, USA
Microcentrifuge Tubes	1.5-ml Boil-Proof Microtubes, Axygen, USA
	0.5-ml Thin Wall Flat Cap PCR tubes, Axygen, USA
	8-tube strips, Axygen, USA
Microwave	Arçelik, Turkey
Micropipettes	Pipet-lite SL10, 20, 100,1000, RAININ, USA
	FINNPIPETTE 0,5-10 µl, 1-10 µl, 10-100 µl, Thermo Scientific, USA
Microscopes	S8APO, Leica, Germany
	S251, Olympus, Japan
Nanodrop	ND-2000c, Thermo Scientific, USA
Pestle	Kontes, pellet peslte ® cordless motor, Thermo Fisher Scientific, USA
	pellet pestle ®, disposible, Sigma-Aldrich, USA

Table 3.8. Equipment used (cont.).

Equipment	**Model/ Company**
Thermocyclers	TC-512, Techne, UK
	Techgene, Techne, UK
	RoboCycler 40 PCR Machine, Stratagene, USA
	LightCycler ® Nano Real-Time PCR System, Roche, Germany
Tips	1000 µl, 200 µl, 100 µl, 10 µl, Universal Fit Filter Tips, Axygen, USA
Tweezer	M5S, Fine Scientific Tools, Switzerland
Vortex	Fisons WhirliMixer, UK
Water Purification	Arium® 611UV Ultrapure Water System, Sartorius, Germany

4. METHODS

Methods compiled in this study are explained in this section.

4.1. Gene Targeting via Homologous Recombination

Homologous recombination (HR) was used to introduce the targeting vectors carrying the desired point mutations (H48R, G37R, G85R and H71Y) on *dSOD* gene. Construction of the targeting vector was done in collaboration with Reenan Lab at Brown University (Deniz, M.Sc. Thesis, Bogazici University, 2011). After the injection of each targeting vector commercially into *Drosophila* embryos via the use of P-element transposition, the randomly integrated transgenic sequence is targeted to its endogenous location through several fly crosses by the activation of FLP/FRT and Cre/LoxP systems (Figure 4.1) (Staber *et al.*, 2011). The vector carrying the selected point mutations, also contained a red eye color gene (*white+*) to be used as selective marker on the white eye background of the transgenic fly.

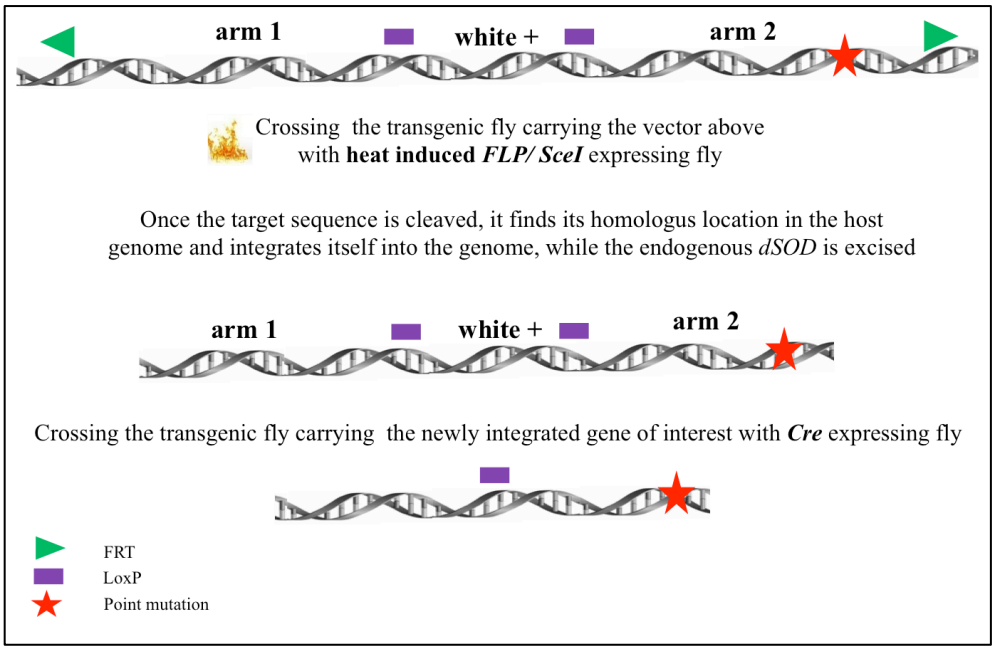

Figure 4.1. HR Scheme.

4.2. Validation of Mutants via PCR

All mutants were subjected to conventional PCR in order to make sure that targeted mutagenesis has occurred properly. This PCR aims to observe the existence of specific point mutations at the desired position for each line.

4.2.1. Genomic DNA Isolation

DNA was isolated from a single fly using Maxwell ® 16 Tissue DNA purification kit, Promega, USA according to manufacturer's protocol. The DNA was eluted in 300 µl elution buffer supplied by the manufacturer.

4.2.2. Validation PCR

PCR primers used for validation were designed to locate the desired mutation and are produce products around 50 base pairs. Reagents used and PCR conditions are listed in the Tables 4.1 and 4.2.

Table 4.1. Validation PCR reagents.

Reagent	Volume (µl)	[Stock]	[Final]
Buffer	10	5X	1X
dNTP	1	10 mM	0.2 mM
Forward Primer	2.5	10 µM	0.5 µM
Reverse Primer	2.5	10 µM	0.5 µM
Phusion Taq	0.25	2U/µl	0.01U/µl

Table 4.2. Validation PCR conditions.

Process	Temperature (°C)	Duration	# of cycles
Initial denaturation	98	2 minutes	1
Denaturation	98	45 seconds	40
Annealing	60	45 seconds	
Extension	72	1 minutes	
Final extension	60	2 minutes	1
	72	7 minutes	1

4.2.3. Visualization and Purification of PCR Products

PCR products were analyzed on a 0.8% agarose gel (0.8 g of agarose dissolved in 100 ml 0.5X TBE buffer), For the visualization of the PCR products under UV light, intercalating dye EtBr was used (final concentration: 0.5 µg/ml). After the addition of EtBr to the dissolved agarose, the mixture was poured to a gel tray with multiple-welled combs and left for polymerization to obtain a homogenous, solid agarose gel.

The total PCR product of 50 µl was mixed with 6X loading dye to a final concentration of 1X. The polymerized gel was placed into an electrophoresis tank containing 0.5X TBE. The DNA samples were loaded into the wells, the gel was run at 80 V for around an hour, visualized under UV light and documented. The desired DNA bands were cut out using a razor blade. The products were purified from agarose with Promega Wizard® SV Gel and the PCR Clean-Up Kit, according to the manufacturer's protocol.

4.2.4. Sequencing

Sequencing of the PCR products was performed using the Applied Biosystems, BigDye ® Terminator v3.1 Cycle Sequencing Kit (Tables 4.3 and 4.4). 100 ng DNA, purified by gel extraction, was used in the sequencing reaction, along with the SODseq3.5.1 as the sequencing primer for all mutants.

Table 4.3. Sequencing reagents.

Reagent	Volume (µl)	[Stock]	[Final]
Big Dye Buffer	2	10X	1X
Primer	2	10µM	1µM
Big Dye	2	-	-

Table 4.4. Sequencing reaction conditions.

Process	Temperature (°C)	Duration	# of cycles
Hot start	94	2 minutes	1
Denaturation	96	10 seconds	25
Annealing	50	5 seconds	
Extension	60	4 minutes	

After the completion of the sequencing reaction, products were subjected to sequence clean up using CleanSeq magnetic beads.

- 6 µl of CleanSeq magnetic beads was added to each sequencing reaction.
- 80 µl of freshly prepared ethanol (80%) was added to the reaction tube and mixed by pipetting.

- The reaction tubes were placed on the Agencourt SPRIPlate 96R-Ring Magnet Plate and incubated for 5 minutes in order to pull down the magnetic beads in the solution. After the incubation, ethanol was removed using a pipette.
- 150 µl of 80% ethanol was added to the tubes and mixed by pipetting. Samples were placed on the magnetic plate this time for 2 minutes.
- Ethanol was again removed and the tubes were kept under the hood for air-drying for 10 minutes.
- 40 µl of water was added to tubes and vortexed until the magnetic beads were suspended back in the solution.
- The tubes were placed on the magnetic plate and incubated for 5 minutes.
- For each tube, 20-µl aliquot was taken and transferred to a clean strip tube for sequencing.

Sequencing analysis was performed at the sequencing facility of University of Wisconsin, Biotechnology Center.

4.3. Observing Genotypic Ratios of Mutant Lines

All validated mutants carry the mutant allele on one of their chromosomes and the wild-type allele on the other chromosome, which also carried the TM3 balancer. In order to observe the genotypic ratios of mutant lines plus the loxP control, heterozygous flies carrying the TM3 balancer on their third chromosome (loxP/TM3, G37R/TM3, H48R/TM3, H71Y/TM3-GFP and G85R/TM3-GFP) were crossed to each other. Since flies carrying the balancer chromosome homozygously will be lethal, this advantage of the balancer chromosomes in *Drosophila* was used to avoid any third genotype in the progeny, other than homozygous (mut/mut) or heterozygous (mut/TM3) mutation carriers.

25 males and females from each line were put in each vial and the genotypes of the progeny were counted by the help of the phenotypic characteristic of the TM3 balancer, which results in short bristle phenotype on the back of the fly. Flies with short

bristles were counted as heterozygotes, and the flies with wild-type bristles were counted as homozygotes, since they were free of the balancer chromosome. Homozygous progeny from these crosses was also crossed with each other in order to observe if all genotypes were fertile.

4.4. Generating Accurate Heterozygote Mutants

To generate accurate models for the disease and to obtain reliable results from all experiments, it is important to have a control animal that has passed all the processes of HR, but does not carry the disease causing point mutations on the *dSod* gene. Our $SOD^{loxP/loxP}$ flies have passed through homologous recombination using an empty vector and carry the same loxP sequence with the mutant flies in their genome, but they are free of mutations. In this thesis, $SOD^{loxP/loxP}$ flies are used both as controls and also to obtain accurate heterozygous flies, e.g., $SOD^{mut/loxP}$.

Validated genotypes loxP/TM3, G37R/TM3, H48R/TM3, H71Y/TM3-GFP and G85R/TM3-GFP were first crossed to themselves in order to obtain homozygote progeny (Figure 4.2). As the outcome of the mut/TM3 X mut/TM3 cross, the progeny had one of the three genotypes: mut/mut, mut/TM3 and TM3/TM3. Since TM3/TM3 is lethal due to the specific property of balancer chromosomes, only the remaining two genotypes were viable. The flies with the mut/mut genotype will not carry the red eye marker (+*white*), that was excised during HR, so they will be white-eyed, where mut/TM3 flies will have the red eye color due to the dominant second allele with wild-type chromosome. By collecting white-eyed flies from these crosses we obtained homozygote mutant flies for the desired mutations.

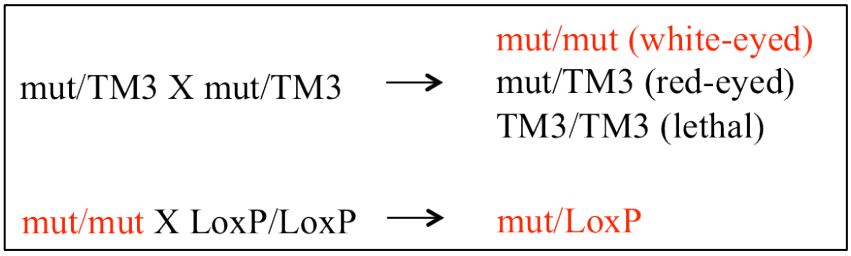

Figure 4.2. Generating Accurate Heterozygote Mutants.

In the second step we aimed to obtain accurate heterozygous mutants ($SOD^{mut/loxP}$) using $SOD^{loxP/loxP}$ homozygotes. $SOD^{loxP/loxP}$ female virgins were collected and for H48R and G37R mutations $SOD^{mut/mut}$ males were crossed to $SOD^{loxP/loxP}$ female virgins and so all the progeny had the $SOD^{mut/loxP}$ genotype (Figure 4.2). For G85R and H71Y mutations female $SOD^{loxP/loxP}$ virgins were crossed with $SOD^{mut/TM3\text{-}GFP}$ males. The white-eyed progeny had the $SOD^{mut/loxP}$ genotype, since both the mut and the loxP alleles do not carry the red eye marker (+*white*). All further experiments were performed on flies obtained from these crosses.

4.5. Life Span Analyses

Life span analyses were performed based on the same experimental set-up with the genotypic ratio observations as each vials contained 25 flies of each sex. Life span analyses were started with newborn flies. Flies in each vial were counted everyday and the number of viable flies of each sex was noted. In order to avoid any death due to bad circumstances in vials (e.g. sticky food), flies were transferred to new vials with fresh food every two days. During the whole analysis fly stocks were kept at 24°C and they were subjected to 12 hours of day light and 12 hours of darkness. The counting process was continued until no viable fly existed in each vial.

4.6. Larval Motility Analyses

Larval motility analysis was performed to observe any possible locomotion defects that occur in the larval stage as an outcome of the mutations introduced. At least 50 L3 stage larvae (third and last larval stage before becoming a pupa) from each genotype were used in this experiment. The number of gridlines spanned by the larva in two minutes was counted under a dissection microscope.

In the experimental set-up, a 2% agarose gel plate was used as a platform for the larva. All larvae subjected to the experiment were washed with PBS in order to avoid any

chemo-attractant that would render the results. For each larva one minute resting time was given prior to two minutes of counting.

4.7. Quantitative PCR (qPCR) Analyses

4.7.1. Collecting Mutant Flies

Only male flies were subjected to qPCR analyses. All flies were collected at the same developmental stage. Since flies that are homozygous for the G85R mutation are lethal as adults, all flies are collected right after eclosing the pupa, before they become complete adults. Three sets from each genotype were collected using Eppendorf tubes, and each set contained 30 flies. Flies were frozen at -80°C in order to sustain tissue integrity.

4.7.2. RNA Isolation

Total RNA isolation for qPCR analysis was performed using 30 flies for each RNA sample. Three sets of RNAs for each genotype were isolated to perform the analyses.

- Heads of 30 flies were dissected using a tweezer and placed in Eppendorf tubes containing 100 µl Trizol. Heads were grinded by using a motorized pestle.
- After grinding, 400 µl Trizol was added to each tube, to add up to the final volume of 500 µl, and the tubes were kept at room temperature for 5 minutes.
- 200 µl chloroform was added to each Eppendorf tube, and the tubes were vortexed for 3-5 seconds.
- Samples were kept at room temperature for 15 minutes, and then centrifuged at 12.000 g for 15 minutes at 4°C.
- Top aqueous phase of each sample was transferred to a new Eppendorf tube.

- 500 µl isopropanol was added to each aqueous phase and mixed by inverting the tubes for several times.
- The samples were stored at room temperature for 10 minutes and then centrifuged at 12.000 g for 8 minutes at 4°C.
- The aqueous phase in each sample was discarded and 1 ml 75% EtOH was added onto the RNA pellets.
- Samples were again centrifuged at 7.500 g for 5 minutes at 4°C.
- Previous two steps were repeated twice.
- Finally, the pellets were left for air-drying in the hood for 10 minutes and dissolved in 50 µl distilled H_20.

The concentrations of RNA samples were measured using a nanodrop instrument, and aliquotes of 100 ng/µl RNA were prepared for each sample. Both the stocks and the aliquots were stored at -80°C until the next step to protect the nucleic acid integrity.

4.7.3. cDNA Synthesis

Construction of cDNA from total RNA by reverse transcription was performed using Transcriptor High Fidelity cDNA Synthesis Kit. After the reaction mixture was prepared, it was incubated at 42°C for 2 hours using a thermocycler (Table 4.5). 10 µl of the isolated RNA (100 ng/µl) was mixed with required reagents for the reverse transcription reaction. A primer mix was prepared using 10 µl from 50 µM oligodT stock and 3 µl from 600 µM hexamer stock. After the reaction was complete, the total volume of 20 µl was increased to 100 µl in order to dilute the cDNA sample.

Table 4.5. cDNA synthesis reaction reagents.

Reagent	Volume (µl)	[Stock]	[Final]
Buffer	4	5X	1X
DTT	2	0.1M	10mM
dNTP	1	10mM each	-
Reverse Transcriptase	1	-	-
Rnase Inhibitor	0.5	2000 units	100 units
Primer mix	0.5	-	-

4.7.4. qPCR

To compare any difference between the gene expressions of mutants, mRNA levels of *dSod* and *dAdar* genes were measured using qPCR. The *Drosophila Gapdh1* (*dGapdh1*) gene was used as the reference/housekeeping gene in the analysis. FastStart Essential DNA Green Master Kit was applied according to the manufacturer's protocol. As the starting material, 2 µl of cDNA was added to the reaction mixture, and the reaction was run using the LightCycler ® Nano Real-Time PCR System.

4.7.5. Data Analysis

In qPCR analyses, a cycle value (ct) for each sample was obtained, which indicates the cycle number, at which the system has started to detect the fluorescence of the amplified product. For data analysis, CopyColor software (Applied Biosystems Inc., USA) was used which operates according to the ΔΔct principle. This principle compares the ct values of the target gene and the reference/housekeeping gene. In this way, the normalized expression of the target gene for each sample was calculated. Three sets of

normalized expression data were subjected to two-tailed *Student's t*-test in order to observe the differences in gene expression levels of mutants and controls.

5. RESULTS

5.1. Validation of Mutant Lines

Four mutations (G37R, H48R, H71Y and G85R) were integrated into the endogenous locus of *dSOD* via homologous recombination (Deniz, M.Sc. Thesis, Bogazici University, 2011). After all HR steps were completed, mutant lines were subjected to PCR and the specific locations of the point mutations were detected (Figure 5.1). As summarized in Section 4.1, mutants that undergo HR, carry the 76bp loxP sequence in the intronic region of *dSOD,* which does not interfere with the expression of the gene and protein sequences. In order to confirm this, the control line LoxP, was also generated and used in all experiments as the accurate experimental control line.

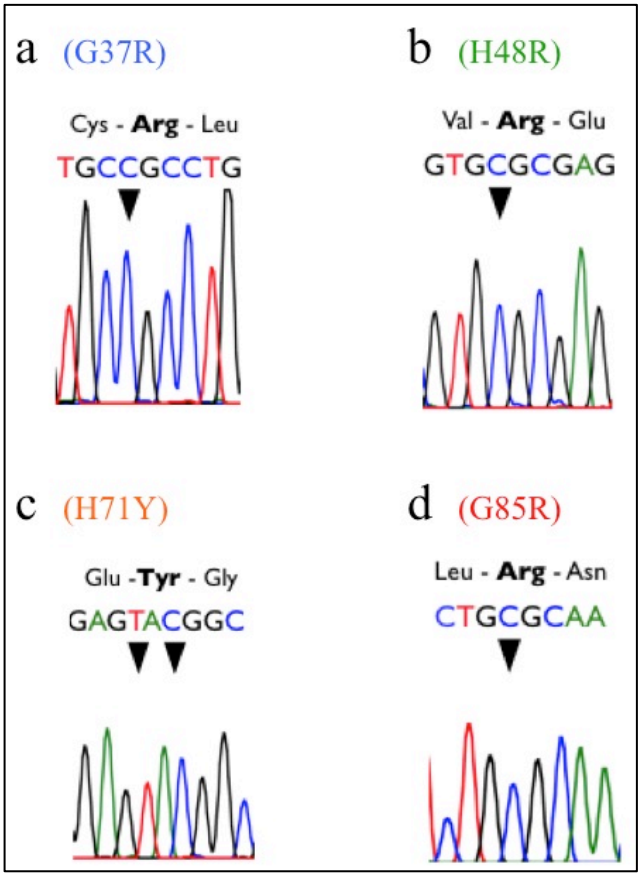

Figure 5.1. Validation of Mutant Lines.

5.2. Phenotypic Properties of Mutant Lines

The phenotypic characteristics of the mutant lines showed variability (Figure 5.2). All mutants were white-eyed as expected, since they did not carry the (*white*+) gene. Canton S. flies that were used in experiments as the wild-type line are red-eyed flies, as seen in Figure 5.2a. LoxP flies had the same phenotypic properties with the Canton S. wild-type flies (e.g. wing structure).

When the homozygote mutant lines were compared with the LoxP control, it was observed that the homozygote lines carrying the G37R and H48R mutations were not different than the control line (Figure 5.2b). H71Y mutants differed from G37R and H48R homozygotes in terms of wing structure. The cricked wing phenotype was present in H71Y homozygotes, for some individuals it was observed that one wing almost lost its function (Figure 5.2c).

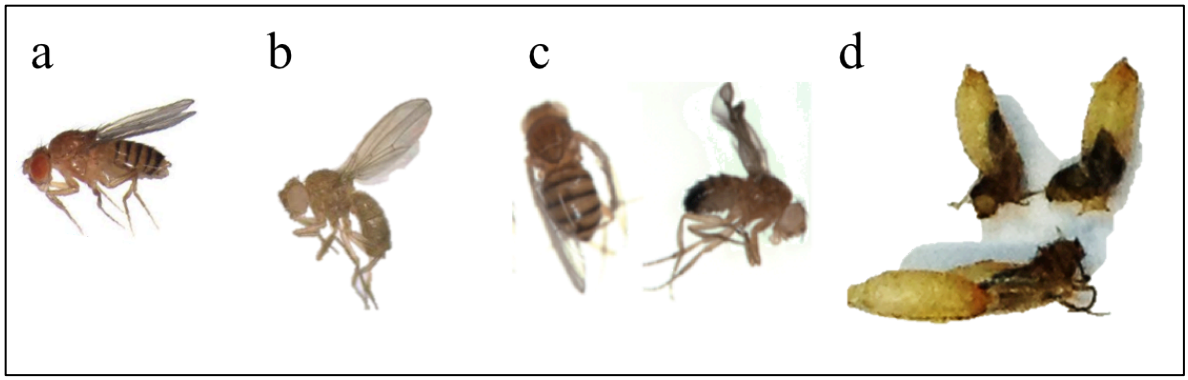

Figure 5.2. Phenotypic characteristics of control and mutant lines. (a) Canton S. (b) $dSOD^{LoxP/LoxP}$, $dSOD^{G37R/G37R}$, $dSOD^{H48R/H48R}$, $dSOD^{G37R/LoxP}$, $dSOD^{H48R/LoxP}$, $dSOD^{H71Y/LoxP}$, $dSOD^{G85R/LoxP}$ (c) $dSOD^{H71Y/H71Y}$ (d) $dSOD^{G85R/G85R}$.

Flies carrying the G85R mutation in homozygous form were not viable as adult flies. A distinct phenotype was observed for these mutants, with which the flies eventually died, while eclosing the pupa or sometimes short after coming out (Figure 5.2d).

All genotypes carrying a mutation on one allele and the loxP sequence on the other allele, were phenotypically normal and shared the characteristics of LoxP homozygotes (Figure 5.2b).

5.3. Genotypic Ratios of Mutant Lines

The fitness of an organism describes its ability to survive and reproduce and depends on a genotype or a phenotype in a certain environment. For example, frequency changes of the alleles over generations may result in total loss of a genotype. Based on this information, genotypic ratios of the mutants, generated in this project, were observed, to see if the mutations have an effect on organismal fitness.

Table 5.1. Genotypic ratios of mutant lines.

Mutation	Homozygote (%)	Heterozygote (%)
LoxP	31	69
G37R	32	68
H48R	39	61
H71Y	11	89
G85R	0	100

Heterozygote flies carrying the TM3 balancer were crossed to each other and the genotypes of the progeny were observed using the phenotypic properties of the TM3 balancer. In a normal heterozygous cross, we would expect to observe a ratio of 1:2:1 (hom:het:hom); however, since flies carrying the balancer homozygously will be lethal, the expected ratio of this experimental process was 1:2:0.

The results revealed a ratio of 1:2.2 for LoxP, 1:2.1 for G37R, 1:1.6 for H48R, 1:8.2 for H71Y lines, and no homozygous progeny was observed for the G85R line

(Table 5.1). The LoxP line, being the experimental control, was consistent with the expected Mendelian ratio. G37R and H48R lines resembled the control line in terms of genotypic ratios, whereas H71Y and G85R lines showed low organismal fitness.

5.4. Life Span Analyses

The results of life span analyses are represented below (Figure 5.3). Flies carrying the G37R and H48R mutation both in homozygous and heterozygous forms had similar survivorship curves with the LoxP/LoxP control. This similarity was also valid for flies that were heterozygous for the H71Y and G85R mutations.

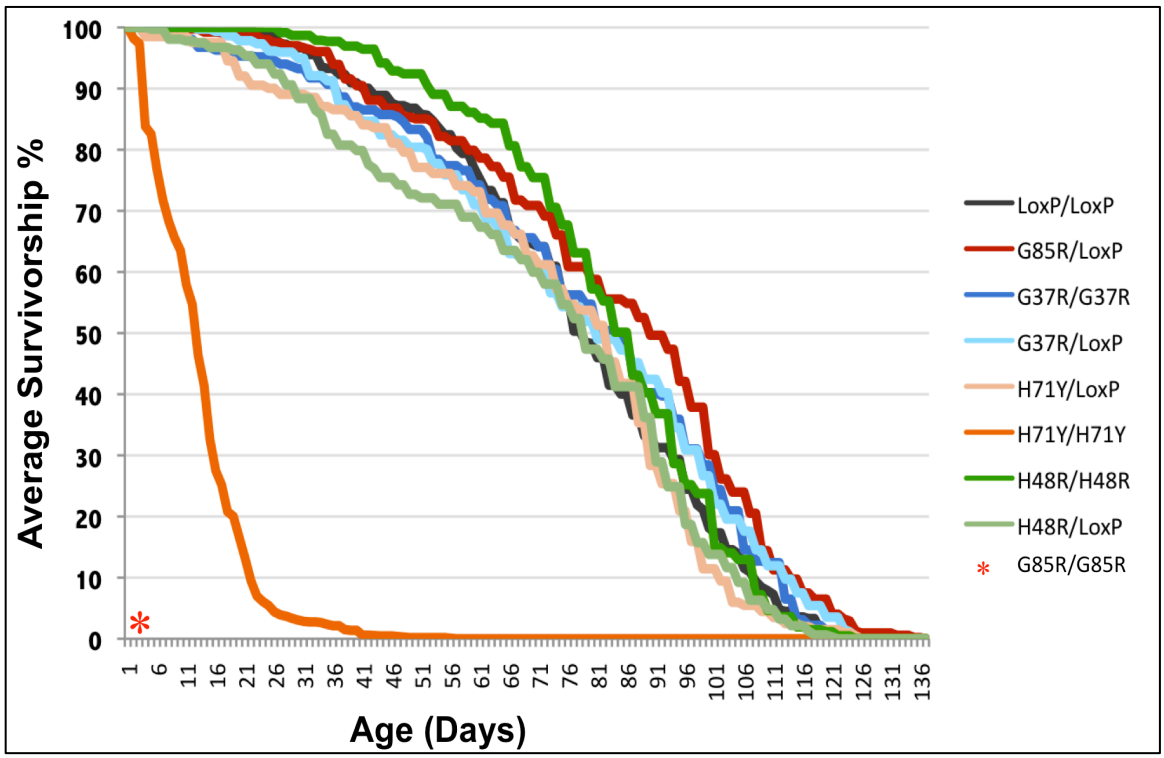

Figure 5.3. Life Span Analyses. Each geneotype is represented with a colored line, except the G85R homozygote line, denoted with an asterix (*), as an indication of its lethality.

The H71Y/H71Y line, which also showed low organismal fitness (1:8.2), displayed a severe phenotype in the life span analysis. The homozygous line carrying the

H71Y mutation represented decreased life span. Only ten per cent of the flies were viable after 20 days and this number was even lowered at around day 40, where the average survivorship of other genotypes were 95% and 80% for days 20 and 40, respectively. The life span analysis was not applicable for the G85R/G85R line, since flies carrying the G85R mutation homozygously were not viable (Figure 5.2).

5.5. Larval Motility Analyses

Larval motility analyses were performed on L3 stage larvae, to observe whether the mutants resemble ALS phenotypes in terms of loss of/decrease in motor function. L3 larva, also known as, third instar larva, is the last larval stage in the *Drosophila* life cycle before the pupa is formed. Viability of all genotypes at this stage enabled us to detect differences between locomotion capabilities of the mutants, considering the lethality of the homozygous G85R mutation at the adult stage.

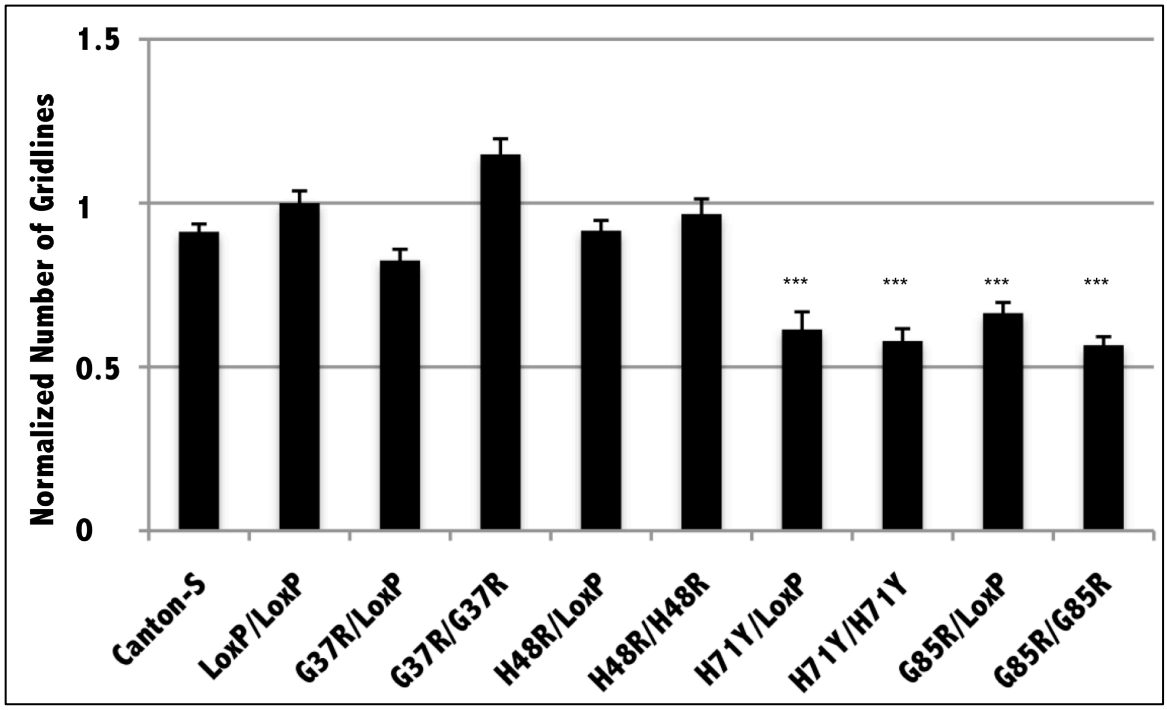

Figure 5.4. Larval Motility Analyses. The figure represents the normalized number of gridlines spanned by the L3 stage larvae for ten different genotypes. Experimental control line LoxP/LoxP was used for normalization.

The results of the larval motility analyses were found to be similar to the previous experimental outcomes for the homozygous mutations. The number of gridlines spanned by the *dSOD*$^{G85R/G85R}$ and the *dSOD*$^{H71Y/H71Y}$ larvae was significantly less than the Canton S., *dSOD*$^{LoxP/LoxP}$, *dSOD*$^{G37R/G37R}$ and *dSOD*$^{H48R/H48R}$ larvae, which gave consistent results (Figure 5.4).

In contrast to life span analyses, in larval motility analyses, heterozygote carriers of the H71Y and G85R mutations were not capable of tolerating the mutations. The *dSOD*$^{G37R/LoxP}$ and *dSOD*$^{H48R/LoxP}$ heterozygote lines once again showed no difference as compared to the wild-type and the experimental control, whereas the *dSOD*$^{H71Y/LoxP}$ and the *dSOD*$^{G85R/LoxP}$, even as heterozygote mutation carriers, presented with significantly low ability to crawl.

5.6. qPCR Analyses

5.6.1. Relative quantification of *dSOD* mRNA levels

qPCR analyses for *dSOD* were performed to observe the proper expression of the *dSOD* mRNA in HR mutants. The *dSOD* mRNA levels of the wild-type line (Canton S.), the experimental control line (LoxP) and the homozygous mutant lines were compared using *dGapdh1* as the reference gene.

To observe the fold differences between the mutants and the controls, normalized expressions were further normalized to Canton S and LoxP lines separately. The results revealed a significant difference between the two controls used in this study (p-value 0.01). The *dSOD* level of the H48R homozygous line was significantly higher when compared to both Canton S and the LoxP controls (p-values 0.02 and 0,03, respectively) (Figure 5.5a and 5.5b). The *dSOD*$^{H71Y/H71Y}$, on the other hand, represented significantly low levels of *dSOD1* mRNA, as compared to Canton S. and the LoxP controls (p-values 0.0001 and 0.001, respectively).

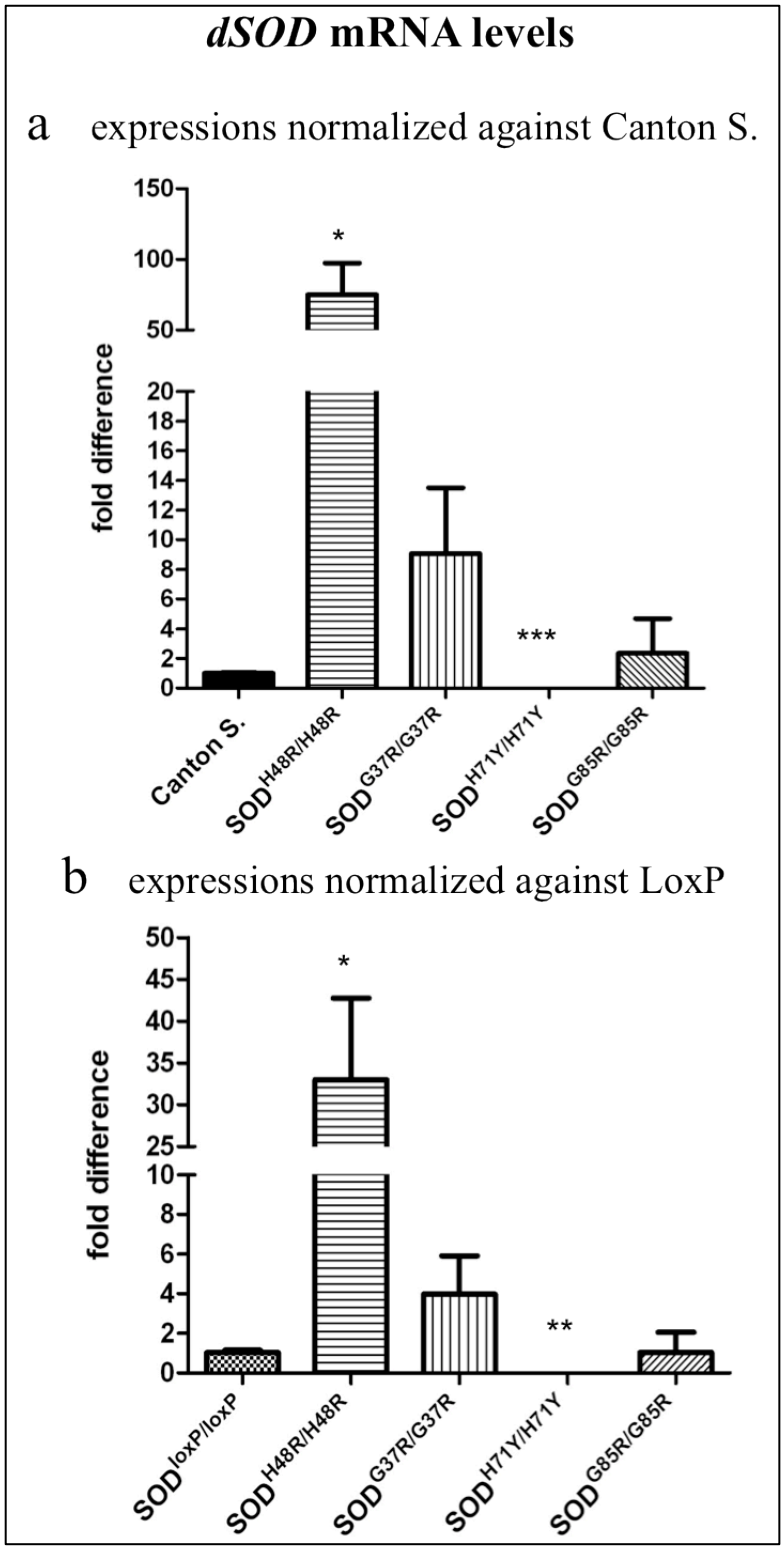

Figure 5.5. *dSOD1* mRNA levels. (a) values normalized against wild-type control Canton S. (b) values normalized against experimental control LoxP.

5.6.2. Relative quantification of *dADAR* mRNA levels

Previously, it was found that the RNA editing enzyme adenosine deaminase acting on RNA 2 (ADAR2), acting on the glutamine/arginine (Q/R) site of the GluA2 pre-mRNA, is downregulated in motor neurons of ALS cases. Inefficient RNA editing of GluA2, a subunit of the L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor resulting in motor neurons, expressing Q/R site-unedited GluA2, was shown to undergo slow death in conditional *ADAR2* knockout mice (Hideyama *et al.*, 2012).

In this study, it was aimed to compare the mRNA levels of *Drosophila* ADAR (*dADAR*) between the wild-type, experimental control and mutant lines. *dGapdh1* was again used to calculate the normalized expression and then these values were normalized separately to the Canton S and LoxP lines. The LoxP control showed lower levels of *dADAR* expression as compared to Canton S line (p-value 0.02).

The analyses revealed that $dSOD^{H48R/H48R}$ and $dSOD^{G37R/G37R}$ lines expressed increased levels of *dADAR* mRNA compared to the wild-type (Canton S) and to the experimental control LoxP (Figure 5.6a and 5.6b). For the H48R homozygotes, p-values were measured as 0.02 and 0.004, when compared to Canton S. and LoxP, respectively. The p-values for G37R mutant were the same being 0.002, when normalized to both controls .

$dSOD^{H71Y/H71Y}$ mutant represented significantly low levels of *dADAR* mRNA, as the result of normalization against both controls, p-values being 0.007, when compared to Canton S. and 0.002, when compared to LoxP lines. In contrast, no significant difference was observed between the expression level of $dSOD^{G85R/G85R}$ and both controls.

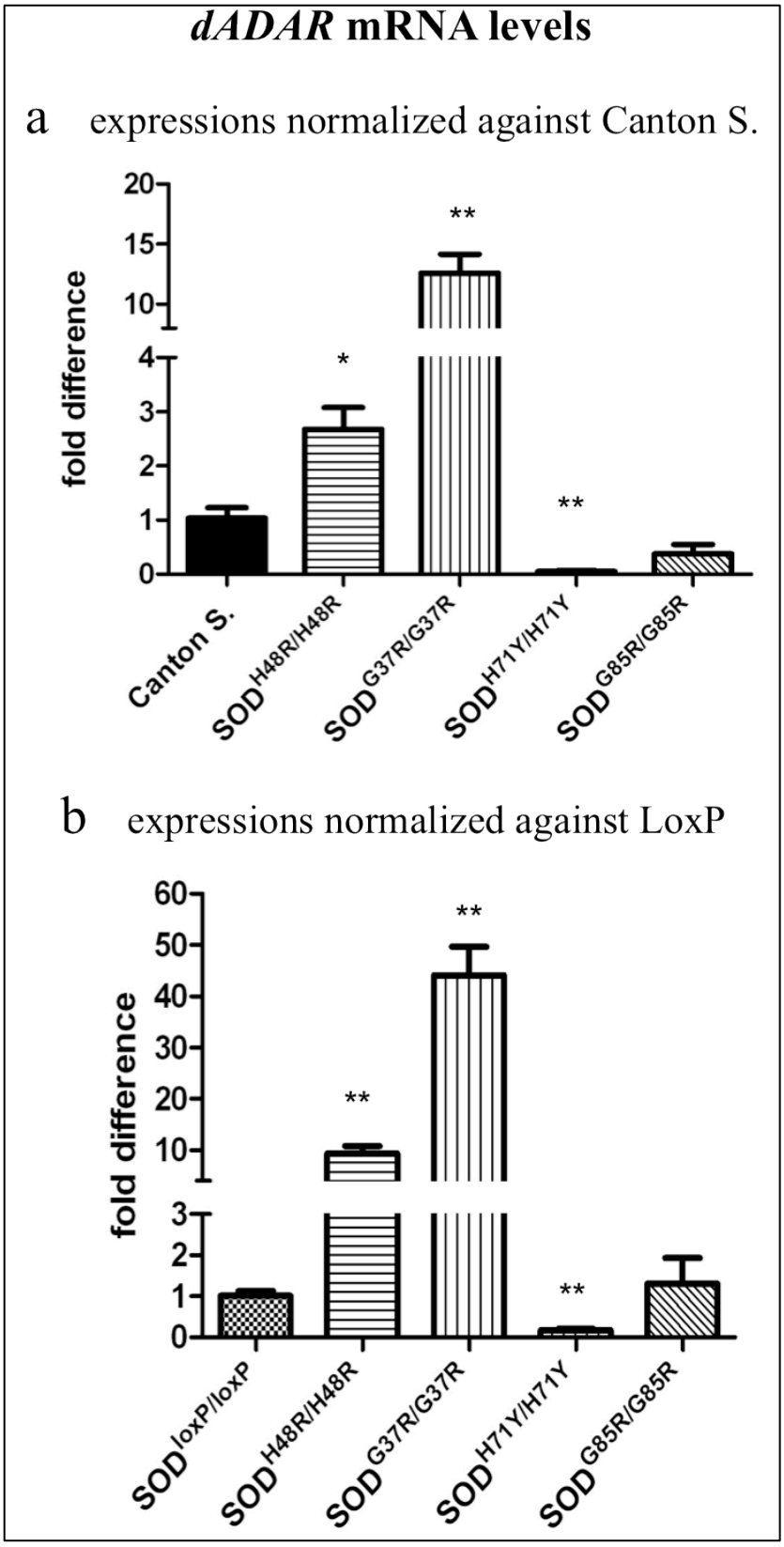

Figure 5.6. *dADAR* mRNA levels. (a) normalized against wild-type control Canton S. (b) normalized against experimental control LoxP.

6. DISCUSSION

Animal model studies targeting diseases are either KO studies, that give insights into the loss-of function (LOF) hypothesis, or overexpression studies that trigger disease phenotypes by overexpressing human protein in the endogenous background. In diseases that occur by the LOF hypothesis, KO or RNA silencing methods are advantageous to mimic the environment, in which the protein function is lost. However, for gain-of toxic function (GOF) diseases, although overexpression studies give insights into disease pathogenesis, the elevated protein levels may also result in false positive phenotypes.

6.1. Different Approaches Targeting ALS

In ALS, known to be a GOF disease, there are several animal models that target the disease from every aspect. For SOD1-based ALS, KO models give clues about the effect of the loss of endogenous enzymatic activity on disease progression, and GOF models investigate the contribution of mechanisms to the disease process. KO models did not show ALS phenotypes; instead, susceptibility to oxidative stress was induced in animals, reducing the fertility and life span. This way, GOF hypothesis behind ALS pathogenesis was confirmed. With GOF models, several other mechanisms playing a role in the disease pathogenesis were discovered as explained earlier in detail (Section 1.3).

Although, the models generated so far, were useful in several aspects, the term ‘accurate’ does not refer to them, since the wild-type endogenous background and the altered protein expression levels contribute to the formation of phenotypes, which usually do not fully resemble ALS. In this work, by generating a knock-in model of SOD1-based disease, using homologous recombination, we aimed to generate an accurate model of SOD1-based ALS and to observe more reliable phenotypes, not dependent on the overexpression of the mutated allele or the presence of the endogenous wild-type copy of SOD1 with full enzymatic activity.

6.2. Selected Mutants and Validation of HR

Validation of four integrated ALS-causing missense mutations into the endogenous locus of *Drosophila* SOD1 (*dSOD)* and the characterization of the mutants was the main goal of this study. Furthermore, molecular analyses were initiated to observe the expression differences of *dSOD* and *dADAR* genes.

The four missense mutations selected for the HR process in this thesis were G37R, H48R, H71Y and G85R. G37 and G85 are wild-type-like mutants, since they do not affect the binding of Cu or Zn ions, whereas H48 and H71, located at the Cu and Zn binding regions of the SOD1 gene, respectively, are classified as metal-binding-region mutants.

The G37R mutation results in reduced mean age of onset in ALS patients, with a broad range in survival time. On the other hand, the G85R mutation is known to delay the disease onset, however resulting in much severe phenotypes and a fast disease progression. The glycine to arginine substitution in general, does not result in change of the hydrophilic nature of the residue, however the amino acid becomes larger in size. The more severe phenotypes caused by the G85R mutation on the other hand, may also be explained by the inefficiency of the dimerization of the SOD1 protein in the presence of this mutation.

The histidine to arginine change at residue 48 results in the loss of the imidazole functional group of histidine, which is known to coordinate ligand binding in metalloproteins and make up the part of the catalytic site in enzymes. Although it is known that most of the *SOD1* mutations do not result in loss of enzymatic activity and the disease is based on GOF, the loss of Cu binding property may still play a role in disease pathogenesis.

H71Y is an uncharacterized novel mutation, recently defined in our lab. The mutation was identified in heterozygous form in a young fALS patient, with an early age

of onset of 19. In the patient, a very fast disease progression with a survival of one year was observed. Prediction tools like PolyPhen-2, SIFT and PROVEAN classify this change in a highly conserved position of the protein as deleterious (Özoğuz et al., paper in preperation). This mutation however, represented incomplete penetrance, since, the father, currently 50 years-old, carries the same mutation without manifesting the disease. In this perspective, this work was particularly important for the characterization of this mutation.

Integration of all four selected ALS-causing missense mutations was successfully validated by conventional PCR. It was shown that the mutants that passed through the HR process carry only the 76 bp foreign sequences in the intronic region of *dSOD* between its two exons. In mutant flies, no other sequence was altered, except the desired point mutations that resulted in the disease-causing amino acid changes. Also the generation of the experimental control line was accomplished, carrying only the 76 bp LoxP sequence, as the result of the injection of the empty vector, which does not contain any disease-causing mutation.

6.3. Properties of Characterized Mutants

The main goal of this study was the characterization of the generated mutants was, in order to show that our mutant flies phenocopy ALS and recapitulate different phenotypes displayed by the different mutations. To be able to achieve this goal, mutant flies were investigated both physically and behaviorally. For each mutant line the results were consistent within the mutation group itself, which enabled a reliable experimental approach.

All flies carrying the G37R and H48R mutations, both heterozygously and/or homozygously, plus the experimental control line, carrying the LoxP sequence homozygously, showed the same phenotypes with the wild-type control Canton S (Section 5.2). These flies had normal wings and their mobility seemed normal both in newborns and adults.

The cricked-wing phenotype observed in H71Y homozygotes is an indication of a severe phenotype. It is yet to be investigated, whether it is a developmental defect or a paralysis phenotype. In addition, the infertility of the H71Y homozygotes was suspected to be emanating from the male flies, since we have observed sexual interaction towards female flies and unfertilized eggs on the fly food. Further studies will help to understand the problem behind the infertility of these mutants. The *dSOD*$^{H71Y/H71Y}$ line represented severe phenotypes in all experiments performed within this study, in accordance with the severe, fast progressive phenotype of our male patient. The low organismal fitness ratio of 1:8.2, showed the decreased viability of the flies carrying this homozygous mutation.

Again in life span assays, it was observed that, about ten per cent of the mutants survived after 20 days. In larval motility analysis, not only the homozygotes, but also the flies carrying the H71Y mutation heterozygously, represented decreased ability to crawl a longer path, when compared to the wild-type Canton S. and experimental control LoxP lines.

Flies carrying the G85R mutation displayed the most severe phenotypes among all mutants, both visibly and in behavioral assays. The *dSOD*$^{G85R/G85R}$ flies were lethal as adult flies and they failed to come out of the pupa. These mutants presented a distinct phenotype, which was unexpected: the flies died when trying to come out. It was possible to observe them viable for about half an hour with their heads out or sometimes even their legs came out of the pupa, however they were unable to come out completely as adult flies. The fact that heterozygote flies, carrying the G85R mutation on one allele and the LoxP sequence on the other allele, were viable indicates that one allele without the G85R mutation is capable of compensating for this lethality.

Like the H71Y heterozygotes, G85R heterozygotes also showed decreased motor function in the larval motility assay. Larval motility assay was very crucial to observe the differences between motor functions of the mutants, since the homozygous carriers of the G85R mutation are lethal. Thus, climbing assays would not be sufficient to compare all genotypes. On the other hand, ALS is an adult onset disease, and based on this, in the

future more assays regarding the motor functions of adult flies could be designed using the viable genotypes.

Combination of organismal fitness calculations and behavioral assays revealed severe phenotypes for the H71Y and G85R mutations. The observed results were correlated with literature, where the G37R and the H48R mutants were reported to have milder phenotypes, as compared to G85R, which represents late disease onset, but fast progression. The uncharacterized H71Y mutation, that showed very fast disease progression in our male patient, also caused severe phenotypes in the *Drosophila* model of the disease. However, the patient carried the mutation heterozygously, while our H71Y heterozygotes did not show as severe phenotypes as the homozygotes.

6.4. Gene Expressions of Mutant Flies

Generating an accurate model of a complex disease has to pass through several steps. After validation of the integrated mutations, it is important to confirm that the mutated gene is still functional. In this perspective, qPCR analyses were performed to observe the *dSOD* mRNA levels of the mutants and to compare these results with the control lines. Our results revealed that the H48R, G37R and G85R mutants expressed proper levels of *dSOD* mRNA, when compared to both CantonS wild-type and the experimental control line LoxP. The significantly high levels of *dSOD* mRNA observed in the H48R homozygotes may be due to the relatively high difference within the cq levels of three different sets of individuals of the same mutation. H71Y homozygotes displaying low levels of *dSOD* mRNA levels with high significance, when compared to both controls, should be further inspected with care. The low mRNA levels of H71Y homozygotes may explain the decreased life span of these mutants, as stated in the literature, due to oxidative stress caused in the absence of *dSOD* (Phillips *et al.*, 1989). Also, there may be an experimental error, which can be excluded/confirmed by repeating the qPCR analysis and performing Western Blot (WB) analyses on mutants. The ongoing WB analyses at Reenan Laboratory/Brown University, RI, USA, may explain the correlation between the *dSOD* mRNA and the protein levels of mutants.

qPCR analyses are also important to further investigate the differences of expression levels of several candidate genes involved in ALS pathogenesis. In this thesis, *dADAR* was chosen as a candidate gene. The fact that our collaborator is specifically interested in RNA editing, results regarding *dADAR* levels, may pave the ways to the understanding of possible role of RNA editing in ALS pathogenesis, in the future. Previously, it was observed in ALS patients that *ADAR2* levels were downregulated, however this finding was related to patients with dementia and TDP-43 pathology and not to SOD1-based ALS (Hideyama *et al.*, 2012). Our results displayed downregulation of *dADAR* only for the H71Y homozygotes with high significance. In contrast, G37R and H48R homozygotes gave contradictory results by showing elevated levels of *dADAR* mRNA and for G85R homozygotes the expression was not significantly different than the control lines. In conclusion, we also did not observe decreased *dADAR* expression in our *dSOD* mutants, however considering the results regarding the uncharacterized H71Y mutation, further experiments on RNA editing efficiencies of mutants is expected to reveal an accordance between *dADAR* levels and SOD1-based ALS.

6.5. Future Goals

Future experimental strategies will include measuring of the superoxide dismutase activity of the mutants, performing immunolocalization studies on dSOD in order to observe possible aggregations and observing the neuromuscular junction integrity by synaptic bouton count.

The superoxide dismutase activity will be measured using a colorimetric assay based on the conversion of WST-1 to WST-1 formazan, a colored product absorbing light at 450 nm. As a result, the relative SOD activity of the sample is calculated from percent inhibition of the rate of WST-1 formazan formation. This experiment will be useful to observe the activity of the mutated enzyme. Especially in metal binding mutants (H48R and H71Y), it will be possible to observe the functionality of the protein. Also for G85R mutants, it is important to see the level of enzymatic activity, since the inefficiency in dimerization was reported previously.

Immunolocatization studies will be useful to observe that the mutant dSOD is properly localized within the motor neurons and will show possible aggregations for dSOD and other proteins involved in ALS pathogenesis. In long-term studies, phenotypic consequences of double mutants can be observed, the synaptic transmission on the giant fiber of *Drosophila* can be measured by electrophysiological studies and suppressor mutation screening may be performed using methane sulphonate (EMS) induced mutagenesis.

This is the first study, which successfully implements homologous recombination, a new and powerful approach to ALS. We hope that genetically accurate models of SOD1-based ALS, generated via homologous recombination, will further help us gain insights into the complex mechanisms involved in neurodegenerative processes.

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