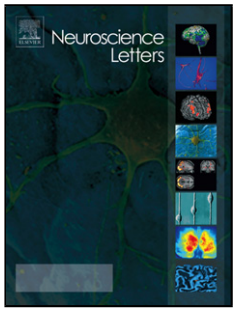

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Author: Xavier Gallart-Palau Chee-Hoe Ng Joan Ribera Siu Kwan Sze Kah-Leong Lim

PII: S0304-3940(16)30297-X
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2016.05.006
Reference: NSL 32025

To appear in: *Neuroscience Letters*

Received date: 4-10-2015
Revised date: 12-2-2016
Accepted date: 5-5-2016

Please cite this article as: Xavier Gallart-Palau, Chee-Hoe Ng, Joan Ribera, Siu Kwan Sze, Kah-Leong Lim, Drosophila expressing human SOD1 successfully recapitulates mitochondrial phenotypic features of familial Amyotrophic Lateral Sclerosis, Neuroscience Letters http://dx.doi.org/10.1016/j.neulet.2016.05.006

Drosophila expressing human SOD1 successfully recapitulates mitochondrial phenotypic features of familial Amyotrophic Lateral Sclerosis

Xavier Gallart-Palau [1,2,3] *, Chee-Hoe Ng [5,6], Joan Ribera [1,2], Siu Kwan Sze [3] and Kah-Leong Lim [4,5,6].

[1]Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida 25198 Lleida, Catalonia, Spain.

[2]Institut de Recerca Biomèdica de Lleida (IRBLLEIDA), 25198 Lleida, Catalonia, Spain.

[3]School of Biological Sciences, Nanyang Technological University, Singapore, 637551.

[4]Department of Physiology, National University of Singapore, Singapore 117543, Singapore.

[5]Department of Research, National Neuroscience Institute, Singapore 308433, Singapore.

[6]Duke-NUS Graduate Medical School, Singapore 169857, Singapore.

*** Correspondence:**

Xavier Gallart-Palau

Facultat de Medicina, IRBLLEIDA, Universitat de Lleida.

Dept. Medicina Experimental.

Av. Rovira Roure 80, 25198 Lleida, Catalonia, Spain.

Phone: +34-973-702427, Fax: +34-973-702426.

E-mail: xavgallart@gmail.com

Highlights

- Expression of SOD1^{G93A} in *Drosophila* thoracic muscles causes FALS phenotype.
- 24B-GAL4-SOD1^{G93A} flies show impaired motor behavior and decreased lifespan.
- 24B-GAL4-SOD1^{G93A} flies successful mimic mitochondrial dysfunction in FALS.
- 24B-GAL4-SOD1^{G93A} represents novel therapeutic opportunities in FALS.

Abstract

Mitochondrial pathology is a basic pathological hallmark of familial amyotrophic lateral sclerosis (FALS) extensively manifested by human patients and mutant SOD1^{G93A} mammalian models. Rodents expressing human FALS-associated mutations successfully mimic several human disease features although they are not as amenable to genetic and therapeutic compound screenings as non-mammalian models. In this study we report a newly generated and characterized *Drosophila* model that expresses human SOD1^{G93A} in muscle fibers. Presence of SOD1^{G93A} in thoracic muscles causes mitochondrial pathology and impairs normal motor behavior in these flies. Use of this new FALS-24B-SOD1^{G93A} fly model holds promise to provide further understanding of the mitochondrial affectation process and new discoveries of therapeutic compounds able to reverse mitochondrial dysfunction in FALS.

Keywords: FALS, SOD1^{G93A}, mitochondria, Drosophila.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating motor neuron disease that involves the progressive loss of motor neurons leading to paralysis and death. Although the etiology of ALS remains unclear, mitochondrial pathology has been found even at pre-symptomatic stages of ALS and is currently considered a converging point of multiple pathological pathways in that disease (Shi et al., 2010). From the apparition of familial amyotrophic lateral sclerosis (FALS) human SOD1 mutants in 1993 (Rosen et al., 1993) research in ALS has been almost exclusively conducted in FALS rodent models. The advantages regarding the use of these animals have been properly and extensively justified although its use dramatically limits the possibilities to perform extensive primary therapeutic compounds screenings in ALS. Prohibitive economic and ethical costs together with discouraging time periods prior to the manifestation of disease hallmarks are the classical disadvantages of rodents for that purpose (Lee et al., 2014).

On the contrary, the fly *Drosophila melanogaster* fulfils the empty space between in vitro models and mammal models for primary drug screenings in neurodegenerative diseases (Tickoo and Russell, 2002, Imai et al., 2012). Use of *Drosophila* implies extremely low costs of maintenance and handling, fast development of disease hallmarks and the possibility to test different forms of administration and toxic effects of promising therapeutic compounds (Matthews et al., 2005, Pandey and Nichols, 2011).

In this study we report a new fly model of ALS expressing the human FALS mutant $SOD1^{G93A}$ under the *24B-Gal4* muscle-specific promoter. Expression of $SOD1^{G93A}$ in thoracic muscles, the region of flies that contains higher presence of mitochondria and higher energetic expenses, shortens lifespan, impairs motor behavior and causes mitochondria dysfunctions in these animals. Characterization of $SOD1^{G93A}$-24B-Gal4

flies opens new venues for genetic and extensive primary drug screenings aimed to treat FALS-associated mitochondrial pathology

Material and Methods

Fly stocks

Gal4/Uas system was used for expression of our transgenes in *Drosophila* flies as previously described (Duffy, 2002).The fly line 24B-Gal4 used in our experiments was obtained from the Bloomington Stock Center (IN., U.S.A.). Flag-tagged wild-type human SOD1 and human FALS-associated mutant $SOD1^{G93A}$ cDNAs were cloned into pUAST expression vectors. Sequencing of cloned products was performed and pUAST generated plasmids were sent to BestGene (CA., U.S.A.) for microinjection. Eight lines of each transgene were generated and at least two transgenic fly lines of each mentioned transgene (male) were used in our experiments.

Immunohistochemistry and western blotting

All antibodies and reagents were purchased from Sigma Aldrich (MO, USA) unless specified otherwise. AJ10 antibody was kindly provided by Esquerda and colleagues. Flies were submerged in a fixative solution (4% PFA in 1x PBS) overnight at 4 °C. Tissues were then cryoprotected and frozen at -80 °C in cryogenic solution buffer as previously described by (Gallart-Palau et al., 2014). Cryostat sectioning at 14 µm was performed (LEICA, Germany) and tissue sections were mounted in gelatinized slides. Immunohistochemistry protocol was followed as previously described by (Gallart-Palau et al., 2014). Mouse anti-flag (1:300), rabbit AJ10 (1:250) and anti-mouse Alexa-488 and anti-rabbit Alexa-594 (1:500) were used as primary and secondary antibodies respectively. Slides were mounted with a drop of Vectashield mounting media

(Vectorlabs CA, USA) prior to the image acquiring in the confocal microscope Fluoview FV-1000 (Olympus, Germany).

Western blotting of flies transgene expression was done in denaturant conditions as previously reported (Gallart-Palau et al., 2015) and 24B-GAL4/+ lines were used as negative controls. Briefly, $SOD1^{WT}$ and $SOD1^{G93A}$ independent pools were formed by a total of 10 flies including 5 flies from each respective line of 24B-GAL4/+, $hSOD1^{WT}$ and $hSOD1^{G93A}$ respectively. Whole flies were homogenized in 1% SDS and proteins were resolved in a SDS-PAGE gel. Mouse anti-flag-HRP (1:1000) and mouse-γ-tubulin (1:1000) were incubated on blotted nitrocellulose membranes as primary antibodies. Anti-mouse-HRP (1:500) was incubated as secondary antibody.

Transmission electron microscopy of thoracic muscles

Preparation of thoracic muscles was performed as previously described by our group (Wang et al., 2007). Briefly, 15d-old flies were deeply anesthetized with CO_2 and their thoraces were dissected under binocular loupe. Muscle tissues were extracted, submerged overnight into a fixative solution (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) and postfixed in 1% OsO_4 for 1 h. Tissues were next dehydrated in ethanol series and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate prior to be observed under a JEM-1230 electron microscope (Jeol, Peabody, MA).

Survival and climbing assays

Male flies were transferred to new food vials every 2-3 days and scored for death. Food vials containing non-yeasted standard food housed a maximum of 20 individuals. A total of 25 individuals per line expressing human wild-type and G93A SOD1 and 24B-GAL4/+ transgenes were CO_2 anesthetized and placed into vertical plastic columns of

15cm length and 1.5 cm diameter for climbing assays. One hour was given to each group for recovery purposes and then they were gently tapped to the bottom of the column and the number of individuals that reached the top at 1 min was scored. Climbing assays were performed in triplicate for each line in intervals of 15 minutes.

Image analysis and statistical analyses

Confocal microscope images were obtained using a Fluoview FV-500 (Olympus, Hamburg, Germany). Scanning parameters were kept constant during the aquisition of all images. Images were analyzed using fluoview software (Olympus, Hamburg, Germany) and imageJ (NIH, MD., USA)(Schneider et al., 2012) and antibody spots were manually counted. Only clearly visible and round shaped spots were quantified. Quantitative data between 24B-GAL4/+, hSOD1^{WT} and hSOD1^{G93A} fly lines was compared by one-way analysis of variance. Kaplan-Meier analysis was performed and survival log rank trends were analyzed by Chi-square. Level of significance in all anlyses was established at $p < 0.05$ unless specified otherwise.

Results and Discussion

hSOD1^{G93A} expression in *Drosophila* thoracic muscles

Mass distribution of muscle fibers in *Drosophila* is highly prominent in thoracic regions to sustain the dynamism of legs and wings (Deak, 1977). A clear advantage of the UAS-GAL4 system is the selective spatial expression of transgenes in specific cells, tissues and body regions (Matthews et al., 2005). We analyzed the expression of human SOD1^{WT} and hSOD1^{G93A} under the 24B promoter and found that was more than 80

percent of hSOD1 expression was confined to thoracic muscle fibers in all transgenic flies (**Figure 1A and 1B**).

Exclusive expression of hSOD1^{G93A} in skeletal muscles of transgenic mice was initially performed by Dobrowolny and colleagues (Dobrowolny et al., 2008). The authors found that this FALS mutant restrictively expressed in skeletal muscles causes reduction of the metabolic activity of muscle fibers, atrophy and sarcolemma in muscle tissues and finally higher accumulation of ROS. Later, restricted expression of FALS mutant SOD1^{G93A} in skeletal muscles promoted motoneuron degeneration via retrograde degeneration of motoneuron axons (Wong and Martin, 2010). These studies suggest that affectation of SOD1^{G93A} in muscle fibers is a crucial event in the triggering of neuropathology in FALS; what justifies the investigation of the effects of SOD1^{G93A} mutant following muscle-restricted expression in a novel *Drosophila* FALS model. Flies expressing neurodegenerative disease-associated mutants under the *24B-GAL4* promoter, a muscle tissue specific driver, have been previously used to study mitochondrial dysfunctions in Parkinson´s Disease (PD) by our group (Ng et al., 2012).

We believed that expression of human SOD1^{G93A} in muscles would cause ALS-associated mitochondrial pathology in *Drosophila* flies. To further confirm expression of SOD1 in our transgenic *Drosophila* lines we analyzed hSOD1^{WT} and hSOD1^{G93A} expression levels by anti-flag antibody image quantification and no significant differences were found on the expression of transgenes between these two lines (**Figure 2H).** Additionally, hSOD1 expression in transgenic lines was validated by WB (**Figure 2I**).

Toxic isoforms of misfolded SOD1 in thoracic fibers of hSOD1^{G93A} flies

Toxicity of hSOD1^{G93A} is associated with the apparition of misfolded isoforms of the protein that can be identified by specific antibodies (Pickles and Vande Velde, 2012). One of these antibodies is AJ10 which was characterized by Sabado and colleagues as able to bind to misfolded and toxic isoforms of hSOD1 (Sábado et al., 2013). We used AJ10 antibody to identify misfolded and toxic isoforms of hSOD1 in our transgenic flies. Positive colocalization (57.6%) **(Figure 2G)** was evidenced in thoracic tissues of hSOD1^{G93A} flies whereas no positive staining was found in hSOD1^{WT} control flies (**Figure 2A-F**).

Expression of hSOD1^{G93A} in thoracic muscles causes mitochondrial pathology

Between the 30 - 40% of our 24B-GAL4-SOD1G93A flies shown an unforeseen upheld wing phenotype unobserved in the 24B-hSOD1^{WT} flies (**Figure 3A)**. A very close phenotype to our findings was previously reported by Deak and colleagues (Deak, 1977) during analysis of upheld mutant flies. According to these authors those mutants held-up their wings vertically and stacked, were flightless and unable to jump as we observed only in our upheld hSOD1^{G93A} flies. Deak and colleagues also observed abnormalities on the legs and cuticle density of upheld mutant flies and these defects were not apparently resembled by our model. Deak and colleagues also found that upheld mutants displayed dramatic disorganization of indirect flight muscles including extreme loss of fibers and abnormal mitochondria distribution whereas no specific mitochondria pathology was reported in those mutants by histological analyses (Deak, 1977). Although muscle disorganization can cause abnormal wings posture in flies (*upheld* or *flapwing*) as referred by Deak, recently, singular alterations in normal mitochondrial dynamics caused by neurodegenerative mutants have been associated to apparition of abnormal wing phenotype without affectation of muscle fibers what could

help to explain our hSOD1^{G93A} encountered phenotype (Deng et al., 2008, Ng et al., 2012, Rai et al., 2014).

It has been shown that toxic and misfolded isoforms of hSOD1 as those identified in our hSOD1^{G93A} flies affect preferentially the mitochondria in FALS models (Pickles and Vande Velde, 2012). In order to characterize the tissues expressing ALS-SOD1^{G93A} in upheld phenotypic flies and hSOD1^{WT} in control flies, thoracic ultrastructural analyses by transmission electronic microscopy (TEM) were performed. ALS-SOD1^{G93A} TEM images showed, in general terms, normal organization of muscle fibers as it was also observed in controls **(Figure 3B-D)**. However, a clear pattern of mitochondrial affectation was observed in ALS fly mutants in concordance to what was previously reported in ALS-SOD1^{G93A} rodent models (Vande Velde et al., 2011, Pickles et al., 2013, Song et al., 2013). Mitochondria in 24B-SOD1^{G93A} flies exhibit abnormal inner structures including cristae, what clearly suggests impairment of the cellular organelle membranes. In addition, elongate mitochondria were often observed together with rounded and swollen organelle profiles **(Figure 3C and 2D**; swollen mitochondria indicated by an asterisk**).** None of these ALS pathological hallmarks were seen in hSOD1^{WT} flies **(Figure 3B)**. In the same light, presence of autophagosome-like features were also observed in 24B-GAL4-SOD1G93A flies mitochondria (**Figure 3D**; dark blue arrow**).** Disorganized and impaired mitochondria as we identified in our ALS mutant flies is related with depletion of energy in thoracic muscle fibers what promotes apparition of the identified upheld phenotype (Ng et al. 2012).

SOD1^{G93A} impairs *Drosophila* motor behavior and shortens lifespan

Upheld wings phenotype arose around 7-10 postnatal days in *24B*-hSOD1^{G93A} and rendered only the affected flies as flightless. Impaired climbing ability was clearly

manifested in one of the analyzed 24B-hSOD1^{G93A} lines at 15 day post-eclosion, whereas in the other 24B-hSOD1^{G93A} line the impaired climbing ability was evidenced around five days later in all cases compared to 24B-hSOD1^{WT} control flies **(Figure 4A)**. Climbing ability of upheld wing 24B-hSOD1^{G93A} flies was highly similar to non-upheld wing flies in the same lines. Additionally, survival studies showed decline in survival for the ALS-mutant flies after 25 days post-eclosion compared to controls although the survival decline was especially apparent in the upheld wing flies **(Figure 4B)**. Despite that significant differences on the expression level of transgenes between transgenic lines were not identified in our study, higher in-line variation as it has been recently described (Huang et al., 2015) may explain some encountered behavioral discrepancies between same transgene lines in our study.

Behavioral assays as climbing and survival assays reflect disease phenotypes in several neurodegenerative transgenic fly models. As has been shown by our 24B-SOD1^{G93A} flies characterized here, the mitochondrial affectation has also been mainly manifested by abnormal wings posture and impaired climbing abilities in PD transgenic models (Ng et al., 2012). Impaired motor ability was observed near the terminal stage of FALS flies, what would be in close accordance to hSOD1^{G93A} mammal models (Gallart-Palau et al., 2014).

Conclusions

We demonstrate here that overexpression of hSOD1^{G93A} in *Drosophila* muscle fibers causes a characteristic pathogenic phenotype that includes flightless upheld wings, climbing defects and mitochondrial dysfunction; features that resemble human FALS. Due to the easy handling, simple genetic modification and highly "druggable" capacity, we believe that the new hSOD1^{G93A} *Drosophila* FALS model reported here will provide significant opportunities to study and rescue the pathogenic interactions between SOD1 mutants and mitochondria in ALS.

The authors declare no conflict of interest regarding to this report.

Acknowledgments

This study was funded in Singapore by grants from the National Medical Research Council-CBRG to K.L.L. and in Europe by doctoral scholarships from the University of Lleida to X.G.P. The authors sincerely thank See Tong Rachel for her critical readings of the manuscript.

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Figure captions

Figure 1. Spatial expression of hSOD1 in *Drosophila* flies. Immunohistochemistry experiments were performed to confirm the spatial expression of FALS transgenes ($hSOD1^{G93A}$ and $hSOD1^{WT}$) in our newly generated transgenic *Drosophila* lines. **A.** Image panel showing Dapi staining, hSOD1 anti-flag staining and the merged image in thoracic and abdominal regions of *Drosophila* body. Scale bar represents 850 µm. **B.** Quantification of hSOD1 expression in thoracic and abdominal regions of *Drosophila* body in our transgenic lines. As shown in the graph thoracic expression of hSOD1 was significantly higher ($p<0.001$) than abdominal expression under the 24B promoter.

Figure 2. Analysis of hSOD1 expression in Drosophila transgenic lines. **A.** Confocal image showing anti-flag hSOD1 antibody signal in thoracic tissues of $hSOD1^{WT}$ transgenic flies. **B.** Confocal image showing anti-AJ10 antibody signal in thoracic tissues of $hSOD1^{WT}$ transgenic flies. **C.** Confocal image showing the merged image of anti-flag and AJ10 signal in thoracic tissues of $hSOD1^{WT}$ transgenic flies. Merged image shows absence of colocalization between both antibodies in WT transgenic flies. **D.** Confocal image showing anti-flag antibody signal in thoracic tissues of $hSOD1^{G93A}$ transgenic flies. **E.** Confocal image showing anti-AJ10 antibody signal in thoracic tissues of $hSOD1^{G93A}$ transgenic flies. **F.** Confocal image showing the merged image of anti-flag and AJ10 signal in thoracic tissues of $hSOD1^{G93A}$ transgenic flies. Merged image shows colocalization (yellow spots) between both antibodies in G93A transgenic flies. Scale bar represents 500 µm. **G.** Percentage quantification of colocalized $hSOD1^{G93A}$ and misfolded and toxic isoforms of hSOD1 stained by AJ10 antibody. **H.** SOD1 expression in WT and G93A flies was quantified and no significant differences

were found on the expression pattern of these transgenes. **I.** hSOD1 expression was validated by WB.

Figure 3. Characterization of the newly generated FALS-24B-hSOD1^{G93A} Drosophila lines. **A.** Image showing the upheld phenotype caused by expression of hSOD1^{G93A} in thoracic muscles of Drosophila flies. Expression of hSOD1^{WT} in Drosophila thoracic muscles does not cause any apparent phenotype. **B.** Ultrastructural image showing the thoracic muscular fibers and mitochondria of Drosophila flies following expression of hSOD1^{WT}. **C.** Ultrastructural image of Drosophila thoracic muscles following expression of the FALS mutant of hSOD1^{G93A}, mitochondria show clear dynamic dysfunction (pro-fusion pattern) and the inner structures are visible showing damage on the mitochondria outer and intermediate membranes. **D.** Ultrastructural image of Drosophila thoracic muscles were swollen mitochondria (pointed by *) and autophagosome-like structures (pointed by blue arrow) are apparent following expression of hSOD1^{G93A} in Drosophila flies. Scale bar in B,C and D ultrastructural images represents 2µm.

Figure 4. Behavioral characterization of FALS-24B-hSOD1^{G93A} Drosophila lines. **A.** Trend line graph showing the climbing assay performance of *Drosophila* lines. 24B-GAL4/+, hSOD1^{WT} and hSOD1^{G93A} fly lines were analyzed and climbing defects were apparent hSOD1^{G93A} transgenic flies from day 10 post-eclosion onwards. Significance level * $p<0.05$. **B.** Kaplan-Meier analysis of survival was performed in 24B-GAL4/+, hSOD1^{WT} and hSOD1^{G93A} fly lines. Significant survival declination peak was found at day 25 post-eclosion. Chi-square log rank trend analysis 13.85, p-value<0.001.

Figure 1

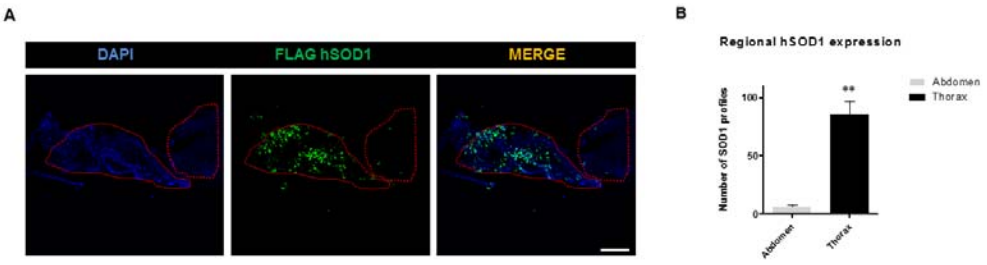

Figure 2

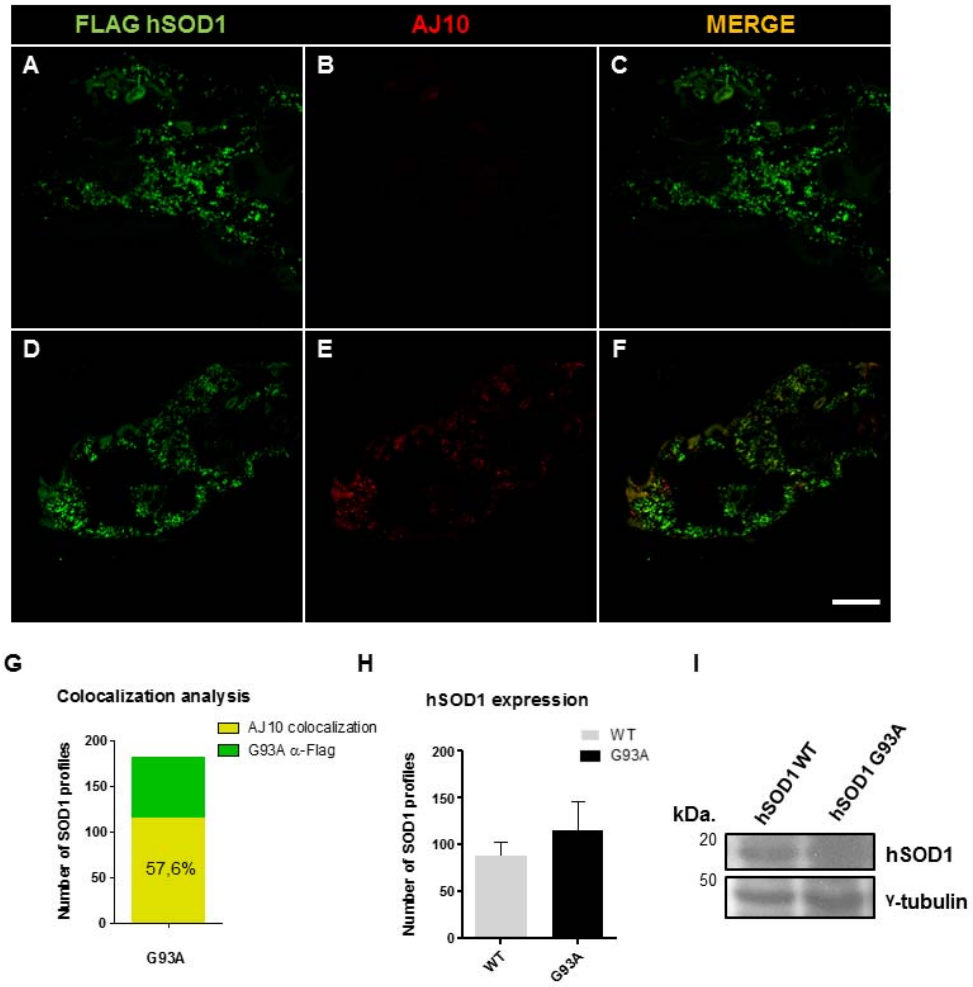

Figure 3

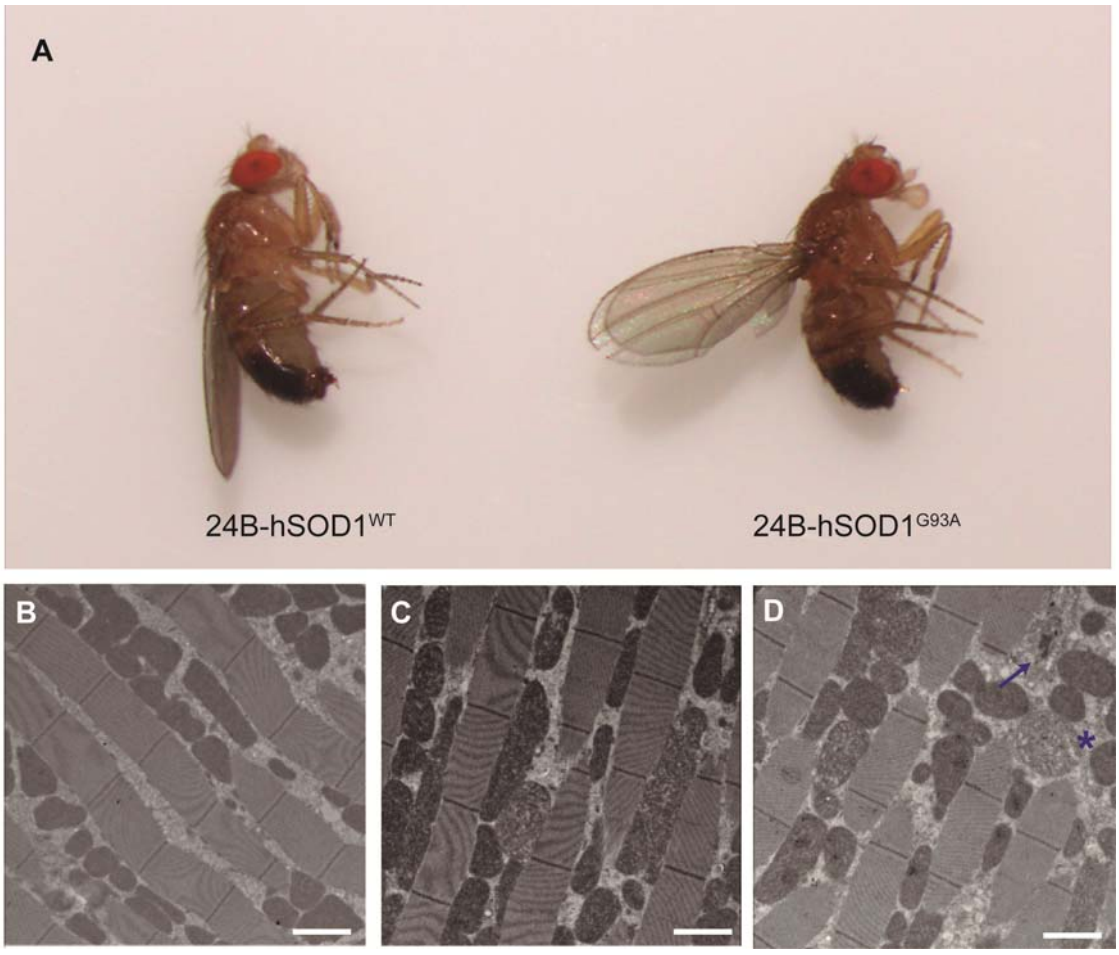

Figure 4