Ph.D Thesis

Studies on *Drosophila* genes related to cancer and their roles in cell metabolism

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Contents

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
ATP	Adenosine Triphosphate
Cabeza	Caz
c-Cb1	Cas-Br-M ecotropic retroviral transforming sequence
COX	cytochrome c oxidase
coxII	cytochrome c oxidase subunit II
DNA	Deoxyribonucleic Acid
DOK2	docking protein 2
dsRNA	Double-stranded Ribonucleic Acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
FUS	Fused in Sarcoma
GRB2	Growth factor Receptor-bound protein 2
JAK1	Janus Kinase 1
JAK2	Janus Kinase 2
JNK signaling	c-Jun N-terminal kinase signaling
mRNA	messenger Ribonucleic Acid
mtDNA	mitochondria Deoxyribonucleic Acid
mtROS	mitochondrial Reactive Oxygen Species
NCK1	NCK adaptor protein 1
nDNA	nuclear DNA
OXPHOS	oxidative phosphorylation

PLC-gamma1	phospholipase C gamma 1
Quantitative RT-PCR	Quantitative Reverse Transcriptase-mediated Polymerase Chain Reaction
RNA	Ribonucleic Acid
SOD1	Superoxide Dismutase 1
RNAi	Ribonucleic Acid interference
ROS	Reactive Oxygen Species
SCO2	Synthesis of Cytochrome c Oxidase 2
SCOX	Synthesis of Cytochrome c Oxidase X
Shc	SHC transforming protein 1
TDP-43	TAR DNA-binding protein of 43kDa

General introduction

1. *Drosophila* **as a model organism of human disease study**

In the past few decades, biomedical researchers got success with valuable information on numerous human diseases basing on genetics studies. However, the limitation of human genetics studies such as complex patterns of inheritance, lack of sufficient family pedigree data and population-based genetic heterogeneity, makes it difficult to analyze genes activities and pathways in detail. In genetics studies, higher animal models such as mice, rats or monkeys have been used. However, now it faces to problems of cost, time, legal and ethical boundaries. Hereby, by using fruit flies, a more powerful model due to its large collection of available genetic tools, short generation time, high reproductive rate, and easily to perform in large-scale genetic screen, can make these problems not be developed.

The fruit fly, *Drosophila melanogaster*, is a little insect with the size about 3mm long which accumulates around the spoiled fruits, has been intensely studied for more than 100 years with thousands of scientists. In the early 1900's, by the results of consideration of T.H. Morgan, *Drosophila* came to the central organism in genetics, especially to identify mutant genes [1]. Many basic biological or physiological functions are conserved between mammals and *Drosophila melanogaster*, and 80% of human disease-causing genes are reported to have a functional homolog and same signaling pathway in *Drosophila* [5-7]. The fly genome, which was completely sequenced in 2001, includes 165 million base pair in length (located on only four pairs of chromosomes) and contains roughly 14,000 predicted and confirmed genes [2-4]. Human has more genes (27,000 human genes) than *Drosophila* but with the similar number of gene families.
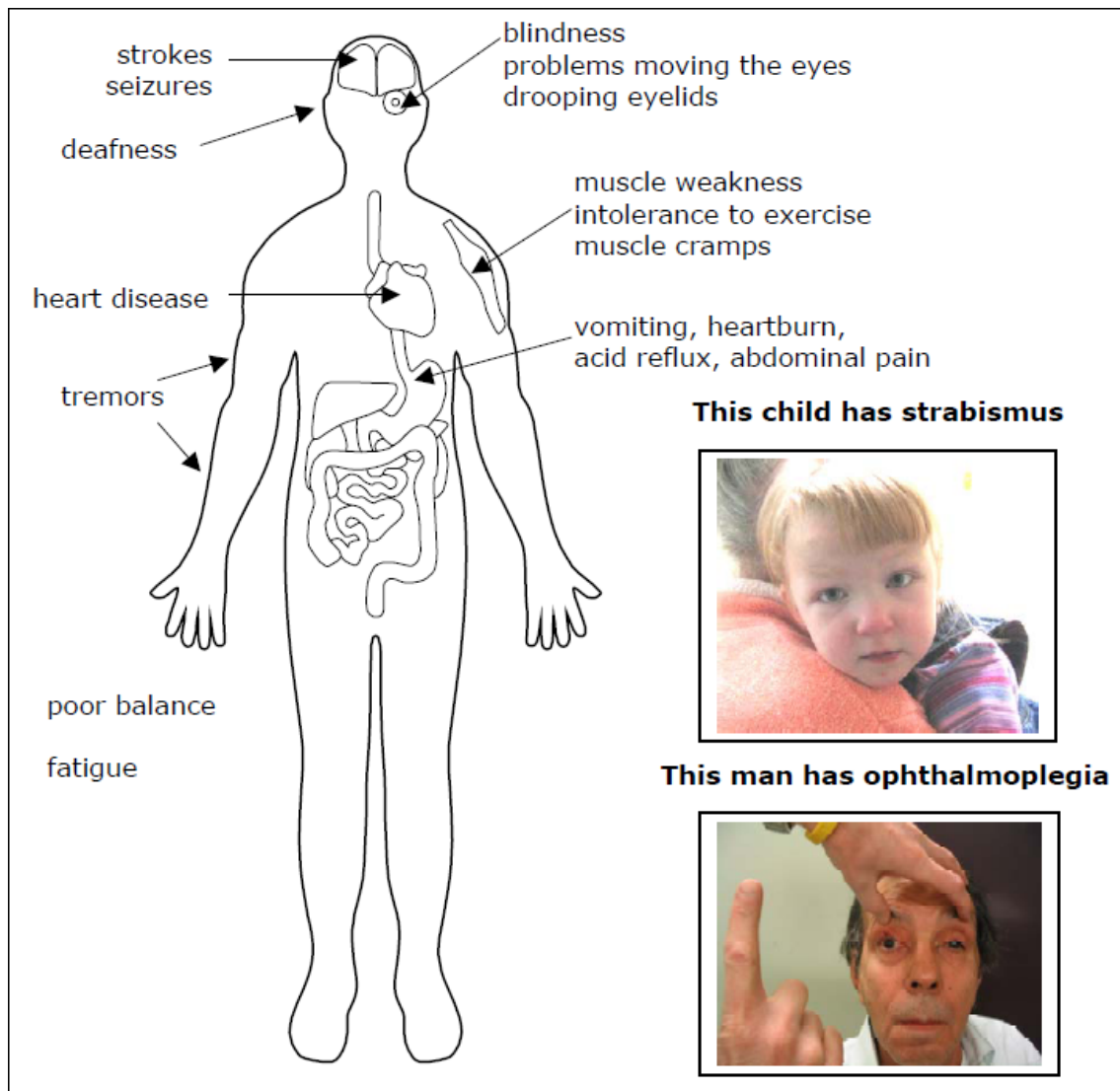
2. Mitochondrial diseases

Figure 1: Symptom of mitochondrial diseases. (Source: *www.mitocanada.org*)

Definition:

Mitochondria are known as the powerhouse of the cell by creating more than 90% of the energy needed by the body to sustain the life and support the growth. When mitochondria fail or less in activities, less and less energy is produced within the cell,

followed by cell injury or cell death. Each cell contains only one nucleus but more than hundreds or thousands of mitochondria and mitochondrial DNA (mtDNA).

Mitochondrial diseases are results of dysfunction of the mitochondrial respiratory chain, due to the mutations in mtDNA or nuclear DNA (nDNA) which lead to altered function of the protein or RNA molecules that normally reside in mitochondria. Although mitochondrial diseases are commonly seen in infants and children, they happen at any age. While some of mitochondrial diseases only affect a single organ, many involve multiple organs and often present with prominent neurologic and myopathic features. Patients who get mitochondrial diseases usually harbor a mixture of normal and mutant mtDNAs as a heteroplasmy. Furthermore, proportion of mutated number of mtDNA affects differently both in among tissues and in age of patients. For example, large numbers of mtDNA deletions in heart, muscle, and brain become fatal in young adulthood such as Kearns-Sayre syndrome, while large numbers of the same mtDNA deletions in blood cause fatal anemia in infancy such as Pearson syndrome.

The most common symptoms are summarized in Figure 1:

- Poor growth, loss of muscle coordination or muscle weakness, neurological problems, autism, visual and/or hearing problems, developmental delays, learning disabilities, heart-liver-kidney diseases, gastrointestinal disorders, diabetes, severe constipation, increase risk of infection, memory loss, autonomic dysfunction … etc.

How common the mitochondria diseases are:

- 1/2000 children in the US will develop mitochondrial disease by the age of 10

- 1/4000 children in the US per year were born with a type of mitochondrial disease.

- In adults, many diseases of aging and diabetes have been found to have defects of mitochondrial function.

Diagnosis and treatment of mitochondrial diseases:

Mitochondrial diseases are difficult to make diagnoses. Furthermore, due to the lack of physician and public awareness, this disease is not often diagnosed. In the present day, diagnosis of mitochondrial diseases can be made through a combination of clinical observation, laboratory evaluation, brain imaging and muscle biopsies. Although new gene techniques such as deep sequencing have been developed but genetic testing is rarely sufficient for the diagnosis of mitochondrial diseases. Besides, there are no highly effective mitochondrial disease treatments until now. Actually, scientists have given their constant efforts in genetics studies to find the real mechanism of these diseases and *Drosophila melanogaster* is used as a convenient animal model for human genetic diseases, including those associated with mitochondrial dysfunction. With advantages in genetics and molecular biology, I hope to be better in understanding mitochondrial diseases. However, the definitive cause of this disease continues to evolve.

3. Epidermal Growth Factor Receptor (EGFR)

Epidermal Growth Factor (EGF) is a small 53 amino acid residue protein that plays important roles in normal cell growth, oncogenesis and wound healing. EGF binds to a specific high-affinity, low-capacity receptor on the surface of responsive cell known as Epidermal Growth Factor Receptor (EGFR). EGFR is a typical cell membrane receptor tyrosine kinase that is activated following ligand biding and receptor dimerization

Moreover, EGFR is also a particular molecular target of high promise on oncology. Mutation of this gene or overexpression of EGFR is reported to be associated with many cancers including lung cancer and breast cancer [9.10]. Understanding the mechanism of this receptor activation and function will provide new lead on the clinical targeting of this receptor in cancer therapy.

It is reported that mitochondrial cytochrome c oxidase subunit II (CoxII) binds EGFR at Tyr(P)-845 in a EGF-dependent manner. However the regulation of this process is now well defined. One of recent studies revealed that CoxII can be phosphorylated by EGFR and EGF stimulation reduces Cox activity and cellular ATP level [11], which suggests that EGFR plays a role in modulating mitochondrial function via its association with, and modification of CoxII.

EGFR signaling pathway is one of the most important pathways which regulate the growth, survival, proliferation and differentiation in mammalian cells. The EGFR receptors signal through Akt, MAPK, and many other pathways to regulate the cell proliferation, migration, differentiation, apoptosis and cell motility [9].

4. Cytochrome c oxidase (COX) and *SCO2* gene

Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain which catalyzes the transfer of electron from reduced cytochrome c to molecular oxygen in eukaryotes and certain prokaryotes. The enzyme is embedded in the inner mitochondrial membrane and is active as a dimer. It has two copper binding sites, two hemes, a magnesium ion and a zinc ion.

Cytochrome c oxidase (COX) deficiency is a genetic condition that affects many parts of the body, with the signs and symptoms begin before age of two. Cytochrome c oxidase (COX) deficiency is frequently fatal in childhood. However, it

may appear later in mildly affected individuals, correlated with the myopathy and hypotonia with no other health problems.

In man, mutations of five human nuclear COX assembly genes, including *SURF1, SCO2, SCO1, COX10* and *COX15,* have been associated with mitochondrial abnormalities and COX deficiency. Human *SCO1* and *SCO2* are paralogous genes that code the metallochaperone proteins that play roles in copper delivery to cytochrome *c* oxidase with the highly conserved potential copper-binding motif, CxxxC. Mutation in the human *SCO2* gene have been found to cause fatal, early onset of hypertrophic cardiomyophathy with encephalopathy [10,11], while mutations in *SCO1* have recently been reported in a family with hepatopathy and ketocaidotic coma, with no cardiac symptoms [12]. Recent studies showed that human SCO2 is also the downstream mediator of the balance between the utilization of respiratory and glycolytic pathways [13] and has a regulatory role in the maintenance of cellular copper homeostasis [14]. Synthesis of cytochrome c oxidase X (SCOX) is a *Drosophila* orthologue of *human* SCO2. Alignment of amino acid sequences of both proteins is shown in Figure 2.

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Human       1    MLLLTRSPTAWHRLSQLKPRVLPGTLGGQALHLRSWLLSRQGPAETGGQGQPQGPGLRTRLLITGLFGAGLG
                                 ::.. .:. ::      : . : .:::
Drosophila  1    ----------------MSRSLQRLVGSCRRWAQQPAIRHYAAPADST-KGK--GPISWRSLAVIGALGAGGV


Human       73   GAWLALRAEKERLQQQKRTEALRQAAVGQGDFHLLDHRGRARCKADFRGQWVLMYFGF THCPDI CPDELEKL
                 : : ...::.. ....: . : ::   :   : : : :   :: :.:.:.::::::::::.:::::::.
Drosophila  54   GFMLYVKSEKDEARMKERQRQLGKAAIG-GSWELVDSQGAVRKSEDFLGKWLLIYFGF THCPDI CPDELEKM


Human       145  VQVVRQLEAEPGLPPVQPVFITVDPERDDVEAMARYVQDFHPRLLGLTGSTKQVAQASHSYRVYYNAGPKDE
                 . :: ..: : : :::.:::::::: :.:::.: :.:::::::.. .. ..:::.:::.::
Drosophila  126  AAVVDEVEKSPQTPAVQPIFITVDPERDSKEVVAKYVKEFSPKLLGLTGTVEQIRKVCKAFRVYFSAGPRDE


Human       217  DQDYIVDHSIAIYLLNPDGLFTDYYGRSRSAEQISDSVRRHMAAFRSVLS-----
                 :.:::::::.: .::.:::: :.:::::.. .:   :.  ..: . :.
Drosophila  198  DNDYIVDHTIIMYLVNPDGEFVDYYGQNRDKDQCVASILVNIAKWNSMNKKGWFS
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Figure 2: Alignment of *human* SCO2 and *Drosophila* SCOX amino acid sequence. Identity is indicated by two-dot place. The copper binding domains including the CXXXC motif are in red box. The histidine residue at position 205 of the *Drosophila* protein, also involved in copper binding [16,17], is indicated by an arrow. The alignment was made by using BLAST and FASTA. The overall identity and the similarity of the amino acid sequences of SCOX and SCO2 are 48.6% and 78.4%, respectively.

5. Amyotrophic lateral sclerosis (ALS)

ALS is a fatal neurodegenerative disease that is characterized by degeneration of motor neurons of the brain and the spinal cord, which leads to progressive muscle weakness and fatal paralysis [18]. Most cases of ALS are sporadic, but some patients have a family history such as a result of mutation in the gene for Cu/Zn superoxide

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dismutase 1 (SOD1) that is located in cytoplasm [19-23]. SOD2 that is not related to ALS is located in mitochondria and may relate to mitochondrial function. Pathology of ALS involve in many mechanisms such as oxidative stress, glutamate excitotoxicity, mitochondrial damage, defective axonal transport, glia cell pathology and aberrant RNA metabolism.

The ALS-related mutant SOD1 have also been found in the intermembrane space, matrix and outer membrane of mitochondria [24-27] and it is believed to cause multiple damages to mitochondria such as loss of mitochondrial membrane potential or swelling of the important organelle [28,29]. The consequences include impaired respiratory complex [30-33], loss of mitochondrial membrane potential, disruption of calcium homeostasis, and impaired axonal transport of mitochondria, potential imbalance of mitochondrial fission and fusion and a decrease of ATP production [32]. Furthermore, apoptosis can be activated [34-36]. Various aspects of the underlying mechanisms as well as functional consequences of mitochondrial dysfunction are observed to believe that mitochondrial dysfunction plays a critical role in mutant SOD1 mediated familial ALS.

5. Outline of this thesis

5.1. Chapter 1: Role of SCOX in determination of Drosophila melanogaster lifespan

In man, COX (cytochrome c oxidase) deficiency is reported to be related to mutation of the *SCO2* (*synthesis of cytochrome c oxidase 2*) gene, which encodes one of the copper-donor chaperones involved in the assembly of mitochondrial cytochrome c oxidase. Such COX deficiency due to the genetic condition leads to heart disease and the Leigh syndrome and is frequently fatal in childhood. S*ynthesis of cytochrome c oxidase X* (*SCOX*) is a *Drosophila* orthologue of human *SCO2*. Here, we generated *SCOX*-knockdown flies and the full length SCOX transgenic flies to investigate the *in vivo* roles of SCOX. Our results demonstrated knockdown of *SCOX* gene in all cells and tissues to be associated with lethality at larval or pupal stages and this correlated with a decrease in ATP level. In contrast, the full length SCOX transgenic flies showed a longer lifespan than wild type flies and control flies carrying *Act5C-GAL4* alone and this correlated with an increase in ATP level. Finally, when cultured on paraquat-added medium, full length SCOX transgenic flies also exhibited an elongated lifespan. Therefore, we hypothesized that SCOX plays an important role in ATP production and consumption, which helps to prevent production of mitochondrial reactive oxidative species and/or impairment of mitochondrial activity under oxidative stress.

5.2. Chapter 2: Genetic link between Cabeza, a Drosophila homologue of Fused in Sarcoma (FUS), and the EGFR signaling pathway

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that causes progressive muscular weakness. Fused in Sarcoma (FUS) that has been identified in familial ALS is a RNA-binding protein that is normally localized in the

nucleus. However, its function in vivo is not fully understood. *Drosophila* has Cabeza (Caz) as a FUS homologue and specific knockdown of *Caz* in the eye imaginal disc and pupal retina using a GMR-GAL4 driver was here found to induce an abnormal morphology of the adult compound eyes, a rough eye phenotype. This was partially suppressed by expression of the apoptosis inhibitor P35. Knockdown of Caz exerted no apparent effect on differentiation of photoreceptor cells. However, immunostaining with an antibody to Cut that marks cone cells revealed fusion of these and ommatidia of pupal retinae. These results indicate that *Caz*-knockdown induces apoptosis and also inhibits differentiation of cone cells, resulting in abnormal eye morphology in adults. Mutation in EGFR pathway-related genes, such as rhomboid-1, rhomboid-3 and mirror suppressed the rough eye phenotype induced by *Caz*-knockdown. Moreover, the rhomboid-1 mutation rescued the fusion of cone cells and ommatidia observed in *Caz* -knockdown flies. The results suggest that Caz negatively regulates the EGFR signaling pathway required for determination of cone cell fate in *Drosophila*.

6. References

[1]   Beller M, Oliver B. One hundred years of high-throughput *Drosophila* research. Chromosome Res, 2006. 14(4):349-362.

[2]   Celniker SE, Rubin GM. The *Drosophila melanogaster* genome. Annu Rev Genomics Hum Genet, 2003. 4:89-117.

[3]   Rubin GM, et al. Comparative genomics of the eukaryotes. Science, 2000. 287(5461):2204-2215.

[4]   Chen D, et al. High-resolution, high-throughput SNP mapping in *Drosophila melanogaster*. Nat Methods, 2008. 5(4):323-329.

[5]   Chien S, et al. Homophila: human disease gene cognates in *Drosophila*. Nucleic Acids Res, 2002. 30(1):149-151.

[6]   Fortini ME, et al. A survey of human disease gene counterparts in the *Drosophila* genome. J Cell Biol, 2000. 150(2):23-30.

[7]   Reiter LT, et al. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. Genome Res, 2001. 11(6):1114-1125.

[8]   Frank H, et al. *Drosophila melanogaster* in the study of human neurodegeneration. CNS Neurol Disord Drug Targets, 2010. 9(4): 504-523.

[9]   Alexander W and Liliana A. The TGFbeta super family signaling pathway. WIRE Dev Biol, 2012. doi:10.1002/wdev.86.

[10]   Jaksch M, Ogilvie I, Yao J, Kortenhaus G, Bresser HG, Gerbitz KD, Shoubridge EA. Mutation in *SCO2* are associated with a distinct form of hypertrophic cardiomyopathy and cytochrome c oxidase deficiency. Hum Mol Genet, 2000. 9(5):795-801.

[11]   Sacconi S, Salviati L, Sue MC, Shanske S, Davidson MM, Bonilla E, Naini BA, De Vivo CD and Dimauro S. Mutation Screening in patients with isolated cytochrome c oxidase deficiency. Pediatric Research, 2003. 53:224-230.

[12]   Valnot I, Osmond S, Gigarel N, Mehaye B, Amiel J, Cormier-Daire V, Munnich, A, Bonnefont JP, Rustin P and Rotig A. Mutations of the *SCO1* gene in mitochondrial cytochrome c oxidase deficiency with neonatal-onset hepatic failure and encephalopathy. Am. J. Hum. Genet, 2000. 67:1104-1109.

[13]   Matoba et al. p53 regulates mitochondrial respiration. Science, 2006. 312(1570):1650-1653.

[14]   Leary et al. The human cytochrome c oxidative assembly factors SCO1 and SCO2 have regulatory roles in maintenance of cellular copper homeostasis. Cell Met, 2007. 5(1):9-20.

[15]   Leary SS, Cobine AP., Nishimura T, Verdijk MR, Ronald de Krijger, René de Coo, Tarnopolsky AM, Winge RD and Shoubridge AE. COX19 mediates the transduction of a mitochondrial redox signal from SCO1 that regulates ATP7A-mediated cellular copper efflux. Mol Biol Cell, 2013. 24(6):683-691.

[16]   Glerum DM, et al. Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem, 1996. 271(24):14504-14509.

[17]   Horng YC, Leary SC, Cobine PA, Young FB, George GN, Shoubridge EA, Winge DR. Human Sco1 and Sco2 function as copper-binding proteins. J Biol Chem, 2005. 280(40):34113-34122. Erratum in: J Biol Chem. 280(49):41122.

[18]   Boilee S, Velde VC, Cleverland WD. ALS: A disease of motor neurons and their nonneuronal neighbors. Neuron, 2006. 52(1):39-59.

[19]   Vande Velde C, Miller MT, Cashman RN, Cleverland WD. Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria. PNAS, 2007. 105(10):4022-4027.

[20]   Gellera C. Genetics of ALS in Italian families. ALSOMND, 2001. 2(1):43-46.

[21]   Orrell RW, Habqood JJ, Malaspina A, Mitchell J, Greenwood J, Lane RJ, deBelleroche JS. Clinical characteristics of *SOD1* gene mutations in UK families with ALS, 1999. 169(1-2):56-60.

[22]   Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature, 1993. 362:59-62.

[23]   Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu Rev Neurosci, 2004. 27:723-749.

[24]   Higgins CM, Jung C, Ding H, Xu Z. Mutant Cu, Zn superoxide dismutase that causes motoneuron degeneration is present in mitochondria in the CNS. J Neurosci, 2002. 22:RC215.

[25]   Vijayvergiya C, Beal MF, Buck J, Manfredi G. Mutant superoxide dismutase 1 forms aggregates in the brain mitochondrial matrix of amyotrophic lateral sclerosis mice. J Neurosci, 2005. 25:2463-2470.

[26]   Vande Velde C, Miller TM, Cashman NR, Cleveland DW. Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria. Proc Natl Acad Sci U S A, 2008. 105:4022-4027.

[27]   Kawamata H, Manfredi G. Different regulation of wild-type and mutant Cu, Zn superoxide dismutase localization in mammalian mitochondria. Hum Mol

Genet, 2008. 17:3303-3317.

[28]   Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron, 1995. 14:1105-1116.

[29]   Kong J, Xu Z. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. J Neurosci, 1998. 18:3241-3250.

[30]   Borthwick GM, Johnson MA, Ince PG, Shaw PJ, Turnbull DM. Mitochondrial enzyme activity in amyotrophic lateral sclerosis: implications for the role of mitochondria in neuronal cell death. Ann Neurol, 1999. 46:787-790.

[31]   Jung C, Higgins CM, Xu Z. A quantitative histochemical assay for activities of mitochondrial electron transport chain complexes in mouse spinal cord sections. J Neurosci Methods, 2002. 114:165-172.

[32]   Ferri A, Cozzolino M, Crosio C, Nencini M, Casciati A, Gralla EB, Rotilio G, Valentine JS. Familial ALS-superoxide dismutases associate with mitochondria and shift their redox potentials. Proc Natl Acad Sci USA, 2006. 103:13860-13865.

[33]   Bowling AC, Schulz JB, Brown RH, Jr., Beal MF. Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. J Neurochem, 1993. 61:2322-2325.

[34]   Pasinelli P, Belford ME, Lennon N, Bacskai BJ, Hyman BT, Trotti D, Brown RH., Jr. Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria.Neuron, 2004. 43:19-30.

[35]   Kirkinezos IG, Bacman SR, Hernandez D, Oca-Cossio J, Arias LJ,

Perez-Pinzon MA, Bradley WG, Moraes CT. Cytochrome c association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. J Neurosci, 2005. 25:164-172.

[36]   Takeuchi H, Kobayashi Y, Ishigaki S, Doyu M, Sobue G. Mitochondrial localization of mutant superoxide dismutase 1 triggers caspase-dependent cell death in a cellular model of familial amyotrophic lateral sclerosis. J Biol Chem, 2002. 277:50966-50972.

[37]   Shi P, Gal J, Kwinter MD, Liu X and Zhu H. Mitochondrial dysfunction in amyotrophic lateral sclerosis. Biochim Biophys Acta, 2011. 1802(1):45-51.

Chapter 1:

Role of SCOX in determination of *Drosophila melanogaster* lifespan

1.1. Introduction

1.1.1. Cancer and ATP

Although humans can live longer nowadays, we have to face so many serious diseases, such as cancers. Nowadays, cancer is a leading cause of disease worldwide. In 2012, 14.1 million of new cases of cancer were estimated in the world: 7.4 million (53%) in males and 6.7 million (47%) in female (Source: *www.cancerresearchuk.org*). The most common cancers are lung, female breast, bowel and prostate, which account for about 42% of all the cases all over the world. Although there are so many invention of cancer treatment reported nowadays, such as surgery, hormone therapy, radiation, chemotherapy, immunotherapy, targeted therapy and so on, it is predicted that there will be 23.6 million new cases worldwide each year by 2030. That is why scientists are taking constant efforts to find the last answer for mechanism of cancer development.

The oldest case of cancer was reported in ancient Egypt, about 3000 B.C with a description of 8 cases of tumors or ulcers of the breast that were removed by cauterization with a tool called a fire drill. And in the ancient description of this disease, it was written that "there is no treatment" for this disease. It was not until 400 B.C that the word "cancer" was firstly credited by the "Father of Medicine" – Greek physician Hippocrates. Cancer begins when cells in a part of the body start to divide and reproduce uncontrollably, to become abnormal cells. The result is a growth called a tumor, which will cause many problems or pressure on the nearby tissues. Serious

malignant tumors can spread throughout the body and invade all the tissues, which blocks the normal activities of these tissues. For examples: if cancer happens in the digestive system, nutrients cannot go through the intestines. When this happens, none of the nutrients can be absorbed. Another example is if cancer affects to the lung, there won't be enough healthy lung tissues to allow adequate oxygen flow. Generally these could cause death in almost cases.

Over the last few decades, a variety of disorders including cancers and numerous neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases), have been proven to be caused by defects in mitochondrial functions [1-4]. Mitochondria are well known to play a central role in the regulation of cellular function including metabolism and cell death in cancer cells. Several functional changes in cancer-cell mitochondria have been observed, such as increased production of mitochondrial reactive oxygen species (mtROS), decreased oxidative phosphorylation, and a corresponding increase in glycolysis [5]. In addition, a high level of mtROS inhibits and damages mitochondrial proteins and lipids in several ways, which impairs mitochondrial functions contributing to aging and disease.

In recent studies, it is reported that some evidence being suggested to prove the relations between cancer and cellular metabolic program. In 1931, with the investigation of the metabolism of tumors and the respiration of cells, Otto Heinrich Warburg got the Nobel Prize in Physiology and Medicine and put the first stone on studying relationship between cell metabolism and oncology. He noted that the cancer cells growing in normal oxygen conditions have an increased rate of glycolysis, compared with the normal cells (the Warburg effect).

ATP, adenosine triphosphate, is a usable form of energy for cells. All living cells make ATP. The oxidative phosphorylation in mitochondria provides most of the ATP in nearly all cell types. In the cellular respiration of normal cells, by using oxygen through an aerobic process, a single molecule of glucose produces 36 to 38 servings of ATP. In contrast, the cancer cells rely mainly in the anaerobic process in which glucose was used to produce 2 servings of ATP. And because cancer cells rely on the glycolysis which only has 2 servings of ATP, they need more glucose for their activity.
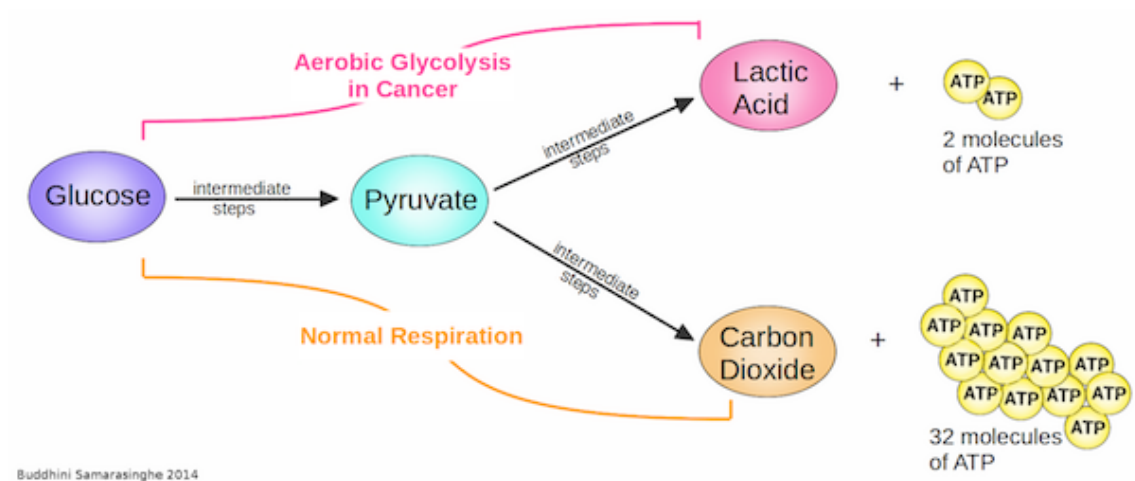
Figure 1: Comparing to normal cells and cancer cells in producing ATP (Source: *http://www.scientificamerican.com*)

1.1.2. COX and SCO2

Cox (Cytochrome *c* oxidase or Complex IV) is the main transmembrane protein complex found in mitochondrion, and acts as the last enzymes of respiratory chains located in the mitochondrial membrane. In human, COX deficiency encompasses a wide and heterogeneous spectrum of mostly severe multisystemic disorders, which primarily affect organs with high energy demand, such as the brain, skeletal muscle,

heart and kidney [7]. COX deficiency is reported to be related to mutation of the *SCO2* (*synthesis of cytochrome c oxidase 2*) gene, which encodes one of the copper-donor chaperones involved in the assembly of mitochondrial cytochrome c oxidase. Such COX deficiency due to the genetic condition leads to heart disease and the Leigh syndrome and is frequently fatal in childhood. In normal cells, it is recognized that the presence of oxygen results in the activation of an oxygen-dependent pathway of oxidative phosphorylation (OXPHOS) and inhibition of glycolysis, but most cancer cells show a higher rate of glycolysis even with only adequate oxygen levels. A high rate of aerobic glycolysis, or the Walburg effect, connects the high rate of glucose fermentation to cancer, and maintains relations with the p53-SCO2-respiration axis. Mutation of mitochondrial inner membrane gene- *SCO2*, one of two human SCO-encoding genes, is reported to result in severe COX deficiency [6]. Mutations in *SCO2* are also reported to be associated with hypertrophic cardiomyopathy and encephalopathy that presents soon after birth [8]. Affected infants have respiratory difficulties and metabolic acidosis, and die within the first year of life. In patients with *SCO2* mutations, neuropathological defects are various, including heterotopia, gliosis, early capillary proliferation, and atrophy [2, 7, 9, 10]. However, no child with *SCO2* mutations has neuropathological defects consistent with the Leigh syndrome, possibly because of their death before manifesting such features [10-12]. Moreover, on survival analysis, high SCO2-expressing breast-cancer patients show a significantly better prognosis than low SCO2-expressing counterparts [13].

Synthesis of cytochrome c oxidase X (SCOX) is a *Drosophila* orthologue of human SCO2. To gain further insight into the *in vivo* roles and genetic functions of *SCO2*, we used *Drosophila melanogaster* as a model system. In *Drosophila*

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melanogaster, a single SCOX-encoding gene (*CG8885*, *scox*) is present. Null mutations of the *scox* gene are reported to be associated with larval lethality while mutations in its 5'UTR are reported to be associated with motor dysfunction and female sterile phenotypes [14]. Here, we generated *SCOX*-knockdown flies and the full length SCOX transgenic flies to investigate the *in vivo* roles of SCOX. *SCOX*-knockdown flies carrying *UAS-SCOXIR* and the full length SCOX transgenic flies carrying *UAS-SCOX* were established. Lifespan, morphology and mobility were examined in each transgenic fly strain, for comparison with wild type and control flies.

1.2. Materials and methods

1.2.1. Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Canton S was used as the wild type strain. In this study, the full length SCOX transgenic fly strain (*Act5C*-GAL4>UAS-*SCOX*) carrying genotype of *w; UAS-SCOX/+; Act5C-GAL4/+* and the *SCOX*-knockdown fly strain (*Act5C*-GAL4>UAS-*SCOXIR*^{20}) carrying the genotype of *w; UAS-SCOXIR*^{20}*/+; Act5C-GAL4/+* were designed in our laboratory. The RNAi transgene on the second chromosome of *Act5C*-GAL4>UAS-*SCOXIR*^{20} fly strain was targeted to the region corresponding to residues 600-964 of *Drosophila* SCOX. The *SCOX*-knockdown fly strain (*Act5C*-GAL4>UAS-*SCOXIR*^{7861}) carrying *w; +; Act5C-GAL4/UAS-SCOXIR*^{7861} (CG8885) was obtained from the Vienna *Drosophila* RNAi center (VDRC). The RNAi transgene on the third chromosome of *Act5C*-GAL4>UAS-*SCOXIR*^{7861} fly strain was targeted to the region corresponding to amino acid residues 584-854 of *Drosophila* SCOX. The Act5C-GAL4 strain was obtained from the Bloomington *Drosophila* stock center.

1.2.2. Oligonucleotides

CG8885XbaI   5'-GCCTCTAGAGTACAACGAAACAACATTTG-3'

CG8885BamHI   5'-GGTCAACGGTGATCCCGATGGATCCTG-3'

CG8885BglII   5'-TTCAGATCTTCCCGCTCCCTGCAACGCTT-3'

CG8885KpnI   5'-TTCGGTCCCTAGCTGAACCATCCCTTTT-3'

1.2.3. Establishment of transgenic flies

PCR was carried out to construct the plasmid pUAS-SCOXIR$_{600-964}$ and pUAS-SCOX with *Drosophila* cDNA as a template.

Construction of pUAS-SCOXIR$_{600-964}$:

To establish the transgenic fly line carrying *UAS-SCOXIR*, a 365-bp fragment of *SCOX* was amplified by PCR using primers CG8885BamlHI and CG8885XbalI, followed by subcloning into the pT7Blue-2vector (Novagen). The obtained DNA fragments were digested with *Xba*I *a*nd *BamH*I and then inserted into the *Bgl*II and *Avr*II sites of pWIZ to create pWIZ-5'-CG8885. Then the 365-bp DNA fragment amplified from pT7-SCOX$_{600-964}$CG8885 was also digested by *Xba*I and inserted into the *Nhe*I and *Xba*I sites of pWIZ-5'-CG8885 to create pUAS-SCOXIR$_{600-964}$.

Construction of pUAS-SCOX containing full length SCOX cDNA:

A 700-bp DNA fragment was amplified by PCR using the CG8885 cDNA as a template and primers CG8885BglII and CG8885KpnI. PCR products were digested with *Bgl*II and *Kpn*I and inserted between the *Bgl*II and *Kpn*I sites of the pUAS-Flag vector.

These plasmids were verified by sequencing and then injected into embryos to obtain stable transformant lines carrying *UAS-SCOXIR* and *UAS-SCOX*. P element-mediated germline transformation was accomplished as described [15] and F1

transformants were selected on the basis of white eye color rescue [15]. Twenty-five lines were established for plasmid pUAS-SCOIR$_{600-964}$ but only line number 20 (UAS-*SCOXIR*[20]) was determined to have an RNAi transgene on the second chromosome while others were determined to have examples on both the second and third chromosomes. Therefore, this line (UAS-*SCOXIR*[20]) was mainly used in this study. Although twenty lines were established for the plasmid pUAS-SCOX, line number 8 (UAS-*SCOX*) showed the severest rough eye phenotype and therefore was primarily used in this study.

To drive expression of *SCOX* double stranded RNA (*SCOX* dsRNA) or SCOX in the whole body of the flies, we crossed the transgenic flies with the *Act5C-GAL4* driver line.

1.2.4. Expression of His-tag fusion proteins

The SCOX full length fragment was transferred to the pCold I expression vector to create pCold-scox which was inserted into *Escherichia coli* (*E. coli*) BL21 to express the His-SCOX full length fusion protein. In brief, lysates of cells were prepared by sonication in PBS containing 1mM phenylmethanesulfonyl fluoride (PMSF) and separated into supernatant and pellets by centrifugation at 12,000g for 20 min at 4°C. Pellets were dissolved in 6M urea buffer (6M urea, 50mMTris-HCl, pH 8.0, 1mM EDTA), and purified by electrophoresis through Ni-NTA agarose (QIAGEN). After purification, the urea buffer was replaced with PBS by dialysis.

1.2.5. Preparation of anti-SCOX antibody

The purified GST-SCOX full length fusion protein was used to elicit polyclonal antibody production in a Guinea pig. The resulting antiserum was used at 1:5000 dilution for Western Blotting Assays.

1.2.6. Longevity assay

Wild-type flies, control flies carrying only *Act5C-GAL4* and *Act5C*-GAL4>UAS-*SCOX* flies were maintained at 28±0.5°C in a tube with standard food. To estimate longevity, newly eclosed adult flies were collected during 24 hours from the onset of explosion for each experimental variant. Young males were separated from females and put into new conical tubes. Flies were transferred to new medium three times per week (each 2-3 days). Dead flies were counted daily. For each experimental variant, three biological replicators were pooled. Survival functions were estimated using the Kaplan-Meier procedure [16] and plotted as survival curves.

1.2.7. Immunoblotting analysis

Protein extracts from the whole bodies of wild type flies, control flies, *Act5C*-GAL4>UAS-*SCOX* flies, and *Act5C*-GAL4>UAS-*SCOXIR*[7861] flies and from *E. coli* producing His-SCOX (full length) fusion protein were prepared in lysis buffer with proteinase inhibitors and then homogenized in a sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and 1.2% β-mercaptoethanol. The homogenates were boiled at 100°C for 5 min, and then centrifuged. The supernatants of extracts were electrophoretic ally separated on SDS polyacrylamide gels containing 12% acrylamide and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Osaka, Japan). The blotted membranes were blocked with TBS/ 0.05% Tween containing 5% skim milk for 1 hour at 25°C, followed by incubation with Guinea pig polyclonal anti-SCOX at a 1:2,000 dilution for 16 hours at 4°C. After washing, the membranes were incubated with HRP-conjugated anti-Guinea pig IgG (GE Healthcare Bioscience, Tokyo, Japan) at 1:10,000 dilution for 2 hours at 25°C. Antibody binding was detected by using ECL Western blotting

detection reagents (GE Healthcare Bioscience) and images were analyzed using a Lumivision Pro HSII image analyzer (Aisin Seiki, Kariya, Japan). To confirm equal amounts of protein loading in each lane, the membranes were also probed with an anti-α-tubulin antibody after stripping the complex of anti-SCOX antibody and HRP-conjugated anti-Guineapig IgG. For the detection of α-tubulin, mouse anti-α-tubulin monoclonal antibody (1:5,000 dilution, Sigma, Tokyo, Japan) and an HRP-conjugated anti-mouse IgG (1:10,000 dilution, GE Healthcare Bioscience) were used as the primary and secondary antibody, respectively.

1.2.8. Quantitative RT-PCR

Total RNAs from whole bodies of *Drosophila* were purified with Trizol (Invitrogene) and 1μg aliquots were reverse transcribed with oligo dT primers using a PrimeScript High Fidelity RT-PCR Kit (Takara). Then, quantitative RT-PCR was performed with a SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara) kit and the Applied Biosystems 7500 quantitative RT-PCR system using 1μg of reverse transcribed sample per reaction. Levels of mRNA in each fly strain were investigated by the C_T comparative method [17]. The *Rp49* was used as an endogenous reference gene. Experiments were performed in triplicate for each of three RNA batches isolated separately.

1.2.9. ATP Assays

A CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) was used to quantify the level of ATP present. Each adult fliy was ground in 100μl Assay Kit buffer and then centrifuged at 12,000 g for 10 min. The supernatant was transferred into a new microtube and 10μl of the supernatant was mixed with 100μl of measure buffer,

followed by incubation for 10 min at 25°C to stabilize luminescent signals before being read on a Lumat LB 9507 luminometer (Berthold).

1.2.10. Climbing Assays

Climbing assays were performed at 25°C and 60% humidity. *Act5C*-GAL4>UAS-*SCOX* flies and wild type flies were placed at 28°C, and newly eclosed adult flies were separated and placed in vials at a density of 20 adult male flies per vial (3 vials was done in each genotype). Flies were transferred, without anesthesia, to a hinge tube with a line at the height of 17.5cm. The tube was tapped to collect the flies to the bottom, and they were then given 5 min to climb the wall. The time when the first fly crossed the line was noted. After 5 min, the number of flies above the line was counted. These climbing assays were carried out at newly elosed time and 3 weeks, and 4 weeks after elosing.

1.2.11. Paraquat Assays

Paraquat assays were carried out in a humidified, temperature controlled incubator at 28°C and 60% humidity under 12-hour light and 12-hour dark cycle culture condition. Instant food with added 10mM paraquat was used. Newly eclosed male adult flies of control flies carrying *Act5C-GAL4* alone, and *Act5C*-GAL4>UAS-*SCOX* transgenic flies were separated and placed in vials at low density (20 flies per vial and in this experiment 3 vials for each genotype were used.). Every 2 days, they were transferred to new tubes containing fresh food and deaths were scored. Survival functions were estimated using the Kaplan-Meier procedure [16] and plotted as survival curves.

1.2.12. Data analysis

All statistical analyses were performed using Microsoft Excel. The Kaplan-Meier procedure was used for assessment of the statistical significance of comparisons between groups of data concerning the median lifespan. For other assays, two-way ANOVA was applied to assess the statistical significance of differences between groups of data. When two-way ANOVA showed significant variation, a Dunnet's test was subsequently used for pairwise comparisons. All data are shown as means ±SEM.

1.3. Results

1.3.1. Specificity of the anti-SCOX antibody and evaluation of SCOX level in transgenic flies carrying UAS-SCOXIR and UAS-SCOX

We raised a polyclonal antibody against SCOX as a tool for studying *in vivo* roles of *SCOX*. To investigate its specificity, Western immunoblotting was carried out with the extracts of adult-male flies of wild type Canton S. A band corresponding to approximately 25 kDa was detected in extracts from Canton S using the anti-SCOX antibody (Figure 2A, lane2). The size of the detected band is nearly identical to the size (28,175.2 kDa) of SCOX protein which is predicted based on its amino acid composition. The faster migrating band was also detected with the Western blot without the first antibody, suggesting the band to be non-specifically detected with the secondary antibody. Moreover, a single band with an apparent molecular weight of 29 kDa was detected in extracts from *E. coli* producing His-SCOX (full length) fusion protein (Figure 2A, lane 1). The data indicate that the prepared antibody is highly specific to the SCOX protein.

To confirm the knockdown or expression of SCOX in the transgenic flies *Act5C*-GAL4>UAS-*SCOXIR*[7861], Western immunoblotting was performed again with anti-SCOX antibody. In extracts from the *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*[7861] flies, no 25kDa SCOX was detected, confirming that SCOX was effectively knocked down in the *Act5C*-GAL4>UAS-*SCOXIR*[7861] flies (Figure 2B, lane 3).

To further examin the knockdown or expression of SCOX in the transgenic flies- *Act5C*-GAL4>UAS-*SCOXIR*[20], Quantitative RT-PCR analysis was performed. In extracts from the *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*[20] flies, a little of SCOX mRNA was detected as compared to control flies carrying *Act5c-GAL4* alone, confirming that SCOX was efficiently knocked down in the *Act5C*-GAL4>UAS-*SCOXIR*[20] flies (Figure 2C). However, there was no significant increase in SCOX protein level in extracts from the transgenic *Act5C*-GAL4>UAS-*SCOX* flies in compared with the wild type flies (Figure 2B, lane 1 and 2). In contrast, the *SCOX* mRNA level was increased 23.8 fold in the transgenic *Act5C*-GAL4>UAS-*SCOX* flies as compared to control flies carrying *Act5C-GAL4* alone (Figure 2D).

1.3.2. Decreased ATP level correlates with lethality in SCOX-knockdown flies

Using the *Act5C-GAL4* driver that expresses GAL4 in the whole body of *Drosophila*, we examined the morphology, the life cycle, and the lifespan of the two independent *SCOX*-knockdown flies, the *Act5C*-GAL4>UAS-*SCOXIR*[7861] flies and the *Act5C*-GAL4>UAS-*SCOXIR*[20] flies, in comparison with wild type flies and control flies carrying *Act5C-GAL4* alone.

No apparent difference in morphology was observed with larvae and adults from these flies. However, *Act5C*-GAL4>UAS-*SCOXIR*[7861] was associated with lethality at the larval stage. Larvae of this knockdown strain survived for 12 days after hatching but demonstrated reduced motility day by day until death. The larvae stayed within the medium with low mobility or no climbing of the walls of culture tubes, in clear contrast to wild type and control flies. The other *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*[20] flies died at the pupal stage. Dissection of the pupae revealed that the knockdown flies grew to the pharate adult stage, but could not hatch.

Previous studies provided evidence that SCOX is required for assembly of the COX complex [18, 19]. In eukaryotic cells, this is crucially important for aerobic respiration in mitochondria, where the majority of molecular oxygen is consumed and high amounts of energy (36-38 ATP) are released. Therefore, decrease of SCOX in the *SCOX*-knockdown flies may lead to decrease in activity of mitochondria, and lowered ATP. To examine this hypothesis, ATP levels were measured in *SCOX*-knockdown flies. That of *Act5C*-GAL4>UAS-*SCOXIR*[7861] larvae was equal to that of wild type flies for the first two days but then significantly decreased from the third day (Figure 3B) ($p < 0.001$) and continued decreasing until death. In contrast, the *Act5C*-GAL4>UAS-*SCOXIR*[20] flies show no significant difference in ATP level as compared with type flies, and the control flies carrying *Act5C-GAL4* alone at the third larval stage (Figure 3A). However, at the pupal stage, a significant decrease in ATP level was observed at days 1 and 3 in compared to the wild type flies (Figure 3C). At the fifth day of the pupal stage (pharate adult stage), a further decrease in ATP level was observed, as comparing to the third day pupal stage (Figure 3C), while wild type flies showed a constant of ATP level. These results showed SCOX to be essential for the

viability of *Drosophila* and the lethal phase coincides with the development stage of the knockdown flies showing the lowest ATP levels.

1.3.3. Increased ATP level correlates with prolonged lifespan in the transgenic flies carrying the full length SCOX

We also examined morphology and lifespan of full length SCOX transgenic flies (*Act5C*-GAL4>UAS-*SCOX*). While the *Act5C*-GAL4>UAS-*SCOXIR*[20] and *Act5C*-GAL4>UAS-*SCOXIR*[7861] knockdown flies showed pupal and larval lethality, respectively, the *Act5C*-GAL4>UAS-*SCOX* flies developed normally and eclosed a little faster than the control flies carrying *Act5C-GAL4* alone. In addition, there was no apparent difference in morphology throughout the developmental stages. However, a longer lifespan of adult flies was observed with the *Act5C*-GAL4>UAS-*SCOX* flies as compared with the control and wild type flies (Figure 4). The median lifespan of wild type flies was 60 days; that of the control flies was 58 days, whereas that of the *Act5C*-GAL4>UAS-*SCOX* flies was an average of 69 days. The differences in lifespan in comparing of the *Act5C*-GAL4>UAS-*SCOX* flies to wild type flies and the control flies were statistically significant ($p < 0.05$ and $p < 0.005$, respectively).

In order to evaluate the functional differences between *Act5C*-GAL4>UAS-*SCOX* and wild type flies, we performed climbing assays with newly eclosed flies and flies at 3-weeks and 4-weeks after eclosion. The *Act5C*-GAL4>UAS-*SCOX* flies showed better mobility than wild type flies at the same age (Figure 5), while there was no significant difference in mobility between wild type flies and the control flies (data not shown). We performed two different climbing assays and in both the *Act5C*-GAL4>UAS-*SCOX* flies showed better mobility than the wild type flies with a faster climbing time and a longer time remaining at elevation (Figure 5).

All of these increases in mobility were statistically significant (p<0.05 and p<0.01, respectively).

To examine the activity of mitochondria at the age in which significant differences in climbing assays were observed in *Act5C*-GAL4>UAS-*SCOX* flies, ATP assays were performed with newly eclosed, 3-week-old and 4-week-old *Act5C*-GAL4>UAS-*SCOX* and wild type flies. The results showed increased ATP levels in the *Act5C*-GAL4>UAS-*SCOX* flies at both time points (p<0.001) (Figure 3D). Moreover, while decrease of ATP level was observed in 4-week as compared with 3-week-old wild type flies (p< 0.05), this was not observed with *Act5C*-GAL4>UAS-*SCOX* flies (Figure 3D).

In summary, on clear contrary to the *SCOX*-knockdown flies, the *Act5C*-GAL4>UAS-*SCOX* flies showed extension of lifespan. These results taken together suggest that decrease of ATP in *SCOX*-knockdown flies leads to the lethality at young age, while increase in the *Act5C*-GAL4>UAS-*SCOX* flies helps to extend their lifespan.

1.3.5. Role of SCOX in alleviating mitochondrial oxidative stress

Decrease of ATP observed in *SCOX*-knockdown larvae apparently correlated with death at young age, suggesting that the defect in SCOX affects activity of mitochondria. In previous studies, mitochondrial ROS (mtROS) production was reported to correlate with the lifespan in *Drosophila*; a decrease in mtROS production increased and increase in decreased the lifespan [17]. In the present study, when newly eclosed male adults of wild type flies and the *Act5C*-GAL4>UAS-*SCOX* flies were collected and fed food containing 10mM paraquat, known to impair the mitochondrial functions by increasing the level of ROS [20-22], the lifespan of

Act5C-GAL4>UAS-*SCOX* flies was observed to be longer than those of wild type and control flies under the same conditions (Figure 6). The median lifespan of both wild type and control flies was 25 days whereas that of *Act5C*-GAL4>UAS-*SCOX* flies was an average of 30 days. These data suggest that SCOX might reduce the level of mtROS and consequently prolong the lifespan.

1.4. Discussion

In a previous study, SCO2 mutation was found to lead to reduced-ATP levels [23]. Here I showed equivalent decrease in ATP in *SCOX*-knockdown flies, pointing to similarity between *Drosophila* SCOX and human SCO2 in functions. Recently, mutations in *SCO2* have been reported in patients with fatal infantile cardioencephalomyopathy and cause cytochrome c oxidase deficiency [11]. In man, cytochrome c oxidase deficiency is a genetic condition that can affect several parts of the body, including the muscles, the heart, the brain and the kidney [7]. Common signs or symptoms of cytochrome c oxidase defects are frequently found in childhood. Cytochrome c oxidase receives an electron from each of four cytochrome c molecules, transfers them to a single oxygen molecule and then converts molecular oxygen to two molecules of H_2O. In addition, it translocates four protons across the membrane, establishing a transmembrane difference of proton-electrochemical potential that allows ATP synthase to synthesize ATP. This means that the lack of functional cytochrome c oxidase disrupts the mechanism, called oxidative phosphorylation, and causes a decrease in energy production which may result in death. In the body of each individual, the brain, the muscle and the heart require the largest amount of energy for them to function. Decrease in energy may lead to decrease in their activity and induce death. In

this study, the *SCOX*-knockdown flies demonstrated pupal or larval lethality which was accompanied by a decrease in ATP level, observed prior to the stage before death. Larval lethality is consistent with the previous report with scox null mutants [14]. Furthermore, full length SCOX transgenic adult flies showed longer lifespan and higher mobility than the wild type flies, accompanied by an increase in ATP level. Our data therefore suggest that SCOX plays an important role in controlling ATP production and/or consumption to keep cells and organs in normal activity.

In the full length SCOX transgenic flies, the SCOX mRNA level was observed to be increased as analyzed by quantitative RT-PCR, but no apparent increase was observed in SCOX protein level as analyzed by Western immunoblotting analysis. However, the full length SCOX transgenic flies show a longer lifespan than the wild type flies. These results suggest a possibility that overexpression of SCOX in the full length SCOX transgenic flies may be transient and some unknown negative feedback mechanism may operate to keep SCOX protein levels constant. Even under these conditions, some genes that act against aging may be affected.

Paraquat, a nonselective herbicide, induces oxidative stress by increasing the production of ROS, which is a toxic by-product of mitochondrial energy production and oxidative phosphorylation in cancer cells [24]. An increase of ROS may lead to signs associated with death and also linked to aging [21]. In our study, the *Act5C*-GAL4>UAS-*SCOX* flies exhibited a longer lifespan than the wild type flies under conditions of 10 mM paraquat feeding. In a previous study, Ca^{2+} was shown to play a role in ATP synthesis and in ROS generation, and most of the mitochondrial effect of Ca^{2+} requires its entry across double-membrane into the matrix. Under physiological conditions, Ca^{2+} is beneficial for mitochondrial functions, but in the

presence of an overriding pathological stimulus, Ca^{2+} may be detrimental which leads to an increase of ROS-associated cell death [25]. The available observations thus suggest that SCOX may help to prevent the formation of Ca^{2+} with pathological stimuli.

The results in the thesis demonstrated knockdown of *SCOX* gene in all cells and tissues to be associated with lethality at larval or pupal stages and this correlated with a decrease in ATP level. In contrast, the full length SCOX transgenic flies showed a longer lifespan than wild type flies and control flies carrying *Act5C-GAL4* alone and this correlated with an increase in ATP level. Finally, when cultured on paraquat-added medium, full length SCOX transgenic flies also exhibited an elongated lifespan. Therefore, we hypothesized that SCOX plays an important role in ATP production and consumption, which helps to prevent production of mitochondrial reactive oxidative species and/or impairment of mitochondrial activity under oxidative stress.

In conclusion, this thesis identified characteristics of the whole bodies of *SCOX*-knockdown and full length SCOX transgenic flies. We propose that SCOX plays an important role in mitochondrial anti-oxidative stress mechanisms or in a mitochondrial death pathway that is important for *Drosophila* lifespan.
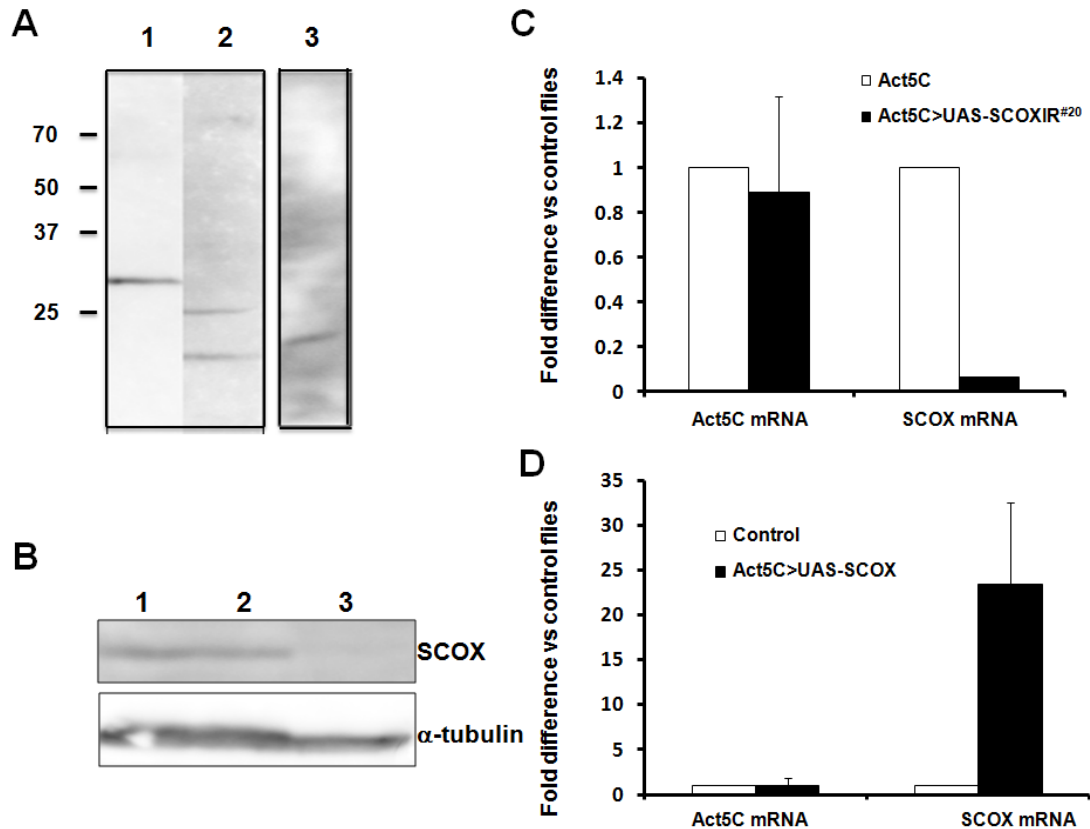
Figure 2: *A*, Western immunoblotting analysis with extracts from Canton S adult-fly and *E. coli* producing His-SCOX (full length) fusion protein. Lane1: *E. coli* extracts; lane 2: Canton S extracts and lane 3: Canton S extracts with only secondary antibody. Positions of size markers are shown in left side (kDa). *B,* Western immunoblotting analysis of the protein extract from whole bodies of adult flies. Lane 1: Canton S, lane 2: *w; UAS-SCOX/+; Act5C-GAL4/+,* and lane 3: *w; +; Act5C-GAL4/ UAS-SCOXIR[7861]*. In the *w; +; Act5C-GAL4/ UAS-SCOXIR[7861]* fly extracts no SCOX band was detectable. *C,* Quantitative RT-PCR for *SCOX* mRNA levels in *w; UAS-SCOXIR[20]/+; Act5C-GAL4/+* and and *w; +; Act5C-GAL4/+* flies. A significant decrease of *SCOX* mRNA expression level in *w; UAS-SCOXIR[20]/+; Act5C-GAL4/+*

flies was observed, compared to *w, +; Act5C-GAL4/+* flies (n=3). **D,** Quantitative RT-PCR for *SCOX* mRNA levels in *w; UAS-SCOX/+; Act5C-GAL4/+* flies and *w; +; Act5C-GAL4/+* flies. An increase of *SCOX* mRNA expression level in *w; UAS-SCOX/+; Act5C-GAL4/+* flies was observed, compared to *w, +; Act5C-GAL4/+* flies (n=3). qPCR-SCOX-forward primer ; 5'-GAGAAGGATGAGGCGAGAATG-3' and qPCR-SCOX-reverse primer; 5'-GCTCCCTGCGAATCAACTAA-3' were used in this assay
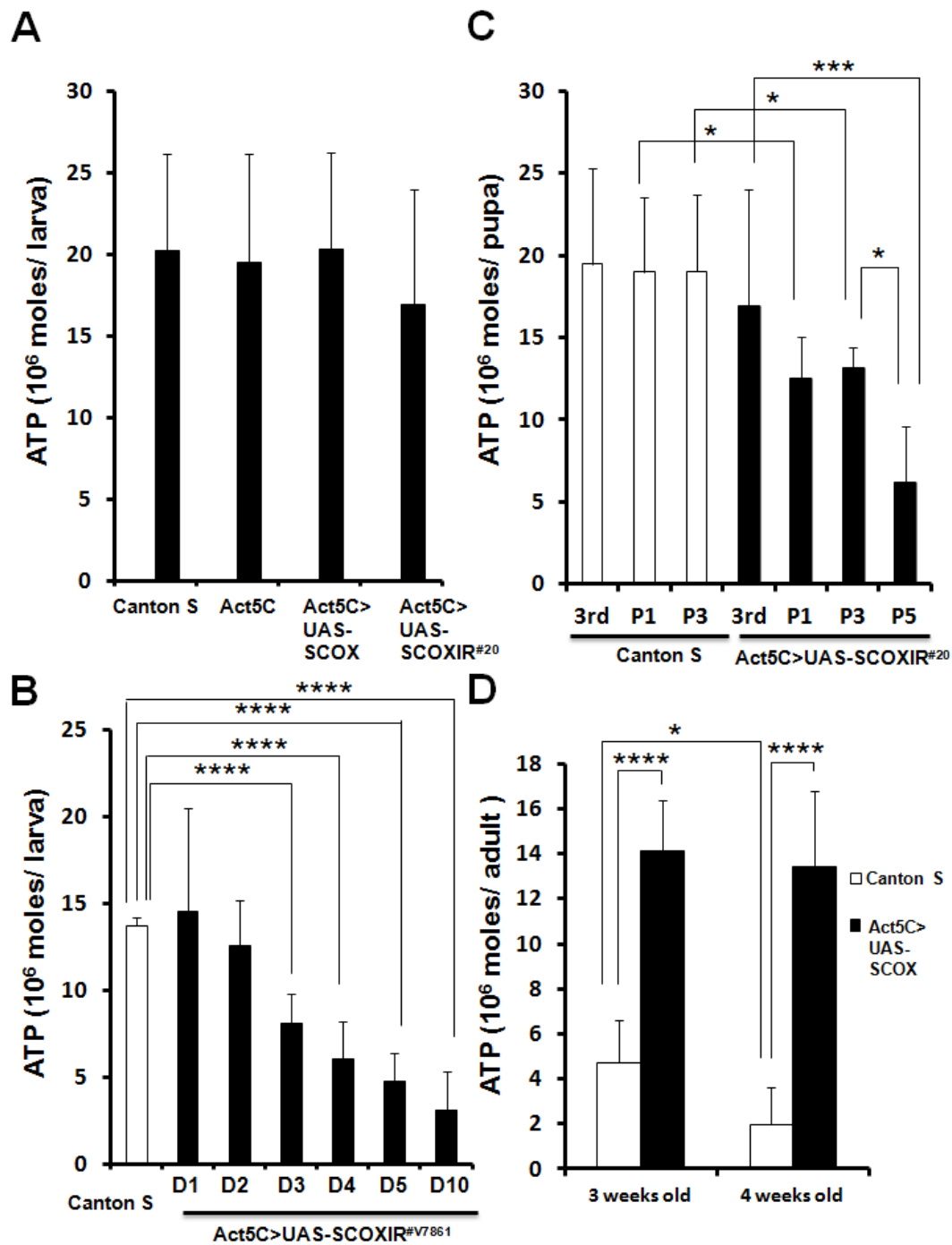
Figure 3: ATP Assay. *A,* ATP levels in third instar larvae. There was no significant difference in ATP levels among the indicated genotypes. *B,* ATP level in the larvae of *w; +; Act5C-GAL4/UAS-SCOXIR^7861* flies. The results showed that there was no significant difference in ATP level in the first two days of the second larval stage, but

decrease was noted from the third day. *C,* ATP level in third instar larvae and pupae with *w; UAS-SCOXIR[20]/+; Act5C-GAL4/+* flies or Canton S flies. A significant decrease was observed in the fifth day of the pupal stage (P5), compared to the third larvae stage (3rd) and the third day of the pupal stage (P3). Canton S flies showed constant levels of ATP. *D,* ATP level of adult flies with *w; UAS-SCOX/+; Act5C-GAL4/+* and Canton S at 3-weeks and 4 weeks of age. An increase in ATP level was observed in *w; UAS-SCOX/+; Act5C-GAL4/+* flies, compared to wild type flies. The horizontal bars indicate the standard errors of the mean values. $****p<0.001$, $***p<0.005$, $**p<0.01$, $*p<0.05$
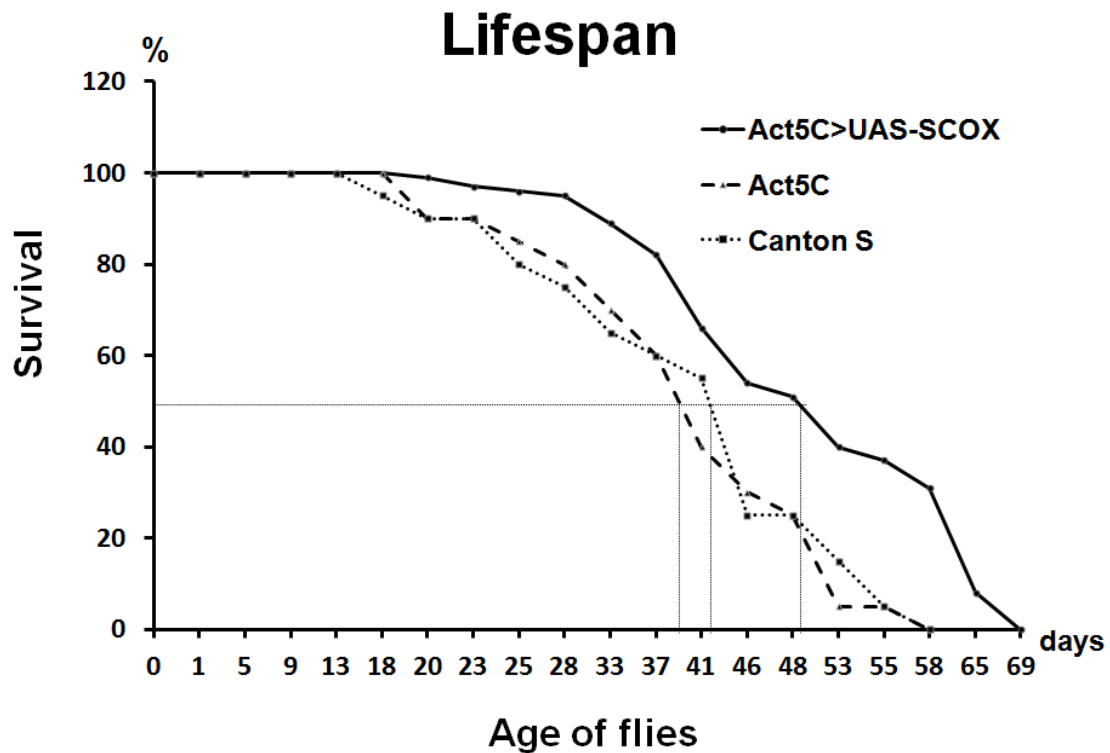
Figure 4: Lifespan analysis of *w; UAS-SCOX/+; Act5C-GAL4/+* flies, *w; +; Act5C-GAL4/+* flies and Canton S flies. Percentage survival of adult-male flies of the indicated genotypes is shown. Flies were collected and divided into 5 vials. The total number of counted flies was n=100 for each fly strain. There was no significant difference in the lifespan of wild type and *w; +; Act5C-GAL4/+* flies, but an extension of lifespan was observed in *w; UAS-SCOX/+; Act5C-GAL4/+* flies. The lifespan differences between *w; UAS-SCOX/+; Act5C-GAL4/+* flies and wild type flies or *w, +; Act5C-GAL4/+* flies were statistically significant ($p<0.05$ and $p<0.005$, respectively). Results were analyzed using OASIS software (http://sbi.postech.ac.kr/oasis)

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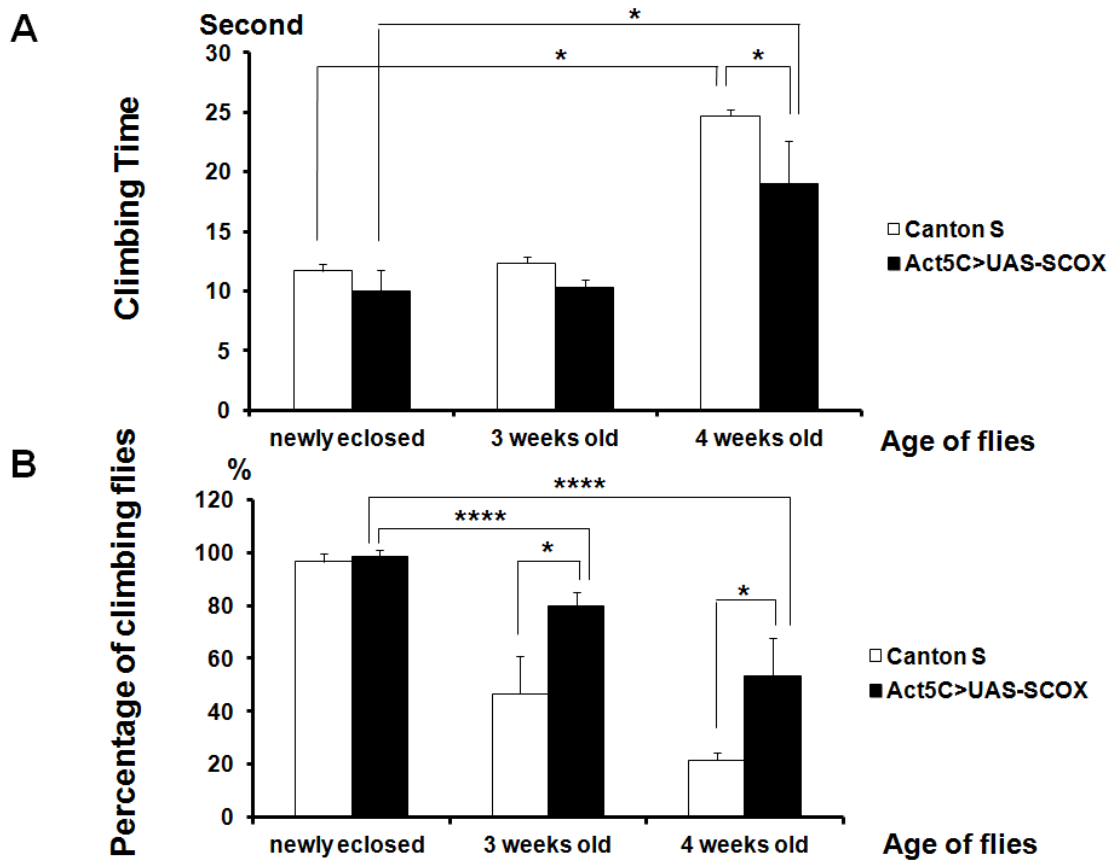
Figure 5: Climbing assay results. Two independent tests were performed for *w; UAS-SCOX/+; Act5C-GAL4/+* flies and Canton S as wild type flies. The total number of counted male-flies was n=60 for each fly strain. ***A,*** Time needed for the first fly climbing to the line (at height of 17.5 cm). There was no significant difference between *w; UAS-SCOX/+; Act5C-GAL4/+* flies and wild type flies at 3-weeks of age. However, with 4-week-old flies, while a longer climbing time was observed in each fly strain, *w; UAS-SCOX/+; Act5C-GAL4/+* flies took less time. ***B,*** Percentage of flies above the line 5 minutes after being put into tube. A decrease was observed at ages of 3-weeks and 4 weeks *w; UAS-SCOX/+; Act5C-GAL4/+* flies. Furthermore, at these latter ages *w; UAS-SCOX/+; Act5C-GAL4/+* flies were observed to keep being above the line longer

than wild type flies. The horizontal bars indicate the standard errors of the mean values.

****p<0.001, ***p<0.005, *p<0.05
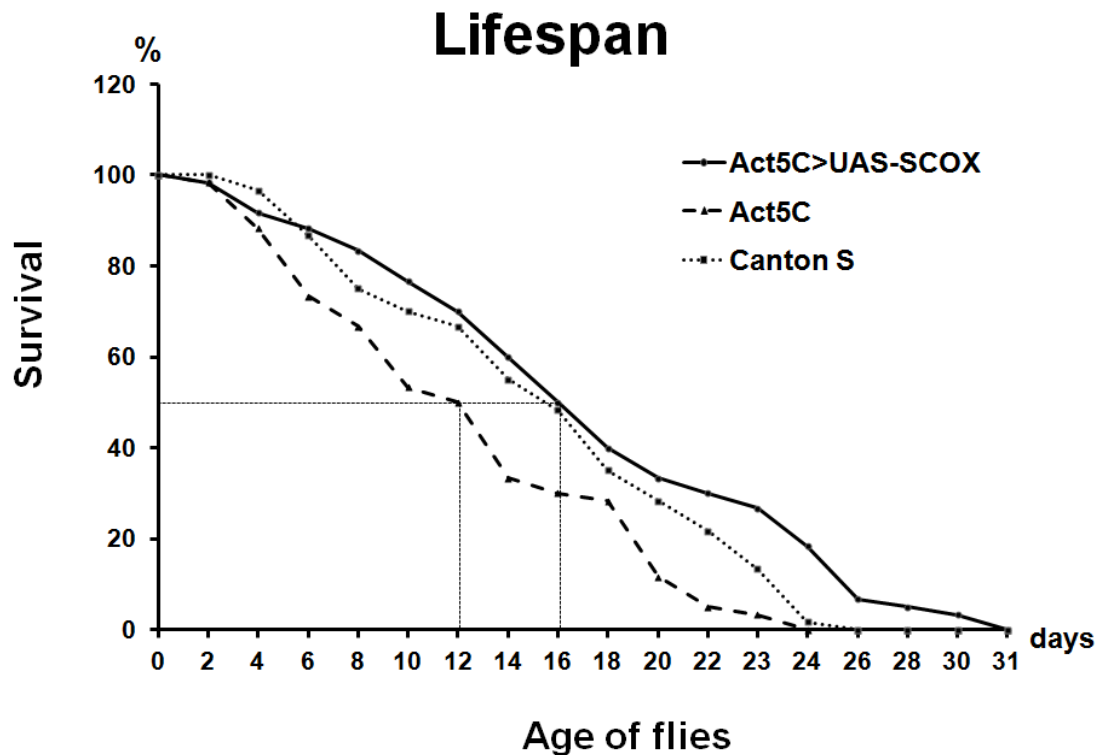
Figure 6: Lifespan analysis of each fly strain in the paraquat assay. Flies were collected and divided into 20 flies per vial (3 vials in total). Adult flies were cultured by food containing 10mM paraquat. The total number of counted flies was n=60 for each fly strain. Percentage survival of adult male-flies of the indicated genotypes is shown. There was no significant differences in the lifespans of wild type and control flies, but an extension was observed in the lifespan of *w; UAS-SCOX/+; Act5C-GAL4/+* flies. The lifespan differences between *w; UAS-SCOX/+; Act5C-GAL4/+*, wild type and *w; +; Act5C-GAL4/+* flies were statistically significant (p<0.005, in both cases).

1.5. References

[1]   Jake GH, Kathleen SM, Jing Z and Thomas JM. Mitochondrial therapeutics in Alzheimer's disease and Parkinson's disease. Alzheimers Res. Ther,   2011. 3: 21.

[2]   Chial H and Craig J. mtDNA and mitochondrial diseases. Nat Edu, 2008. 1(1): 217.

[3]   Aleksandra M, Katarzyna GW, Ireneusz S and Cerazy Z. Mitochondrial DNA in pathogenesis of Alzheimer's and Parkinson's diseases. Acta Neurobiol, 2006. 66: 153-176.

[4]   Timothy H and Gino C. A mitochondria DNA clone is associated with increased risk for Alzheimer disease. Proc. Natl. Acad. Sci. USA, 1995. 92: 6892-6895.

[5]   Chen Y, Cairns R, Papandreou I, Koong A, Denko NC. Oxygen consumption can regulate the growth of tumors, a new perspective on the Warburg effect. PLoS One, 2009. 4:e7033.

[6]   Jaksch M, Paret C, Stucka R, Horn N, Muller-Hocker J, Horvarth R, Trepesch N, Stecker G, Freisinger P, Thirion C Muller J, Lunkwitz R, Rodel G, Shoubridge AE, Lockmuller. Cytochrome *c* oxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts. Oxford Journal, 2001. 10:3025-3035.

[7]   Zeviani M, Tiranti V and Piantadosi C. Mitochondrial disorders. Medicine (Baltimore), 1998. 77:59-72.

[8]   Sanz A, Fernández-Ayala DJ, Stefanatos RK, Jacobs HT. Mitochondrial ROS production correlates with, but does not directly regulate lifespan in *Drosophila.* Aging, 2010. 2: 200-223.

[9]   Papadopoulou LC, Sue CM, Davidson MM, Tanji K, Nishino I, Sadlock JE, Krishna S, Walker W, Selby J, Glerum DM, Van Coster R, Lyon G, Scalais E, Lebel R, Kaplan P, Shanske S, De Vivo DC, Bonilla E, Hirano M, DiMauro S,Schon EA. Fatal infantile cardioencephalomyopathy with COXdeficiency and mutations in *SCO2*, a COX assembly gene. Nat Genet, 1999. 23: 333-337.

[10]   Sue CM, Karadimas C, Checcarelli N, Tanji K, Papadopoulou LC, Pallotti F, Guo FL, Shanske S, Hirano M, De Vivo DC, van Coster R, Kaplan P, Bonilla E, DiMauro S. Differential features of patients with mutations in two COX assembly genes, *SURF-1* and *SCO2*. Ann Neurol, 2000. 47: 589-595.

[11]   Vesela K, Hansikova H, Tesarova M, Martasek P, Elleder M, Houstek J, Zeman J. Clinical, biochemical and molecular analyses of six patients with isolated cytochrome c oxidase deficiency due to mutations in the *SCO2* gene. Acta Paediatr, 2004. 93: 1312-1317.

[12]   Bohm M, Pronicka E, Karczmarewicz E, Pronicki M, Piekutowska-Abramczuk D, Sykut-Cegielska J, Mierzewska H, Hansikova H, Vesela K, Tesarova M, Houstkova H, Houstek J and Zeman J. Retrospective, multicentric study of 180 children with cytochrome c oxidase deficiency. Pediatrian Res, 2006. 59: 21-26.

[13]   Won KY, Lim SJ, Kim GY, Kim YW, Han SA, Song JY, Lee DK. Regulatory role of p53 in cancer metabolism via SCO2 and TIGAR in human breast cancer. Human Pathology, 2011. 43: 221-228.

[14]   Porcelli D, Oliva M, Duchi S, Latorre D, Cavaliere V, Barsanti P, Villani G, Gargiulo G, Caggesse C. Genetic, functional and evolutionary characterization of *scox*, the *Drosophila melanogaster* ortholog of human *SCO1* gene. Mitochondrion, 2010. 10: 433-448.

[15]   Spradling AC. P-element-mediated transformation. In: Robertsd.B. (Ed.), *Drosophila*: A Practical Approach. Oxford (IRL Press) 1986. pp. 175-197.

[16]   Yang JS, Nam HJ, Seo M, Han SK, Choi Y, Nam HG, Lee SJ, Kim S. OASIS: Online application for the survival analysis of lifespan assays performed in aging research. PloS ONE, 2011. 6:e23525.

[17]   Morrison TB, Weiss JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques, 1998. 24: 954-958, 960, 962.

[18]   Diaz F. Cytochrome c oxidase deficiency: patients and animal models. Biochim Biophys Acta, 2009. 1802: 100-110.

[19]   Leary SC, Kaufman BA, Pellecchia G, Guercin GH, Mattman A, Jaksh M, Shoubridge EA. Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. Hum Mol Genet, 2004. 13: 1839-1848.

[20]   Ramiro L, Nacira M, German R, Marianela R, Mariana M, Victorio T and Gaston Q. Paraquat: an oxidative stress inducer, Herbicides - Properties, Synthesis and Control of Weeds. Dr. Mohammed Nagib Hasaneen (Ed.), ISBN, 2012: 978-953-307-803-8, InTech: pp. 135-148.

[21]   McCarthy S, Somayajulu M, Sikorska M, Borowy-Borowski H, Pandey S. Paraquat induces oxidative stress and neuron cell death; neuroprotection by water-solube coenzyme Q10. Toxicology and applied pharmacology, 2004. 201: 21-31.

[22]   Bus JS, Aust SD, Gibson JE. Paraquat toxicity: Proposed mechanism of action involving lipid peroxidation. Environ Health Perspect, 1976. 16: 139-146.

[23]   Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurey PJ, Bunz F, Hwang PM. p53 regulates mitochondrial respiration. Science, 2006. 312: 1650-1653.

[24]   Vijay P, Stewart F, Alan GL and Blanka R. The effect of age on radiation resistance and oxidative stress in adult *Drosophila melanogaster*. Radiation Research, 2008. 169: 707-711.

[25]   Brookes, Paul S, Yisang Y, James LR, Anders MW, Shey-Shing S. Calcium, ATP, and ROS: a mitochondrial love-rate triangle. Am J Physiol Cell Physiol, 2004. 287: 817-833.

Chapter 2:

Genetic link between *Cabeza*, a *Drosophila* homologue of *Fused in Sarcoma* (*FUS*), and the EGFR signaling pathway

2.1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterized by degeneration of upper and lower motor neurons of the brain and the spinal cord, which leads to progressive muscle weakness and fatal paralysis [1]. Most cases of ALS are sporadic, but some patients have a familial history as a result of a mutation in the gene for Cu/Zn superoxide dismutase (*SOD1*) [2].

The family of MAPKs includes ERK, p38 and JNK. Each MAPK signaling pathway consists of at least three components, a MAPK kinase kinase, a MAPK kinase and a MAPK. Deviation from strict control of MAPK signaling pathways has been implicated in the development of human neurodegenerative diseases including Alzheimer's, Parkinson's and ALS [3]. Recently it was reported that aberrant expression and activation of p38 in motor neurons and microglia play important roles in ALS progression [4]. Persistent activation of p38 correlates with degeneration of motor neurons in transgenic mice expressing a mutant *SOD1* [5,6]. Moreover a p38 inhibitor was demonstrated to prevent the apoptosis of motor neurons induced by a mutant *SOD1* [7]. Thus a possible link between MAPK signaling and ALS has been suggested.

A substantial number of proteins linked to ALS are directly or indirectly involved in RNA processing [8]. Among RNA-binding proteins, mutations in the *TAR DNA-binding protein of 43kDa* gene (*TDP-43*) and *fused in sarcoma* (*FUS*) gene have

been identified as major genetic causes in both familial and sporadic ALS [9-18]. TDP-43 and FUS are implicated in multiple aspects of RNA metabolism including transcriptional regulation, mRNA splicing and mRNA shuttling between the nucleus and the cytoplasm [19,20].

Drosophila melanogaster has a single orthologue of human FUS, named Cabeza (Caz). *In situ* hybridization and immunohistochemistrical analyses demonstrated that Caz mRNA and protein are enriched in the brain and central nervous system (CNS) during embryogenesis, and the Caz protein has been detected in the nuclei of several larval tissues and in imaginal discs [21,22]. The full-length recombinant Caz protein and its RRM domain are capable of binding RNA in vitro [21]. These findings suggest that Caz is a nuclear RNA binding protein that may play an important role in the regulation of RNA metabolism during *Drosophila melanogaster* development.

In the previous studies using neuron specific *Caz*-knockdown flies, it was demonstrated that Caz functions in neuronal cell bodies and/or axons of the CNS and is involved in elongation of synaptic branches of motoneurons [22]. However, contributions of Caz during development of various tissues in *Drosophila melanogaster* are not fully understood. As a first step toward clarification, we investigated the effect of knockdown of *Caz* on eye development and revealed a rough eye phenotype, accompanied by apoptosis, abnormal differentiation of cone cells and defects in ommatidia rotation. In addition, a *Rhomboid-1* mutant could be shown to rescue the fusion of cone cells and mutations of *rhomboid-3* and mirror significantly suppressed the rough eye phenotype of the *Caz*-knockdown flies. Since *rhomboid-1, rhomboid-3,* and *mirror* are EGFR pathway-related genes, these results indicate genetic links between Caz and EGFR signaling.

2.2. Materials and Methods

2.2.1. Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Canton S was used as the wild type. *w; UAS-Caz-IR363-399; +* (CG3606) and *UAS-rho-IR^{28690}* was obtained from Vienna *Drosophila* RNAi Center (VDRC). The RNAi of this strain was targeted to the region corresponding to residues 363-399 of *Drosophila Caz (UAS-Caz-IR$_{363-399}$)*. Four and seven transgenic strains carrying *UAS-Caz-IR$_{1-167}$* and *UAS-Caz-$_{IR180-346}$* were established, respectively [22]. Each transgenic strain showed a consistent phenotype. Alleles of the following genes were obtained from the Bloomington *Drosophila* stock center: *mirror^{SaiD3}, ru^{1}, rho^{7M43} and rho^{AA69}*. Enhancer trap lines carrying the lacZ markers AE127 (inserted into *seven-up*) [23] and P82 (inserted into *deadpan*) [24] were obtained from Y. Hiromi. These lines express the β-galactosidase marker in photoreceptor cells (R) of R3/R4/R1/R6 and R3/R4/R7, respectively. *hspFlp; +; tub1> FRT cd2 FRT> GAL4, UAS-GFP/ TM3* was a kind gift from A. Plessis. Establishment of lines carrying GMR-GAL4 was as described earlier [25]. Act5C-GAL4/ TM6B flies were also obtained from the Bloomington *Drosophila* stock center.

2.2.2. Generation of RNAi clones in retinae

RNAi clones in retinae were generated with the flip-out system [26]. Female flies with *hspFlp; +; tub1> FRT cd2 FRT> GAL4, UAS-GFP/ TM3* were crossed with *w; UAS-Caz-IR$_{363-399}$; +* male flies and clones were marked by the presence of GFP. Flip-out was induced 24-48 h after egg laying with a 60 min heat shock at 37°C.

2.2.3. Immunostaining

For immunohistochemistory, larval eye imaginal discs and pupal retinae were dissected, and fixed in 4% paraformaldehyde/ PBS for 15 min and 30 min at 25°C, respectively. After washing with PBS containing 0.3% Triton X-100, the samples were blocked with PBS containing 0.15% Triton X-100 and 10% normal goat serum for 30 min at 25°C, and incubated with diluted primary antibodies in PBS containing 0.15% Triton X-100 - 7 - and 10% normal goat serum for 16h at 4°C. The following antibodies were used: mouse anti-LacZ (1:500, Developmental Studies Hybridoma Bank [DSHB], 40-1a), mouse anti-Elav (1:200 DSHB 9F8A9), mouse anti-Cut (1:500, DSHB 2B10), and mouse anti-Discs large (1:500) (DSHB) anf anti-di-phospho ERK (dpERK) (1:500) (Sigma). After extensive washing with PBS containing 0.3% Triton X-100, samples were incubated with secondary antibodies labeled with either Alexa 546 or Alexa 488 (1:400, Invitrogen) for 3h at 25°C. Alexa 488-conjugated phalloidin (200 units/ ml) was used for the detection of F-actin. After extensive washing with PBS containing 0.3% Triton X-100, samples were mounted in Vectashield (Vector Laboratories Inc.) and analyzed by confocal laser scanning microscopy (Olympus Flouview FV10i).

2.2.3. Western immunoblot analysis

Protein extracts from the whole pupae of Drosophila carrying *Act5C-GAL4/+* or *Act5C-GAL4/+; UAS-Caz-IR$_{363-399}$/+* were prepared as previously described [22]. The homogenates were boiled at 100 °C for 5 min, and then centrifuged. The supernatants (extracts) were electrophoretically separated on SDS-polyacrylamide gels containing 12% acrylamide and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The blotted membranes were blocked with TBS/0.05% Tween containing 5% skim milk for 1 h at 25 °C, followed by incubation with rabbit polyclonal

anti-Caz at a 1:5,000 dilution for 16h at 4°C. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (GE Healthcare Bioscience) at 1:10,000 dilution for 2 h at 25°C. Antibody binding was detected using ECL Western blotting detection reagents (GE Healthcare Bioscience) and images were analyzed using a Lumivision Pro HSII image analyzer (Aisin Seiki).

2.2.3. Apoptosis assay

Third instar larvae or 42APF pupae were dissected in PBS and the eye imaginal discs or pupal retinae were fixed in 4% paraformaldehyde in PBS for 30 min at 25°C. After being washed with 0.3% PBST, the samples were permeabilized by incubation in 0.25% PBST for 20 min in 25°C. After washing with H_2O, the TUNEL reaction was carried out using a Click-iT TUNEL Alexa Fluor 594 Imaging Assay Kit (Life Technologies) according to the manufacture's recommendations.

2.2.4. Scanning electron microscopy

Adult flies were anesthetized, mounted on stages, and observed under a scanning electron microscope (SEM) VE-7800 (Keyence Inc.) in the low vacuum mode. The eye phenotype of at least five adult male flies (3 to 5 days old) of each line was examined in each experiment and the experiments were done in triplicate. No significant variation in eye phenotype was observed among the five individuals.

2.2.5. Data analysis

Quantification of intensity of Caz signals was carried out with six to nine different samples by using Meta Morph software (Molecular Devices). For the statistical analysis, Microsoft Excel 2007 was used. P-values were calculated using Welch's t-test and the error bars represent Standard Errors from Means.

2.3. Results

2.3.1 Knockdown of Caz in eye imaginal discs induces morphologically aberrant rough eyes

In order to investigate in vivo functions of Caz, we examined the effect of reduction of Caz protein *in vivo* using a combination of the GAL4-UAS targeted expression system and the RNAi method. Knockdown of *Caz* in all tissues by the Act5C-GAL4 driver strain resulted in late pupal lethality in transgenic lines carrying *UAS-Caz-IR$_{1-167}$* (data not shown). Knockdown of *Caz* in eye imaginal discs by the GMR-GAL4 driver strain, in which *Caz* double-stranded RNA (dsRNA) was expressed in the region posterior to the morphogenetic furrow, induced morphologically aberrant rough eyes. SEM images showed fusion of ommatidia and a lack of bristles (Data not shown). Flies carrying GMR-GAL4 alone exhibited apparently normal eye morphology (Data not shown). Moreover, to eliminate the possibility of off-target effects, we established eleven independent transgenic fly lines carrying *UAS-Caz-IR* targeted to the different regions of the Caz mRNA assessed in a previous study [22]. Phenotypes of the established transgenic fly lines crossed with the GMR-GAL4 driver strain are reported [27]. Each independent strain showed the similar rough eye phenotype as the flies carrying *UAS-Caz-IR* targeted to different regions of the Caz mRNA (Data not shown). These results suggest that the rough eye phonotype observed in *Caz*-knockdown flies is not due to a possible insertional mutation or off-target effect but rather to reduction of the Caz protein level. Throughout the following studies, we utilized the strain CG3606 carrying *UAS-Caz-IR$_{363-399}$*.

To investigate whether the expression of *Caz* dsRNA efficiently reduces the level of Caz protein, we performed immunostaining of pupal retinae at 42 h after pupal

formation (APF) with anti-Caz antibodies. We utilized the flip-out system to produce the RNAi clone so that the level of Caz could be directly compared within a single retina. We used *UAS-Caz-IR$_{363-399}$* line, since it apparently showed the severest rough eye (Figure S1E and F). Within the RNAi clone of *Caz* marked by the presence of GFP signals, Caz signals marked by Red were reduced by 50 % (Figure 1A to G). Double immunostaining of pupal retinae with anti-cut antibody and ant-Caz antibody revealed relatively high expression of Caz in cone cells (Figure 1J) and the Caz signal was reduced by 62% in flies expressing Caz dsRNA drived by GMR-GAL4 (Figure 1K and N). Moreover, effective knockdown of *Caz* in pupae of *UAS-Caz-IR$_{363-399}$* line was demonstrated by the Western immunoblot analysis with anti-Caz antibody (Figure 1O). All of these results further indicate that the rough eye phenotype observed in RNAi flies of *Caz* is due to reduction of the Caz protein level.

2.3.2. Knockdown of Caz induces apoptosis in pupal retinae

Extensive apoptosis could be considered as one factor causing fused ommatidia in the adult compound eye, since it is frequently accompanies this phenotype. We therefore examined if excessive cell death might occur during eye development in Caz-knockdown flies by crossing *GMR-GAL4; UAS-Caz-IR$_{363-399}$; +* with flies expressing a broad specificity Caspase inhibitor P35 encoded by the baculovirus *Autographa californica* [28]. Partial suppression of the rough eye phenotype induced by knockdown of *Caz* was observed in flies co-expressing P35 (Figure 2A, a-f). Expression of P35 alone exerted no apparent effect on the compound eye morphology (Figure 2A, g and h). Moreover, we monitored apoptotic cells in third instar larval eye imaginal discs by TUNEL assay. However, no detectable apoptotic signals in eye imaginal discs of these flies was observed (Figure 2B, a, d, g and j). We therefore next examined pupal

retinae at 42 h APF by TUNEL assay. Apoptotic cells detected in the *Caz*-knockdown retinae were significantly reduced in flies expressing P35 (Figure 2B, c, f and i). These results indicate that knockdown of *Caz* induces apoptosis in some cells in pupal retinae

2.3.3. Knockdown of Caz interferes with cell differentiation in pupal retinae

Photoreceptor cells are known to be generated in a stereotype order: R8 is generated first, with movement posterior from the morphogenetic furrow, then cells are added pair wise (R2 and R5, R3 and R4, and R1 and R6), and R7 is the last photoreceptor to be added to each cluster [29]. To investigate whether *Caz*-knockdown inhibits differentiation of photoreceptor cells, we crossed flies expressing *Caz* dsRNA with two enhancer trap lines, AE127 and P82, to specifically mark photoreceptor cells of R3/R4/R1/R6 and R3/R4/R7, respectively and then immunostained the eye imaginal discs with anti-β-galactosidase antibodies (Data not shown). In parallel, we carried out immunostaining of eye imaginal discs with anti-Elav antibodies (Data not shown). Elav, a pan-neuronal marker is normally expressed in the posterior portion of the eye imaginal discs. In eye imaginal discs of *Caz*-knockdown flies, all eight photoreceptor cells and their neuron appeared to differentiate normally (Data not shown).

We next examined the pattern formation in pupal ommatidia of *Caz*-knockdown flies. Differentiation of photoreceptors, cone cells, and 1°, 2°, and 3° pigment cells was completed by about 42 h APF at 28°C. However, in pupal retinae of *Caz*-knockdown flies, immunostaining with an anti-Cut antibody that marks cone cells showed that some of these were fused in flies expressing *Caz* dsRNA (Figure 3D and H). Flies carrying GMR-GAL4 alone exhibited apparently normal cone cells (Figure 3A and H). The quantified data indicate that occurrence of cone cell fusion was increased by 5.7 fold in the *Caz*-knockdown retinae.

Furthermore, we monitored apical cell junctions in pupal retina at 42 h APF by immunostaining with anti-Discs large. The results showed cells in pupal retinae of *Caz*-knockdown flies to be attached loosely, the orientation of ommatidia to be irregular, and the size of ommatidia to vary (Figure 4C). In addition some ommatidia were apparently fused (Figure 4C). These data suggest that knockdown of *Caz* disrupts differentiation of pupal ommatidial cell types, especially cone cells, and 1°, 2°, and 3° pigment cells, probably by repressing or enhancing expression of genes involved in differentiation processes.

2.3.4. Genetic link between Caz and rhomboid

The epidermal growth factor-receptor (EGFR) signaling pathway, evolutionarily conserved from *C. elegans* to man, controls a variety of different cellular processes. In *Drosophila melanogaster*, these include proliferation, patterning, cell-fate determination, migration, and survival [30]. Contributions to cone cell-fate and ommatidial rotation have also been documented [30-33]. One of the rate limiting components of *Drosophila* EGFR signaling is Rhomboid [32-34]. We therefore examined the effects of mutations that might modify the Caz-induced rough eye phenotype, especially focusing on the *rhomboid* gene.

Half dose reduction *rhomboid-1 (rho)* significantly suppressed the rough eye phenotype (Figure 5A, a, b, c and d) and rescued the fusion of cone cells (Figure 3E and H) in pupal retinae. Rescue of the fusion of the cone cells was also observed with overexpression of P35 (Figure 3G and H), suggesting that induction of apoptosis is also responsible for this phenotype. Two different alleles of *rho* showed suppression of the rough eye (Figure 5A, a, b, c and d). In addition, similar extent of suppression of the rough eye was observed by knockdown of *rho* (Figure 5A, m and n), but not by

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expression of dsRNA for GFP (Fig. 5A, k and l). Rhomboid-1 is a seven membrane-spanning serine protease, undergoing cleavage of Spitz to release the secreted form as an EGFR ligand from the Golgi apparatus [32-35]. In the eye, Rhomboid-3, also known as Roughoid, cooperates with Rhomboid-1 [32, 33]. Expectedly, the Rhomboid-3 hypomorph mutant ru^1 also demonstrated suppression of the rough eye phenotype induced by knockdown of *Caz* (Figure 5A, g and h). Moreover, a *mirror* loss of function mutant $mirr^{SaiD3}$ similarly suppressed the rough eye phenotype (Fig. 5A, e and f). The *mirror* gene encodes a homeodomain-containing transcription factor that is thought to activate transcription of *rhomboid* [36]. In addition, immunostaining of eye imaginal discs with anti-dpERK antibody also pointed activation of ERK signals in the *Caz*-knockdown flies (Figure 5B). These results indicate a genetic link between Caz and the EGFR signaling pathway.

2.4. Discussion

In this study, I found that *Caz*-knockdown in eye imaginal discs induces a rough eye phenotype associated with apoptosis, abnormal differentiation of cone cells and pigment cells, and defects in ommatidia rotation in pupal retinae. However, apoptosis and differentiation of photoreceptor cells were not affected in larval eye imaginal discs expressing *Caz* dsRNA. Why did *Caz*-knockdown in eye imaginal discs affect pupal retinae but not third instar larval eye discs? *In situ* hybridization and immunohistochemistrical analyses demonstrated that *Caz* mRNA and protein are enriched in the brain and CNS during embryogenesis, and Caz protein was detected in the nuclei of several larval tissues and in imaginal discs [21]. However, the expression

level of Caz is higher in adult eyes than in larval eye discs (Flybase). Thus, it is possible that Caz plays a more important role in eye development in the pupal stage.

The observation that the rough eye phenotype of *Caz*-knockdown flies was significantly suppressed by co-expression of P35 and that apoptotic cells detected by immunostaining with anti-cleaved Caspase-3 antibody were significantly increased in pupal retinae of flies expressing *Caz* dsRNA suggests that induction of apoptosis at least partially contributes to the rough eye phenotype. It is reported that the number of dying cells increases dramatically if interactions between cells are disrupted, for instance upon cell ablation [37]. Therefore, one possible explanation is that *Caz*-knockdown disrupts interactions between cells in pupal retinae, as evidenced with anti-Cut immunostaining, that results in induction of apoptosis. In addition, it is well known that apoptosis is induced by JNK or p38 signaling [38-40]. It is also reported that persistent activation of the JNK or p38 signaling pathways mediates neuronal apoptosis in ALS [3-7,41], and that TDP-43 is related to JNK signaling [42]. Thus, another possible explanation is that *Caz*-knockdown induces JNK or p38 signaling, resulting in increase of apoptosis in pupal retinae.

We found a genetic interaction between *Caz* and *Rhomboid*, a rate-limiting component of the EGFR signaling pathway. Appropriate levels of EGFR signaling are required for cone cell-fate and ommatidial rotation [30-33]. Knockdown of *Caz* in eye imaginal discs and pupal retinae induced abnormal differentiation of cone cells and defects in ommatidia rotation that eventually resulted in the rough eye phenotype in adults. The *rhomboid-1* mutant rescued the fusion of cone cells and mutations of *rhomboid-3* and *mirror* significantly suppressed the rough eye phenotype of *Caz*-knockdown flies. In contrast, mutations of *egfr* did not suppress the rough eye

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phenotype induced by knockdown of *Caz* (data not shown). These apparently contradictory results might be explained as follows. Once activated, the signaling cascade could be amplified progressively, so that only a half reduction of some components of pathway such as *egfr* may not be sufficient to suppress the effects of over-activation of the initiator such as *rhomboid*. In any event, the present study suggests that Caz negatively regulates EGFR signaling. Since the expression level of Caz is much higher in adult eyes than larval eye discs, negative regulation of EGFR signaling by Caz may play a role in controlling EGFR signaling less reactive to oxidative stress during adulthood. It should be noted that a hallmark of ALS is chronic neuronal exposure to oxidative stress and inflammation.

In summary, the results have shown that knockdown of *Caz* in the *Drosophila* retina induces a rough eye phenotype associated with increased apoptosis, abnormal differentiation of cone cells and pigment cells, and defects in ommatidia rotation. Here I provide the first definitive evidence that Caz plays an important role in regulation of the EGFR signaling pathway. It should be noted that the neurodegeneration occurring in ALS can be accounted for deviation from strict control of MAPK signaling [3]. Thus, the *Caz*-knockdown flies used in the present study should provide a useful tool for elucidating functions of FUS and pathological mechanisms of associated ALS.
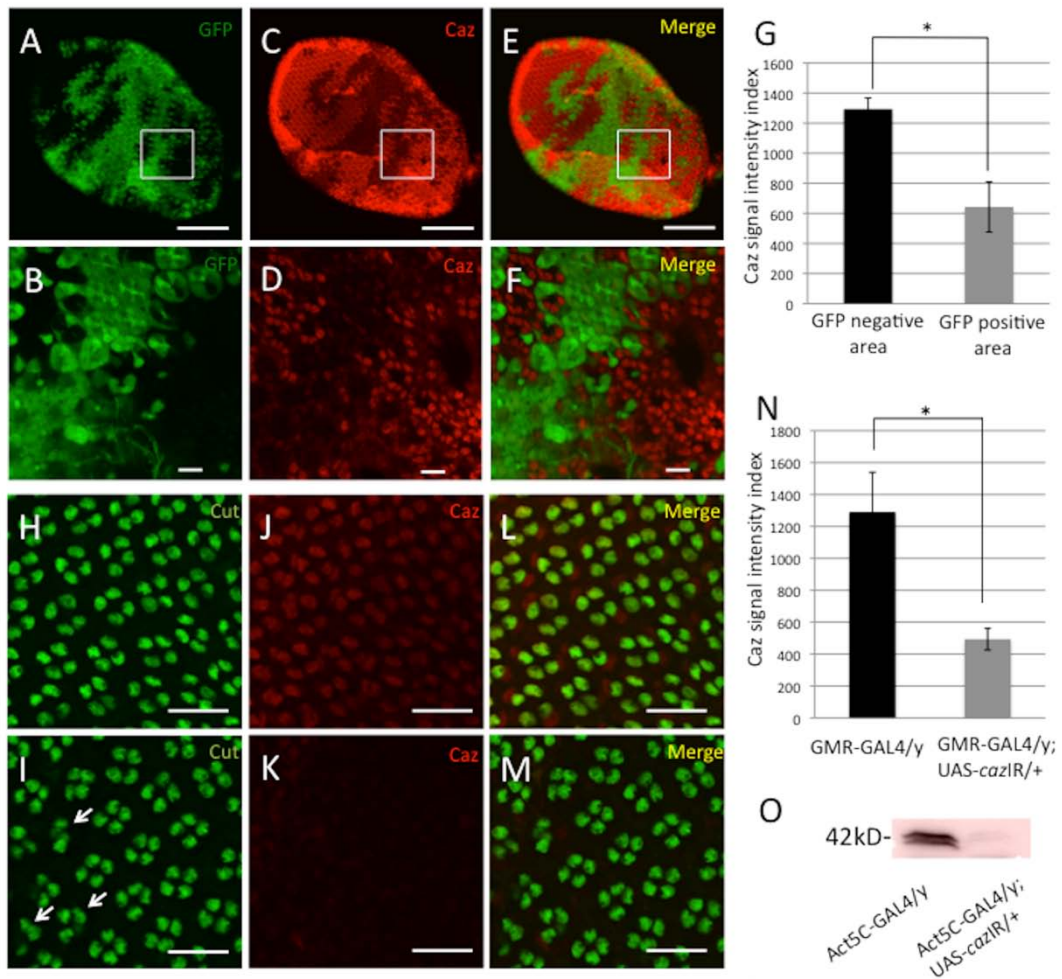
Figure 1: The level of anti-Caz signals is reduced in Caz dsRNA-expressing areas. Immunostaining of retinae at 42h APF with anti-Caz antibody (C, D, J and K). RNAi clones in retinae were generated with the flip-out system (A to F). Female flies with *hspFlp; +; tub1> FRT cd2 FRT> GAL4, UAS-GFP* were crossed male flies with *w; UAS-Caz-IR₃₆₃₋₃₉₉; +*. The *Caz* dsRNA-expressing area is positively marked with GFP (A, B). (E and F): Merged images. Panels B, D and F show higher magnification images of the regions marked with squares in panels A, C and D. (G) Quantification of intensities of Caz-signals in GFP-positive and –negative areas. Mean intensities with standard deviation from six pupal retinae are shown. *P<0.05. (H to M)

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Immunostaining of retinae at 42h APF with anti-cut (H and I) and anti-Caz (J and K) antibodies. (L and M) Merged images. (H, J and K) GMR-GAL4/y. (I, K and M) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$*. (N) Quantification of intensities of Caz-signals. Mean intensities with standard deviation from nine pupal retinae are shown. *P<0.05. The bars indicate 1 0 0 μm (A, C and E), 1 0 μm (B, D and F) and 2 0 μm (H to M), respectively. (O) Western immunoblotting analysis. Protein extracts were prepared from the whole pupae of *Drosophila* carrying *Act5C-GAL4/+* (left lane) or *Act5C-GAL4/+; UAS-Caz-IR$_{363-399}$/+* (right lane). The blot was probed with anti-Caz antibody.
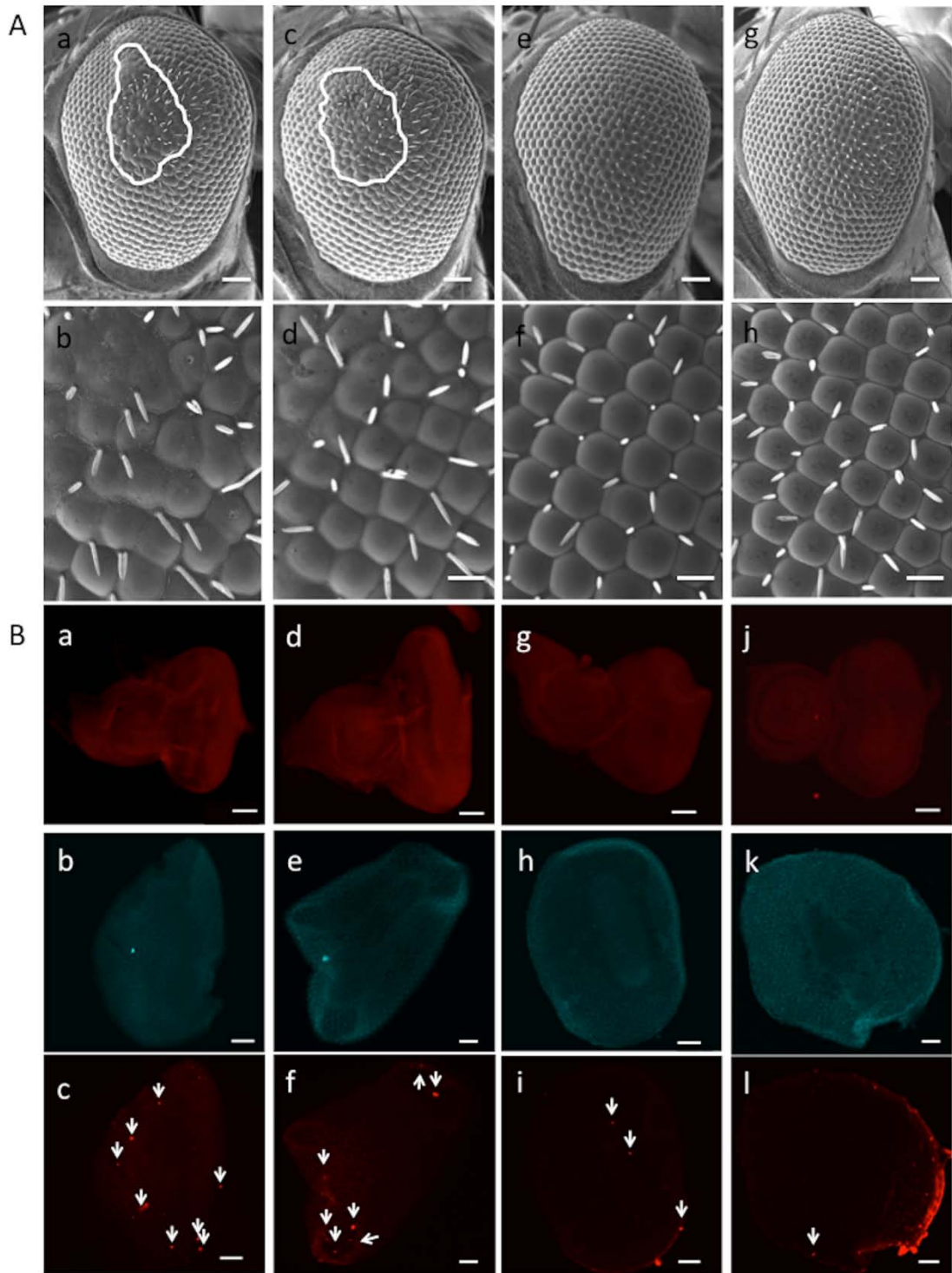
Figure 2: Knockdown of *Caz* in eye imaginal discs induces apoptosis. (A) Over-expression of P35 suppresses the rough eye phenotype, as shown by scanning electron micrographs of adult compound eyes. (a, b) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/*

+; +, (c, d) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-GFP/+,* (e, f) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-P35/+.* (g, h) *GMR-GAL4/y; +/+; UAS-P35/+.* The flies were developed at 28°C. The eye phenotype of at le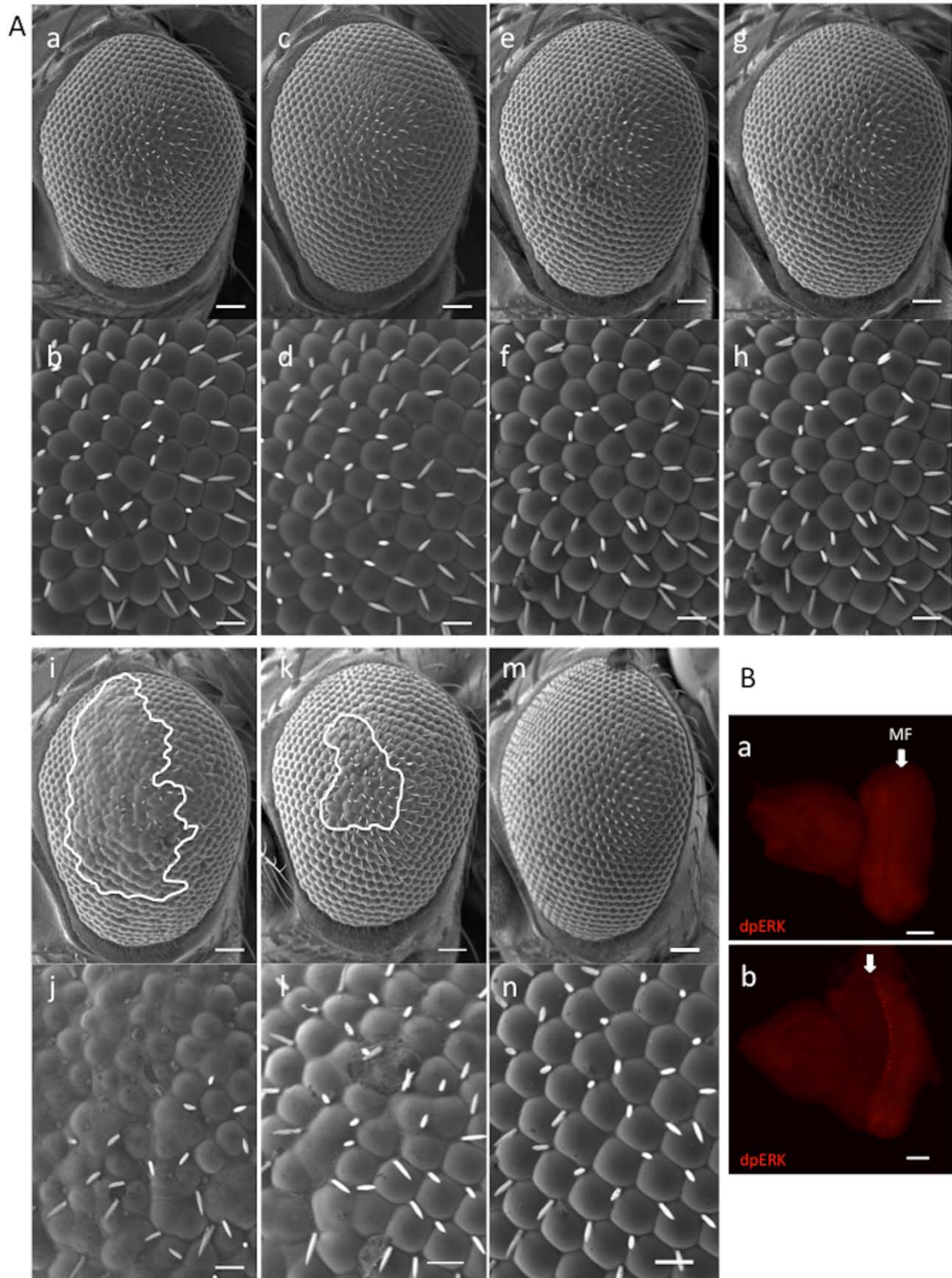ast five adult male flies (3 to 5 days old) of each line was examined and the experiments were done in triplicate. No significant variation in eye phenotype was observed among the five individuals. The rough area of the compound eye was circled as an index of the rough eye phenotype. The bars indicate 50μm (a, c, e and g), and 14.2μm (b, d, f and h), respectively. (B) Detection of apoptotic cells in third larval eye imaginal discs (a, d, g and j) and pupal retinae (c, f, i and l) by TUNEL assay. Pupal retinae were also stained with DAPI (b, e, h and k). (a to c) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/ +; +.* (d to f) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-GFP/+.* (g to i) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-P35/+.* (j to l) *GMR-GAL4/y; +/+; UAS-P35/+.* The flies were developed at 28°C. The bars indicate 100μm.
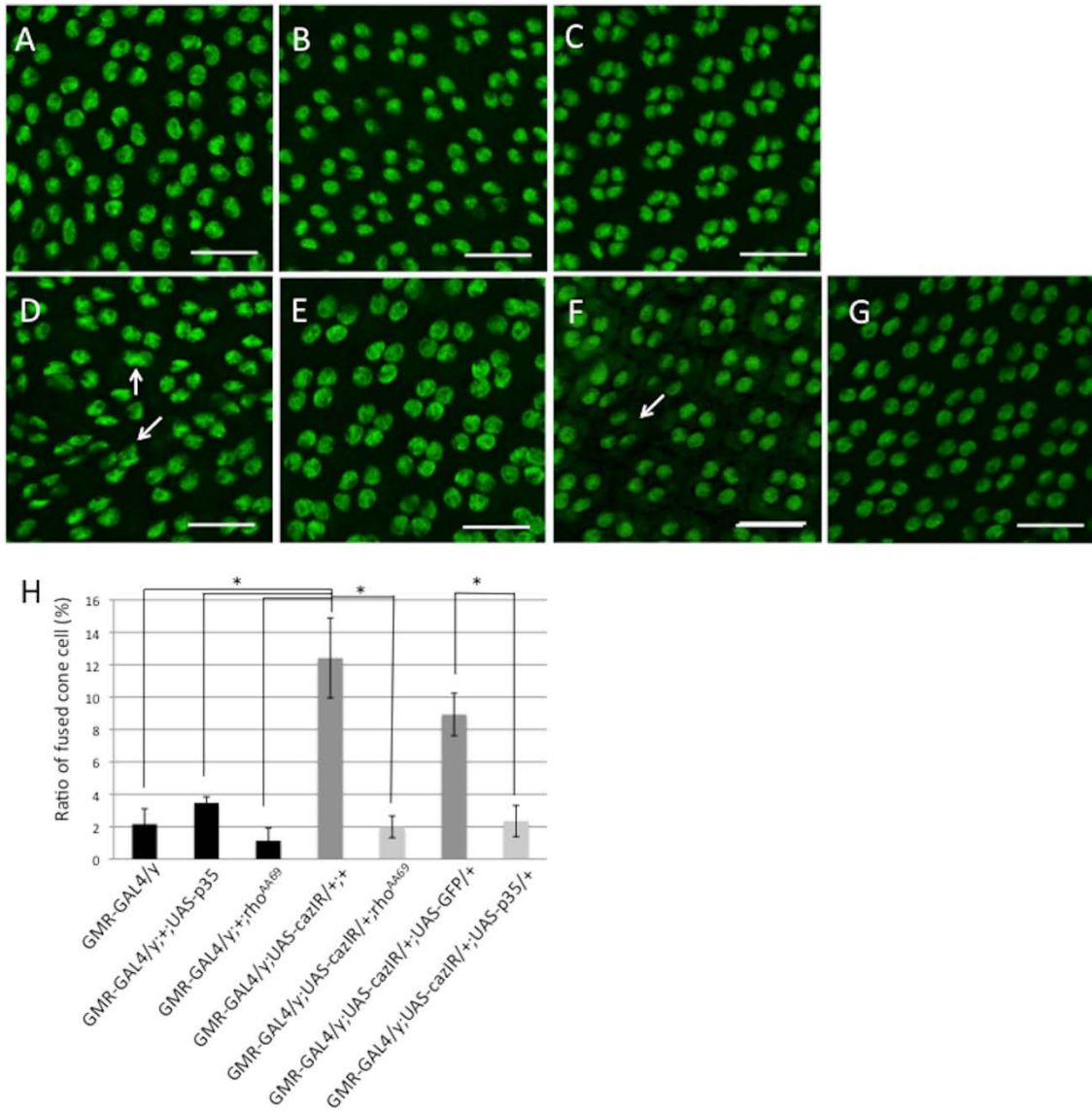
Figure 3: Knockdown of *Caz* interferes with differentiation of cone cells. Immunostaining of retinae at 42 h APF with an antibody to Cut that marks cone cells (green). (A) *GMR-GAL4/y.* (B) *GMR-GAL4/y; +; UAS-P35/+.* (C) *GMR-GAL4/y; +; rhoAA69/+.* (D) *GMR-GAL4/y; UAS-Caz-IR$^{363-399}$/+; +.* (E) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; rho^{AA69}/+.* (F) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-GFP/+.* (G) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-P35/+.* The flies were developed at 28°C.

Arrows show fused cone cells and fused ommatidia are circled. The bar indicates 20μm (A-G). (H) Quantification of fused cone cells in pupal retinae. Ratio of fused cone cells in each retina us shown (%). Mean values with standard deviation from six pupal retinae are shown. *$P<0.05$. (Left to Right) *GMR-GAL4/y, GMR-GAL4/y; +; UAS-P35/+, GMR-GAL4/y; +; rho^{AA69}/+, GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; +, GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; rho^{AA69}/+, GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-GFP/+* and *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-P35/+.*
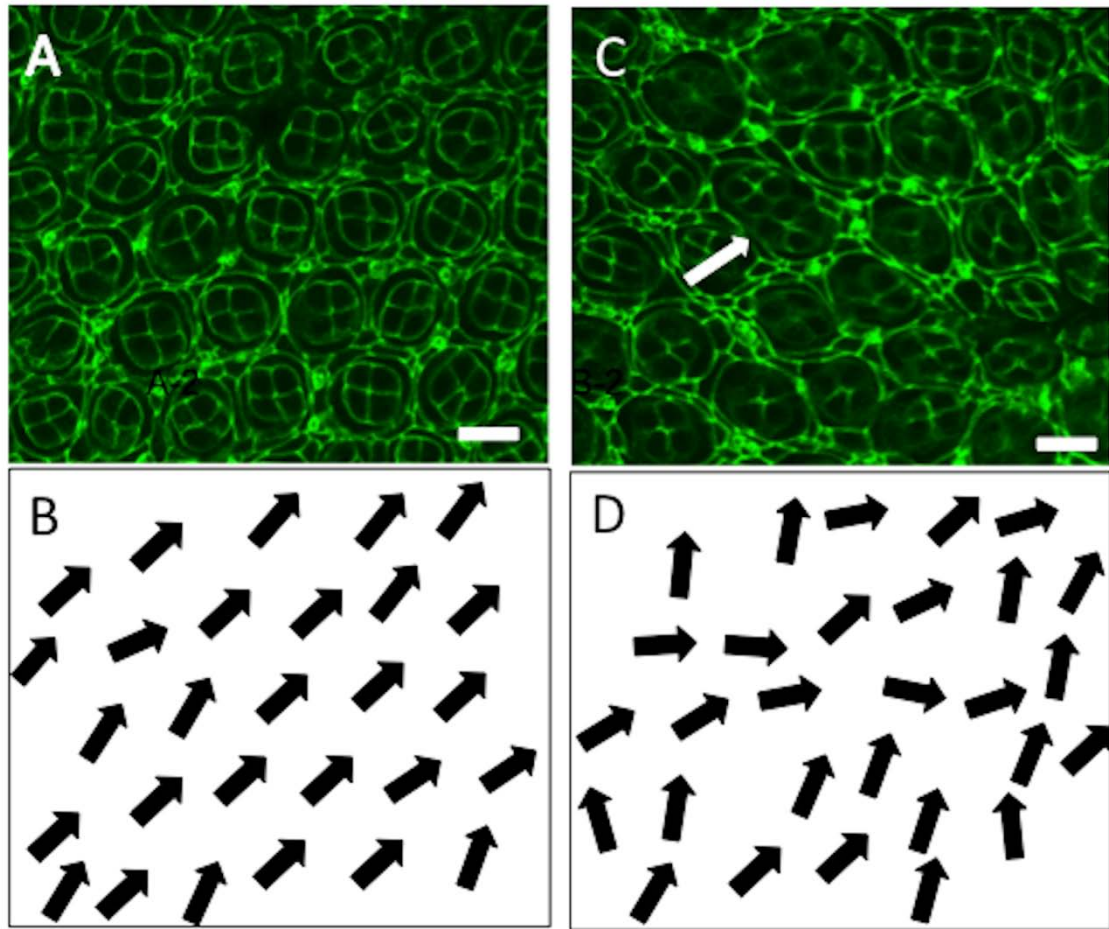
Figure 4: Effects of *Caz*-knockdown on morphogenesis of pupal retinae. Confocal sections stained with anti-Discs large (A, C). (A) *GMR-GAL4/y*. (C) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; +*. Black arrows in the lower panels (B, D) indicate the orientation of the ommatidia. Note disruption in the *Caz*-knockdown flies (D) in compared to control (B). The size of ommatidia is also irregular, and cell attachment appears to be loose (compare panels A and C). The white arrow in panel C indicates an example of the fused ommatidia.

Figure 5: Scanning electron micrographs of adult compound eyes. (A) Female

flies expressing *Caz* dsRNA (*GMR-GAL4/GMR-GAL4; UAS-Caz-IR_{363-399} ; +*) were

crossed *with UAS-GFP-IR, UAS-rho-IR^{28690}, rho^{7M43}* (amorph), rho^{AA69} (undetermined),

mirror^{SaiD3} (loss of function), or *ru^1* (hypomorph) male flies, and then F1 progeny were developed at 28°C without balancer chromosomes and used for inspection of the eye phenotype. The eye phenotype of at least five adult male flies (3 to 5 days old) of each line was examined and the experiments were done in triplicate. No significant variation in eye phenotype was observed among the five individuals. The rough area of the compound eye was circled as an index of the rough eye phenotype. (a, b) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; rho^{7M43}/+.* (c, d) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; rho^{AA69}/+.* (e, f) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/ +; mirror^{SaiD3}/ +* (g, h) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/ +; ru^1/ +.* (i, j) *GMR-GAL4/ y; UAS-Caz-IR$_{363-399}$/+; +.* (k, l) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-GFP-IR/+,* (m, n) *GMR-GAL4/y; UAS-Caz-IR$_{363-399}$/+; UAS-rho-IR^{28690}/+.* The flies were developed at 28°C. The bar indicates 50 μm (a, c, e, g, i, k, m) or 14.2μm (b, d, f, h, j, l, n). (B) Immunostaining of eye imaginal discs with anti-dpERK antibody. (a) *GMR-GAL4/y.* (b) *GMR-GAL4/ y; UAS-Caz-IR$_{363-399}$/+; +.* Arrows indicate morphogenetic furrows (MF). The bars indicate 100μm.

2.5. References

[1]   Boillee S, Vande Veldev C, Cleveland DW. ALS: a disease of motorneurons and their nonneuronal neighbors. Neuron, 2006. 52: 39-59.

[2]   Vucic S, Kiernan MC. Pathology of neurodegeneration in familial amyotrophic lateral sclerosis. Curr Mol Med, 2009. 9: 255-272.

[3]   Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim. Biophys. Acta, 2010. 1802: 396-405.

[4]   Bendotti C, Bao Cutrona C, Cheroni C, Grignaschi G, Lo Coco D, Peviani M, Tortarolo M, Veglianese P, Zennaro E. Inter- and intracellular signaling in amyotrophic lateral sclerosis: role of p38 mitogen-activated protein kinase. Neurodegener, 2005. 2:128-134.

[5]   Tortarolo M, Veglianese P, Calvaresi N, Botturi A, Rossi C, Giorgini A, Migheli A, Bendotti C. Persistent activation of p38 mitogen-activated protein kinase in a mouse model of familial amyotrophic lateral sclerosis correlates with disease progression. Mol. Cell. Neurosci, 2003. 23:180-192.

[6]   Holasek SS, Wengenack TM, Kandimalla KK, Montano C, Gregor DM, Curran GL, Poduslo JF. Activation of the stress-activated MAP kinase, p38, but not JNK in cortical motor neurons during early presymptomatic stages of amyotrophic lateral sclerosis in transgenic mice. Brain Res, 2005. 1045:185-198.

[7]   Dewil M, Dela Cruz VF, Van Den Bosch L, Robberecht W. Inhibition of p38 mitogen activated protein kinase activation and mutant SOD1(G93A)-induced motor neuron death. Neurobiol, 2007. 26:332-341.

[8]   Lemmens R, Moore MJ, Al-Chalabi A, Brown Jr. RH, Robberecht W. RNA metabolism and the pathogenesis of motor neuron diseases. Trends Neurosci, 2010. 33: 249-258.

[9]   Mackenzie IR, Rademakers R, Neumann M. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. Lancet. Neurol, 2010. 9:995-1007

[10]   Gitcho MA, Baloh RH, Chakraverty S, Mayo K, Norton JB, Levitch D, Hatanpaa KJ, White CL, Bigio EH, Caselli R, et al. TDP-43 A315T mutation in familial motor neuron disease. Ann. Neurol, 2008. 63:535-538.

[11]   Yokoseki A, Shiga A, Tan CF, Tagawa A, Kaneko H, Koyama A, Eguchi H, Tsujino A, Ikeuchi T, Kakita A, et al., TDP-43 mutation in familial amyotrophic lateral sclerosis. Ann. Neurol, 2008. 63:538-542.

[12]   Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Vande Velde C, Bouchard JP, Lacomblez L, Pochigaeva K, Salachas F, et al. TARDBP mutations in individuals with sporadic and familial amyotrophic lateralsclerosis. Nat. Genet, 2008. 40:572-574.

[13]   Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, Ackerley S, Durnall JC, Williams KL, Buratti E, et al. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science, 2008. 319:1668-1672.

[14]   Pesiridis GS, Lee VM, Trojanowski JQ. Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. Hum. Mol. Genet, 2009. 18:156-162.

[15]   Kwiatkowski Jr. TJ, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, et al. Mutations in the

FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science, 2009. 323:1205-1208.

[16]   Vance C, Rogelj B, Hortobagyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, et al. Mutations in FUS, an RNA processing - 18 -protein, cause familial amyotrophic lateral sclerosis type 6. Science, 2009. 323:1208-1211

[17]   Hewitt C, Kirby J, Highley JR, Hartley JA, Hibberd R, Hollinger HC, Williams TL, Ince PG, McDermott CJ, Shaw PJ. Novel FUS/TLS mutations and pathology in familial and sporadic amyotrophic lateral sclerosis. Arch. Neurol, 2010. 67:455-461.

[18]   Rademakers R, Stewart H, Dejesus-Hernandez M, Krieger C, Graff-Radford N, Fabros M, Briemberg H, Cashman N, Eisen A, Mackenzie IR. *Fus* gene mutations in familial and sporadic amyotrophic lateral sclerosis. Muscle Nerve, 2010. 42:170-176.

[19]   Zinszner H, Sok J, Immanuel D, Yin Y, Ron D. TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling. J. Cell Sci, 1997;110:1741-1750.

[20]   Lagier-Tourenne C, Polymenidou M, Cleveland DW. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Hum. Mol. Genet, 201. 19:46-64.

[21]   Stolow DT, Haynes SR. *Cabeza*, a *Drosophila* gene encoding a novel RNA binding protein, shares homology with *EWS* and *TLS*, two genes involved in human sarcoma formation. Nucleic Acids Res, 1995. 23:835-843.

[22]   Sasayama H, Shimamura M, Tokuda T, Azuma Y, Yoshida T, Mizuno T, Nakagawa M, Fujikake N, Nagai Y, Yamaguchi M. Knockdown of the *Drosophila*

Fused in Sarcoma (*FUS*) homologue causes deficient locomotive behavior and shortening of motoneuron terminal branches. PLoS One, 2012. 7: e39483.

[23]   Mlodzik M, Hiromi Y, Weber U, Goodman CS, Rubin GM. The *Drosophila* seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. Cell, 1990. 60: 211-224.

[24]   Kramer S, West SR, Hiromi Y. Cell fate control in the *Drosophila* retina by the orphan receptor seven-up: its role in the decisions mediated by the ras signaling pathway. Development, 1995. 121:1361-1372.

[25]   Takahashi Y, Hirose F, Matsukage A, Yamaguchi M. Identification of three conserved regions in the DREF transcription factors from *Drosophila melanogaster* and *Drosophila virilis.* Nucleic Acids Res,1999. 27: 510-516.

[26]   Sun J, Tower J. FLP recombinase-mediated induction of Cu/Zn superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster*. Mol. Cell. Biol, 1999. 19:216-228.

[27]   Azuma Y, Tokuda T, Shimamura M, Kyotani A, Sasayama H, Yoshida T, Mizuta I, Mizuno T, Nakagawa M, Fujikake N, Ueyama M, Nagai Y, Yamaguchi M. Identification of ter94, Drosophila VCP, as a strong modulator of motor neuron degeneration induced by knockdown of *Caz, Drosophila FUS*. Hum. Mol. Genetics (2014) in press.

[28]   Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J. Virol, 1993. 67:2168-2174.

[29]   Zipursky SL, Rubin GM. Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. Annu. Rev. Neurosci, 1994. 17:373-397.

[30]   Gaengel K, Mlodzik M. Egfr signaling regulates ommatidial rotation and cell motility in the *Drosophila* eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. Development, 2003. 130:5413-5423.

[31]   Fu W, Noll M. The Pax2 homolog sparkling is required for development of cone and pigment cells in the *Drosophila* eye. Genes Dev, 1997. 11:2066-2078.

[32]   Wasserman JD, Urban S, Freeman M. A family of rhomboid-like genes: *Drosophila rhomboid-1* and *roughoid/rhomboid-3* cooperate to activate EGF receptor signaling. Genes. Dev, 2000. 14:1651-1663.

[33]   Shilio BZ. Regulating the dynamics of EGF receptor signaling in space and time. Development, 2005. 132:4017-4027.

[34]   Yogev S, Schejter ED, Shilo BZ. *Drosophila* EGFR signaling is modulated by differential compartmentalization of Rhomboid intramembrane proteases. EMBO J, 2008. 27:1219-1230.

[35]   Urban S, Lee JR, Freeman M. *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell, 2001. 107:173-182.

[36]   Jordan KC, Clegg NJ, Blasi JA, Morimoto AM, Sen J, Stein D, McNeill H, Deng WM, Tworoger M, Ruohola-Baker H. The homeobox gene mirror links EGF signalling to embryonic dorso-ventral axis formation through notch activation, Nat. enet, 2000. 24:429-433.

[37]   Hidalgoa A, Constant CF. The control of cell number during central nervous system development in flies and mice. Mech. Dev, 2003. 120:1311-1325.

[38]   Agnè F, Suzanne M, Noselli S. The *Drosophila* JNK pathway controls the morphogenesis of imaginal discs during metamorphosis. Development, 1999. 126:5453-5462.

[39]   Tare M, Modi RM, Nainaparampil JJ, Puli OR, Bedi S, Fernandez-Funez P, Kango-Singh M, Singh A. Activation of JNK signaling mediates amyloid-β-dependent cell death. PLoS One, 2011. 6:e24361.

[40]   Correa SA, Eales KL. The role of p38 MAPK and its substrates in neuronal plasticity and neurodegenerative disease. J. Signal. Transduct, 2012. 2012:649079.

[41]   Ackerley S, Grierson AJ, Banner S, Perkinton MS, Brownlees J, Byers HL, Ward M, Thornhill P, Hussain K, Waby JS, Anderton BH, Cooper JD, Dingwall C, Leigh PN, Shaw CE, Miller CC. p38α stress-activated protein kinase phosphorylates neurofilaments and is associated with neurofilaments pathology in amyotrophic lateral sclerosis. Mol. Cell. Neurosci, 2004. 26:354-364.

[42]   Meyerowitz J, Parker SJ, Vella LJ, Ch. D Ng, Price KA, Liddell JR, Caragounis A, Li QX, Masters CL, Nonaka T, Hasegawa M, Bogoyevitch MA, Kanninen KM, Crouch PJ, White AR. C-Jun N-terminal kinase controls TDP-43 accumulation in stress granules induced by oxidative stress. Mol. Neurodegener, 2011. 6:57.

Conclusions and perspectives

For a long time, *Drosophila* has been used as an efficient genetic model organism for studying on molecular mechanism of human diseases such as cancer, human neurodegenerative diseases. The most important reason is that how similar *Drosophila* is to human. Although there are important differences between flies and humans such as the circulatory is much simpler than in the fly but in general, cell cycle regulation, synaptogenesis, membrane trafficking, cell death are similar in *Drosophila* and human. Furthermore, *Drosophila* is easy and inexpensive to culture in laboratory conditions with a much shorter life cycle than vertebrate models. Currently, it is estimated that there are around 14000 genes in *Drosophila* and all information including sequence, mutations and related literature are shown in database of Flybase. It is no doubt to believe that *Drosophila* is the key advances in study about genetics and human diseases.

Since the first mitochondrial dysfunction was described in 1960s, researchers have advanced understating the role of mitochondria playing in health, disease, and aging. A wide range of unrelated human diseases including cancers and neuronal degeneration is reported to result in mitochondrial dysfunction. However, since symptoms vary from case to case, age of onset, rate of progression, it is difficult to diagnose when mitochondrial dysfunction first appears. The more we understand about the mechanisms related to mitochondrial dysfunction, the more chance we have to discover the best treatment being tailored specifically to each patient, to alleviate the symptoms of the disorders and to slow down the progression of the diseases.

In the study about *in vivo* roles of SCOX, I obtained the results that knockdown of *SCOX* in all cells is associated with lethality in larvae or pupae, while the full length

of SCOX transgenic flies showed a longer lifespan correlated with the decrease and increase in ATP level, respectively. Besides, when cultured on paraquat-added medium, full length SCOX transgenic flies inhibited an elongated lifespan. I therefore hypothesized that SCOX plays an important role in ATP consumption and production. However, until now, it is unclear to answer where SCOX targets in ATP production and consumption in cells and if SCOX have other *in vivo* roles or not. To get the answer of these questions, more *in vivo* and *in vitro* experiments will be done in future to find out the genetic link between SCOX and other mitochondrial genes, especially ATP production and consumption-related genes. Moreover, the study of Rossato et al (1999) on sperm got the result that sperm treatment with extracellular ATP increases fertilization rates in in vitro fertilization for male factor infertility. Mitochondria have effect on all biochemical pathways including the pathway that drives sperm mobility. Sperms require a substantial amount of energy to swim fast enough to reach the oviduct during fertilization, and the decrease of ATP production and consumption may lead to male infertility. I hypothesized that SCOX plays role in ATP production and consumption so SCOX also may play important roles in infertility. Both *in vivo* and *in vitro* experiments in future are necessary to prove this hypothesis.

In the other part of my study on *Cabeza*, I obtained the results indicating that *Caz*-knockdown induces apoptosis and inhibits differentiation of cone cells. Moreover, mutation of EGFR pathway-related genes suppressed the rough eye phenotype induced by *Caz*-knockdown and the *rhomboid-1* mutation rescued the fusion of cone cells and ommatidia observed in *Caz*-knockdown flies. These results suggest that Caz negatively regulates the EGFR signaling pathway in *Drosophila melanogaster*. In the future, more *in vivo* and *in vitro* experiments will be done to clarify other genetic functions of Caz

and to further elucidate the mechanism of mitochondrial dysfunction and neurodegenerative disorders. Deep understanding about genetic functions of Caz may lead us to discover more effective treatments of ALS.

List of publications

Role of SCOX in determination of *Drosophila melanogaster* lifespan

Thanh Binh Nguyen, Hiroyuki Ida, Mai Shimamura, Daishi Kitazawa, Shinichi Akao, Hideki Yoshida, Yoshihiro H Inoue, Masamitsu Yamaguchi

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Genetic link between *Cabeza,* a *Drosophila* homologue of *Fused in Sarcoma* (*FUS*), and the EGFR signaling pathway

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