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

Mai Shimamura^{1, 2, #}, Akane Kyotani^{1, 2}, Yumiko Azuma³, Hideki Yoshida^{1, 2}, Thanh Binh Nguyen^{1, 2}, Ikuko Mizuta³, Tomokatsu Yoshida³, Toshiki Mizuno³, Masanori Nakagawa⁴, Takahiko Tokuda^{3, 5*} and Masamitsu Yamaguchi^{1, 2*}

¹Department of Applied Biology and ²Insect Biomedical Research Center, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan; ³Department of Neurology, ⁴North Medical Center and ⁵Department of Molecular Pathobiology of Brain Diseases, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan

*Corresponding authors: M. Yamaguchi, Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan; Tel: +81-75-724-7781, Fax: +81-75-724-7799; E-mail: myamaguc@kit.ac.jp and T. Tokuda, Department of Molecular Pathobiology of Brain Diseases (Neurology), Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan; Tel: +81-75-251-5793, Fax: +81-75-211-8645; E-mail: ttokuda@koto.kpu-m.ac.jp

[#]Present address: Environmental Research Laboratory of Public Health, Kankyo Eisei Yakuhin Co. Ltd., 3-6-2, Hikaridai, Seika-cho, Soraku-gun, Kyoto, Kyoto 619-0237, Japan

Abstract

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 an 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 retinæ. 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*.

Key words: Amyotrophic Lateral Sclerosis, Cabeza, retina, EGFR pathway, *Drosophila*

Abbreviations: *Caz*, Cabeza; ALS, Amyotrophic Lateral Sclerosis; FUS, Fused in

Sarcoma; EGFR, Epidermal growth factor-receptor; SOD1, Cu/Zn superoxide dismutase; TDP-43, TAR DNA-binding protein of 43kDa gene; CNS, central nervous system; APF, after pupal formation; ERK, Extracellular signal-related kinase.

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 has a single orthologue of human FUS, named Cabeza (Caz). *In situ*

hybridization and immunohistochemical 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* development.

In our previous studies using neuron specific *Caz* knockdown flies, we 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* 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.

Materials and Methods

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-IR*₁₈₀₋₃₄₆ 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 was also obtained from the Bloomington *Drosophila* stock center.

Generation of RNAi clones in retinæ

RNAi clones in retinæ 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.

Immunostaining

For immunohistochemistry, larval eye imaginal discs and pupal retinæ 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

and 10% normal goat serum for 16 h 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), mouse anti-Discs large (1:500) (DSHB) and 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 3 h 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).

Western immunoblot analysis

Protein extracts from the whole pupae of *Drosophila* carrying *Act5C-GAL4/+* or *Act5C-GAL4/+; UAS-Caz-IR₃₆₃₋₃₉₉/+* 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 16 h 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).

Apoptosis assay

Third instar larvae or 42APF pupae were dissected in PBS and the eye imaginal discs or pupal retinæ were fixed in 4% paraformaldehyde in PBS for 30 min at 25°C. After being washed with 0.3% PBST, the sample were permeabilized by incubation in 0.25% PBST for 20 min in 25°C. After washing with H₂O, 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.

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.

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.

Results

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-IR₁₋₁₆₇* (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 (Fig. S1C-F). Flies carrying GMR-GAL4 alone exhibited apparently normal eye morphology (Fig. S1A, B). 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 (Fig. S1C-F). These results suggest that the rough eye phenotype 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-IR₃₆₃₋₃₉₉*.

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-IR₃₆₃₋₃₉₉* line, since it apparently showed the severest rough eye (Fig. 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 % (Fig. 1A to G). Double immunostaining of pupal retinae with anti-cut antibody and anti-Caz antibody revealed relatively high expression of Caz in cone cells (Fig. 1J) and the Caz signal was reduced by 62% in flies expressing Caz dsRNA driven by GMR-GAL4 (Fig. 1K and N). Moreover, effective knockdown of Caz in pupae of *UAS-Caz-IR₃₆₃₋₃₉₉* line was demonstrated by the Western immunoblot analysis with anti-Caz antibody (Fig. 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.

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-IR₃₆₃₋₃₉₉; +* 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 (Fig. 2A, a-f). Expression of P35 alone exerted no apparent effect on the compound eye morphology (Fig. 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 (Fig. 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 (Fig. 2B, c, f and i). These results indicate that knockdown of *Caz* induces apoptosis in some cells in pupal retinae.

Knockdown of *Caz* interferes with cell differentiation in pupal retinae

Photoreceptor cells are known to be generated in a stereotypic 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 (Fig. S2, A-B and D-E). In parallel, we carried out immunostaining of eye imaginal discs with anti-Elav antibodies (Fig. S2, C, F). 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 (Fig. S2).

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 (Fig. 3D and H). Flies carrying GMR-GAL4 alone exhibited apparently normal cone cells (Fig. 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 (Fig. 4C). In addition some ommatidia were apparently fused (Fig. 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.

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*, 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 (Fig. 5A, a, b, c and d) and rescued the fusion of cone cells (Fig. 3E and H) in pupal retinæ. Rescue of the fusion of the cone cells was also observed with overexpression of P35 (Fig. 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 (Fig. 5A, a, b, c and d). In addition, similar extent of suppression of the rough eye was observed by knockdown of *rho* (Fig. 5A, m and n), but not by 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 *ru¹* also demonstrated suppression of the rough eye phenotype induced by knockdown of *Caz* (Fig. 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 (Fig. 5B). These results indicate a genetic link between *Caz* and the EGFR signaling pathway.

Discussion

In this study, we 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 immunohistochemical 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 retinæ 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 retinæ, 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 retinæ.

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 retinæ 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 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, we 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 we 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.

Acknowledgements

We thank Dr. Y. Hiromi and Dr. A. Plessis for supplying fly lines, and Dr. M. Moore for comments on the English in the manuscript. This study was partially supported by a scholarship and grants from JST and the Ministry of Education, Science, Sports and Culture of Japan and the JSPS Core-to-Core Program, B. Asia-Africa Science Platforms.

References

- [1] S. Boillee, C. Vande Velde, D.W. Cleveland, ALS: a disease of motoneurons and their nonneuronal neighbors. *Neuron* 52 (2006) 39-59.
- [2] S. Vucic, M.C. Kiernan, Pathology of neurodegeneration in familial amyotrophic lateral sclerosis. *Curr Mol Med.* 9 (2009) 255-272.
- [3] E.K. Kim, E.-J. Choi, Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta* 1802 (2010) 396-405.
- [4] C. Bendotti, M. Bao Cutrona, C. Cheroni, G. Grignaschi, D. Lo Coco, M. Peviani, M. Tortarolo, P. Vegliane, E. Zennaro, Inter- and intracellular signaling in amyotrophic lateral sclerosis: role of p38 mitogen-activated protein kinase. *Neurodegener. Dis.* 2 (2005) 128-134.
- [5] M. Tortarolo, P. Vegliane, N. Calvaresi, A. Botturi, C. Rossi, A. Giorgini, A. Migheli, C. Bendotti, Persistent activation of p38 mitogen-activated protein kinase in a mouse model of familial amyotrophic lateral sclerosis correlates with disease progression, *Mol. Cell. Neurosci.* 23 (2003) 180–192.
- [6] S.S. Holasek, T.M. Wengenack, K.K. Kandimalla, C. Montano, D.M. Gregor, G.L. Curran, J.F. Poduslo, 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.* 1045 (2005) 185–198.
- [7] M. Dewil, V.F. dela Cruz, L. Van Den Bosch, W. Robberecht, Inhibition of p38 mitogen activated protein kinase activation and mutant SOD1(G93A)-induced motor neuron death, *Neurobiol. Dis.* 26 (2007) 332–341.
- [8] R. Lemmens, M.J. Moore, A. Al-Chalabi, R.H. Brown Jr., W. Robberecht, RNA metabolism and the pathogenesis of motor neuron diseases, *Trends Neurosci.* 33 (2010) 249-258.

- [9] I.R. Mackenzie, R. Rademakers, M. Neumann, TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia, *Lancet. Neurol.* 9 (2010) 995-1007.
- [10] M.A. Gitcho, R.H. Baloh, S. Chakraverty, K. Mayo, J.B. Norton, D. Levitch, K.J. Hatanpaa, C.L. White, 3rd., E.H. Bigio, R. Caselli, et al. TDP-43 A315T mutation in familial motor neuron disease, *Ann. Neurol.* 63 (2008) 535-538.
- [11] A. Yokoseki, A. Shiga, C.F. Tan, A. Tagawa, H. Kaneko, A. Koyama, H. Eguchi, A. Tsujino, T. Ikeuchi, A. Kakita, et al., TDP-43 mutation in familial amyotrophic lateral sclerosis, *Ann. Neurol.* 63 (2008) 538-542.
- [12] E. Kabashi, P.N. Valdmanis, P. Dion, D. Spiegelman, B.J. McConkey, C. Vande Velde, J.P. Bouchard, L. Lacomblez, K. Pochigaeva, F. Salachas, et al., TARDBP mutations in individuals with sporadic and familial amyotrophic lateralsclerosis, *Nat. Genet.* 40 (2008) 572-574.
- [13] J. Sreedharan, I.P. Blair, V.B. Tripathi, X. Hu, C. Vance, B. Rogelj, S. Ackerley, J.C. Durnall, K.L. Williams, E. Buratti, et al., TDP-43 mutations in familialand sporadic amyotrophic lateral sclerosis, *Science* 319 (2008) 1668-1672.
- [14] G.S. Pesiridis, V.M. Lee, J.Q. Trojanowski, Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. *Hum. Mol. Genet.* 18 (2009) R156-R162.
- [15] T.J. Kwiatkowski Jr., D.A. Bosco, A.L. Leclerc, E. Tamrazian, C.R. Vanderburg, C. Russ, A. Davis, J. Gilchrist, E.J. Kasarskis, T. Munsat, et al., Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis, *Science* 323 (2009) 1205-1208.
- [16] C. Vance, B. Rogelj, T. Hortobagyi, K.J. De Vos, A.L. Nishimura, J. Sreedharan, X. Hu, B. Smith, D. Ruddy, P. Wright, et al., Mutations in FUS, an RNA processing

- protein, cause familial amyotrophic lateral sclerosis type 6, *Science* 323 (2009) 1208-1211.
- [17] C. Hewitt, J. Kirby, J.R. Highley, J.A. Hartley, R. Hibberd, H.C. Hollinger, T.L. Williams, P.G. Ince, C.J. McDermott, P.J. Shaw, Novel FUS/TLS mutations and pathology in familial and sporadic amyotrophic lateral sclerosis, *Arch. Neurol.* 67 (2010) 455-461.
- [18] R. Rademakers, H. Stewart, M. Dejesus-Hernandez, C. Krieger, N. Graff-Radford, M. Fabros, H. Briemberg, N. Cashman, A. Eisen, I.R. Mackenzie, Fus gene mutations in familial and sporadic amyotrophic lateral sclerosis, *Muscle Nerve*, 42 (2010) 170-176.
- [19] H. Zinszner, J. Sok, D. Immanuel, Y. Yin, D. Ron, TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling, *J. Cell Sci.*, 110 (1997) 1741-1750.
- [20] C. Lagier-Tourenne, M. Polymenidou, D.W. Cleveland, TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration, *Hum. Mol. Genet.* 19 (2010) R46-R64.
- [21] D.T. Stelow, S.R. Haynes, 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.* 23 (1995) 835-843.
- [22] H. Sasayama, M. Shimamura, T. Tokuda, Y. Azuma, T. Yoshida, T. Mizuno, M. Nakagawa, N. Fujikake, Y. Nagai, M. Ymaguchi, Knockdown of the *Drosophila* Fused in Sarcoma (FUS) homologue causes deficient locomotive behavior and shortening of motoneuron terminal branches, *PLoS One*, 7 (2012) e39483.
- [23] M. Mlodzik, Y. Hiromi, U. Weber, C.S. Goodman, G.M. Rubin, The *Drosophila* *seven-up* gene, a member of the steroid receptor gene superfamily, controls

- photoreceptor cell fates, *Cell* 60 (1990) 211–224.
- [24] S. Kramer, S.R. West, Y. Hiromi, 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* 121 (1995) 1361–1372.
- [25] Y. Takahashi, F. Hirose, A. Matsukage, M. Yamaguchi, Identification of three conserved regions in the DREF transcription factors from *Drosophila melanogaster* and *Drosophila virilis*, *Nucleic Acids Res.* 27 (1999) 510-516.
- [26] J. Sun, J. Tower, FLP recombinase-mediated induction of Cu/Zn superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster*, *Mol. Cell. Biol.* 19 (1999) 216-228.
- [27] Y. Azuma, T. Tokuda, M. Shimamura, A. Kyotani, H. Sasayama, T. Yoshida, I. Mizuta, T. Mizuno, M. Nakagawa, N. Fujikake, M. Ueyama, Y. Nagai, M. Yamaguchi, 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] N.E. Crook, R.J. Clem, L.K. Miller, An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* 67(1993) 2168-2174.
- [29] S.L. Zipursky, G.M. Rubin, Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*, *Annu. Rev. Neurosci.* 17 (1994) 373-397.
- [30] K. Gaengel, M. Mlodzik, Egfr signaling regulates ommatidial rotation and cell motility in the *Drosophila* eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6, *Development* 130 (2003) 5413-5423.
- [31] W. Fu, M. Noll, The *Pax2* homolog *sparkling* is required for development of cone and pigment cells in the *Drosophila* eye, *Genes Dev.* 11 (1997) 2066-2078.

- [32] J.D.Wasserman, S. Urban, M. Freeman, A family of rhomboid-like genes: *Drosophila rhomboid-1* and *roughoid/rhomboid-3* cooperate to activate EGF receptor signaling, *Genes. Dev.*, 14 (2000) 1651-1663.
- [33] B.Z. Shilio, Regulating the dynamics of EGF receptor signaling in space and time. *Development*, 132 (2005) 4017-4027.
- [34] S. Yogev, E.D. Schejter, B.Z. Shilo, *Drosophila* EGFR signaling is modulated by differential compartmentalization of Rhomboid intramembrane proteases. *EMBO J.* 27 (2008) 1219-1230.
- [35] S. Urban, J. R. Lee, M. Freeman, *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases, *Cell* 107 (2001) 173-182.
- [36] K.C. Jordan, N.J. Clegg, J.A. Blasi, A.M. Morimoto, J. Sen, D. Stein, H. McNeill, W.M. Deng, M. Tworoger, H. Ruohola-Baker, The homeobox gene *mirror* links EGF signalling to embryonic dorso-ventral axis formation through notch activation, *Nat. Genet.* 24 (2000) 429-433.
- [37] A. Hidalgo, C.F. Constant, The control of cell number during central nervous system development in flies and mice, *Mech. Dev.*, 120 (2003) 1311-1325.
- [38] F. Agnè, M. Suzanne, S. Noselli, The *Drosophila* JNK pathway controls the morphogenesis of imaginal discs during metamorphosis, *Development* 126 (1999) 5453-5462.
- [39] M. Tare, R.M. Modi, J.J. Nainaparampil, O.R. Puli, S. Bedi, P. Fernandez-Funez, M. Kango-Singh, A. Singh, Activation of JNK signaling mediates amyloid- β -dependent cell death, *PLoS One* 6 (2011) e24361.
- [40] S.A.Correa, K.L. Eales, The role of p38 MAPK and its substrates in neuronal plasticity and neurodegenerative disease, *J. Signal. Transduct.* 2012 (2012) 649079.

- [41] S. Ackerley, A.J. Grierson, S. Banner, M.S. Perkinton, J. Brownlee, H.L. Byers, M. Ward, P. Thornhill, K. Hussain, J.S. Waby, B.H. Anderton, J.D. Cooper, C. Dingwall, P.N. Leigh, C.E. Shaw, C.C. Miller, p38 α stress-activated protein kinase phosphorylates neurofilaments and is associated with neurofilaments pathology in amyotrophic lateral sclerosis. *Mol. Cell. Neurosci.* 26 (2004) 354-364.
- [42] J. Meyerowitz, S.J. Parker, L.J. Vella, D.Ch. Ng, K.A. Price, J.R. Liddell, A. Caragounis, Q.X. Li, C.L. Masters, T. Nonaka, M. Hasegawa, M.A. Bogoyevitch, K.M. Kanninen, P.J. Crouch, A.R. White, C-Jun N-terminal kinase controls TDP-43 accumulation in stress granules induced by oxidative stress, *Mol. Neurodegener.* 6 (2011) 57.

Figure legends

Fig. 1-The level of anti-Caz signals is reduced in Caz dsRNA-expressing areas.

Immunostaining of retinæ at 42h APF with anti-Caz antibody (C, D, J and K). RNAi clones in retinæ 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 retinæ are shown. *P<0.05. (H to M) Immunostaining of retinæ 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-IR₃₆₃₋₃₉₉*. (N) Quantification of intensities of Caz-signals. Mean intensities with standard deviation from nine pupal retinæ are shown. *P<0.05. The bars indicate 100µm (A, C and E), 10µm (B, D and F) and 20µm (H to M), respectively. (O) Western immunoblot analysis. Protein extracts were prepared from the whole pupae of *Drosophila* carrying *Act5C-GAL4/+* (left lane) or *Act5C-GAL4/+*; *UAS-Caz-IR₃₆₃₋₃₉₉/+* (right lane). The blot was probed with anti-Caz antibody.

Fig. 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-IR₃₆₃₋₃₉₉/+*; *UAS-GFP/+*, (e, f) *GMR-GAL4/y*;

UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-P35/+. (g, h) *GMR-GAL4/y; +/+; UAS-P35/+*. The flies were developed at 28°C. 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. 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 retinæ (c, f, i and l) by TUNEL assay. Pupal retinæ were also stained with DAPI (b, e, h and k). (a to c) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; +*. (d to f) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-GFP/+*. (g to i) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-P35/+*. (j to l) *GMR-GAL4/y; +/+; UAS-P35/+*. The flies were developed at 28°C. The bars indicate 100µm.

Fig. 3-Knockdown of *Caz* interferes with differentiation of cone cells.

Immunostaining of retinæ 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; +; rho^{AA69}/+*. (D) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; +*. (E) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; rho^{AA69}/+*. (F) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-GFP/+*. (G) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; 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 retinæ. Ratio of fused cone cells in each retina is shown (%). Mean values with standard deviation from six pupal retinæ 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-IR₃₆₃₋₃₉₉/+; +*, *GMR-GAL4/y;*

UAS-Caz-IR₃₆₃₋₃₉₉/+; rho^{AA69}/+, GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-GFP/+ and GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-P35/+.

Fig. 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-IR₃₆₃₋₃₉₉/+; +*. 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.

Fig. 5-Scanning electron micrographs of adult compound eyes. (A) Female flies expressing *Caz* dsRNA (*GMR-GAL4/GMR-GAL4; UAS-Caz-IR₃₆₃₋₃₉₉; +*) were crossed with *UAS-GFP-IR*, *UAS-rho-IR²⁸⁶⁹⁰*, *rho^{7M43}* (amorph), *rho^{AA69}* (undetermined), *mirror^{SaiD3}* (loss of function), or *ru¹* (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-IR₃₆₃₋₃₉₉/+; rho^{7M43}/+*. (c, d) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; rho^{AA69}/+*. (e, f) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; mirror^{SaiD3}/+* (g, h) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; ru¹/+*. (i, j) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; +*. (k, l) *GMR-GAL4/y; UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-GFP-IR/+*, (m, n) *GMR-GAL4/y;*

UAS-Caz-IR₃₆₃₋₃₉₉/+; UAS-rho-IR²⁸⁶⁹⁰/+. 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-IR₃₆₃₋₃₉₉/+; +*. Arrows indicate morphogenetic furrows (MF). The bars indicate 100µm.

Figure1

Figure2

Figure3

Figure4

Figure5