**Knockdown of the *Drosophila* FIG4 induces deficient locomotive behavior,
shortening of motor neuron, axonal targeting aberration, reduction of life span and
defects in eye development**

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ABSTRACT

Mutations in *Factor-Induced-Gene 4 (FIG4)* gene have been identified in Charcot-Marie-Tooth disease type 4J (CMT4J), Yunis-Varon syndrome and epilepsy with polymicrogyria. FIG4 protein regulates a cellular abundance of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), a signaling lipid on the cytosolic surface of membranes of the late endosomal compartment. PI(3,5)P₂ is required for retrograde membrane trafficking from lysosomal and late endosomal compartments to the Golgi. However, it is still unknown how the neurodegeneration that occurs in these diseases is related to the loss of FIG4 function. *Drosophila* has *CG17840 (dFIG4)* as a human *FIG4* homologue. Here we specifically knocked down *dFIG4* in various tissues, and investigated their phenotypes. Neuron-specific knockdown of *dFIG4* resulted in axonal targeting aberrations of photoreceptor neurons, shortened presynaptic terminals of motor neurons in 3rd instar larvae and reduced climbing ability in adulthood and life span. Fat body-specific knockdown of *dFIG4* resulted in enlarged lysosomes in cells that were detected by staining with LysoTracker. In addition, eye imaginal disc-specific knockdown of *dFIG4* disrupted differentiation of pupal ommatidial cell types, such as cone cells and pigment cells, suggesting an additional role of dFIG4 during eye

development.

Keywords

CMT4J, FIG4, *Drosophila*, neuron, eye

1. Introduction

Factor-Induced-Gene 4 (FIG4), also known as *SAC3*, was firstly identified in a human immature myeloid cell line (Nagase et al., 1996). FIG4 is one of the five proteins carrying a sac phosphatase domain (SAC1, SJL1/INP51, SJL2/INP52, SJL3/INP53, FIG4) found in *Saccharomyces cerevisiae* (Guo et al., 1999; Hughes, 2001). This domain carries seven conserved motifs that define a phosphoinositide phosphatase enzymatic activity (Guo et al., 1999; Hughes et al., 2000), although FIG4 protein by itself appears to have no phosphoinositide phosphatase activity *in vitro* (Guo et al., 1999; Gary et al., 2002).

Previous studies suggested that the FIG4 protein forms a complex with Vac14 at plasma membrane and works as a phosphatase that regulates production of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) preferentially (Gary et al., 2002; Rudge et al., 2004; Duex et al., 2006; Winters et al., 2011). However, later studies indicate that FIG4 complexed with Vac14 can activate the 5'-kinase of PI(3)P known as Fab1 (Jin et al., 2008; Ikononov et al., 2009). This PAS complex mediates the conversion of PI(3)P to PI(3,5)P₂ (Sbrissa et al., 2007; Sbrissa, D et al., 2008; Huotari and Helenius, 2011). In this manner, FIG4 can decrease PI(3,5)P₂ levels via its phosphatase action and

also promote PI(3,5)P₂ synthesis by acting as a secondary scaffold for the PAS. However, the latter function appears to be dominant, since loss of FIG4 results in a reduction of PI3,5P₂ (Chow et al 2007). PI(3,5)P₂ is well known to be associated with intracellular vesicles of the early and late endosome (Winters et al., 2011).

It is reported that mutations in the *FIG4* gene are associated with, Yunis-Varon syndrome (YVS) (Campeau et al., 2013), epilepsy with polymicrogyria (Baulac et al., 2014) and Charcot-Marie-Tooth disease (CMT) (Chow et al., 2007; Zhang et al., 2008; Abe et al., 2011; Li et al., 2013). YVS is an autosomal-recessive disorder with cleidocranial dysplasia, digital anomalies, and severe neurological involvement (Campeau et al., 2013). Enlarged vacuoles are found in neurons, muscle, and cartilage. Homozygous *FIG4*-null mice exhibit features of YVS, including neurodegenerative and enlarged vacuoles in neurons (Campeau et al., 2013). FIG4 is also reported to be a causative gene for the familial epilepsy with polymicrogyria associated with temporo-occipital polymicrogyria, psychiatric manifestations and epilepsy (Baulac et al., 2014). *FIG4*-null mouse brain shows neurodevelopmental impairment in the hippocampus, cortex and cerebellum as well as impaired cerebellar gyration (Baulac et al.,

2014).

CMT is one of the most common forms of inherited neuropathies and affecting both motor and sensory peripheral nerves (Abe et al., 2011). It is known that mutations in the *FIG4* gene are a cause of Charcot-Marie-Tooth disease type 4J (CMT4J) (Chow et al., 2007; Zhang et al., 2008). CMT4J is a peripheral neuropathy characterized during childhood with accelerated limb weakness and muscle atrophy during the teen or adult years that is typically asymmetric and can involve both distal and proximal limb muscles (Li et al., 2013). In addition, the *FIG4* gene is reported to be a genetic susceptibility factor for Amyotrophic lateral sclerosis (ALS) (Chow et al., 2009). ALS is a devastating neurodegenerative disease that is characterized by degeneration of motor neurons of the brain and the spinal cord, which leads to progressive muscle weakness and eventually fatal paralysis, typically within 1 to 5 years after disease onset (Boillée et al., 2006). Up to now there is no effective therapy for these neurodegenerative diseases.

Although previous studies showed the functions of FIG4 protein to be metabolism of PI(3,5)P₂, how mutation of *FIG4* could cause pathogenesis of these neurodegenerative diseases are not fully understood yet. Considering the advantages of *Drosophila* as a

useful model organism, development of *Drosophila* models for these diseases would contribute to understand their pathogenic mechanism. There is a single ortholog for human *FIG4* in *Drosophila*, named *Drosophila FIG4/dFIG4* (CG17840) that is located on 24F4 region of the 2nd chromosome.

In the present study, we found that neuron-specific knockdown of the *dFIG4* gene reduces the climbing abilities of adult flies as well as anatomical defects at neuromuscular junctions, such as a reduced length of synaptic branches, in presynaptic terminals of motor neurons in third instar larvae. The decreased expression of the *dFIG4* may be therefore sufficient for the degeneration of motor neurons and for the deficient locomotive behavior. Moreover, eye disc-specific knockdown of *dFIG4* disrupted differentiation of cone cells and pigment cells in pupal retinae, suggesting an additional role of dFIG4 during compound eye development.

2. Materials and Methods

2.1. Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5%

glucose and 7% dry yeast. The flies carrying $w; UAS-dFIG4-IR_{605-714}; + (CG17840)$ was obtained from the Vienna Drosophila RNAi center (VDRC). The fly lines carrying $w; UAS-dFIG4-IR_{516-523}; + (CG17840)$ and $w; P[GAL4-elav.L]^3$ were obtained from Bloomington Drosophila Stock Center at Indiana University. FB-GAL4 driver ($w; P[w^{+mW.hs}=GawB]FB+SNS$) (Gronke et al., 2009) was kindly supplied from Dr R. P. Kuhnlein.

2.2. Comparison of amino acid sequences of human FIG4 and *Drosophila* FIG4

The amino acid sequence of *Drosophila* FIG4 was retrieved from the Flybase (<http://flybase.org>). The identity and the similarity of *Drosophila* FIG4 and human FIG4 were compared using BLAST (<http://blast.genome.jp/>) and FASTA (<http://fasta.genome.jp/>). FASTA was used for comparison of the whole sequences, and BLAST was used for comparison of each of the corresponding domains between dFIG4 and human FIG4.

2.3. Production of rabbit anti-dFIG4 antibody

The dFIG4 peptide, H-CTSQRPPHPHEDRYKRMFQN-OH corresponding to amino acid residues (aa) 131-aa149 of dFIG4 (the underlined N terminal residue C was an added residue) were individually conjugated with Keyhole limpet hemocyanin (KLH). This KLH-conjugated peptide was mixed with Freund's complete adjuvant to provide a suspension, which was injected subcutaneously into rabbit (Japanese White) kept under SPF conditions. The rabbits were then boosted with inoculations of an immunogen of the same quality once a week for 7 weeks, and a terminal bleed was performed to collect the maximum amount of serum (Sigma-Aldrich). The IgG was purified from the serum by using a Protein A Mag SepharoseTM Xtra (GE healthcare).

2.4. Western immunoblotting analysis

Protein extracts from the central nervous system (CNS) of *Drosophila* adults carrying *UAS-GFP-IR/+*; *elav-GAL4/+*, *w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+* or *w*; *UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+* were prepared. Briefly, the CNS was excised from newly eclosed adult male flies and homogenized in a sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol

blue and 1.2% β -mercaptoethanol. The homogenates were boiled at 95 °C for 5 min, and then centrifuged. The supernatants (extracts) were electrophoretically separated on SDS-polyacrylamide gels containing 8% acrylamide and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The blotted membranes were blocked with TBS/0.05% Tween 20 containing 1% blocking solution (GE healthcare) for 1 h at 25 °C, followed by incubation with rabbit anti-dFIG4 IgG at a 1:500 dilution for 16 h at 4 °C. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (GE healthcare) at 1:10,000 dilution for 2 h at 25 °C. Antibody binding was detected using ECL Western blotting detection reagents (GE healthcare) and images were analyzed with a AE-9300H Ez-Capture MG (ATTO). To ensure equal protein loading in each lane, the membranes were also probed with an anti- α -tubulin antibody after stripping the complex of anti-dFIG4 antibody and HRP-conjugated anti-rabbit IgG. For the detection of α -tubulin, mouse anti- α -tubulin monoclonal antibody (1:5,000 dilution, Sigma,) and HRP-conjugated anti-mouse IgG (1:10,000 dilution, GE healthcare) were used as the primary and secondary antibodies, respectively.

2.5. Immunostaining

For immunohistochemistry, larval eye imaginal discs and both pupal retinæ and adult heads 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; rabbit anti-FIG4 IgG (1:50), 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 (Dlg) (1:500, DSHB 4F3) and mouse anti-Chaoptin (1:100, DSHB 24B10). 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 or Alexa 594 (1:400, Invitrogen) for 3 h at 25°C. After extensive washing with PBS containing 0.3% Triton X-100, samples were mounted in the Vectashield (Vector laboratories) and analyzed by a confocal laser scanning microscopy (Olympus Flouview FV10i).

2.6. Visualization of neuromuscular junction

For visualization of neuromuscular junction (NMJ), third instar larvae carrying *w*; *UAS-GFP-IR*/+; *elav-GAL4*/+, *w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+ and *w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+ were dissected in HL3 saline (Stewart et al., 1994) and fixed in 4% paraformaldehyde in PBS for 30 min at 25°C. After blocking with PBS containing 2% bovine serum albumin (BSA) and 0.1% TritonX-100, FITC-conjugated goat anti-HRP (1:1,000, MP biochemicals) was applied to the sample as the detection antibody. The stained samples were mounted and inspected under a Zeiss LSM510 confocal laser-scanning microscope. MN4 (Ib) in muscle 4 in abdominal segment 2 was quantified. Images were acquired using a Zeiss LSM510 by merging 0.2-1.0 µm interval Z-sections onto a single plane. Nerve terminal branch lengths were measured using MetaMorph software (Molecular devices).

2.7. Visualization of lysosomes with LysoTracker

For visualization of lysosomes, fat body was obtained by dissecting third instar

larvae carrying *w;UAS-GFP-IR/FB-GAL4* or *w; UAS-dFIG4-IR₆₀₅₋₇₁₄/FB-GAL4* in PBS and stained with 100 nM of LysoTracker Blue DND22 (Invitrogen) for 1 min. The stained samples were washed twice with PBS and then mounted in the Vectashield (Vector laboratories) and analyzed by a confocal laser scanning microscopy (Olympus Flouview FV10i).

2.8. Viability assay

Viability assays were carried out in a humidified, temperature controlled incubator at 25 °C and 60% humidity on a 12 h light and 12 h dark cycle on standard fly food. Flies carrying *w; UAS-GFP-IR/+; elav-GAL4/+*, *w; UAS-dFIG4-IR₆₀₅₋₇₁₄/+; elav-GAL4/+* and *w; UAS-dFIG4-IR₅₁₆₋₅₂₃/+; elav-GAL4/+* were placed at 28 °C, and newly eclosed adult male flies were separated and placed in vials at a low density (10-20 flies per vial). Every 3 days, they were transferred to new tubes containing fresh food and deaths were scored. Survival rate was determined by plotting a graph of the percentage of surviving flies versus days.

2.9. Climbing assay

Climbing assays were performed as described previously (Feiguin et al., 2009). Flies carrying *w*; *UAS-GFP-IR*/+; *elav-GAL4*/+, *w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+ and *w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+ were placed at 28 °C, and newly eclosed adult male flies were separated and placed in vials at a density of 25-30 flies per vial. Flies were transferred, without anesthesia, to a conical tube, then the tubes were tapped to collect the flies to the bottom, and were then given 30 s to climb the wall. The flies were collected at the bottom by tapping of the tube, and were again allowed to climb for 30 s. The similar procedures, all of which were videotaped, were repeated five times in total. For all of the climbing experiments, the height to which each fly climbed was scored as follows (score (height climbed)); 0 (less than 2 cm), 1 (between 2 and 3.9 cm), 2 (between 4 and 5.9 cm), 3 (between 6 and 7.9 cm), 4 (between 8 and 9.9 cm) and 5 (greater than 10 cm). The climbing index of each fly strain was calculated as follows; the sum of the products of each score multiplied by the number of flies for which that score was recorded, was calculated, and this number was then divided by five times the total number of flies examined. These climbing assays were carried out at day 3, day 7 and day 14 after

eclosion.

2.10. Data analysis

For the statistical analysis of NMJ length, Microsoft Excel 2007 was used. P-values were calculated using Welch's t-test and the error bars represent Standard Errors from Means. For the statistics of climbing assay, the SPSS software (IBM) was used. All data are shown as means $\pm$ SEM. For the statistics of viability assay, the GraphPad Prism version 6.02 –Survival of Three groups– software was used.

3. Results

3.1. Comparison of the amino acid sequence of human FIG4 and *Drosophila* FIG4

The amino acid sequence of *Drosophila* FIG4 (CG17840) was retrieved from the Flybase and was compared with that of human FIG4 using FASTA and BLAST (Figure 1). The identity and the similarity of the amino acid sequences of dFIG4 and human FIG4 are 41.6% and 66.8%, respectively. Regarding conservation of specific FIG4 domains, the Sac domain, which is known to have a phosphatase activity, is highly conserved

between human FIG4 and *Drosophila* FIG4, showing 49% identity. The similarity of the human and *Drosophila* Sac domain is as high as 75% (Figure 1). High conservation of amino acid sequences between dFIG4 and human FIG4 suggests that dFIG4 is truly a *Drosophila* homologue of human FIG4.

3.2. Specificity of the anti-dFIG4 antibody and evaluation of knockdown efficiency

We raised a polyclonal antibody against a peptide corresponding to aa131-aa149 of *Drosophila* FIG4 for immunological studies (Figure 2). In order to confirm the specificity of this antibody, we used this polyclonal antibody for immunoblotting analyses with extracts of adult heads carrying *w*; *UAS-GFP-IR*/+; *elav-GAL4*/+, *w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+ and *w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+ (Figure 2A). A major band with an apparent molecular weight of 97,000 was detected on immunoblots of the flies carrying *w*; *UAS-GFP-IR*/+; *elav-GAL4*/+ using the anti-dFIG4 antibody, although a few minor bands are also detected (data not shown). The molecular size of this band is in the similar range as that predicted from the amino acid sequences of dFIG4 (100.66 kDa). The intensity of this band was reduced to 18.9 % in flies carrying *w*;

UAS-dFIG4-IR₆₀₅₋₇₁₄/+; elav-GAL4/+ and to 21.3 % in those carrying *w*;

UAS-dFIG4-IR₅₁₆₋₅₂₃/+; elav-GAL4/+ in compared with its intensity in the control fly

carrying *w*; *UAS-GFP-IR/+; elav-GAL4/+* (Figure 2B). These data also confirmed that

dFIG4 is effectively knocked down in flies carrying both *w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+;*

elav-GAL4/+ and *w*; *UAS-dFIG4-IR₅₁₆₋₅₂₃/+; elav-GAL4/+*.

The anti-dFIG4 antibody was used for immunostaining of central nervous system (CNS) of larvae. *Drosophila* FIG4 was strongly expressed in larval CNS (Figure 3A) and

the dFIG4 signals were reduced in the *dFIG4*-knockdown fly carrying *w*;

UAS-dFIG4-IR₆₀₅₋₇₁₄/+; elav-GAL4/+ (Figure 3B). The dFIG4 signals do not merge with

DAPI signals, indicating that dFIG4 specifically localizes in cytoplasm (Figure 3A, a-2 to c-2).

3.3. Neuron-specific *dFIG4* knockdown causes short life span and mobility defects in adults

To analyze the effect of *dFIG4*-knockdown on fly phenotypes, we first investigated whether fly viability was affected by neuron-specific knockdown of *dFIG4*. Using an

elav-GAL4 driver that expresses GAL4 in the nervous system of the fly, we analyzed the phenotypes of flies in which *dFIG4* double-stranded RNA (dsRNA) was expressed throughout the nervous system. Efficient knockdown of *dFIG4* has been confirmed as described above (Figure 2). To examine the effects of neuron-specific *dFIG4*-knockdown on the fly life span, we determined the life span of flies carrying *w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+, *w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+ and the control *w*; *UAS-GFP-IR*/+; *elav-GAL4*/+ (Figure 4). We examined adult flies until 120 days after eclosion. The median life span of the control flies (n = 116) was 51 days, whereas flies carrying *w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+ (n = 110) or *w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+ (n = 103) lived an average of 39 days and 36 days, respectively. The differences between control flies and two independent knockdown flies are about 10 days, respectively. These reductions in life span were statistically significant ($p < 0.001$). Life spans of neuron-specific *dFIG4*-knockdown flies were thus significantly shorter than those of the control flies.

In order to further evaluate the functional effects of neuron-specific *dFIG4*-knockdown, we then performed climbing assays of the *dFIG4*-knockdown adult

flies (Figure 5). The flies carrying *w; UAS-dFIG4-IR₆₀₅₋₇₁₄/+; elav-GAL4/+* showed reduced mobility on day 3 (10.9%), day 7 (28.9%) and day 14 (55.3%) in compared to the control flies carrying *w; UAS-GFP-IR/+; elav-GAL4/+*. Similarly flies carrying *w; UAS-dFIG4-IR₅₁₆₋₅₂₃/+; elav-GAL4/+* showed reduced mobility both on day 3 (7.1%) and day 14 (27.1%). All of these reductions in mobility were statistically significant ($p < 0.05$). More prominent reductions in mobility were observed in aged flies (compare day 3 and day 14 in Figure 5, $p < 0.05$). These results indicate that *dFIG4* is required for locomotion.

3.4. *dFIG4* regulates the formation of motor neurons at presynaptic terminals in the NMJ

Based on the observations that *dFIG4*-knockdown flies showed mobility deficits in the climbing assays, together with the reports indicating that *FIG4*, the human counterpart of *dFIG4*, is involved in CMT4J that impairs motor neurons (Chow et al., 2007; Zhang et al., 2008; Abe et al., 2011; Li et al., 2013). Furthermore, mutations in *Drosophila glycyl-tRNA synthetase* gene that is another CMT-causing gene induced

defects in motor neuron morphology at NMJ (Niehues et al., 2015). We therefore decided to analyze the morphology of motor neuron presynaptic terminals at NMJs in these flies. Since most motor neurons of the adult fly originate from larval motor neurons, we compared the NMJ structure in muscle 4 of the larvae of *w; UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+* and *w; UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+* flies with that of larvae of control flies carrying *w; UAS-GFP-IR/+*; *elav-GAL4/+* (Figure 6A). Measurement of the total length of synaptic branches of motor neurons in these larvae indicated that the total branch length was significantly decreased in *w; UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+* ($66.5 \pm 26.2 \mu\text{m}$, $p < 0.05$) and *w; UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+* ($79.4 \pm 16.9 \mu\text{m}$, $p < 0.05$) flies compared to that of the *w; UAS-GFP-IR/+*; *elav-GAL4/+* ($116.2 \pm 31.8 \mu\text{m}$) flies (Figure 6B). These results suggest that *dFIG4* regulates the formation and/or maintenance of motor neurons at presynaptic terminals in the NMJ. However, since the observed defect in the NMJ may simply reflect a part of motor neuron degeneration, more detailed analyses on the whole motor neurons would be necessary.

3.5. Knockdown of *dFIG4* in eye imaginal discs induces morphologically aberrant

rough eyes

In order to investigate *in vivo* functions of dFIG4, we examined the effect of *dFIG4*-knockdown with the other GAL4 drivers. Knockdown of *dFIG4* in eye imaginal discs by the *GMR*-GAL4 driver strain, in which *dFIG4* 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 (Figure 7). Both of two different RNAi strains targeted to different region of the dFIG4 exhibited the rough eye phenotype, eliminating the possible off-target effects, although extent of the rough was severer with the flies carrying *GMR-GAL4; UAS-dFIG4-IR₆₀₅₋₇₁₄/+* (Figure 7E, F). Flies expressing GFP dsRNA exhibited apparently normal eye morphology (Figure 7A, B).

Photoreceptor cells are known to be generated in a stereotype order. R8 photoreceptor cell is generated first, 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 (Zipursky and Rubin, 1994). To examine whether *dFIG4*-knockdown inhibits differentiation of photoreceptor cells, we crossed the *dFIG4*-knockdown flies 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 discs with anti-LacZ IgG (Figure 8A). In eye imaginal discs of *dFIG4*-knockdown flies, all eight photoreceptor cells appeared to differentiate normally (Figure 8A).

3.6. Knockdown of *dFIG4* induces axonal targeting aberrations of photoreceptor neuron

In *Drosophila* eye, the axonal projections from photoreceptor cell neurons innervate the centers of the brain to generate visual connections. The differentiating photoreceptor cell neurons of the developing fly eye form an axonal bundle, which targets the different layers of the brains. Each ommatidium of the *Drosophila* eye comprises of eight photoreceptors (R1-R8) where R1-R6 innervates lamina and R7-R8 extends into medulla of the brain. The Chaoptin serves as a marker for retinal axons and their projections to the brain. In the control eye imaginal disc photoreceptor cell neuron innervate both medulla and lamina in the brain and thus makes a characteristic “inverted cap” like orientation (Figure 9, A-a and B-a). Although, in the eye imaginal discs of flies carrying

GMR-GAL4; UAS-dFIG4-IR₆₀₅₋₇₁₄, the axonal targeting aberrations appeared to be marginal (Figure 9, A-b), those of flies carrying *UAS-dFIG4-IR₆₀₅₋₇₁₄; elav-GAL4* were severely impaired as their innervation points in the brain are aberrant (Figure 9, B-b). These results suggest that dFIG4 plays a role in axonal targeting.

3.7. Knockdown of *dFIG4* interferes with cell differentiation in pupal retinae

We firstly examined eight photoreceptor cells in pupal retinae by immunostaining with anti-Chaoptin IgG to mark eight photoreceptor cell membranes. In pupal retinae of *dFIG4*-knockdown flies, again all eight photoreceptor cells were detected normally (Figure 10A-c and B-c).

We next examined the pattern formation in pupal ommatidia of *dFIG4*-knockdown flies. In the wild type flies, differentiation of photoreceptors, cone cells, and primary (PPC), secondary (SPC), and tertiary pigment cells (TPC) was completed by about 42 h APF at 28°C. However, in pupal retinae of *dFIG4*-knockdown flies, immunostaining with an anti-Cut antibody that marks cone cells showed that one out of four cone cells per ommatidium were lost in many ommatidia of flies expressing *dFIG4* dsRNA (Figure

10B-a). Flies expressing GFP dsRNA exhibited apparently normal cone cells (Figure 10A-a). The quantified data indicate that occurrence of loss of cone cell was increased by 5.7 fold in the *dFIG4*-knockdown retinæ (Figure 10C).

Furthermore, we monitored apical cell junctions in pupal retina at 42 h APF by immunostaining with the anti-Dlg antibody. In pupal retinæ of *dFIG4*-knockdown flies, loss of cone cell was again observed in many ommatidia (Figure 10B-b). In addition extra SPC- and/or TPC-like cells surrounding cone cells were observed (Figure 10B-b). These data suggest that knockdown of *dFIG4* disrupts differentiation of pupal ommatidial cell types, especially cone cells, and pigment cells, probably by repressing or enhancing expression of genes involved in differentiation processes.

3.8. Knockdown of *dFIG4* induces enlargement of lysosomes in Fat body

It has been reported that homozygous *FIG4* mutations in cultured mouse cells induce enlarged lysosomes (Zou et al., 2015). We therefore examined effect of *dFIG4*-knockdown on the size of lysosome. Fat body appears to be suitable for this analysis and the tissues of the control fly or *dFIG4*-knockdown fly were stained with

LysoTracker to monitor lysosomes (Figure 11). As expected, significantly increased size of lysosome was evident in fat body of the *dFIG4*-knockdown fly (Figure 11B). These results suggest that dFIG4 plays a role in dynamic regulation of lysosome size.

4. Discussion

In order to clarify whether or not disruption of the physiological functions of dFIG4 are critical for the development of neurodegeneration, we have established fly models in which the *dFIG4* gene is knocked down. We demonstrated that neuron-specific knockdown of *dFIG4* affected the life span of the *dFIG4*-knockdown flies and reduced the climbing abilities of adult flies, and also caused anatomical defects in presynaptic terminals of motor neurons in third instar larvae. These results suggest that a decrease in *dFIG4* expression is sufficient for the development of defects in locomotive abilities and for a decrease in the total length of synaptic branches of motor neurons at the NMJs in *Drosophila*. Although we focused on defects in presynaptic terminals of motor neurons in the present study, the observed defects may simply reflect a part of motor neuron degeneration. More detailed analyses on the whole motor neurons would be therefore

necessary to further address that the *Drosophila* model established in this study faithfully represents the pathology of CMT4J.

To eliminate the possibility of off-target effects of our RNAi constructs, we used two different dFIG4 inverted repeat constructs (*UAS-dFIG4-IR*₆₀₅₋₇₁₄ and *UAS-dFIG4-IR*₅₁₆₋₅₂₃) whose target sequences did not overlap with each other. When these transgenic flies were crossed with the *elav*-GAL4 line to specifically express *dFIG4* dsRNA in neuronal tissues, each independent fly strain showed essentially the same phenotype. These results indicate that the phenotypes observed with the neuron-specific *dFIG4*-knockdown flies are not due to an off-target effect but rather to a reduction in dFIG4 protein levels.

Mutations in the *FIG4* gene are associated with CMT4J (Chow et al., 2007; Zhang et al., 2008; Abe et al., 2011; Li et al., 2013), YVS (Campeau et al., 2013) and epilepsy with polymicrogyria (Baulac et al., 2014). In this study, we demonstrated that neuron-specific knockdown of *dFIG4*, the *Drosophila* *FIG4* homologue, could induce a short life span, a defect in fly locomotive abilities, degeneration of motoneurons at NMJs and axonal targeting aberrancy of photoreceptor neurons. These phenotypes are at least partially

similar to pathology of **CMT4J** patients. Our results also suggest that the loss of physiological FIG4 function is likely to be the fundamental pathogenic mechanism that causes FIG4-related **CMT4J**. To elucidate the molecular mechanisms leading to neuronal dysfunction in FIG4-associated **CMT4J** are necessary to develop the disease-modifying therapies.

Knockdown of *dFIG4* also induced enlargement of lysosomes that are frequently observed in **CMT4J** and YVS patients, although further analyses are necessary to address relevancy of the *dFIG4*-knockdown fly as YVS model. In any event, the *dFIG4*-knockdown fly developed in this study can be used for genome-wide screen of genes that genetically interact with the *dFIG4* gene. Once the *dFIG4*-interacting gene(s) are identified in *Drosophila*, their human homologue(s) could be a candidate(s) for a novel diagnosis marker(s) and/or therapeutic targets of **CMT4J** and/or YVS. In addition, the *dFIG4*-knockdown fly characterized in the present study, at least partially representing features of human **CMT4J**, would also be suitable for the screening of chemicals that can modify these pathogenic processes leading to the degeneration of motor neurons in **CMT4J**.

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Figure legends

Figure 1. Sequence alignment of FIG4 proteins from human and *Drosophila*

melanogaster. Human and *Drosophila* FIG4 consist of 903 amino acids and 853 amino acids, respectively. Identical amino acids are dark shaded and similar or related amino acids are light shaded. The highly conserved Sac domain is enclosed.

Figure 2. Western immunoblot analysis. (A) Proteins were extracted from adult heads carrying *elav-GAL4>UAS-GFP-IR* (*w*; *UAS-GFP-IR/+*; *elav-GAL4/+*), *elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃* (*w*; *UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+*) and *elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄* (*w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+*). The blots were probed with a rabbit anti-dFIG4 antibody. The band with an apparent molecular weight of 97,000 corresponds to dFIG4 protein. (B) Quantification of the 97 kDa band with an image analyzer AE-9300H Ez-Capture MG. α -Tubulin was used as a loading control.

Figure 3. Immunocytochemical localization of dFIG4 in larval central nervous system. (A, B) Larval central nervous systems were stained with DAPI (a-1 and a-2), anti-dFIG4 IgG (b-1 and b-2). Merged confocal images are shown in c-1 and c-2. Higher magnification images of boxed regions in a-1 to c-1 are shown in a-2 to c-2. The enlarged images of boxed regions in a-2 to c-2 are shown in insets. Arrowheads indicate the

DAPI-stained nucleus in the single cell. (A) *elav-GAL4>UAS-GFPIR* (*w*; *UAS-GFP-IR/+*; *elav-GAL4/+*). (B) *elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄* (*w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+*). Bars indicate 50 μ m (a-1 to c-1) and 20 μ m (a-2 to c-2).

Figure 4. Life span analyses. Percentage survivals of adult male flies carrying *elav-GAL4>UAS-GFPIR* (*w*; *UAS-GFP-IR/+*; *elav-GAL4/+*, n=116), *elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃* (*w*; *UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+*, n=103) and *elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄* (*w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+* n=110) are shown with SE bars. The median lifespan for each strain is indicated with red line. The reductions in life span of dFIG4-knockdown flies are statistically significant ($p < 0.001$).

Figure 5. Climbing assays. Five independent tests were performed for each genotype.

The total number of flies counted was as follows: *elav-GAL4>UAS-GFPIR* (*w*; *UAS-GFP-IR/+*; *elav-GAL4/+*, n=135); *elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃* (*w*; *UAS-dFIG4-IR₅₁₆₋₅₂₃/+*; *elav-GAL4/+*, n=155); *elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄* (*w*; *UAS-dFIG4-IR₆₀₅₋₇₁₄/+*; *elav-GAL4/+*, n=155). Flies carrying

elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃ or elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄ showed significantly reduced ability to climb up in compared to the control flies carrying elav-GAL4>UAS-GFPIR. The horizontal bars indicate standard errors of mean values. *P<0.05.

Figure 6. Confocal images of anti-HRP staining of muscle 4 synapse in third instar

larvae. (A) A representative image of anti-HRP staining of muscle 4 synapses in third instar larvae with elav-GAL4>UAS-GFPIR (*w*; *UAS-GFP-IR*/+; *elav-GAL4*/+, panel a), elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃ (*w*; *UAS-dFIG4-IR*₅₁₆₋₅₂₃/+; *elav-GAL4*/+, panel b), or elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄ (*w*; *UAS-dFIG4-IR*₆₀₅₋₇₁₄/+; *elav-GAL4*/+, panel c). Bars indicate 50 μm. **(B)** Total branch length of the NMJ from muscle 4 for each of the indicated genotypes. Compared to the total length of synaptic branches of motoneurons in the control elav-GAL4>UAS-GFPIR larvae, those in elav-GAL4>UAS-dFIG4IR₅₁₆₋₅₂₃ larvae (*P<0.05, n=10) and elav-GAL4>UAS-dFIG4IR₆₀₅₋₇₁₄ (*P<0.05, n=10) are significantly decreased.

Figure 7. Knockdown of *dFIG4* in eye imaginal discs induces a rough eye phenotype.

Scanning electron micrographs of adult compound eyes. (A, B)

GMR-GAL4>UAS-GFP_{IR} (*GMR-GAL4/y;UAS-GFP-IR/+*); (C, D)

GMR-GAL4>UAS-dFIG4_{IR516-523} (*GMR-GAL4/y;UAS-dFIG4-IR516-523/+*) ; (E, F)

GMR-GAL4>UAS-dFIG4_{IR605-714} (*GMR-GAL4/y;UAS-dFIG4-IR605-714/+*). 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. Anterior is to the left and dorsal to the top. The flies

were developed at 28°C. The bars indicate 50µm (A, C, E) and 14.2µm (B, D, F),

respectively.

Figure 8. Knockdown of *dFIG4* exerts no effect on differentiation of photoreceptors

in eye imaginal discs. Immunostaining of eye imaginal discs with anti-LacZ IgG (A, B).

(A, a-1 and a-2) *GMR-GAL4/y; UAS-GFP_{IR}/+; AE127-lacZ/+*. (B, a-1 and a-2)

GMR-GAL4/y; deadpan-lacZ/ UAS-GFP_{IR}. (A, b-1 and b-2) *GMR-GAL4/y;*

UAS-dFIG4-IR605-714/+; AE127-lacZ/+. (B, b-1 and b-2) *GMR-GAL4/y;*

deadpan-lacZ/*UAS-dFIG4-IR*₆₀₅₋₇₁₄. AE127 and p82 express β -galactosidase in R3/R4/R1/R6 (marked as 3,4,1,6) and R3/R4/R7 (marked as 3,4,7), respectively. Panels a-2 and b-2 show higher magnification images of the regions panels a-1 and b-1. The flies were developed at 28°C. The bars indicate 50 μ m (a-1, b-1) and 10 μ m (a-2, b-2).

Figure 9. Knockdown of *dFIG4* induces axonal targeting aberration of photoreceptor neuron. Immunostaining of photoreceptor neurons with anti-Chaoptin IgG (24B10) (A,B). (A-a) *GMR-GAL4; UAS-GFP-IR* (*GMR-GAL4/y; UAS-GFP-IR*). (A-b) *GMR-GAL4; UAS-dFIG4IR*₆₀₅₋₇₁₄ (*GMR-GAL4/y; UAS-dFIG4-IR*₆₀₅₋₇₁₄). (B-a) *elav-GAL4>UAS-GFP-IR* (*w; UAS-GFP-IR/+; elav-GAL4/+*). (B-b) *elav-GAL4>UAS-dFIG4IR*₆₀₅₋₇₁₄ (*w; UAS-dFIG4-IR*₆₀₅₋₇₁₄/*+*; *elav-GAL4/+*). Arrows indicate lamina where R1 to R6 axons innervate. Arrowheads indicate the end of medulla region where R7 and R8 extend. The bars indicate 50 μ m (a, b).

Figure 10. Knockdown of *dFIG4* interferes with differentiation of cone cells and pigment cells. Immunostaining of retinae at 42 h APF with anti-Cut IgG that marks cone

cells (a), anti-Dlg IgG (b) and anti-Chaoptin IgG (c). (A) GMR-GAL4>UAS-GFP-IR (*GMR-GAL4/y; UAS-GFP-IR*). (B) GMR-GAL4>UAS-*dFIG4IR*₆₀₅₋₇₁₄ (*GMR-GAL4/y; UAS-dFIG4-IR*₆₀₅₋₇₁₄). The flies were developed at 28°C. Ommatidia with three cone cells are shown by arrows (B-a). The missing cone cells are encircled (B-b). R1 to R8 photoreceptor cells are indicated as 1 to 8 (A-c and B-c). The bar indicates 10 µm (A-a,b and B-a,b) and 20 µm (A-c and B-c). (C) Quantification of ommatidia with three cone cells in pupal retinæ. Ratio of ommatidia with three cone cells in each retina is shown (%). Mean values with standard deviation from ten pupal retinæ are shown. *P<0.05.

Figure 11. Knockdown of *dFIG4* induces enlarged lysosomes in Fat body. Larval Fat body was stained with LysoTracker to visualize lysosomes. (A)FB-GAL4>UAS-GFP-IR (*w; FB-GAL4/ UAS-GFP-IR*). (B)FB-GAL4>UAS-*dFIG4IR*₆₀₅₋₇₁₄ (*w; FB-GAL4/ UAS-dFIG4-IR*₆₀₅₋₇₁₄). Enlarged images of lysosomes are shown in insets. The bar indicates 10 µm.

Figure1

human	1	-----MPTAAAPIISSVOKLVLYETRARYFLVGSNNAETKYRVLKIDRTEPKDLVIIDDR	55
Drosophila	1	MNNDNPNIIVFNPLISSIQKVLYETRARLYLVGSNNRETRFRLLTIDRLAHNRLSIEENA	60
human	56	HVYTOQEVRELLGRLDLGNRTKMGQKGSSGLFRAVSAFGVVGFRFLEGYYIVLITKRRK	115
Drosophila	61	NEFNSLEIRRFVASL-----SGSPKVT SAYGVLGFRFLEGYYLLLVTKRKC	107
human	116	MADIGGHAIYKVEDTNMIYIPN-DSVRVTHPDEARYLRIFONVDLSSNFYFSYSYDLSHS	174
Drosophila	108	CAHIGRHLVYTIKDTVMVRVNEVTSQRPPHPHEDRYKRMFQONIDLRSNFYFSYSYDLTRT	167
human	175	LQYNLTVLRMPLEMLKSEMTQNRQESFDIFEDEGLITQGGSGV-----FGICSEPYMKYV	229
Drosophila	168	LQYNESAPRY-----VGAKVDLDRDEPL---PDWNTLTSNVDKAHERVDYAFRSDSRKRFV	220
human	230	WNGELLDIIKSTVHRDWLLYIIHGFCGQSKLLIYGRPVYVTLIARRSSKFAGTRFLKRG	289
Drosophila	221	WNAYLLQPMEGIMLKDWLLEVTGGEVSQSCISIFGRHVNVC LVARRSSRFAGTRFLKRG	280
human	290	NCEGDVANEVETEQILCDASVMSFTAGSYSSYVQVRGSVPLYWSODISTMMPKPPITLDO	349
Drosophila	281	NFQGDVANEVETEQIVSDGQRIC-----AFTQMRGSI PSHWSQDISKMVPKPKQIQLDI	333
human	350	ADPFAHVAALHFDOMFORFGSPITIIILNLVKEREKRKHHERILSEELVAAVTYLNQFLPPEH	409
Drosophila	334	CDPYAQTPSLHFERLLFHYGAPLIMLNLVKKRERRKHESIISKELEYSIRYLNQFLPPPH	393
human	410	TIVYIPWDMAKYTKSKLCNVLDRLNVIAESVVKKTGFFVNRPDSYCSILRPDEKWNELGG	469
Drosophila	394	RMKHIIHFDMARQSRLSGGNVMEQLAIAESIVQMTGMFFKAAGS-----EPG-	440
human	470	CVIPTGRLOTGILRTNCVDCLDRNTAQFMVGKCALAYOLYSLGLIDKPNLOFDTDAVRL	529
Drosophila	441	-----LQTGIVRTNCVDCLDRNTSAQFAIGKCALGHQLERLGFVKSACLEFDSDCVTM	493
human	530	FEELYEDHGD TLSLOYGGSQLVHRVKTYRKIAPWTOHSKDIMOTLSRYYSNAFSDADROD	589
Drosophila	494	LENLYEEHGD TLALQYGGSQLVHRIKTYRKTA PWGSQGSQSDVMQTL SRYYSNTFSDTEKQH	553
human	590	SINLFLGVFHP--TEGKPHLWELPTDFYLHHKNTMRLLPTRRSYTYWWTPEVIKHLPLPY	647
Drosophila	554	SINLFLGIYKPSLTKQGPPIWELQTDYDMHNAFVPR--ADSKAITDWVRHKVRACL PYS-	610
human	648	DEVICAVNLKKLIVKKFHKYE---EEIDIHNEFFRYPYELSSFDDTFCLAMTSSARDFMPK	704
Drosophila	611	-----CA-DSNKLVKELFRVHSSGLEMIDAYSNYHQSFKWTDFSEHIAFEISQLALRYMP-	665
human	705	TVGIDPSPF-----TVRK-----PDETGKSVLGNKSNREEAVLQRKTAASAPPPPSEEAVS	755
Drosophila	666	TFRTNFSPPFORQIQTSRKARQNP SMTGQSSTGS-----MNSNSSSSS-----	707
human	756	SSSEDDSGTDREEEGSVSQRSTPVKMTDAGDSAKVTENVVQP-MKELYGINLSDGLSEED	815
Drosophila	708	--EGDDSSSDEELSASF AEKE--ANQTESTEPAAATLATGLPSMEEIYGCTINPP-SKQS	762
human	816	FSIYSRFVOLGOSQHKQDKNSQ-----OPCSRCSQDGVIKLTPISAFSODNIYEVOPPR	868
Drosophila	763	MAVYKKYVQMGKLSGGARPAQTAVAQRDQELAKIMRG-ITLRPLSDYGTDSYLSVRPPV	821
human	869	VDRKSTEIFQAHIQASQGIMQPLGKEDSSMYREYIRNRYL	903
Drosophila	822	VPRKSLTIYAEYCRTRSTFNAPVKLEEFDVLYQYVQKL--	853

Figure2

A

B

Figure3

Figure4

Figure5

Figure6

Figure7

Figure8

Figure9

A GMR-GAL4;UAS-GFPIR

GMR-GAL4;UAS-*dFIG4IR*₆₀₅₋₇₁₄

B elav-GAL4>UAS-GFPIR

elav-GAL4>UAS-*dFIG4IR*₆₀₅₋₇₁₄

Figure10

A GMR-GAL4;UAS-GFP^{IR}

B GMR-GAL4;UAS-*dFIG4R*₆₀₅₋₇₁₄

Figure11

FB-GAL4>UAS-GFP_{IR}

FB-GAL4>UAS-*dFIG4*_{IR}⁶⁰⁵⁻⁷¹⁴