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Lipid Phosphate Phosphatase gene *CG11426* regulates glia cell numbers through Hippo signaling pathway

인지질 탈인산화효소 유전자 *CG11426*의 Hippo signaling pathway를 통한 신경교세포의 세포 수 조절

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ABSTRACT

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Glial cells are known to have various functions such as supporting neuron nutrients, defending neuronal environments, and axon pathfinding. Among them, *Drosophila* glial cells are highly similar to human glial cells. Therefore, studying *Drosophila* glial cells is important to understand human neurodegeneration. I investigated a *Drosophila* gene which encodes *lipid phosphate phosphatase (LPP) gene*. The functions of the *Drosophila LPP gene* are known as germ cell migration, septate junction formation, and trachea development. In this study, i discovered a novel function of the *Drosophila* gene. Using the GAL4/UAS system, i suppressed the *LPP gene* in the *Drosophila* glia and eye. A mutation in the *LPP* gene caused a proliferation of glial cells in the embryo and eye imaginal disc at the postembryonic. Also i found that mutants of *CG11426* gene have an abnormal phenotype. Mutants of the *LPP* gene fly showed an ommatidia aggregation and trachea defects. These results suggest that the *CG11426* genes were possibly involved in glial cell development.

Keywords: Lipid phosphate phosphatase, glia, proliferation, Hippo signaling pathway, GPCRs signaling, spingosine-1-phosphate

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I. Introduction

1. Glia cell conserved in vertebrate and *Drosophila*

The nervous system consists of two types of cells: neurons and glia. Neurons convey information, while glia cells support neurons. There are 10 to 50 times as many glia cells as neurons but their function is still unclear. Generally, Glia cell provides trophic support, synaptic modulation, neuronal ensheathment, cellular maintenance, and immune surveillances (Barres, 2008; Edwards and Meinertzhagen, 2010; Freeman and Doherty, 2006).

The glia cell is well-conserved in *Drosophila*. The main classes of glia in vertebrates - Astrocyte, Oligodendrocyte, Microglia, and Schwann cell - exhibit functional and morphological homology to their *Drosophila* counterparts (Freeman and Doherty, 2006). Astrocyte is similar to Cortex glia, Oligodendrocyte to Neurophil glia, Microglia to CNS glia cell (Cortex, Neurophil, and Surface glia), and Schwann glia cell to Peripheral glia cell (Table 1). Thus, the investigation of glia cells in *Drosophila* is good genetic tool for study of mammals' glia cells.

Table 1. Comparison of vertebrate and *Drosophila* glial subtypes (Freeman and Doherty, 2006).

Vertebrate glial subtype [7]	Primary functions [7]	Distribution [7]	Comparable *Drosophila* glial subtype[a]
Astrocytes	Trophic support of neurons, synapse modulation	Embedded in CNS cell cortex, ensheathing synapses, CNS surface	Cortex glia (and a subset of surface glia)
Oligodendrocytes	Neuronal ensheathment, trophic support of neurons, myelination	Ensheathing axons in CNS	Neuropil glia
Microglia	Immune surveillance, macrophage function	Throughout CNS	Cortex, surface and neuropil glia
Schwann cells	Ensheathment and support of peripheral nerves, myelination	Ensheathing PNS nerves	Peripheral glia

2. Hippo signaling pathway

The regulation of glia cell numbers is important, but the controlling mechanism of glia cell numbers during development is still unclear. Furthermore, the understanding of the over-growth of glia cells is related to the understanding of brain tumor. Hippo signaling pathway, a well-known signaling pathway in *Drosophila* regulating glia cell numbers, was isolated at first in genetic screening as the regulation of the wing and eye development (Saucedo and Edgar, 2007). When the signaling pathway was inactive, a severe overgrowth of wing and eye tissues occurred. After finding out the Hippo signaling pathway, some studies have reported that the Hippo signaling pathway was related to *Drosophila* glia tumorigenesis and had an orthologue in mammals.

In mammal, MST1/2 activates LATS1/2 which phosphorylates YAP/TAZ, which itself is an oncogene transcription factor. During the YAP/TAZ phosphorylation, YAP/TAZ cannot pass through the nucleus membrane and the expression of oncogene is suppressed (Pan, 2010; Zhao et al., 2007). Thus, an active MST1/2 works as a tumor suppressor. The mechanism of MST1/2 activation is well-known in mammals. There are several studies concerning the activation of MST1/2. Expanded-Hippo signaling mainly involves a Merlin, Kibra, and Expanded. These proteins activate MST1/2 together. Other studies report that MST1/2 is also activated by GPCRs related signaling pathway. The signaling molecules Lysophosphatidic acid (LPA) or Sphingoshine-1-phosphate (S1P) activate GPCRs coupled to $G\alpha_{12/13}$ or $G\alpha_{q/11}$ (Juan and Hong, 2016; Miller et al., 2012). GPCRs act through Rho-GTPase and F-actin and they inhibit LATS1/2. This GPCRs mechanism is independent from MST1/2 activation (Yu et al., 2012).

Likewise, *Drosophila* has similar pathways that suppress tumor or cell

proliferation. In *Drosophila*, Hippo activates Warts which phosphorylates yki. A phosphorylated yki cannot locate in the nucleus and therefore cannot act as an oncogene transcription factor (Harvey et al., 2003; Udan et al., 2003). *Drosophila* Hippo is also activated by Expanded, Merlin, and Kibra (Ling et al., 2010; Robinson et al., 2010; Yu et al., 2010). Yet, there are still another mechanisms that remains unclear and a GPCRs linked mechanism has not been reported yet.

A well-conserved function of the Hippo signaling pathway in both mammals and *Drosophila*, including yki as transcription factor, is the regulation of apoptosis. In *Drosophila*, yki activates DIAP1 which is a transcription factor encoding the inhibition of apoptosis. DIAP1 inactivates the caspases Dronc and Ice (Kumar, 2004; Schwerk and Schulze-Osthoff, 2003). Generally, the activation of caspases leads to a cell death. Thus, activating yki leads to cell proliferation and tumorigenesis.

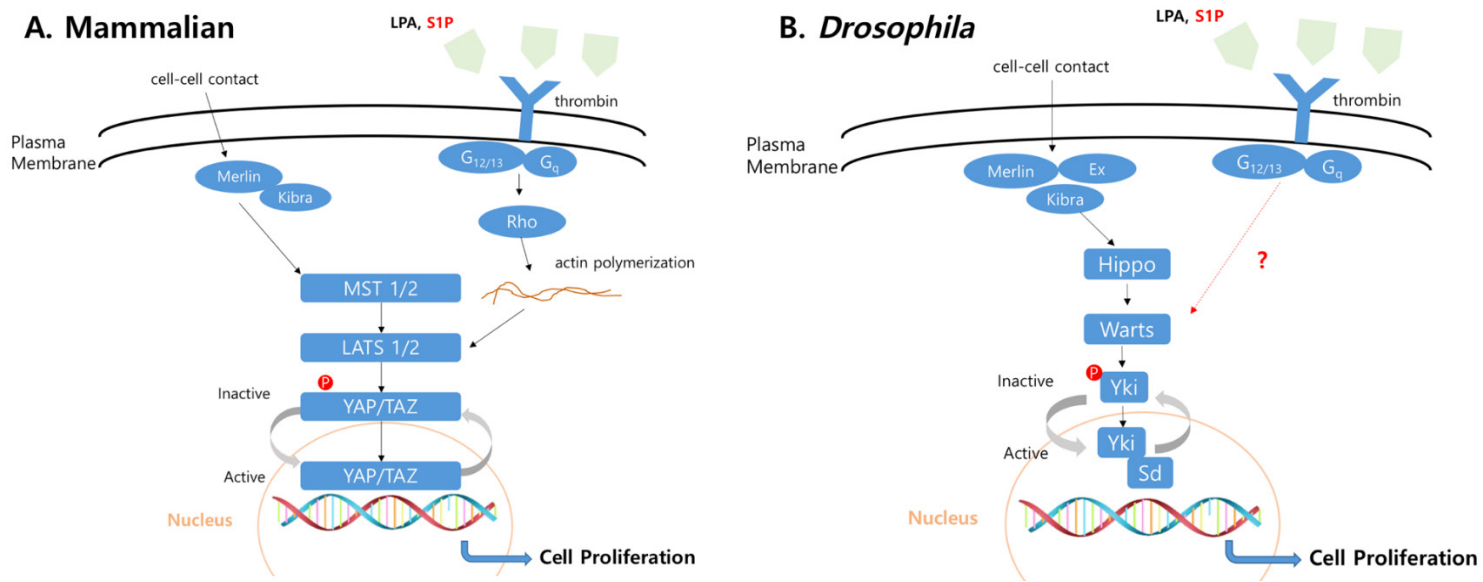

Figure 1. Hippo signaling pathway in Human and *Drosophila*. A) In mammalian Hippo signaling pathway B) in *Drosophila* Hippo signaling pathway.

3. Role of Lipid Phosphate Phosphatase (LPP)

Lipid phosphate phosphatase (LPP), an integral membrane enzyme, consists of six transmembrane α-helices structure. LPP's C- and N-terminal are located on the cytoplasmic side, and catalytic domain is located on the extracellular side (Zhang et al., 2000)**.** This location allows the dephosphorylation of lipid phosphates outside of the cells. The LPP has three catalytic domains; C1, C2, and C3. C1 enables the recognition of substrate for reaction. On the other hand, C2 and C3 have amino acids, which are necessary for phosphotransferase reaction (Neuwald, 1997; Sigal et al., 2005).

LPP dephosphorylates the extracellular Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). LPA and S1P are important for the regulation of cell division, migration, and cell survival (Moolenaar et al., 2004). The regulation of S1P concentration is especially important. S1P has a bioactivity and plays an important role in cell growth, migration, and cell survival. There are several reports that S1P promotes tumor growth, neovascularization and inflammation (Pyne and Pyne, 2010). In addition, various cancers show an increase in Sphingosine kinase, which highlights the importance of regulating S1P and sphingosine concentration.

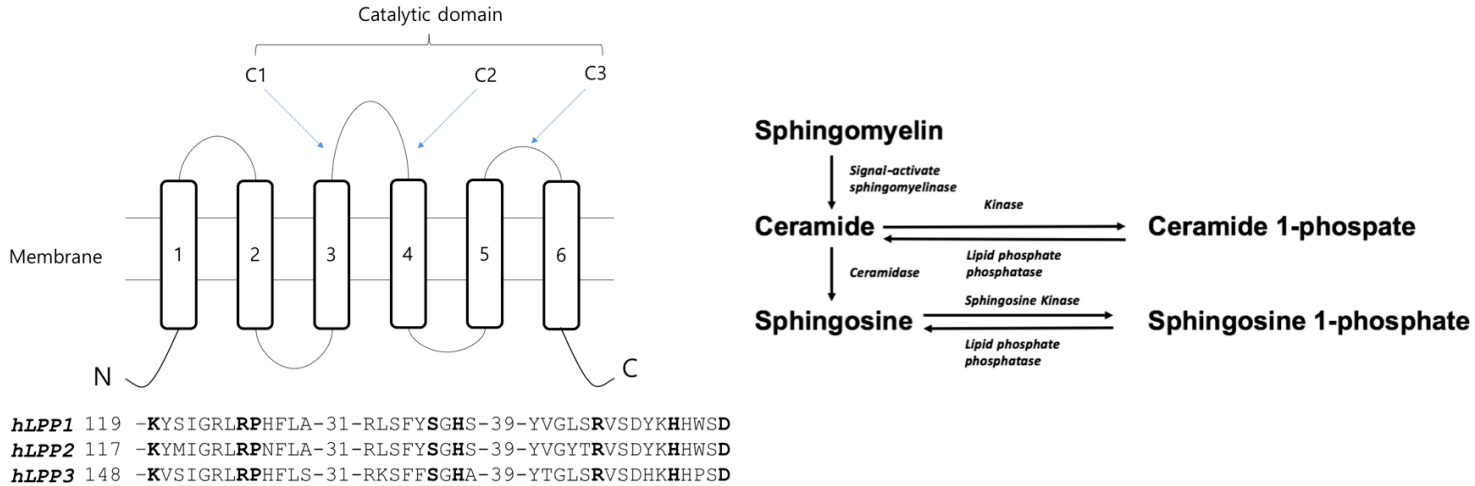

Figure 2. Lipid phosphate phosphatase. LPP has six transmembrane α-helices structure. There are three catalytic domains which well-conserved in three LPP isotypes. LPP converts ceramide-1-phosphate and sphingosine-1-phosphate to ceramide and sphingosine.

4. Lipid Phosphate phosphatase gene in mammals and *Drosophila*

The three isotypes of LPPs are well-known in mammals. These three LPP have well-conserved catalytic domains (Fig. 2). However, they have different functions and non-redundant effects on several diseases and signaling pathways (Brindley et al., 2002; Tang et al., 2015).

LPP1 seems to regulate the cycle of circulating LPA. Mice with a *LPP* overexpression showed a 50% decrease in their birth weight as well as hair abnormalities. However, there was no change of LPA concentration. In contrast, mice with a *LPP* knockdown showed higher LPA concentration (Yue et al., 2004).

LPP2 seems to regulate the cell cycle. *LPP2* knockdown mice showed a delay of S-phase entry and Cyclin A expression in fibroblasts. *LPP2* overexpression had the opposite effect. These *LPP1* and *LPP2* knockout mice showed to be fertile and viable (Flanagan et al., 2009; Tang et al., 2015; Zhang et al., 2000).

LPP3 regulates the vascular system. *LPP3* knockout mice did not form a chorio-allantotic placenta and yolk sac vasculature. This phenotype is related to *Wnt* signaling. Knockout *LPP3* gene decreases the TCF/β-catenin transcription (Escalante-Alcalde et al., 2003).

Drosophila has *LPP* genes known as *Wunen* 1 and *Wunen* 2 which are homologs of human *LPPs*. *Wunen1* and *Wunen2* have highly conserved phosphatase domains (Burnett and Howard, 2003). *Wunens* are important to germ cell migration and survival during embryogenesis (Renault et al., 2010; Starz-Gaiano et al., 2001; Zhang et al., 1996). The loss of *Wunen1* and *Wunen2* in germ cells leads to the death of germ cells, and in somatic cells, lead to mismigration (Renault et al., 2004). Also,

Wunen1 and *Wunen2* were reported to have essential tissue-autonomous roles in trachea development. The loss of *Wunen1* and *2* mutant results in a failure to locate the luminal components necessary for forming the septate junction (Ile et al., 2012).

5. Lipid Phosphate Phosphatase gene *CG11426* expression location is similar to *repo* gene

To find the genes that affect Glia cells, 100 gene candidates were selected with reference to the *flybase* database, and the repo patterns were compared. *repo* which determines the fate of glia cells is expressed in every glia cells except in midline glia cells. *repo* is used as a marker for glia cell studies. In *Drosophila, repo* is expressed in the sensory system, and nervous system at embryonic stage 13. Fig. 3 shows how repo is localized. Among the 100 genes, the gene expression pattern of *CG1146* was most similar to repo. The co-expression rate between *repo* and *CG11426* is 81.54%. Specifically, *CG11426* is strongly expressed in surface glia and longitudinal glia cell. Based on these results, I expected that CG11426 gene might be related to glia development and proceeded with the research.

Here, I analyzed *CG11426* which encodes Lipid phosphate phosphatase and found its functions during neuronal development. *CG11426* has well-conserved LPP catalytic domains and is a homolog of *Wunen*. But the functions of *CG11426* have not been reported yet. To find out *CG11426*'s role, I used *CG11426* knockdown mutant using the GAL4/UAS system. As a result, I found an increased number of glia cells and an ommatidia defect. This indicates that *CG11426* gene is related to glia development. In addition, a knockdown in adult flies showed a higher expression level of *yki* and *Hpo*. This suggests that *CG11426* is possibly involved in the Hippo signaling pathway.

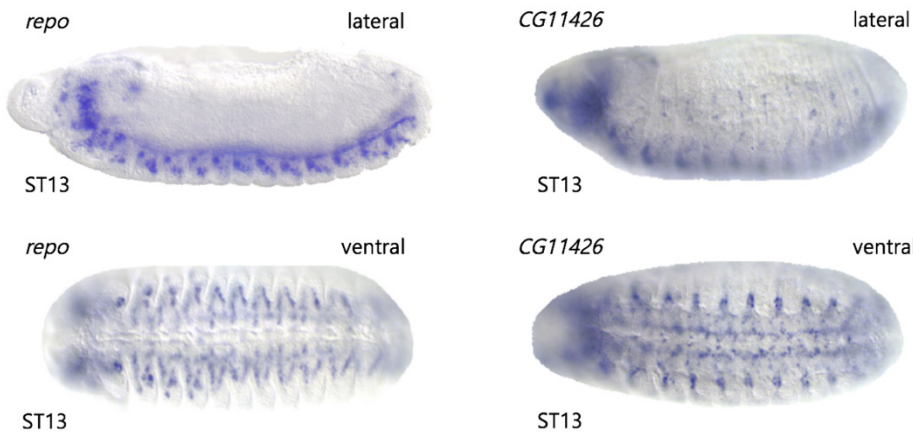

Figure 3. *CG11426* in situ pattern is highly similar to *Repo*. *CG11426* expression location is highly similar to *Repo*. *Repo* gene is expressed in glia cell, sensory system, and nervous system at embryonic stage 13. Likewise, *CG11426* is expressed in surface glia, midline glia, and brain cell at embryonic stage 13. The *in situ* data is from *flyexpression.*

II. Materials and Methods

1. Fly Strains

All flies were copulated and bred on the standard environment at 25°C. The expression of *CG11426* was regulated by TRiP line contribution to the Harvard TRiP project. The TRiP lines (stock number #43281 and #53879) encode UAS-*CG11426* RNAi and UAS-*Dcr* (Bloomington *Drosophila* stock center). *yki*-TRiP (#34067 from Bloomington *Drosophila* stock center) is also contributed by Harvard TRiP project. *CG11426* mutant from Bloomington *Drosophila* stock center (contribution by Exelixis, Inc, stock number #19222) was used. To analyse knockdown phenotypes, GAL4 line; UAS-Dcr2; *Repo*-Gal4/TTG, and ok107-GAL4 (#854 from Bloomington *Drosophila* stock center) were used.

Table 1 shows the scheme for *CG11426* mutant line using RNAi line and repo-GAL4 system. The results in Table 1 are RNAi (*CG11426*)/Cyo ; *Repo*-GAL4/TM3.Sb. Table 2 shows the scheme for *CG11426* RNAi double dose. The results in Table 2 are RNAi (*CG11426*)/Cyo ; RNAi (*CG11426*). Table 3 shows the *CG11426* rescue scheme using *yki*-RNAi and *CG11426*-RNAi. The results in Table 3 are RNAi (*CG11426*)/Cyo ; RNAi (*yki*).

Table 2. Scheme for getting *CG11426* mutant.

-RNAi (*CG11426*)/Cyo ; *Repo*-GAL4/TM3.Sb

P	$\frac{Gla}{CTG} ; \frac{+}{+}$ Ⓧ $\frac{+}{+} ; \frac{Ly}{TM3.Sb}$	P	$\frac{bl}{Cyo} ; \frac{+}{+}$ Ⓧ $\frac{+}{+} ; \frac{repo-GAL4}{TTG}$
F1	$\frac{+}{CTG} ; \frac{+}{TM3.Sb}$	F1	$\frac{+}{Cyo} ; \frac{+}{TTG}$
P	$\frac{+}{CTG} ; \frac{+}{TM3.Sb}$ Ⓧ $\frac{RNAi(CG11426)}{Cyo} ; \frac{+}{+}$	P	$\frac{+}{Cyo} ; \frac{+}{TTG}$ Ⓧ $\frac{+}{+} ; \frac{repo-GAL4}{TM3.Sb}$
F1	$\frac{RNAi(CG11426)}{CTG} ; \frac{+}{TM3.Sb}$	F1	$\frac{+}{Cyo} ; \frac{repo-GAL4}{TTG}$
P	$\frac{RNAi(CG11426)}{CTG} ; \frac{+}{TM3.Sb}$ Ⓧ $\frac{+}{Cyo} ; \frac{repo-GAL4}{TTG}$		
F1	$\frac{RNAi(CG11426)}{Cyo} ; \frac{repo-GAL4}{TM3.Sb}$		

Table 3. Scheme for getting *CG11426* RNAi double dose.

- RNAi (*CG11426*)/Cyo ; RNAi (*CG11426*)

P $\frac{Gla}{CTG};\frac{+}{+}$ ⓧ $\frac{+}{+};\frac{Ly}{TM3.Sb}$	P $\frac{bl}{Cyo};\frac{+}{+}$ ⓧ $\frac{+}{+};\frac{repo-GAL4}{TTG}$
F1 $\frac{+}{CTG};\frac{+}{TM3.Sb}$	F1 $\frac{+}{Cyo};\frac{+}{TTG}$
P $\frac{+}{CTG};\frac{+}{TM3.Sb}$ ⓧ $\frac{RNAi(CG11426)}{Cyo};\frac{+}{+}$	P $\frac{+}{Cyo};\frac{+}{TTG}$ ⓧ $\frac{+}{+};\frac{RNAi(CG11426)}{RNAi(CG11426)}$
F1 $\frac{RNAi(CG11426)}{CTG};\frac{+}{TM3.Sb}$	F1 $\frac{+}{Cyo};\frac{RNAi(CG11426)}{TTG}$
P $\frac{RNAi(CG11426)}{CTG};\frac{+}{TM3.Sb}$ ⓧ $\frac{+}{Cyo};\frac{RNAi(CG11426)}{TTG}$	
F1 $\frac{RNAi(CG11426)}{Cyo};\frac{RNAi(CG11426)}{TM3.Sb}$	
F2 $\frac{RNAi(CG11426)}{Cyo};\frac{RNAi(CG11426)}{RNAi(CG11426)}$	

Table 4. Scheme for getting rescue fly.

- RNAi (*CG11426*)/Cyo ; RNAi (*yki*)

P $\frac{Gla}{CTG};\frac{+}{+}$ Ⓧ $\frac{+}{+};\frac{Ly}{TM3.Sb}$	P $\frac{bl}{Cyo};\frac{+}{+}$ Ⓧ $\frac{+}{+};\frac{repo-GAL4}{TTG}$
F1 $\frac{+}{CTG};\frac{+}{TM3.Sb}$	F1 $\frac{+}{Cyo};\frac{+}{TTG}$
P $\frac{+}{CTG};\frac{+}{TM3.Sb}$ Ⓧ $\frac{RNAi(CG11426)}{Cyo};\frac{+}{+}$	P $\frac{+}{Cyo};\frac{+}{TTG}$ Ⓧ $\frac{+}{+};\frac{RNAi(yki)}{RNAi(yki)}$
F1 $\frac{RNAi(CG11426)}{CTG};\frac{+}{TM3.Sb}$	F1 $\frac{+}{Cyo};\frac{RNAi(yki)}{TTG}$
P $\frac{RNAi(CG11426)}{CTG};\frac{+}{TM3.Sb}$ Ⓧ $\frac{+}{Cyo};\frac{RNAi(yki)}{TTG}$	
F1 $\frac{RNAi(CG11426)}{Cyo};\frac{RNAi(yki)}{TM3.Sb}$	
F2 $\frac{RNAi(CG11426)}{Cyo};\frac{RNAi(yki)}{RNAi(yki)}$	

2. Antibody Staining

In order to dechorionate eggs, 50% bleach was used for 3min, and the screen was rinsed using dH_2O. For egg fixation, 4% paraformaldehyde and heptane were used for 25 min, and the screen was rinsed using PT. To remove the vitelline membrane, cold methanol was used for 25 min in a shaker. All the primary antibodies were provided from the *Developmental Studies Hybridoma Bank* (*DSHB*) and all primary and secondary antibodies were diluted using the same proportions of PT; Repo (1:20), Elav (1:20), 2A12 (1:5), and HRP (1:1000).

3. Dissection of eye imaginal disc

The stock of flies used was raised at 25 °C in a 12 hr/12 hr day/night cycle. I used the late 3rd instar larvae that stopped feeding and wandered on the walls of the food vial after about 96 hours since the eggs had been laid. The larvae were dissected in 1X PBS, and fixed for 30 min in 4% of paraformaldehyde. After the fixation, 4% of paraformaldehyde was replaced with 1x PBS and rinsed twice using 1x PBS. The samples were washed in PT for 15 min (on a shaker, at room temperature) and i performed an antibody staining.

4. Quantitative Real-time PCR(qRT)

All the flies used in qRT analyses were bred at 25°C and i selected 30 male flies whthin 24 hours since their birth. All the flies were frozen and extracted with an RNA isolation kit (*Macherey-Nagel*). RNA samples with a concentration higher than 1.8 were selected and cDNA was polymerized using PCR. All-in-one RT master mix (*abm*) was used to synthesize cDNA. The polymerized cDNA samples were used in qRT templates.

rp49, a reference gene which encodes ribosomal protein, was used, and *yki*, and *Hpo* were used as interest genes. All the primer sequences used are presented in Table 5.

Table 5. qRT primer which used to qRT analysis.

Gene	Forward primer	Reverse primer
Rp49 (control)	TACAGGCCCAAGATCGTGAA	TCTCCTTGCGCTTCTTGGA
yki	AGTGCCCAAGAGTTCCCC	TGCGACATGCAGGTGTTCA
Hpo	CGAGCCATCTTTATGATTC	GGCACTTGCTCACGAAGTCAAT

III. Results

1. *CG11426* ; Lipid phosphate phosphatase coding gene

CG11426 is located in 3L: 22,470,556..22,473,509 [+]. About 3Kbp *CG11426* gene has 1023bp CDS and only one transcript (Fig. 4A). *CG11426* mutant inserted with P-element, stock number #19222, is found in 5'-UTR region. There are several reports that C*G11426* presents Lipid phosphate phosphatase activity (Garcia-Murillas et al., 2006). Furthermore, the result of protein to protein blast indicates that *CG11426* is very highly orthologue with *Wunen* gene, which is well-known as a *Drosophila lipid phosphate phosphatase* gene (Fig. 4B). The positivity of these two genes is 69% and the error rate is $2e^{-82}$. In addition, *CG11426* has well-conserved LPP catalytic domains. According to Long et al (2008)., LPP has three catalytic domains. *CG11426* is very well-conserved in all these three catalytic domains (Fig. 4C).

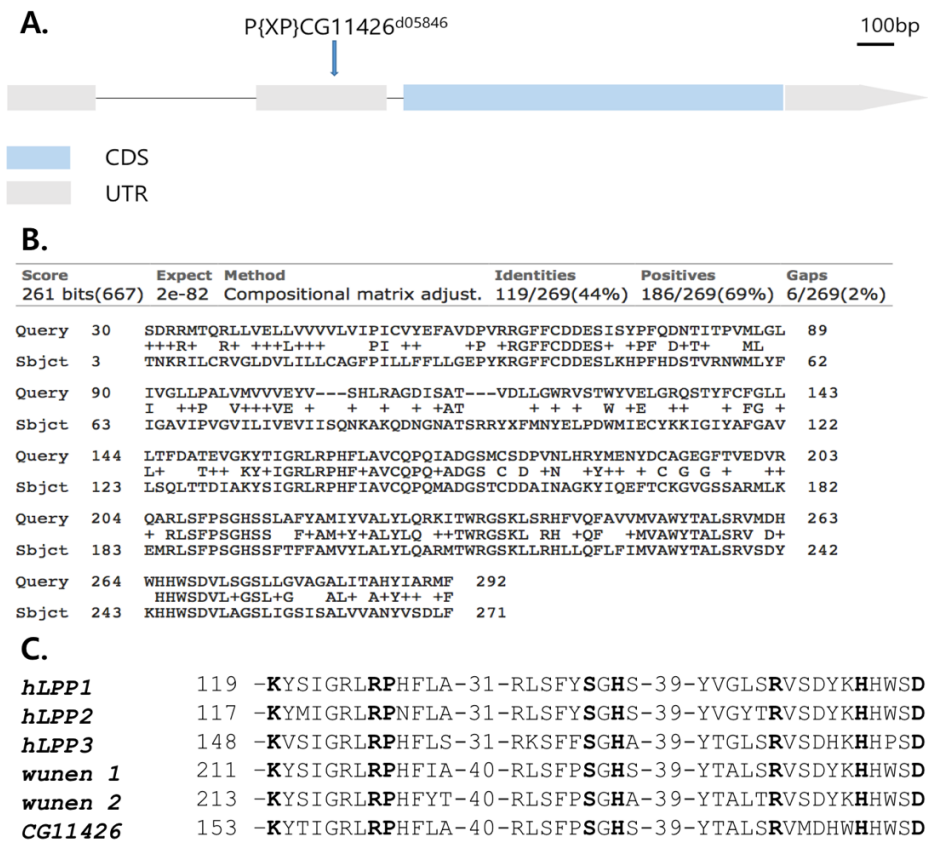

Figure 4. *CG11426* genetic information. A) *CG11426* genetic map. *CG11426* only has one transcript. Stock number #19222 mutant was inserted with P-element in 5'-UTR region. Total gene size is around 3Kbp. B) Protein-Protein blast result. *CG11426* is highly similarity to *Wunen* which is well-known as *Drosophila LPP* gene. C) Comparison of catalytic domains; *hLPP1-3* are human LPP genes; *Wunen1, 2* are *Drosophila LPP* gene.

2. *CG11426* expression region from the embryonic stage to adulthood

CG11426 is expressed after embryonic stages 11. It appears in posterior spiracle primordium and is expressed generally after embryonic stages 13. At embryonic stages 13-16, *CG11426* is expressed in the embryonic surface glia, head sensory system, nervous system, and optic lobe primordium (Fig. 5A) *CG11426* is also expressed in larvae and adults. After the embryonic stage, *CG11426* is expressed in the eye, Malpighian tubules, and thoracic-abdominal ganglion. Notably, *CG11426* is highly expressed in the eye and Malpighian tubules (Fig. 5B).

A.

Transcript Expression	
Stage	Tissue/Position
embryonic stage 11-12 embryonic stage 13-16	Posterior spiracle primordium
	Dorsal sensory complex proper primordium
	Embryonic central brain surface glia
	Embryonic head sensory system
	Embryonic/Larval nervous system
	Labral sensory complex
	Lateral cord surface glial cell
	Midline ventral glial cell
	Ventral midline
	Ventral nerve cord
	Ventral sensory complex primordium
	Embryonic optic lobe primordium

B.

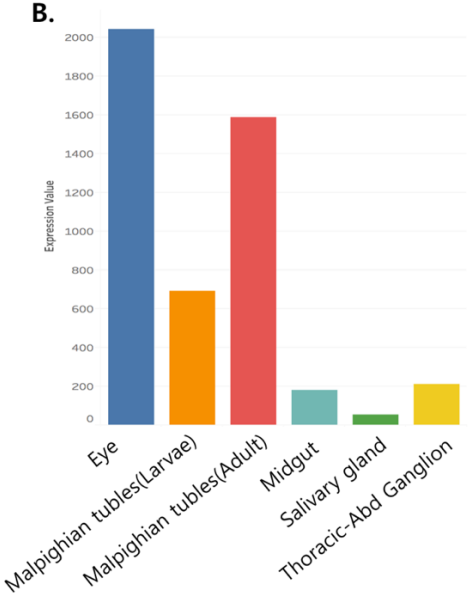

Figure 5. *CG11426* expression data. A) *CG11426* Transcript expression at embryonic stage. *CG11426* is expressed at late embryonic stage. *CG11426* is expressed in surface glia, sensory system, nervous system, and optic lobe primordium. The table was reported by *flybase* database. B) *CG11426* anatomical expression data at larvae and adult. *CG11426* is most highly expressed in the eye, and then in the Malpighian tubules. It is expressed in very high levels in adults and larvae. The next ones are the thoracic-abdominal ganglion cell, midgut, and salivary gland. The expression data was reported by *FlyAtlas.*

3. *CG11426* regulates glia cell numbers in embryonic development

To study *CG11426* related in glial cell development, an immunohistochemistry staining was performed in *CG11426* mutant and knockdown flies. *CG11426* mutant (stock number #19222) is can reach the embryonic stage, but never reach into adulthood with two copies of *CG11426* mutation. After selecting homozygote *CG11426* embryo by florescence (*twi-GAL4, UAS-GFP*), an immunohistochemistry staining with Repo was performed. *CG11426* mutant showed an increased glial cell numbers (Fig. 6A). At embryonic stage 16, the 1st abdominal segment to the 3rd abdominal segments of mutant had more glial cells (about 160, Fig. 6B) than wild-type embryos (about 122, Fig. 6B).

Knockdown flies also have the same phenotype. After down regulating *CG11426* gene expression level using *repo-GAL4; UAS-CG11426 RNAi* line, knockdown flies had an increased number of glial cells (about 141, Fig. 6B) at embryonic stage 16. Thus, a knockdown *CG11426* expression level in glial cell led to, an increase in the number of glia cells. This phenotype suggests that *CG11426* is involved in regulating glial cell development

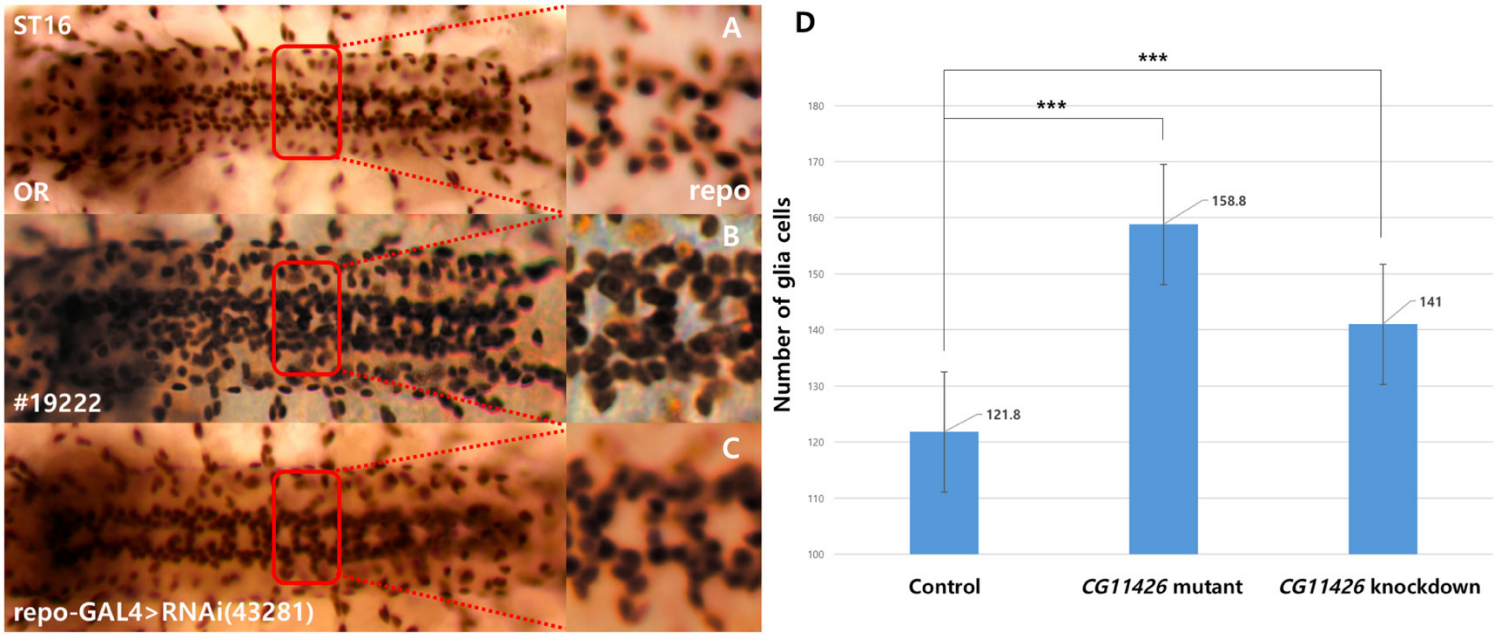

Figure 6. Glial cell numbers in embryonic stage. A-C) Repo antibody staining at embryonic stage 16. Red squares indicate the 3rd Abdominal segment. A: Control (*OregonR*) B: *CG11426* mutant embryo (stock number #19222) C: *CG11426* knockdown embryo using *Repo*-GAL4/UAS-*CG11426* RNAi (stock number #43281) D) Glial cell numbers. Control has about 121.8 glial cells from the 1st Abdominal to the 3rd Abdominal. On the other hand, *CG11426* mutant has 158.8 glial cells, *CG11426* knockdown embryo has 141 glial cells. These difference in glial cell numbers is statistically significant.

4. *CG11426* highly expressed in eye tissue

The *Drosophila's* eye has an ommatidia unit consisting of approximately 800 unit eyes (Kumar, 2012). These ommatidium contain eight photoreceptor neuron and pigment cells (Edwards and Meinertzhagen, 2010). The lamina, the basement of photo receptor, is populated by six distinct glia subtypes: Fenestrated, Pesudocartridge, Distal and Proximal satellite, epithelial, and marginal glia.

As already mentioned in Fig. 5, *CG11426* is highly expressed in the adult eye. During *CG11426* knockdown using GAL4/UAS system, flies' eyes showed an integration and aggregation of ommatidia units (Fig. 7). These phenotypes got worse as flies got older. While the data is not shown here, knockdown flies using *Repo*-GAL4 also showed the same phenotypes. These phenotypes are considered to be caused by a mutation in the eye pigment development, in the especially glia cell development.

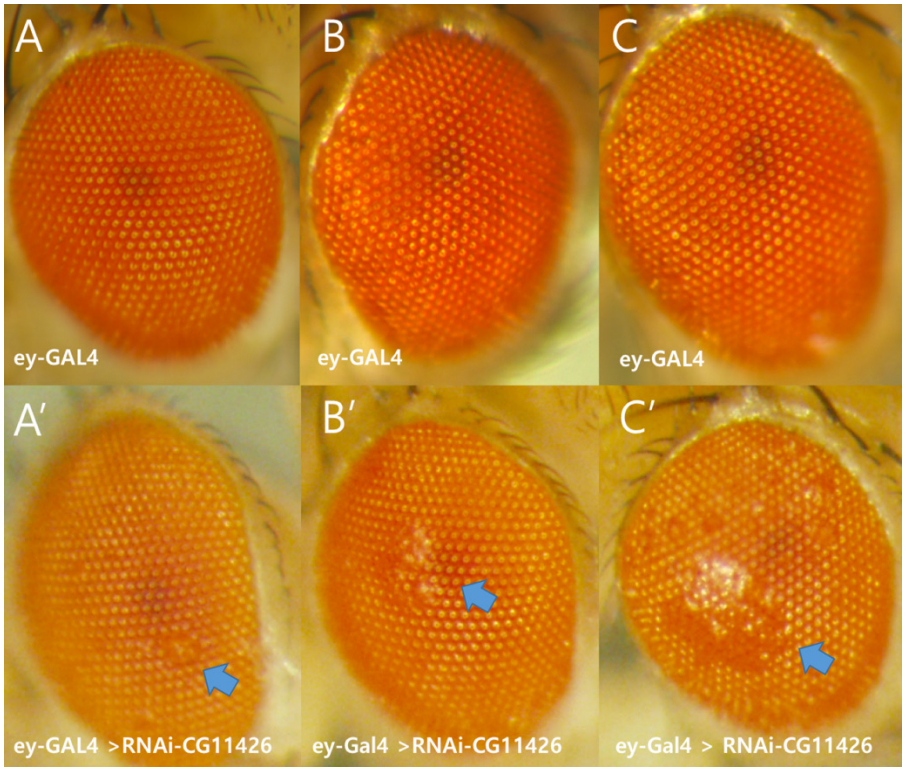

Figure 7. Knockdown *CG11426* in eye cell. Using ey-GAL4/UAS-*CG11426* RNAi, *CG11426* expression levels were down-regulated in the eye. Getting older, ommatidia aggregation phenotypes got more severe. A-C) Control (3, 7, and 10 Days) A'-C') ey-GAL4 > UAS-*CG11426* RNAi (3, 7, and 10 Days)

5. *CG11426* regulates glia cell numbers in eye imaginal disc

To investigate in more details, I looked into the eye imaginal disc at 3rd larvae. The eye imaginal disc is a region to become a portion of adult eyes. To quantify glia cell numbers in the eye imaginal disc, I performed immunohistochemistry staining using Repo and HRP. Analyses have been performed at 3rd larvae having 10-12 rows of photoreceptor cells. In wild types, the number of glial cells at 3rd larvae were on average 144, whereas *CG11426* knockdown larvae had on average 189 glia cells (Fig. 8). These observations establish that *CG11426* is related to the regulation of glia cell numbers.

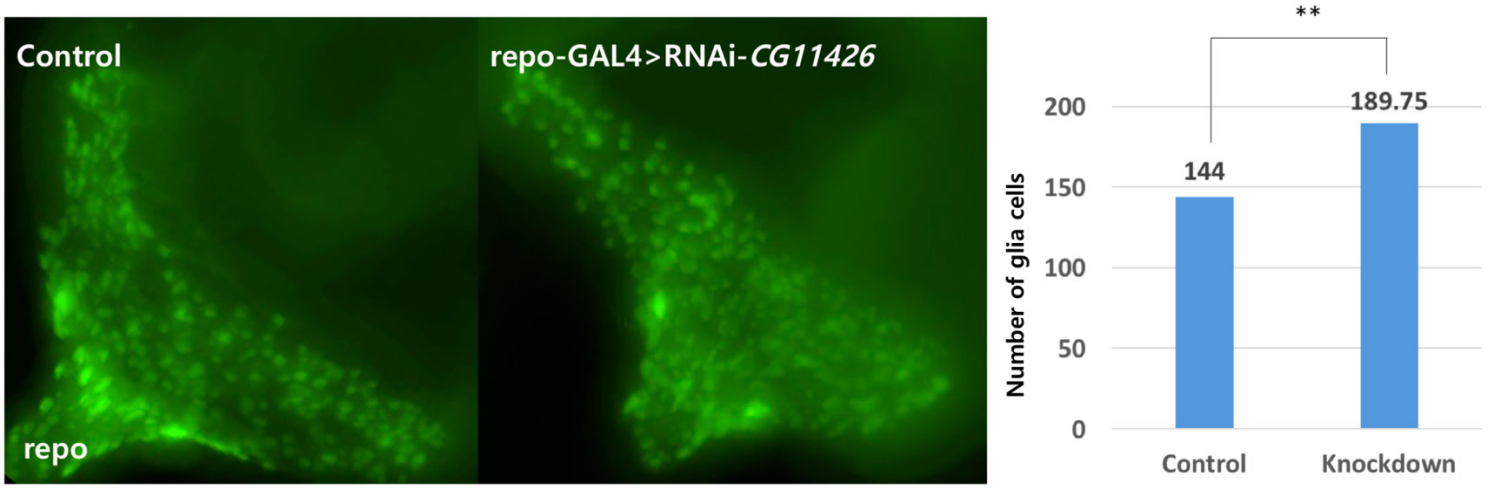

Figure 8. Eye imaginal disc of knockdown *CG11426* larvae. *CG11426* knockdown mutants using *Repo*-GAL4 in larvae stage have an increased number of glia cells in the eye imaginal disc. Control has about 144 and Knockdown *CG11426* mutant has about 190.

6. *CG11426* regulates Hippo signaling pathway

CG11426 homozygotic mutant flies are lethal. However, knockdown flies using GAL4/UAS system can reach the adult stage. To investigate how *CG11426* regulates glia proliferation, i checked the gene expression in knockdown flies. After, I performed an extraction of mRNA, synthesis of cDNA and qRT analysis. The result of qRT analysis shows that *yki* and *Hpo* expression levels have significantly increased (Fig. 9). These results indicate that *CG11426* regulates glia proliferation through the Hippo signaling pathway.

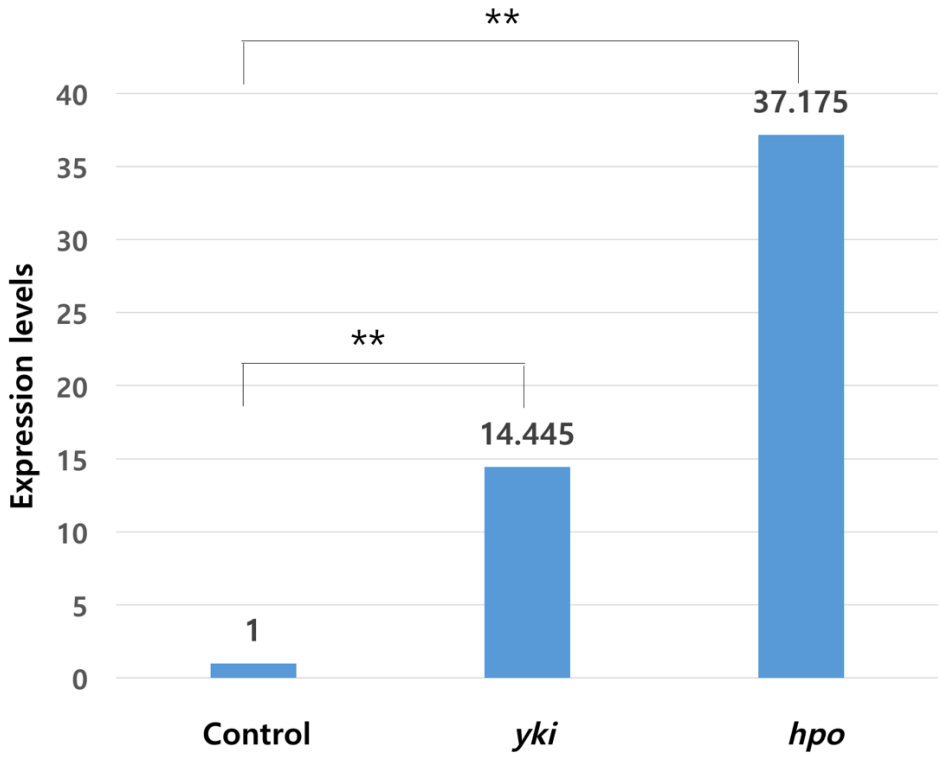

Figure 9. *CG11426* knockdown flies qRT result. *CG11426* knockdown mutants have a significantly increased *yki* and *Hpo* gene expression. Control (*Repo*-GAL4/TTG). The expression levels were down regulated by *Repo*-GAL4 and RNAi line (stock number #43281).

IV. Discussion

It is important to understand the regulation of glia cell numbers. An overgrowth of the glia cell numbers leads to neuronal cancer and abnormal glia growth which causes neurodegeneration. It is well-known that mutants of Hippo signaling pathway have a strong tissue overgrowth phenotype in *Drosophila* and that various cancers present an has abnormal MST1/2 concentration in mammals (Juan and Hong, 2016; Nakayama et al., 2015; Pan, 2010). In addition, there are several studies show that human *LPP* genes are involved in cancer (Nakayama et al., 2015; Pyne and pyne, 2010) and embryonic development.

CG11426 has been reported as *Wunen*-like gene or *LPP*-like gene. (Garcia-Murillas et al., 2006; Morrison et al., 2000) Comparing sequence result (Fig. 4) supports that *CG11426* has three catalytic domains of LPP. But it seems to have a different function from *Wunen1* or *Wunen2*. *Wunen1* and *Wunen2* are expressed mainly in embryonic stage 10-13, whereas, *CG11426* is expressed after embryonic stage 11 but mainly works after embryonic stages 13. The expression region is also quite different. *Wunen1* and *Wunen2* are expressed in the midgut primordium or hindgut primordium whereas *CG11426* is expressed in the nervous system.

According to some studies (Hooks et al., 1998; LEUNG et al., 1998), *Wunen1* and *Wunen2* are similar to human *PLPP1* (match scores 12/12) and *PLPP3* (match scores 8/12). On the other hand, *CG11426* is an orthologue of human *PLPP2* (match scores 7/12). In mammals, *LPP2* has the function of regulating the cell cycle in fibroblast (Flanagan et al., 2009; Tang et al., 2015; Zhang et al., 2000). Likewise, In *Drosophila*, *CG11426* seems to be involved in regulating the cell cycle. During *CG11426* knockdown using *Repo*-GAL4 in the embryonic stage, the number of glia

cells increased (Fig. 6 and Fig. 8) and defected ommatidia (Fig. 7). These results indicate that *CG11426* regulates glia cell proliferation and formation of the neuronal visual system.

In mammals, LPP genes share the same function of converting S1P to sphingosine, but different phenotypes appear when these genes are knocked out. Therefore, one might speculate that LPP genes in *Drosophila* also share the same functions, but are non-redundant. However, results disprove this speculation, since LPP genes in *Drosophila* actually differ from those in other mammals According to Hooks et al. (1998) and LEUNG et al. (1998), *Wunen1* is closer to the human LPP1 than LPP3, well-known for its roles in the vascular system. However, Wunen1 presents more strongly expressed phenotypes of trachea development in *Drosophila*. Besides, the data is not shown here because of the rare frequency, *CG11426* mutant also be involved in the trachea development. Therefore, *Drosophila LPP* genes seem to share the same functions with each other, but do not seem to conserve an strict non-redundant property.

There are many reports of the importance of S1P concentration outside of the cell. S1P is expressed in many cell types. S1P has a biological activity and promotes tumor growth, neovascularization, and inflammation (Pyne and Pyne, 2010). Some studies report that S1P binds to S1P receptor and regulates proliferation, migration, and survival in CNS (Soliven et al., 2011). Others studies report that S1P binds to GPCRs and inhibits LAST1/2 through Rho-GTPase and F-actin (Juan and Hong, 2016; Miller et al., 2012). The inhibition of LATS1/2 triggers yki nucleus localization and causes cell proliferation.

In the case of *CG11426*, it seems that S1P works through GPCRs. During the knockdown of *CG11426* using *Repo*-GAL4, the expression level of *yki* in adult flies was about 14 times higher than control flies (Fig. 9). The expression level of *Hpo*

also support this hypothesis. *CG11426* knockdown flies have a *Hpo* expression level about 37 times higher than control flies. According to Yu et al. (2012), proliferation through GPCRs is independent from MST1/2 activation. If proliferation works through ordinary Expanded-Hippo signaling, the *yki* level should be lower than in Fig. 9. Therefore, the expression of *Hpo* is independent from MST1/2 and a high level expression of *Hpo* seems to have a negative feedback for the activation of *Hpo* and inhibition of yki (Ikmi et al., 2014; Jukam et al., 2013).

In summary, I showed that *CG11426* has novel functions and roles that have never been reported yet. *CG11426* has LPP activity and is a homolog of the human *LPP2* gene and *Wunen*. It has well-conserved catalytic domains of human and *Drosophila* LPP. It also regulates glia cell numbers during development. *CG11426* knockdown flies and mutant flies have an increased glia cell numbers in the embryo and eye imaginal disc.

CG11426 seems to regulate glia cells through GPCRs. The Hippo signaling pathway is well-known in *Drosophila,* but there are not as many types of Hippo signaling pathway known for *Drosophila* as in other mammals. *CG11426* knockdown mutants have had higher expression levels of *Hpo* and *yki* than the control flies. This indicates that *CG11426* does not allow the general Hippo signaling pathway and shows that *CG11426* seems to be related to Hippo signaling pathway through GPCRs. To clarify these results, the following studies are required.

Firstly, the relation between *Wunen* and *CG11426* needs to be clarified, although *Wunen1* and *Wunen2* are different isotypes from *CG11426*. In the case of humans, isotypes have the same function but are non-redundant (Brindley et al., 2002 Tang et al., 2015). However, it is known that mammalian *LPP1* contributes to tumor microenvironment and therefore to cancer seeding (Nakayama et al., 2015) and Ile et al (2012) report that double mutants of Wunen1 and Wunen2 have more strongly

expressed phenotypes. Therefore, it is important to understand their interaction. A double mutant of *Wunen* and *CG11426* is expected to have a more severe phenotype.

Secondly, the relation between Hippo signaling pathway gene and *CG11426* needs to be clarified. A change in the expression level of *yki* and *Hpo* indicates that *CG11426* is related to the Hippo signaling pathway. To further clarify how this works, *CG11426* and *Hpo* double mutant, and *CG11426* and *yki* double mutant need to be studied.

Thirdly, GPCRs activation and Rho-GTPase activity need to be checked. One needs to know whether *Drosophila* also have a GPCRs linked hippo signaling pathway. *Drosophila* and mammalian Hippo signaling pathways are well-conserved, but there is no report of Hippo signaling pathway through GPCRs. Checking GPCR activity in *CG11426* mutant may provide a clue in finding out a new Hippo signaling pathway through GPCRs in *Drosophila*.

References

Barres, B.A. (2008). The mystery and magic of glia: A perspective on their roles in health and disease. Neuron *60*, 430-440.

Brindley, D.N., English, D., Pilquil, C., Buri, K., and Ling, Z.-C. (2002). Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids *1582*, 33-44.

Burnett, C., and Howard, K. (2003). Fly and mammalian lipid phosphate phosphatase isoforms differ in activity both in vitro and in vivo. EMBO Reports *4*, 793-799.

Edwards, T.N., and Meinertzhagen, I.A. (2010). The functional organisation of glia in the adult brain of *Drosophila* and other insects. Progress in Neurobiology *90*, 471-497.

Escalante-Alcalde, D., Hernandez, L., Le Stunff, H., Maeda, R., Lee, H.-S., Sciorra, V.A., Daar, I., Spiegel, S., Morris, A.J., and Stewart, C.L. (2003). The lipid phosphatase LPP3 regulates extra-embryonic vasculogenesis and axis patterning. Development *130*, 4623-4637.

Flanagan, J.M., Funes, J.M., Henderson, S., Wild, L., Carey, N., and Boshoff, C. (2009). Genomics screen in transformed stem cells reveals RNASEH2A, PPAP2C, and ADARB1 as putative anticancer drug targets. Molecular Cancer Therapeutics *8*, 249-260.

Freeman, M.R., and Doherty, J. (2006). Glial cell biology in *Drosophila* and vertebrates. Trends in Neurosciences *29*, 82-90.

Garcia-Murillas, I., Pettitt, T., Macdonald, E., Okkenhaug, H., Georgiev, P., Trivedi, D., Hassan, B., Wakelam, M., and Raghu, P. (2006). lazaro encodes a lipid phosphate phosphohydrolase that regulates phosphatidylinositol turnover during *Drosophila* phototransduction. Neuron *49*, 533-546.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The *Drosophila* Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457-467.

Hooks, S.B., Ragan, S.P., and Lynch, K.R. (1998). Identification of a novel human phosphatidic acid phosphatase type 2 isoform. FEBS letters *427*, 188-192.

Ikmi, A., Gaertner, B., Seidel, C., Srivastava, M., Zeitlinger, J., and Gibson, M.C. (2014). Molecular evolution of the Yap/Yorkie proto-oncogene and elucidation of its core transcriptional program. Molecular biology and evolution, msu071.

Ile, K.E., Tripathy, R., Goldfinger, V., and Renault, A.D. (2012). *Wunen*, a *Drosophila* lipid phosphate phosphatase, is required for septate junction-mediated barrier function. Development *139*, 2535-2546.

Juan, W.C., and Hong, W. (2016). Targeting the Hippo signaling pathway for tissue regeneration and cancer therapy. Genes *7*, 55.

Jukam, D., Xie, B., Rister, J., Terrell, D., Charlton-Perkins, M., Pistillo, D., Gebelein, B., Desplan, C., and Cook, T. (2013). Opposite feedbacks in the Hippo pathway for growth control and neural fate. Science *342*, 1238016.

Kumar, J.P. (2012). Building an ommatidium one cell at a time. Developmental Dynamics *241*, 136-149.

Kumar, S. (2004). Migrate, differentiate, proliferate, or die: Pleiotropic functions of an apical" apoptotic caspase". Science Signaling *2004*, pe49.

Leung, D.W., Tompkins, C.K., and White, T. (1998). Molecular cloning of two alternatively spliced forms of human phosphatidic acid phosphatase cDNAs that are differentially expressed in normal and tumor cells. DNA and Cell Biology *17*, 377-385.

Ling, C., Zheng, Y., Yin, F., Yu, J., Huang, J., Hong, Y., Wu, S., and Pan, D. (2010). The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. Proceedings of the National Academy of Sciences *107*, 10532-10537.

Long, J.S., Pyne, N.J., and Pyne, S. (2008). Lipid phosphate phosphatases form homo-and hetero-oligomers: catalytic competency, subcellular distribution and function. Biochemical Journal *411*, 371-377.

Miller, E., Yang, J., DeRan, M., Wu, C., Su, A.I., Bonamy, G.M., Liu, J., Peters, E.C., and Wu, X. (2012). Identification of serum-derived sphingosine-1-phosphate as a small molecule regulator of YAP. Chemistry & biology *19*, 955-962.

Moolenaar, W.H., van Meeteren, L.A., and Giepmans, B.N. (2004). The ins and outs of lysophosphatidic acid signaling. Bioessays *26*, 870-881.

Morrison, D.K., Murakami, M.S., and Cleghon, V. (2000). Protein kinases and phosphatases in the *Drosophila* genome. The Journal of cell biology *150*, F57-F62.

Nakayama, J., Raines, T.A., Lynch, K.R., and Slack-Davis, J.K. (2015). Decreased peritoneal ovarian cancer growth in mice lacking expression of lipid phosphate phosphohydrolase 1. PloS one *10*, e0120071.

Neuwald, A.F. (1997). An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases. Protein science *6*, 1764-1767.

Pan, D. (2010). The hippo signaling pathway in development and cancer. Developmental cell *19*, 491-505.

Pyne, N.J., and Pyne, S. (2010). Sphingosine 1-phosphate and cancer. Nature Reviews Cancer *10*, 489-503.

Renault, A., Sigal, Y., Morris, A., and Lehmann, R. (2004). Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. Science *305*, 1963-1966.

Renault, A.D., Kunwar, P.S., and Lehmann, R. (2010). Lipid phosphate phosphatase

activity regulates dispersal and bilateral sorting of embryonic germ cells in *Drosophila*. Development *137*, 1815-1823.

Robinson, B.S., Huang, J., Hong, Y., and Moberg, K.H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in *Drosophila* via the FERM-domain protein Expanded. Current Biology *20*, 582-590.

Saucedo, L.J., and Edgar, B.A. (2007). Filling out the hippo pathway. Nature Review Molecule Cell Biology *8*, 613-621.

Schwerk, C., and Schulze-Osthoff, K. (2003). Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochemical Pharmacology *66*, 1453-1458.

Sigal, Y.J., Mcdermott, M.I., and Morris, A.J. (2005). Integral membrane lipid phosphatases/phosphotransferases: common structure and diverse functions. Biochemical Journal *387*, 281-293.

Soliven, B., Miron, V., and Chun, J. (2011). The neurobiology of sphingosine 1-phosphate signaling and sphingosine 1-phosphate receptor modulators. Neurology *76*, S9-S14.

Starz-Gaiano, M., Cho, N.K., Forbes, A., and Lehmann, R. (2001). Spatially restricted activity of a *Drosophila* lipid phosphatase guides migrating germ cells. Development *128*, 983-991.

Tang, X., Benesch, M.G., and Brindley, D.N. (2015). Lipid phosphate phosphatases

and their roles in mammalian physiology and pathology. Journal of lipid research *56*, 2048-2060.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nature cell biology *5*, 914-920.

Yu, F.-X., Zhao, B., Panupinthu, N., Jewell, J.L., Lian, I., Wang, L.H., Zhao, J., Yuan, H., Tumaneng, K., and Li, H. (2012). Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. Cell *150*, 780-791.

Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.-M., and Pan, D. (2010). Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Developmental cell *18*, 288-299.

Yue, J., Yokoyama, K., Balazs, L., Baker, D.L., Smalley, D., Pilquil, C., Brindley, D.N., and Tigyi, G. (2004). Mice with transgenic overexpression of lipid phosphate phosphatase-1 display multiple organotypic deficits without alteration in circulating lysophosphatidate level. Cellular signalling *16*, 385-399.

Zhang, N., Sundberg, J.P., and Gridley, T. (2000). Mice mutant for *Ppap2c*, a homolog of the germ cell migration regulator *wunen*, are viable and fertile. Genesis *27*, 137-140.

Zhang, N., Zhang, J., Cheng, Y., and Howard, K. (1996). Identification and genetic analysis of *wunen*, a gene guiding *Drosophila melanogaster* germ cell

migration. Genetics *143*, 1231-1241.

Zhang, Q.X., Pilquil, C.S., Dewald, J., Berthiaume, L.G., and Brindley, D.N. (2000b). Identification of structurally important domains of lipid phosphate phosphatase-1: implications for its sites of action. Biochemical Journal *345*, 181-184.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., and Li, L. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes & Development *21*, 2747-2761.

국문초록

인지질 탈인산화효소 유전자 *CG11426*의 Hippo signaling pathway를 통한 신경교세포의 세포 수 조절

신경교세포는 뉴런 영양소를 공급하고, 신경을 보호하며, 엑손의 길 찾기와 같은 다양한 기능을 가지고 있다. 초파리의 신경교세포는 인간의 신경교세포와 매우 유사하기 때문에 초파리에서 신경교세포를 연구하는 것은 궁극적으로 인간의 신경 퇴화를 연구하는 데 의의가 있다. 본 논문은 기존에 연구되지 않은 인지질 탈인산화효소 유전자, *CG11426*을 발견하고 그 기능에 대해 조사하였다. 초파리에서 인지질 탈인산화효소는 생식 세포 이동, 정점 접합 형성 및 기관 발달로 알려져 있는데, 이 연구에서는 기존에 보고되지 않은 새로운 기능을 발견하였다. GAL4 / UAS 시스템을 사용하여 신경교세포와 눈에서 인지질 탈인산화효소 유전자를 억제하였고, 그 결과 배아와 눈이 될 예정조직에서 신경교세포의 증식을 확인하였다. 이것은 *LPP* 유전자가 신경교세포 발달에 관여할 가능성이 있음을 시사한다.

주요어: 인지질 탈인산화효소, 신경교세포 증식, Hippo signaling pathway, GPCRs signaling, spingosine-1-phosphate

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