Dissection of the Mechanisms Coordinating Neurons and Glia Development in the *Drosophila* Visual System

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Abstract

The establishment of neuronal circuits during embryonic development requires the coordinated development of neurons and glia. Although studies in the past two decades have made significant progress in understanding the underlying mechanisms, many key issues remain unaddressed. My Ph.D. research utilizes the developing *Drosophila* visual system as a model to answer two important questions about the coordinated development of photoreceptor (R cells) neurons and glial cells in sub-retinal space (i.e. sub-retinal glia). The first question is, what are the mechanisms that direct the coordinated exit of photoreceptor (R cells) axons and wrapping glial (WG) membrane from the eye disc into the optic stalk? And the second question is, do sub-retinal glia produce signals that regulate the timing of R-cell differentiation in the developing eye disc? In Chapter 2, I describe my work to answer the first question. By taking a combination of immunohistological and genetic approaches, I identify the first cell-surface receptor (i.e. Mys/Scb integrins) on R-cell axons for mediating the exit of R-cell axons. My results also show that the extension of WG membrane from the eye disc into the optic stalk is dependent on its association with R-cell axons. In Chapter 3, I describe the research to address the second question. By interfering with EGFR signaling in sub-retinal glia, I identify sub-retinal glia as an important source of signals that activate insulin signaling pathway in eye precursor cells for the timing of R-cell differentiation. And my results from cell-type-specific knockdown, epistasis analysis, and transgene rescue suggest that in response to EGFR activation, sub-retinal glia produce ILP3 and ILP6 to activate insulin signaling in eye precursor cells for temporal control of R-cell differentiation.

My findings shed new light on the mechanisms coordinating the development of R cells and sub-retinal glia. Since integrins and insulin pathways are conserved throughout evolution, it is highly likely that similar mechanisms are utilized in mammals for the control of circuit development. Thus, my work on integrin and insulin pathways in the *Drosophila* visual system should provide important clues to the mechanisms controlling the coordinated development of neurons and glia in mammals and help the development of novel therapeutic strategies to treat brain diseases associated with alterations in these pathways.

Résumé

L'établissement des circuits neuraux au cours du développement embryonnaire dépend du développement coordonné des neurones et des glies. Même si les études des deux dernières décennies ont permis des progrès significatifs dans notre compréhension des mécanismes sous-jacents, des questions fondamentales restent encore en suspens. Ma thèse de doctorat utilise le système visuel en développement de la *Drosophile* comme modèle pour répondre à deux questions sur le développement coordonné des neurones photorécepteurs (cellules R) et des cellules gliales sous-rétiniennes. Premièrement, quels sont les mécanismes qui coordonnent la sortie des axones photorécepteurs et des membranes de la glie enveloppante du disque oculaire jusqu'à la tige optique ? Deuxièmement, est-ce que les glies sous-rétiniennes produisent des signaux contrôlant quand les cellules R vont se différencier dans le disque oculaire ? Dans le deuxième chapitre, je décris le travail que j'ai réalisé afin de répondre à la première question. Utilisant une combinaison d'approches immuno-histologiques et génétiques, j'ai tout d'abord identifié un récepteur de surface cellulaire (i.e Mys/Scb integrins) sur les axones des cellules R. De plus, mes résultats montrent que l'extension de la membrane de la glie enveloppante, du disque oculaire jusqu'à la tige optique, dépend de son association avec les axones des cellules R. Dans le troisième chapitre, j'aborde les expériences que j'ai réalisées pour répondre à la deuxième question. En interférant avec le signal EGFR des glies sous rétiniennes, j'ai pu identifier que ces glies sont les sources majeures de signaux activant la signalisation de l'insuline dans les précurseurs oculaires, permettant ainsi le contrôle temporel de la différentiation des cellules R. Les résultats obtenus par des expériences d'ablation de cellules spécifiques suivies d'analyse d'épistasie et de sauvetage transgénique suggèrent qu'en réponse à l'activation par EGFR, les glies sous-rétiniennes produisent ILP3 et ILP6, qui vont activer la signalisation de

l’insuline dans les cellules précurseurs oculaires pour le contrôle temporel de la différentiation des cellules R.

Mes découvertes mettent en évidence des mécanismes coordonnant le développement des cellules R et les glies sous-rétiniennes. Etant donné que les voies des intégrines et de l’insuline sont conservées au cours de l’évolution, il est vraisemblable que des mécanismes similaires soient utilisés chez les mammifères pour le contrôle du développement des circuits. Ainsi, mon travail dans le système visuel de la *Drosophile* pourrait offrir des pistes pour l’étude des mécanismes contrôlant le développement coordonné des neurones et glies chez les mammifères et ainsi aider à développer de nouvelles stratégies thérapeutiques pour traiter les maladies affectant ces processus dans le cerveau.

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Preface

My Ph.D. study utilizes the *Drosophila* visual system as a model to understand the mechanisms controlling the coordinated development of neurons and glia in the developing nervous system. My work led to two major discoveries. Firstly, I identify the first cell-surface receptor (i.e. Mys/Scb integrins) on photoreceptor axons that plays a specific role in mediating the exit of photoreceptor axons and wrapping glia membrane from the eye disc. And secondly, I show for the first time that sub-retinal glia is an important source of signals for regulating the timing of photoreceptor differentiation. I also provide evidence supporting that sub-retinal glia-derived insulin-like peptides ILP3 and ILP6 activate insulin receptor in eye precursor cells to positively regulate the timing of photoreceptor differentiation.

This thesis is based on a peer-reviewed paper and a submitted manuscript. It is composed of four chapters. Chapter 1 consists of a literature review of relevant fields, the rationale, and objectives for this study. Chapter 2 includes a published paper, and Chapter 3 contains a submitted manuscript. Both Chapter 2 and 3 are made up of five sections, including Abstract, Introduction, Materials and Methods, Results, and Discussion. Chapter 4 is comprised of the general discussion, conclusion, and future direction sections.

Original Contributions to Knowledge

Chapter 2: This Chapter has been published in Molecular Brain: *Ren Q, Rao Y. The exit of axons and glial membrane from the developing Drosophila retina requires integrins. Mol Brain 2022; 15(1):2.* My supervisor Dr. Yong Rao and I contributed to the development of the rationale, the experiment design, the data analysis and wrote the paper.

Chapter 3: This chapter has been submitted to the Journal of Neuroscience: *Qian Ren, Wen-Tzu Chang, and Yong Rao. Glia-derived Insulin-like Peptides Regulate the Timing of Photoreceptor Differentiation in the Drosophila Visual System.* Dr. Yong Rao and I designed the experiments, analyzed the data, and wrote the manuscript. Ms. Wen-Tzu Chang helped me generate the Ilp3 and Ilp6 expression construct.

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List of Abbreviations Used

ABS3	Actin-binding sites 3
ADNF	Activity-dependent neurotrophic factor
Aos	Argos
Ato	Atonal
Bdl	Borderless
bHLH	Basic helix-loop-helix
bks	*brakeless*
BN	Bolwig's nerve
CNS	Central nervous system
Dpp	Decapentaplegic
ECM	Extracellular matrix
EG	Edging glial cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elp	*Ellipse*
ERK	Extracellular signal-regulated kinase

Ey	Eye disc
FAK	Focal adhesion kinase
FGF	Fibroblast growth factors
FOXO	Forkhead box O
gcm	*glial cell missing*
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
Hh	Hedgehog
Htl	Heartless
IAPs	Integrin-associated proteins
IBS	Integrin-binding sites
if	*inflated*
IGFBP	IGF-binding protein
IGF-IR	Insulin-like growth factor I receptor
IGFs	Insulin-like growth factors
IGR3	Leucine-rich repeat-containing G protein-coupled receptor 3
ILK	Integrin-linked protein kinase
ILPs	Insulin-like peptides

InR	Insulin receptor
INSL3-6	Insulin-like peptide 3-6 (in mammals)
IPCs	Insulin-producing cells
IRS	Insulin receptor substrate
ISP	Insulin signal pathway
La	Lamina
Lz	Lozenge
MAPK	Mitogen-activated protein kinase
Me	Medulla
mew	*multiple edematous wings*
MF	Morphogenetic furrow
mys	*myospheroid*
Ol	Optic lobe
ORNs	Olfactory receptor neurons
Os	Optic stalk
PDK1	Phosphoinositide-dependent protein kinase-1
PG	Perineurial glia
Phyl	Phyllopod

PI3K	Phosphoinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-diphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PNS	Peripheral nervous system
PTB	Phosphotyrosine-binding
Pyr	Pyramus
R cells	Photoreceptor neurons
Repo	Reversed polarity
RGD	Arginine-glycine-aspartic acid
RNAi	RNA interference
Ro	Rough
RPC	Retinal progenitor cells
RTK	Receptor tyrosine kinase
scb	*scab*
Sev	Sevenless
Sgg	Shaggy
SH2	Src homology 2
Shh	Sonic hedgehog

SPG	Subperineurial glia
Spitz	Spi
Svp	Seven-up
SVZ	Subventricular zone
TGF	Transforming growth factor
Ths	Thisbe
TOR	Target of rapamycin
TOR-C2	TOR-complex 2
TSC1/2	Tuberous sclerosis complex 1/2
Tutl	Turtle
VCAM-1	Vascular cell adhesion molecules 1
VIP	Vasoactive intestinal polypeptide
VUM	Ventral unpaired median
wg	*wingless*
WG	Wrapping glia

Chapter 1 : Introduction and Literature Review

1.1 The Coordinated Development of Neurons and Glia

1.1.1 The Reciprocal Neuron-Glia Interaction in Mammals

The functionality of the nervous system requires reciprocal neuron-glia interaction. Growing evidence during the past decade indicates that neurons actively promote the proliferation and differentiation of glia in mammals (He & Lu, 2013; Stipursky et al., 2011). For example, the neuronal-derived excitatory neurotransmitter glutamate influences the expression of the glial fibrillary acidic protein (GFAP) in astrocytes (Stipursky et al., 2011). Several signaling pathways such as Sonic Hedgehog (Shh), modulate the progression of oligodendrocyte specification and differentiation in a specific spatial and temporal manner (He & Lu, 2013).

It has become increasingly obvious that glial cells, such as astrocytes, also produce various signals that influence neuronal development in the central nervous system (CNS) (Blondel et al., 2000; Lim & Alvarez-Buylla, 1999; Song et al., 2002). For example, the extensive neurogenesis of subventricular zone (SVZ) cells was observed when coculturing dissociated adult SVZ or postnatal cells with astrocyte monolayers *in vitro* (Lim & Alvarez-Buylla, 1999). Consistently, *in vivo* observations showed that astrocytes induced neurogenesis of SVZ cells (Lim & Alvarez-Buylla, 1999), suggesting that astrocytes provide signals to activate neuroblasts. It is also reported that astrocytes from the postnatal hippocampus were capable of releasing factors to modulate the proliferation and differentiation of adult-derived stem cells (Song et al., 2002).

Synaptic formation and transmission are also influenced by molecules secreted by astrocytes (Blondel et al., 2000; Iino et al., 2001; Oliet et al., 2001). For instance, in response to vasoactive intestinal polypeptide (VIP) stimulation, astrocytes released activity-dependent neurotrophic factor (ADNF) to promote the differentiation of hippocampal neurons through

regulating glutamate responses on neurons (Blondel et al., 2000). Additionally, ADNF has been an intriguing therapeutic candidate for intractable neurodegenerative diseases since it exhibits neuroprotection against neurotoxins (Brenneman & Gozes, 1996).

1.1.2 The Interaction between Neurons and Glia in *Drosophila* CNS

Compared to the complex mammalian nervous system, invertebrates like *Drosophila* have a much simpler nervous system comprised of a limited number of neurons and glia. Based on the development of neurons and glia, the interactions between neurons and glia are divided into four classes (Edenfeld et al., 2005). Firstly, glial cell fate is modulated by intrinsic transcription factors, while glial cell numbers are regulated by neuronal-derived signals (Edenfeld et al., 2005). For instance, the *glial cell missing* (*gcm*) gene and the closely related *gcm2* can determine neural or glial cell fates. The transient expression of Gcm and lower level of Gcm2 in glia precursors ensure their cell fate change from the neuronal default to glia (Akiyama et al., 1996; Alfonso & Jones, 2002). These transcription factors activate the homeodomain protein, reversed polarity (Repo) that promotes glial differentiation and the expression of the BTB-Zn finger protein, Tramtrackp69, which stabilizes glial differentiation (Akiyama et al., 1996; Alfonso & Jones, 2002; Cohen et al., 2003; Kammerer & Giangrande, 2001; Klaes et al., 1994). Recently, it is reported that Repo repressed neuronal differentiation by synergizing with Tramtrackp69 (Yuasa et al., 2003). In glial lineages with low Gcm expression, Prospero, another transcription factor, can upregulate the levels of Gcm to maintain these glial cell fates and ensure normal glial numbers (Freeman & Doe, 2001; Griffiths & Hidalgo, 2004). The number of glial cells is modulated by signals from neurons that regulate apoptosis (Fichelson & Gho, 2003). Glial apoptosis is often observed in the peripheral nervous system (PNS), where the survival of some lateral glia depends on axon contact and the *Drosophila* neuregulin homolog, the neuronal-

derived epidermal growth factor receptor (EGFR) ligand Vein (Hidalgo & ffrench-Constant, 2003; Hidalgo et al., 2001; Kinrade et al., 2001).

Secondly, neurons and glia interact during the process of glial cell migration (Edenfeld et al., 2005). After the specification of glial cells, many of them must migrate long distances to their destination in the developing nervous system (Jacobs et al., 1989). Some axonal guidance cues, or cues derived from axons, also guide glial cells along the path of axonal projection (Choi & Benzer, 1994; Kinrade et al., 2001; Klämbt et al., 1991). In *Drosophila melanogaster*, for example, longitudinal glial cells reached their proper position in the nerve cord by using the axonal guidance signal Slit (Kinrade et al., 2001). Similarly, axons of ventral unpaired median (VUM) midline neurons provided a way for the traveling of midline glia (Klämbt et al., 1991).

Thirdly, glial cells play a role in the sorting of axons and the modification of boundary formation (Edenfeld et al., 2005). Glial cells could guide the formation of discrete axonal fascicles in the periphery (Oland & Tolbert, 2003). For example, in *Drosophila*, the olfactory receptor neurons (ORNs) encounter the so-called sorting-zone glia during development (Tucker & Tolbert, 2003). In this zone, the contact of axons with glial cells pauses the advancement of growth cone, and then modify its morphology. Subsequently, the ORN axons are sorted into distinct fascicles, which terminate in specific glomeruli (Rössler et al., 1999; Tucker et al., 2004). It is also reported that when reaching a specific region, axons appeared to be separated by glia for boundary formation (Salecker & Boeckh, 1996). Similarly, in the *Drosophila* nervous system, the subdivision of specific neuronal compartments is shaped by glia (Younossi-Hartenstein et al., 2003).

Fourthly, glial cells play an essential role in neuronal remodeling (Edenfeld et al., 2005). One such example is the local modulation of the efficacy of synaptic connections by glia

(Deitmer, 2000), where glutamate transporters in glia clear the neurotransmitter L-glutamate to prevent oxidative stress and neuropil degeneration (Rival et al., 2004; Soustelle et al., 2002). Another example is axon pruning, which is a process of removing exuberant projections in target regions (Kantor & Kolodkin, 2003). For instance, the mushroom body within the nervous system of adult flies is responsible for learning and memory behaviors. In the γ-neurons of the mushroom body, ecdysone induces extensive axon pruning during metamorphosis, and the removal of degenerating axonal fragments is mediated by surrounding glia (Watts et al., 2003; Zhu et al., 2003).

While accumulated evidence supports the importance of neuron-glia interaction in the establishment of neural networks during embryonic development, our understanding of underlying molecular and cellular mechanisms remains incomplete. To comprehensively understand the coordinated development of neurons and glia, it is important to identify and characterize novel players. The eye disc of *Drosophila* has emerged as an excellent model for such studies. The focus of my Ph.D. thesis was to understand the mechanisms controlling the coordinated development of photoreceptor neurons in the eye-disc epithelium, and glial cells in the sub-retinal space in the developing *Drosophila* compound eye.

1.2 The *Drosophila* Visual System as a Model

The relative simplicity of the *Drosophila* visual system and the powerful *Drosophila* genetics greatly facilitate the study of the mechanisms regulating the coordinated development of neurons and glia in the nervous system. Below, I describe key events, such as neuron differentiation and projection, glial cells in sub-retinal space (i.e. sub-retinal glial) migration, and neurons-glia interaction in the development of the *Drosophila* compound eye.

1.2.1 Neurogenesis of Eye Imaginal Disc at the Third-instar Larval Stage

The adult *Drosophila* visual system consists of the compound eye and the optic lobe. The compound eye is built of about 800 identical photoreceptor (R cell) clusters called ommatidia. Each ommatidial unit is composed of eight photoreceptor neurons (R1-R8), cone cells, and pigment cells (Ready et al., 1976).

The origin of the *Drosophila* compound eye was traced to the developing embryo by classical embryological studies and clonal analysis (Ready et al., 1976). During embryogenesis, about 20 cells of the embryonic blastoderm, which are set aside in the embryo, continually divide to yield monolayer epithelia named eye imaginal discs (Garcia-Bellido & Merriam, 1969; Ready et al., 1976; Wolff, 1993). This disc is a flattened sac of epithelium. One surface of it develops into a simple squamous epithelium, known as the peripodial membrane, while the other surface becomes a columnar epithelium (or eye-disc epithelium)(Cho et al., 2000; McClure & Schubiger, 2005). Later, neurogenesis takes place in the eye-disc epithelium, which everts and gives rise to the compound eye (Ready et al., 1976; Wolff, 1993).

Cell numbers in the columnar epithelium increase logarithmically in the course of the second-instar larval stage (Becker, 1957). At the end of the first-instar larval stage, the number of cells is about 130 cells and increases to 1300-1600 at the beginning of the third-instar larval

stage (Becker, 1957; Meinertzhagen, 1993; Wolff & Ready, 1991). Then, mitotic activity continues to generate around 10,000 cells in the eye field at the end of the third-instar larval stage (Silies et al., 2010). The optic stalk at the posterior end of the eye disc connects it to the optic lobe throughout larval life (Silies et al., 2010; Wolff & Ready, 1991).

1.2.2 Morphogenetic Furrow (MF) Initiation and Progression

Unlike other imaginal discs, differentiation in the eye disc shows a progressive pattern, which moves anteriorly across the disc from the posterior end in a wave-like manner (Wolff & Ready, 1991). The initiation of eye-disc differentiation is marked by the formation of an indentation in the disc known as the morphogenetic furrow (MF), which is caused by apical constriction and apical-basal contraction (Ready et al., 1976). Cells within the MF are initially arrested at the G_1 stage of the cell cycle (Thomas et al., 1994), and then differentiate as "preclusters", which give rise to future photoreceptor R8, R2/R5 pairs, and R3/R4 pairs (Freeman, 1996, 1997).

Hedgehog (Hh) signalling is essential to the initiation and progression of the MF (Borod & Heberlein, 1998; Domínguez & Hafen, 1997; Royet & Finkelstein, 1997)(Fig 1.1A and B). At the early third-instar stage, *hh* is expressed at the posterior margin of the eye disc, where it is normally required for the initiation of R cell differentiation (Domínguez & Hafen, 1997). A recent study suggests that deletions of an enhancer element of the *hh* gene caused the loss of Hh expression and a failure of MF progression (Rogers et al., 2005). Hh activates the expression of the proneural gene *atonal* (*ato*), which subsequently promotes differentiation (Domínguez, 1999). *ato* encodes for a basic helix-loop-helix (bHLH) protein and is specially required for inducing the formation of R8-ommatidial founder photoreceptor cell (Jarman et al., 1994).

Decapentaplegic (Dpp) signaling is also essential for the initiation of differentiation

(Chanut & Heberlein, 1997; Pignoni & Zipursky, 1997)(Fig 1.1A and B). For instance, misexpression of *dpp* could trigger the ectopic initiation of MF from the anterior margin (Chanut & Heberlein, 1997). Consistent with a function specific to the MF initiation, clones of cells defective for genes encoding components of the Dpp signaling pathway caused a failure of differentiation initiation as they touch the posterior margin of the eye disc (Burke & Basler, 1996; Das et al., 1998; Wiersdorff et al., 1996). Additionally, defective Dpp signaling pathway has a more significant impact on differentiation initiation than MF movement (Burke & Basler, 1996; Wiersdorff et al., 1996). For instance, *dpp* null alleles still displayed normal R-cell recruitment and progression of MF (Wiersdorff et al., 1996). Moreover, it is suggested that Dpp signaling is essential to maintain the normal rate of progression rather than MF progression. For example, when Dpp signaling was blocked, Hh signaling could replace the role of Dpp to trigger the undifferentiated cells to adopt pre-proneural states, but was not as efficient as Dpp signaling, thereby resulting in delayed MF progression (Greenwood & Struhl, 1999).

wingless (*wg*) is expressed in the anterior dorsal and ventral margins of the eye disc before the initiation of MF (Baker, 1988)(Fig 1.1A and B). Loss of *wg* resulted in ectopic Dpp expression and premature MF initiation, whereas ectopic expression of *wg* at the posterior margin of the eye disc prevented the initiation of MF (Heslip et al., 1997; Treisman & Rubin, 1995). Taken together, these studies suggest that *wg* and *dpp* antagonize each other to specify the correct initial position of the MF and to shape the furrow as a linear wave. Recently, it is suggested that Upd signaling also inhibited the transcription of *wg* to regulate MF initiation (Tsai et al., 2007).

1.2.3 EGFR Signaling Directs Serial Induction of Photoreceptor Cell Fate.

The epidermal growth factor receptor (EGFR) is involved in many processes such as cell cycle control, cell proliferation, cell fate specification, cell maintenance, and programmed cell death (Boonstra et al., 1995; Domínguez et al., 1998; Kumar, 2002). Altered EGFR signaling has been associated with a wide range of cancers in mammals (Normanno et al., 2006). EGFR belongs to the superfamily of transmembrane receptors with tyrosine kinase activity (Boonstra et al., 1995; Sabbah et al., 2020). Each member of this family consists of a cytoplasmic region containing the kinase domain, a single transmembrane segment, and an extracellular ligand-binding domain (Hubbard, 1999; Livneh et al., 1985; Tan & Kim, 1999; Weiss et al., 1997).

The initial evidence for the involvement of EGFR signaling in eye development is obtained by analyzing *Ellipse* (*Elp*) mutants (Baker & Rubin, 1989). These mutations are dominant "eye-specific" *EGFR* alleles that severely disrupted the compound eye. *Elp* mutant eyes are small, and the ommatidia are abnormally spaced (Baker & Rubin, 1989). And analysis of *EGFR* null alleles shows that photoreceptors are absent in mosaic clones of *EGFR* null eye tissues (Baker & Rubin, 1989; Xu & Rubin, 1993). These studies indicate that EGFR activity is critical for the formation of individual photoreceptors within the ommatidia.

EGFR signaling is also required for photoreceptor recruitment during ommatidial assembly as demonstrated by studies on the role of Spitz (Spi) in the developing eye disc (Freeman, 1994b; Tio et al., 1994; Tio & Moses, 1997). Spi is an epidermal growth factor (EGF)-like family member, and the fly homolog of human transforming growth factor (TGF)-α. The binding of diffusible ligand Spi to the extracellular domain of EGFR stimulates its dimerization, subsequently leading to trans-autophosphorylation of its cytoplasmic domain (Bogdan & Klämbt, 2001). It is suggested that Spi activates EGFR signaling during oogenesis

(Stevens, 1998). In mosaic *spi* eye clones, although the specification of the founder cell R8 or the spacing between developing ommatidial clusters appeared normal, the progression of ommatidial differentiation was disrupted, which led to the arrest of differentiation at the single-neuron (i.e. R8) stage (Tio & Moses, 1997). This suggests that Spi is required for the progression of ommatidial differentiation after the R8-only stage, but not required for the differentiation of the founder cell R8 (Tio & Moses, 1997). Consistently, it is shown that R8 specification and spacing remained normal in *EGFR* mutants (Domínguez et al., 1998; Xu & Rubin, 1993).

It appears that the progression of ommatidial differentiation after the R8-only stage requires R8 to release Spi and Argos (Aos), another EGF-like molecule that antagonizes Spi (Freeman, 1994a; Freeman et al., 1992; Tio & Moses, 1997). Spi and Aos iteratively activate EGFR in precursor cells to control the addition of other R-cell subtypes (Doroquez & Rebay, 2006; Freeman, 1996, 1997; Jin et al., 2000; Klein et al., 2004). Aos blocked the binding of Spitz and the extracellular domain of EGFR by competing with Spi, thereby preventing the dimerization of EGFR (Jin et al., 2000). However, a recent biochemical study shows that Aos combined with Spitz to form complexes, which sequestered Spi away from EGFR to negatively regulate EGFR signaling (Klein et al., 2004).

1.2.4 R-cell Specification in the *Drosophila* Eye

The initiation of ommatidial differentiation begins at the posterior disc margin. Cells anterior to the MF are undifferentiated and proliferative, while those posterior to the MF are ommatidial cells that differentiate into dorso-ventral columns (Tomlinson, 1985).

Within each ommatidium, the specification of R8, the founder cell, occurs first within column 0 (Hsiung & Moses, 2002). R8 plays a central role in retinal development, as it determines the number and position of all other ommatidial cells (Kumar, 2012; Tomlinson &

Ready, 1987). The mechanisms controlling R8 specification appear to be different from that of other R-cell subtypes (Yang & Baker, 2001). First, R8-inductive signals seem to act over a long distance (Baker, 2002; Greenwood & Struhl, 1999). Second, several signals act to inhibit nearby cells from accepting an R8 fate (Roignant & Treisman, 2009).

R8 specification requires the function of *atonal* (*ato*) (Jarman et al., 1994; Jarman et al., 1995). *ato* expression begins in a stripe of cells just ahead of the MF in response to the Hh signal from differentiated R cells posterior to MF (Jarman et al., 1994; Jarman et al., 1995). As the MF progresses, the expression of *ato* is briefly retained by about 12 cells called intermediate clusters (Baker et al., 1996; Dokucu et al., 1996; Jarman et al., 1994), and then restricted to three cells that are defined as the "R8 equivalence group" from which R8, R2, R5 are specified (Dokucu et al., 1996). Subsequently, *ato* is only expressed in the single precursor cell that will give rise to the future R8 (Jarman et al., 1995). The dynamic expression of Ato is modulated by the combined action of Hh and Notch. It is reported that Hh produced by differentiating R cells posterior to the furrow positively regulates the expression of Ato (Borod & Heberlein, 1998; Greenwood & Struhl, 1999), while Notch signaling inhibits the expression of Ato (Baker & Yu, 1998). Consistently, *Notch* mutants show a dramatic increase in the number of cells adopting an R8 fate (Baker & Zitron, 1995).

R2 and R5 photoreceptors differentiate along the side of R8 precursors about 2-3h afterwards (Saint et al., 1988; Tomlinson et al., 1988). Rough (Ro) protein plays an important role in R2/R5 cell specification and acts as a homeobox domain transcription factor that is exclusively expressed in the eye imaginal disc (Saint et al., 1988; Tomlinson et al., 1988). Loss of *ro* caused the transformation of R2/R5 precursor cells into R1/R3/R4/R6 fate. Additionally, the appearance of 1-2 additional R8 neurons in each ommatidium was observed in *ro* mutants

(Heberlein et al., 1991; Van Vactor et al., 1991). It is reported that Ro and Ato act antagonistically during the early stages of precluster formation. Misexpression of *ato* or removal of *ro* caused the discrete R8 "equivalent group" to differentiate into R8s. And loss of *ro* resulted in ectopic Ato expression in R8 (Dokucu et al., 1996). However, a recent report suggests that in *ro* mutants, the abnormal differentiation of R2 and R5 precursors into R8 was independent of the aberrant expression of Ato, and the selection of R8 was instead decided by a loop between *sevenless* (*sev*) and *ro* (Pepple et al., 2008).

The specification of R3/R4 occurs lastly within the preclusters. The nuclear receptor Seven-up (Svp) is essential for the specification of R3/R4 (Hiromi et al., 1993; Mlodzik et al., 1990). Svp is initially expressed in R3/R4, and later also expressed in R1/R6 in more mature clusters (Mlodzik et al., 1990). It is shown that loss of *svp* transformed R3/R4/R1/R6 into the R7 fate. Conversely, ectopic expression of Svp led to complex cell fate changes (Hiromi et al., 1993). Additionally, loss of *Svp* specifically in R3 and R4 precursor cells also led to the appearance of extra photoreceptor cells in each mutant ommatidium (Mlodzik et al., 1990).

The differentiation of the R3/R4 pairs in more mature ommatidium is important for the establishment of ommatidial polarity since the R3/R4 pairs maintain asymmetric positions that create a chirality for the cluster (Zheng et al., 1995). Loss of *Notch* transformed the R3/R4 pairs into non-chiral R3/R3, while gain of function in *Notch* caused the R3/R4 pairs to assume non-chiral R4/R4 (Cooper & Bray, 1999; Fanto & Mlodzik, 1999). It is also shown recently that Spalt, a zinc-finger transcription factor, plays a role in regulating R3/R4 specification and polarity (Domingos et al., 2004).

The differentiation of other R-cell subtypes (i.e. R1/R6 and R7) and cone cells occurs after the first mitotic wave (Kumar, 2012). Several cell-type-specific transcription factors are

involved in the process (Chang et al., 1995; Daga et al., 1996; Higashijima et al., 1992). Bar is required for the specification of R1/R6 cells (Higashijima et al., 1992). The expression of Bar in R1 and R6 requires another transcription factor Lozenge (Lz), which is broadly expressed in the precursor cells, including all undifferentiated cells posterior to the MF (Daga et al., 1996; Flores et al., 2000; Xu et al., 2000). In *lz* mutants, the expression of Bar in R1/R6 was lost, leading to the failure of R1/R6 differentiation (Daga et al., 1996; Higashijima et al., 1992). The differentiation of R1/R6 also requires Svp, as loss of *svp* led to the transformation of R1/R6 into the R7 fate (Mlodzik et al., 1990). Another nuclear protein expressed in R1/R6 is Phyllopod (Phyl). In *phyl* mutants, R1/R6 did not differentiate properly (Chang et al., 1995; Dickson et al., 1995).

The last R-cell subtype to be recruited into the ommatidial cluster is R7 (Kumar, 2012). Mutations in *sevenless* (*sev*) encoding for a receptor tyrosine kinase (RTK) specifically disrupted R7 differentiation (Tomlinson et al., 1987). In *sev* mutants, the presumptive R7 cell was transformed into a cone cell fate (Tomlinson & Ready, 1987). The Sev receptor in R7 is activated by its ligand Bride of Sevenless (Boss) which is specifically expressed in the R8 cell (Krämer et al., 1991; Van Vactor et al., 1991). In *boss* mutants, like that in *sev* mutants, ommatidia were missing R7 cells (Reinke & Zipursky, 1988). Additionally, the activation of Sev increased the expression of Prospero (Pros) in R7 cells (Kauffmann et al., 1996).

The differentiation of R7 cells also requires inductive signals from the R1/R6 pairs (Tomlinson & Struhl, 2001). It is reported that loss of the Notch ligand Delta in R1 and R6 caused the transformation of R7 cells into an R1/R6 cell type (Cooper & Bray, 2000; Tomlinson & Struhl, 2001). And constitutive activation of Notch signaling in presumptive R1/R6 precursor cells directed them toward an R7-like fate (Tomlinson & Struhl, 2001). Together, these studies

support that the R7 specification requires at least two inductive signals: Boss on R8 to activate Sev, and Delta on R1/R6 to activate Notch.

1.2.5 Diversity of Glial Cell Types in the Eye Imaginal Disc

At the third-instar larval stage, there are four main subtypes of glial cells in the sub-retinal space: subperineurial glial cells (SPG), perineurial glial cells (PG), wrapping glial cells (WG), and edging glial cells (EG) (Silies et al., 2007). In each eye disc, only two gigantic SPG (i.e. carpet cells) are found. The membrane of SPG extends below the eye-disc epithelium in the differentiated posterior region, and its anterior margin follows MF. The posterior margin of the SPG membrane extends from the optic stalk into the lamina of the optic lobe. PG are the major sub-retinal glial subtype (Silies et al., 2010; Silies et al., 2007). Apical PG are located in between differentiating R cells and the SPG, while basal PG are located below the SPG (Tsao et al., 2020). When apical PG encounter the nascent R-cell axons, it dissociates from the SPG. PG is triggered by reprogramming from a migratory cell type to a differentiating WG (Silies et al., 2007; Tsao et al., 2020). WG then ensheath the nascent R-cell axons and follow the axons through the optic stalk towards the optic ganglia in the brain lobes (Silies et al., 2007). The least characterized sub-glial subtype is EG that localised in the eye-disc margins (Silies et al., 2007).

All sub-retinal glial cells in the third-instar larval eye disc originate from the optic stalk (Choi & Benzer, 1994; Rangarajan et al., 1999). The optic stalk is first observed at the second-instar larval stage. Before R-cell differentiation in the eye disc, there are only 6 to 20 glial cells in the stalk (Silies et al., 2010; Silies et al., 2007). At the early third-instar larval stage when R-cell differentiation initiates, the number of glial cells in the optic stalk increase to about 150 (Silies et al., 2010; Silies et al., 2007). As R cell differentiation sweeps across the eye disc from the posterior to anterior region, glial cells migrate from the optic stalk into the eye disc where

they undergo proliferation and differentiation. The number of glial cells continue to increase during the migration process. At the end of larval development, there are about 325 glial cells in each eye disc (Silies et al., 2010; Silies et al., 2007).

1.2.6 Migration of Glial Cells into the Eye Imaginal Disc

Silies et al. in 2007 proposed a "sequential differentiation model" for the migration and differentiation of glia from the optic stalk to the eye disc (Silies et al., 2007). According to this model, SPG separate immature PG in the basal layer, which gradually migrate through the optic stalk into the eye imaginal disc. Thereby, PG have no opportunity to contact photoreceptors. As the eye image disc grows, the number of PG will increase and then extend their filopodia, which come into contact with nascent photoreceptors. Subsequently, PG will differentiate into WG, which ensheath R-cell axons and follow the axons over the optic stalk into the optic lobe (Silies et al., 2007)(Fig 1.2).

Silies et al. in 2007 provided three pieces of evidence to support their hypothesis. First, blocking mitosis only in the outer layer (basal layer of SPG) decreased glia number, whereas blocking mitosis in the inner layer (apical layer of SPG) did not impact glia number (Cafferty, 2008; Silies et al., 2007). These results indicate that older glia cells are in the inner region of the disc, whereas immature, mitotically active glia are found in the outer region of the disc. Next, they labeled the outer glial cell and traced the trajectory (Silies et al., 2007). The results show that the outer glia eventually migrated to the inner position and wrapped axons, suggesting that the inner glial cells were from the outer glia. Finally, it is shown that other glia cells moved further into the eye disc epithelium when SPG were ablated only (Silies et al., 2007), suggesting that SPG function in preventing ectopic glial cell migration into the eye disc. Together, these results support the model that SPG separate the outer PG and the inner WG, and restrict their

migration. The inner WG are from the outer layer of PG cells. Consistently, the location of the labeled three glial layers in the optic stalk is displayed: the internal WG, the intermediate SPG, and the outmost PG (Xie et al., 2014).

Unexpectedly, a recent study suggests that the outer and inner layer glia (basal and apical layers of SPG) all include PG, but the differentiation from PG to WG only takes place in the inner layer (Tsao et al., 2020). In this scenario, SPG do not play a role as the physical barrier which prevent the outer PG from contacting the inner R-cells (Tsao et al., 2020). Moreover, the difference between outer and inner PG is predetermined before they reach the final location. In other words, before PG contact the SPG membrane, their lineages have determined if they go under or above the SPG membrane (Tsao et al., 2020). These segregation processes occur in the eye disc long before the third instar larval stage. Their cell fate is determined by their lineage rather than by their location in the sub-retinal space (Tsao et al., 2020).

1.2.7 FGF Signaling for Glia Migration and Differentiation.

During the migration process of glial cells from the optic stalk into the eye disc, a switch from glial-glia interaction to glial-neuron interaction occurs. And the fibroblast growth factor (FGF) signaling plays a role in the switching process (Franzdottir et al., 2009). There are two FGF8-like ligands, including Thisbe (Ths) (Gryzik & Müller, 2004; Kadam et al., 2009; Stathopoulos et al., 2004), and Pyramus (Pyr) (Ho et al., 2019), both of which activate the FGF receptor Heartless (Htl) (Franzdottir et al., 2009).

Initially, glial-derived Pyr acts to regulate glial cell motility and cell numbers (Franzdottir et al., 2009). Downregulating *pyr* by using a hypomorphic allele or performing pan-glial knockdown led to a reduction in glial cell numbers (Franzdottir et al., 2009). And downregulating Pyr signaling in the entire eye disc disrupted glial migration. In contrast,

migration of glia over the MF was observed when small patches of cells expressing Pyr were generated ahead of the MF (Franzdottir et al., 2009). It is also reported that in Pyr-expressing clones, glial cells ectopically migrated into the apical side of the eye disc in the apical periodical membrane (Franzdottir et al., 2009).

Subsequently, neuronal-derived Ths stops glial migration and initiates the differentiation of PG to WG (Franzdottir et al., 2009; Klambt, 2009)(Fig 1.2). Loss of *ths* caused a glial over migration phenotype, suggesting that *ths* is involved in stopping glial migration (Franzdottir et al., 2009). Moreover, reduction of neuronal Ths expression in *ths* mutant larvae resulted in severe differentiation phenotypes (Franzdottir et al., 2009). Conversely, increased neuronal expression of Ths promoted glial differentiation (Franzdottir et al., 2009).

Htl appears to be the receptor for Pyr and Ths in the regulation of glial migration and differentiation (Franzdottir et al., 2009). Manipulating the function of Htl in glia causes phenotypes similar to that observed in mutants defective in *pyr* or *ths*. For example, interfering with the function of Htl disrupted glial migration and also caused a decrease in the number of glial cells. Conversely, hyperactivating Htl signaling greatly increased the number of glial cells and led to abnormal glial migration (Franzdottir et al., 2009).

Htl acts to regulate glial migration and differentiation by modulating downstream targets such as the adapter protein Dof (Downstream of FGFR), the small GTPase Ras and the transcription factor Pointed (Franzdottir et al., 2009; Rangarajan et al., 1999). Genetic studies suggest that these downstream components of Htl may regulate the development of sub-retinal glia differently (Franzdottir et al., 2009). Dof may be responsible for the regulation of glial cell number, while Ras (Rap1) and Pointed are involved in the regulation of glial migration and differentiation, respectively. Like FGF signaling, it is also suggested that Dpp and Hh signaling

are responsible for the proliferation, differentiation, and motility of the sub-retinal glia (Hummel et al., 2002; Rangarajan et al., 2001; Yuva-Aydemir et al., 2011).

1.2.8 Projection of R-cell Axons into the Optic Lobe

One focus of my thesis work is to understand the mechanism regulating the exit of R-cell axons from the eye disc. R-cell axons from the same ommatidial cluster fasciculate with each other to form a single bundle that migrates from the eye disc through the optic stalk into the optic lobe (Hadjieconomou et al., 2011; Tayler & Garrity, 2003). In order to arrive at their appropriate target regions in the optic lobe, R-cell axons from each ommatidium must make multiple decisions along the path of axonal outgrowth (Nériec & Desplan, 2016). When R-cell axons reach the basal layer of the eye disc, they make a 90-degree turn and then grow posteriorly (Cagan & Ready, 1989). After exiting the eye disc at the most posterior end, R-cell axons migrate through the optic stalk into the developing optic lobe, where they terminate in distinct layers and establish a topographic map in the target layer (Nériec & Desplan, 2016; Tayler & Garrity, 2003). R1-R6 axons terminate in the outermost lamina layer, and R7 and R8 axons project through the lamina into the distal medulla. R8 axons establish synaptic connections with the targets within the M3 sublayer, while R7 axons terminate within the deeper M6 sublayer (Silies et al., 2010; Tayler & Garrity, 2003).

It is reported that the exit of R-cell axons from the eye disc requires the presence of glial cells (Choi & Benzer, 1994; Rangarajan et al., 1999). In the absence of sub-retinal glia, when the migration of glia into the eye disc was blocked, R-cell axons failed to leave the eye into the optic stalk (Rangarajan et al., 1999). Interestingly, the presence of only a few glia at the posterior end of the eye disc was shown to be sufficient for the exit of R-cell axons from the eye disc (Rangarajan et al., 1999), suggesting that sub-retinal glia at the posterior end of the eye disc may

present important guidance information for directing the extension of R-cell axons from the eye disc into the optic stalk. Consistently, it is shown that the ectopic positions of sub-retinal glial cells cause the misprojection of R-cell axons (Hummel et al., 2002; Rangarajan et al., 2001; Xie et al., 2014).

The termination of R1-R6 axons at their intermediate target in the lamina requires the presence of glial cells in the target region (Poeck et al., 2001; Winberg et al., 1992). At the third-instar larval stage, R1-R6 axons terminate in between epithelial and marginal glial layers (Winberg et al., 1992). That these glial cells may provide a stop signal for the termination of R1-R6 axons is supported by genetic analysis of the *nonstop* gene, which encodes for a ubiquitin-specific protease (Poeck et al., 2001). *nonstop* is specifically required in glia, but not in R-cell axons or lamina neurons, for the termination of R1-R6 axons. In *nonstop* mutants in which epithelial and marginal glial layers were absent, R1-R6 axons failed to stop at the lamina termination site and projected ectopically into the medulla (Poeck et al., 2001). These results suggest that *nonstop* upregulates the stop signal on the glia for R1-R6 termination. The molecular identity of the glia-derived stop signal, however, remains elusive.

The molecular identity of the cell-surface receptor on R1-R6 axons that recognizes the glia-derived stop signal also remains unknown. The expression of this unknown receptor may be regulated by the transcription factor Brakeless (Bks). In *bks* mutants, although the projections of R7 and R8 axons occurred normally, most R1-R6 axons failed to stop in the lamina and projected abnormally into the medulla (Rao et al., 2000; Senti et al., 2000). Genetic mosaic analysis and transgene rescue showed that *bks* is specifically required in R-cell axons. Since *bks* encodes for a nuclear protein (Rao et al., 2000; Senti et al., 2000), it is possible that Bks controls

the expression of a cell-surface receptor on R1-R6 axons that recognizes the glia-derived signal for the termination of R1-R6 axons.

While recent studies have made solid progress in defining the molecular mechanisms underlying the coordinated development of R cells and glia in the developing visual system, our understanding of the underlying mechanisms remains incomplete. My recent results reveal important roles for integrins (Chapter 2) and insulin-like peptides (ILPs) (Chapter 3) in the developing eye disc, which provide novel and important insights into the mechanisms coordinating the development of neurons and glia. In the following sections, I will provide some background about integrins and ILPs and recent studies about them in *Drosophila* visual system.

1.3 Integrin Pathway

1.3.1 Integrin Family Proteins in Mammals

Integrins, consisting of α and β subunits, are heterodimeric transmembrane proteins (Barczyk et al., 2010). In humans, there are at least 18 α and 8 β subunits (Shimaoka et al., 2002). These subunits assemble into 24 distinct integrins (Barczyk et al., 2010; Hynes, 2002). Integrin α subunit and β subunit are quite different and have no detectable homology (Barczyk et al., 2010). However, the sequence among α subunits has about 30% similarity, and different β subunits exhibit about 45% similarity, indicating their gene families are largely evolved by gene duplication (Barczyk et al., 2010; Takada et al., 2007). The structure of α and β integrin is highly conserved throughout evolution, strongly suggesting that they are derived from a common ancestral gene (Huhtala et al., 2005; Takada et al., 2007).

The ectodomain of the integrin α subunit contains four or five domains, including two calf domains, a seven-bladed β-propeller, and a thigh domain (Campbell & Humphries, 2011). The fifth extracellular domain (I domain) exists in some subtypes of the α subunit (Campbell & Humphries, 2011; Takada et al., 2007)(Fig 1.3). Among 18 integrin α subunits, nine have I-domain, including α11, α10, α2, α1, αX, αL, αM, αE, and αD (Huhtala et al., 2005; Takada et al., 2007), which acts in mediating extracellular adhesion and ligand binding (Huhtala et al., 2005). Among non-I-domain α subunits, arginine-glycine-aspartic acid (RGD)-recognizing integrin α subunits αV, α8, α5, and αIIb are closely related to each other. And laminin-binding integrin α subunits contain α3, α6, and α7, the orthologues of which are observed in pufferfish (Huhtala et al., 2005). Integrin α4 and α9 recognize the ligand-vascular cell adhesion molecule-1 (VCAM-1)(Huang et al., 2000; Yang et al., 1995).

The ectodomains of the β subunit has seven domains, including the β-I domain, Plexin-semaphorin-integrin (PSI) domain, a hybrid domain, and four cysteine-rich EGF modules (Campbell & Humphries, 2011)(Fig. 1.3). The α-I domain is homologous to the β-I domain (Campbell & Humphries, 2011; Takada et al., 2007). There are two conformations for the I domain outside cells: an open (active) or a closed (inactive) conformation. And the conformation change affects its interaction with ligands (Takada et al., 2007; Wegener et al., 2007).

While the cytoplasmic tails of α-subunits are dramatically different, the cytoplasmic tails of β-subunits are strikingly homologous (Takada et al., 2007; Wegener et al., 2007). The cytoplasmic tails interact with cytoskeletal and signaling proteins to mediate signal transduction (Campbell & Humphries, 2011; Wegener et al., 2007). For example, the Talin phosphotyrosine-binding (PTB) domain interacts with the integrin β tails to activate integrins (Wegener et al., 2007).

In mammals, some integrins are expressed specifically in certain tissues or cell types, such as α4β7 in a group of memory T cells; αEβ7 in dendritic cells and T cells; αIIbβ3 in platelets (Takada et al., 2007). Some integrins are widely distributed. For example, αVβ3 is expressed in the endothelium (Hoshiga et al., 1995; Takada et al., 2007). Genetic studies have revealed diverse roles of integrins in different tissues, such as αIIbβ3 and αV in thrombus formation, α9β1in lymphangiogenesis, α6β4 in the integrity of the skin, α4β1 in the survival of retina neurons (Hynes, 2002; Leu et al., 2004) .

Integrins play important roles in the development of the nervous system (Clegg et al., 2003). Integrins regulate cell-cell recognition and specific adhesion interactions among neurons, glia, and the surrounding ECM (Park & Goda, 2016). For example, integrins modulate different aspects of neuronal migration and neuron-glial interaction during the developing lamina of the

cerebral cortex in mammals (Anton et al., 1999). During the process of cell migration, there are dynamic interactions between integrins and the actin cytoskeleton in different parts of a cell. At the leading edge of the migratory cells, the binding of ECM by integrins allows the rearrangement of the actin cytoskeleton, which promotes membrane protrusion. However, at the rear end of the migrating cells, the detachment and dissociation of integrins from the ECM and actin cytoskeleton take place separately, and then integrins are recycled to the front of the cell (Ballestrem et al., 2001; Brakebusch & Fässler, 2003; Laukaitis et al., 2001). Integrins connect the dynamic changes of ECM with synapses to modify their structure and regulate their function (Barros et al., 2009; Câmara et al., 2009; Chavis & Westbrook, 2001; Huang et al., 2006; Lee et al., 2006). For instance, integrins act as sensors for the changes in ECM to modulate the cytoskeleton of synapses in both the embryonic and adult neurogenic regions (Campos, 2005; Graus-Porta et al., 2001).

1.3.2 Integrins in *Drosophila*

Drosophila integrins include five α subunits (αPS1, αPS2, αPS3, αPS4, αPS5) and two β-subunits (βPS and β_v)(Broadie et al., 2011; Brown et al., 2000; Gotwals, Paine-Saunders, et al., 1994). The common β-subunit βPS, encoded by the *myospheroid* (*mys*) gene, can associate with any of three α-subunits including αPS1 encoded by the *multiple edematous wings* (*mew*) gene, αPS2 encoded by *inflated* (*if*), and αPS3 encoded by *scab* (*scb*) (Gotwals, Paine-Saunders, et al., 1994). Integrins is widely expressed in CNS (Xie & Auld, 2011; Xie et al., 2014). For example, βPS-integrin is broadly expressed between the interface of distinct glial cell types in *Drosophila* peripheral nerves, while αPS3 and αPS2 are expressed in the innermost WG and the outermost PG, respectively (Xie & Auld, 2011). Unlike widely expressed βPS, β_v is only expressed in the midgut endoderm (Yee & Hynes, 1993).

Drosophila integrin βPS is mostly homologous to the vertebrate β1 subunit (Huhtala et al., 2005; MacKrell et al., 1988). αPS1 shares most of its identity with vertebrate α3, α6, and α7 subunits (Huhtala et al., 2005), which belong to the subfamily of laminin-binding integrins (Hynes, 1992). αPS2 is homologous to RGD-binding integrins in vertebrates, such as α5, α8, αV, and αIIb (Gotwals, Paine-Saunders, et al., 1994; Huhtala et al., 2005).

Genetic approaches have been used to study the roles of *Drosophila* integrins in embryonic development (Brabant & Brower, 1993; Brown et al., 1993; Gotwals, Paine-Saunders, et al., 1994). It is reported that null mutations in *mys* (*βPS*) caused embryonic lethality (Brown et al., 1993). *if* (*αPS2*) mutants also displayed embryonic lethality (Brabant & Brower, 1993). Interestingly, the developmental phenotypes of *if* mutants are less severe than that of *mys* mutants (Brown, 1994). Compared to that in *mys* mutants, muscle detachment appeared later and the failure of dorsal closure never happened in *if* mutants (Brabant & Brower, 1993). These results support that embryonic development also requires the action of other integrin heterodimers in which Mys (βPS) associates with other types of α subunits.

Integrins are also required for eye development at the third-instar larval stage. Immunohistological analysis showed that integrins are expressed in the optic stalk (Xie et al., 2014). Mys (βPS) was detected at the interface of the PG layer and the ECM, the interface between PG and SPG, as well as the two SPG (Xie et al., 2014). While Scb (αPS3) was detected at the interface of PG and SPG, If (αPS2) was observed between the PG and ECM (Xie et al., 2014), and was also reported to be expressed in WG in the eye disc (Tavares et al., 2015). Mew (αPS1), however, is not expressed in the eye disc or optic stalk (Xie et al., 2014).

Glia-specific knockdown studies show that the presence of *mys* (*βPS)* in glia is required for glial migration and R-cell axonal projections (Xie et al., 2014). Knocking down *mys* in glia

prevented the migration of glia into the sub-retinal space of the eye disc and also caused R-cell axon stalling (Xie et al., 2014). In contrast, glia-specific knockdown of β_v did not cause any obvious phenotype (Tavares et al., 2015), which is consistent with the specific expression of β_v in the midgut (Yee and Hynes, 1993).

if (*αPS2*), but not *scb* (*αPS3),* is also required for the migration of glial cells from the optic stalk into the eye disc at the third-instar larval stage (Xie et al., 2014). Interestingly, like *mys* knockdown, glia-specific knockdown of *if* and *scb* caused R-cell axon stalling (Tavares et al., 2015; Xie et al., 2014). Thus, it appears that the Mys/If heterodimer acts in glia to mediate the migration of glia from the optic stalk into the eye disc, while both Mys/If and Mys/Scb heterodimers are required in glia for R-cell axonal projections.

1.3.3 Talin, An Intracellular Partner for Integrins

Talin (~270kDa) is a large intracellular protein consisting of a large flexible C-terminal rod domain (>200 kDa) and a globular N-terminal head (~50kDa) (Klapholz & Brown, 2017; Molony et al., 1987). The Talin rod is composed of at least two actin-binding sites (ABS2-3) and a second integrin-binding site (IBS2)(Klapholz & Brown, 2017). The head of Talin contains a FERM domain (residues 86-400), which consists of F1, F2, and F3 domains (Goult et al., 2010). A recent study shows that the 85 amino-acid residues preceding F1 form a ubiquitin-like fold, which is referred to as the F0 domain (Goult et al., 2010). The F3 domain interacts with the cytoplasmic domains of β-integrins as well as many integrin-associated proteins (IAPs), such as focal adhesion kinase (FAK) (Calderwood et al., 2002; García-Alvarez et al., 2003; Klapholz & Brown, 2017). The orientation of the β-integrin is regulated by Talin, which induces the prolonged open conformation of the integrin ectodomains (Shattil et al., 2010)(Fig. 1.4). The F0 domain in the Talin head binds Rap1 proteins (Rap1a and/or Rap1b)(Goult et al., 2010; Plak et

al., 2016). The head of Talin can lie along the plasma membrane with an extended conformation (Elliott et al., 2010).

In *Drosophila*, Rhea (i.e. the *Drosophila* ortholog of vertebrate Talin) is required for the recruitment of IAPs for strengthening or modifying the adhesion (Zervas et al., 2011). Talin is only activated when binding to other proteins, and thus acts as a classic adaptor protein (Klapholz & Brown, 2017). When available and activated for interaction, Talin are assembled and concentrated at adhesion sites to activate integrins and initiate a cascade of events (Klapholz & Brown, 2017). Talin can be recruited to the locations of integrin-mediated adhesion in multiple ways, such as through rod (R1-R4), the IBS1 in the head and IBS2 in the rod, or via ABS3 (Himmel et al., 2009; Kanchanawong et al., 2010; Klapholz & Brown, 2017; Moes et al., 2007; Parsons et al., 2008; Rossier et al., 2012; Tanentzapf & Brown, 2006; Tremuth et al., 2004).

Several signaling pathways can regulate integrin activation, and the binding of Talin to the tail of the β subunit is often the final step (Shattil et al., 2010; Tadokoro et al., 2003; Tanentzapf & Brown, 2006). The binding of Talin is important for the inside-out integrin activation (Wegener et al., 2007)(Fig 1.4). Mutations in the single *Drosophila talin* gene caused phenotypes similar to that in *mys* (*βPS*) null mutant embryos, such as a failure of germ band retraction and muscle detachment (Brown et al., 2000). And mutations affecting three critical residues L325, R358, and W359 in the vertebrate Talin (equivalent residues in *Drosophila* Talin are L334, R367, and W368) impaired the activation of integrins (García-Alvarez et al., 2003; Klapholz & Brown, 2017; Kopp et al., 2010; Stefanini et al., 2014). It is shown that like *mys* knockdown, glia-specific knockdown of *talin* caused a failure of R-cell projection into the optic stalk (Tavares et al., 2015).

1.3.4 Outside-in and Inside-Out Signaling

In the absence of stimulation, integrins αβ heterodimers maintain a closed inactive conformation (Hynes, 2002). The binding partners from outside or inside convert the conformation from an inactive to active state (Liddington & Ginsberg, 2002; Luo et al., 2007). Ligands binding with the large extracellular domains open the inner membrane clasp, which changes the structure of β- and α- subunits (Kim et al., 2011). This allows the recruitment of a number of linker proteins for relaying the extracellular signals into the interior of a cell, a process known as outside-in signaling (Park & Goda, 2016).

On the contrary, the binding of intracellular proteins to the short intracellular tails of integrins would allow the opening of the outer membrane clasp, which facilitates its interaction with the extracellular matrix (ECM) (known as inside-out signaling) (Luo et al., 2007; Park & Goda, 2016). One such intracellular binding partner that activates integrins is Talin (see 1.3.3), which also binds actin filament (Liddington & Ginsberg, 2002). Other intracellular adaptor proteins such as integrin-linked protein kinase (ILK) and kindlins, can also act as activators of integrins (Honda et al., 2009; Moser et al., 2008). Additionally, there are intracellular binding partners of integrins that act as inhibitors to prevent the activation of integrins, such as filamin and integrin-cytoplasmic domain-associated protein 1 (Brakebusch & Fässler, 2003; Critchley, 2000; Geiger et al., 2001; Liu et al., 2000).

Upon binding with ECM, the clustering of integrins with opening conformation mediates strong adhesion (Kechagia et al., 2019). The density of ECM proteins can influence the activation and signaling degree of integrins (Kechagia et al., 2019). And the difference in mechanical force on the ECM also modulates integrin signaling (Amschler et al., 2014; Arnold et al., 2004; Kechagia et al., 2019; Kim et al., 2011). Together, these characteristics allow integrins

to transmit bi-directional signaling across the plasma membrane (Giancotti & Ruoslahti, 1999; Liddington & Ginsberg, 2002).

1.4 ILPs Involved in Neuron-Glia Interactions

1.4.1 Discovery of Insulin family

Insulin is one of the most broadly explored peptide hormones and maintains normal blood glucose levels. Insulin deficiency causes diabetes and obesity (Cheatham & Kahn, 1995). Insulin was discovered in 1921 by Frederick Banting, Charles Best, and John Macleod at the University of Toronto in Canada (Mayer et al., 2007; Rosenfeld, 2002), representing a major breakthrough in the treatment of diabetes. Because of this discovery, Banting and Macleod were awarded the Nobel prize in Physiology or Medicine in 1923 (Rosenfeld, 2002). Later studies suggest the presence of insulin-like substances in the serum (Martin et al., 1958; Renold et al., 1960). Molecular characterization of these insulin-like substances leads to the discovery of insulin-like peptides (ILPs) (Rinderknecht & Humbel, 1978a, 1978b).

In humans, the insulin family consists of one insulin, two insulin-like growth factors (IGFs)-IGFI and IGFII, and seven members of the relaxin-like peptides family, which contain gene 1-3 relaxin and insulin-like peptide 3-6 (INSL3-6) (Shabanpoor et al., 2009). IGFs and the seven relaxin peptides are produced in a variety of cell types or tissues, such as the liver, heart, and brain, while insulin is produced from β cells of the pancreas (Shabanpoor et al., 2009). Furthermore, a number of ILPs have also been discovered in insects, such as one ILP in the locusts, 8 ILPs (DILP1-8) in *Drosophila* (Antonova et al., 2012), and 38 ILPs in the silkmoth *Bombyx mori* (Badisco et al., 2008; Lagueux et al., 1990; Nässel & Vanden Broeck, 2016; Yoshida et al., 1998).

In addition to the similarity in their amino acid sequence (Antonova et al., 2012), most vertebrate and invertebrate insulin-family members also show conserved structural features, such as the formation of dimeric peptides in which A and B chains are connected by disulphide bonds

(Grönke et al., 2010; Nässel & Vanden Broeck, 2016; Shabanpoor et al., 2009; Yoshida et al., 1998). While most ILPs contain A and B chains due to the cleavage of the C peptide, IGFs retain the C peptide and thus only have a single chain in which internal cysteine bridges are formed (Antonova et al., 2012; Brogiolo et al., 2001; Mizoguchi & Okamoto, 2013; Nässel & Vanden Broeck, 2016).

1.4.2 Mammalian IGFs in Neural Development

Mammalian IGFs play important roles in the developing nervous system (Broughton & Partridge, 2009; Folli et al., 1996; Kappeler et al., 2008). IGF-I and IGF-II share significant homology with proinsulin, and IGF-I has been characterized in greater detail (Berger, 2001). While the liver is the primary source of IGF-I which is quickly secreted into the serum in response to growth hormone (GH), IGF-I is also produced in all major cell types in the CNS (Blum et al., 2003; Gourmelen et al., 1994). At the early stages of development, IGF-I is principally present in cephalic regions, and is mainly expressed in glial cells and neurons at a later stage (Bondy, 1991; de Pablo et al., 1993). The diverse patterns of IGF-I expression are coordinated with neuronal cell differentiation, proliferation, and synaptogenesis in specific regions (Andersson et al., 1988; Bach et al., 1991; Baker et al., 1993; Pérez-Villamil et al., 1994).

Genetic studies support the role of IGF-I in neural development. It is reported that IGF-I deficient mice displayed both brain and somatic growth retardation, dysfunctional synapse formation, fewer oligodendrocytes, as well as less myelin in brain white matter (Baker et al., 1993; Beck et al., 1995; Cheng et al., 2003; Ye et al., 2002). Similarly, mutant mice defective in IGF-binding protein (IGFBP) or IGF-I receptor (IGF-1R) had fewer neurons and oligodendrocytes due to increased neuronal apoptosis and decreased neuronal proliferation, and showed abnormal spine formation (Divall et al., 2010; Gutiérrez-Ospina et al., 1996;

Holzenberger et al., 2000; Liu et al., 1993; Zeger et al., 2007). Conversely, transgenic mice overexpressing IGF-I in the brain had increased numbers of neurons and glia (Hodge et al., 2004). Overexpressing astrocyte-derived IGF-I in transgenic mice also caused an increase in the levels of GFAP (Ye et al., 2004), consistent with that IGF-I promotes astroglial differentiation (Ballotti et al., 1987).

Accumulated evidence also suggests important roles for IGF-I in the developing mammalian eye (Hellstrom et al., 2001; Mellough et al., 2015; Pinzon-Guzman et al., 2015; Pinzon-Guzman et al., 2011; Vanhaesebrouck et al., 2009; Wang et al., 2018). IGF-1 is expressed in the retina at the developmental and adult stages (Zeilbeck et al., 2016). Treatment of cultured human embryonic stem cells with IGF-I promoted the formation of three-dimensional eye structures (Mellough et al., 2015). It is also reported that IGF-1 induced rod photoreceptor differentiation in mouse retinal explants (Pinzon-Guzman et al., 2011). Furthermore, treatment of cultured mouse retinal progenitor cells (RPC) with IGF-I accelerated the proliferation of RPC and promoted the differentiation of RPC into photoreceptors and other retinal neurons (Wang et al., 2018).

IGF-I mediates cellular signaling by activating its receptor IGF-IR, which is a membrane-bound glycoprotein consisting of two α and two β subunits (Massagué & Czech, 1982; Pollak, 2008). Upon IGF-I binding, IGF-1R is activated and subsequently initiates a cascade of signaling events for regulating cell proliferation and differentiation (Escott et al., 2013; Inoki et al., 2003; Kooijman, 2006; Taniguchi et al., 2006; Xu et al., 2012). The canonical insulin signaling pathway (ISP) will be discussed in section 1.4.4.

The function of IGF-I is modulated by IGF binding proteins (IGFBPs), which include IGFBP 1-6 (Boisclair et al., 2001; Hashimoto et al., 1997; Twigg & Baxter, 1998). IGFBPs are

expressed by a variety of cells, including cerebral blood vessels, neurons, astroglial cells, and choroid plexus epithelial cells (Lee et al., 1993; Lee et al., 1997), and show a high-binding affinity for IGF-I (Baxter, 2000; Baxter, 2014). IGFBPs act by modulating the transportation and stabilization of IGF-I in plasma, the localization of IGF-I, and the interactions between IGF-I and its receptors (Firth & Baxter, 2002).

1.4.3 ILPs in *Drosophila*

There are eight *ilp* (*ilp*1-8) genes in the *Drosophila* genome, each encodes for a single ILP (Nässel & Vanden Broeck, 2016). Different ILPs show distinct expression patterns during development (Colombani et al., 2012; Ikeya et al., 2002; Slaidina et al., 2009). ILP2, 3, and 5 are major ILPs in the circulation, as they are produced predominantly by a set of insulin-producing cells (IPCs) in the brain and released to the hemolymph in response to internal and external cues (Nässel & Vanden Broeck, 2016). ILPs are also produced by a variety of cell types in other tissues (Nässel et al., 2015). For example, ILP6 is produced by adipocytes of the fat body (Bai et al., 2012), WG in the eye disc (Fernandes et al., 2017), and the thoracic/abdominal ganglia (Antonova et al., 2012).

Drosophila ILPs play important roles in regulating carbohydrate and lipid metabolism, cell growth and differentiation, and lifespan (Taguchi & White, 2008). In the developing nervous system, for example, it is reported that ILP2, 3, 5, and 6 are involved in regulating the timing of neuroblast reactivation (Chell & Brand, 2010; Sousa-Nunes et al., 2011). Loss of ILP 2, 3, 5 and 6 delayed neuroblast reactivation. And glia-specific overexpression of *dilp6* promoted neuroblast activation in the absence of nutrition (Chell & Brand, 2010). In the developing visual system, the production of ILP6 from WG in the eye disc is reported to be required for the initiation of lamina neuronal differentiation in the developing optic lobe (Fernandes et al., 2017).

In *Drosophila*, there are two receptors for ILPs. The receptor for ILP1-7 is the insulin receptor (DInR), and the receptor for ILP8 is the leucine-rich repeat-containing G protein-coupled receptor 3 (LGR3) (Brogiolo et al., 2001; Garelli et al., 2015). While LGR3 shows a more specific expression pattern (Colombani et al., 2015; Kong et al., 2010), DInR is expressed broadly in all examined tissues and displays little variation (Nässel & Vanden Broeck, 2016; Veenstra, 2009; Veenstra et al., 2008). Like mammalian insulin family members, ILPs also activate the canonical ISP (see the following section).

1.4.4 Insulin Signaling Pathway (ISP)

The insulin signaling pathway (ISP) is highly conserved throughout evolution (Okada et al., 2010; Taniguchi et al., 2006; Wu & Brown, 2006)(Fig 1.5). The binding of insulin/ILPs to the α subunit of the InR induces its conformational change leading to autophosphorylation in tyrosine residues at the cytosolic side of the β subunit (Taniguchi et al., 2006). The activation of InR subsequently stimulates intracellular signaling proteins, such as the mitogen-activated protein kinase (MAPK) in the extracellular signal-regulated kinase (ERK) pathway, insulin receptor substrate (IRS)/phosphoinositol 3-kinase (PI3K)/Akt, and components of the target of rapamycin (TOR) pathway (Antonova et al., 2012; Belfiore et al., 2009; Taniguchi et al., 2006).

The phosphorylation of the adaptor scaffold protein IRS by activated InR is a key step in the transduction of insulin/ILPs signals (Araki et al., 1994; Withers et al., 1998). In *Drosophila*, IRS consist of two proteins: Lnk, an orthologue of the src homology 2B (SH2B) adaptor protein 1, and Chico, an orthologue of mammalian IRS1 (Böhni et al., 1999; Werz et al., 2009). Mutant flies with defective in *lnk* or *chico* were smaller in size, and showed increased lipid levels in the hemolymph, supporting that Chico and Lnk are two critical components of the ISP in *Drosophila* (Teleman, 2009; Werz et al., 2009). The docking sites of IRS bind to downstream signaling

proteins containing SH2 domains (Taniguchi et al., 2006). Among them, the adaptor protein Shc and PI3K are particularly important, as their interactions with IRS trigger the activation of the PI3K/Akt and MAPK signaling pathway (Taniguchi et al., 2006).

The binding of the regulatory subunit of PI3K (p60 in *Drosophila*; p85 in mammals) to tyrosine-phosphorylated IRS via its SH2 domain activates the catalytic subunit of PI3K (p110), which then converts PIP_2 (phosphatidylinositol 4,5-diphosphate) to PIP_3 (phosphatidylinositol (3,4,5)-triphosphate) (Leevers et al., 1996; Taniguchi et al., 2006). PIP_3 at the plasma membrane, in turn, binds to pleckstrin homology-domain-containing proteins Akt (also known as protein kinase B) and 3-phosphoinositide-dependent protein kinase-1(PDK1), thus allowing PDK1 to phosphorylate and partially activate Akt (Taniguchi et al., 2006). Full activation of Akt also requires the serine residue Ser 505 in the hydrophobic motif of Akt to be phosphorylated by a rapamycin-insensitive companion of TOR kinase (Rictor) in the TOR-complex 2 (TOR-C2) (Hietakangas & Cohen, 2007). In addition, the serine residue Ser 473 in Akt can be potentially phosphorylated by over 90 kinases, such as protein kinase C, DNA damage response kinase, and choline kinase (Chua et al., 2009; Sarbassov et al., 2004; Sarbassov et al., 2005).

The activation of Akt modulates multiple signaling pathways, which are involved in the process of protein synthesis, glucose metabolism, cell proliferation and survival, neuroendocrine, and stress response (Taniguchi et al., 2006). For example, Akt phosphorylates the *Drosophila* orthologue of Shaggy (Sgg) involved in glycogen synthesis (Frame et al., 2001). Akt inhibits the function of tuberous sclerosis complex (TSC1/2) by phosphorylation, and thus prevents TSC1/2 from inhibiting the TOR kinase pathway (Taniguchi et al., 2006) for the regulation of protein synthesis, metabolism, and cell growth (Harris & Lawrence, 2003). Forkhead box O (FOXO) transcription factors are also well-known downstream targets of Akt. The phosphorylation of

FOXO by Akt prevents FOXO from entering the nucleus to turn on target gene expression (Antonova et al., 2012). FOXO regulates the transcription of many target genes, which modulate cell differentiation, fate, metabolism and survival, and detoxification (Lam et al., 2006).

In the developing *Drosophila* visual system, ISP is required for the proper timing of photoreceptor differentiation in the eye disc (Bateman & McNeill, 2004), the pathfinding of R-cell axons in the optic lobe (Song et al., 2003), and the differentiation of lamina neurons in the optic lobe (Fernandes et al., 2017). For example, reducing InR signaling in eye precursor cells delayed R-cell differentiation, while hyperactivating InR signaling caused precocious R-cell differentiation (Bateman & McNeill, 2004).

1.5 Rationale and Objectives for This Study

Recent studies have made solid progress in defining the molecular mechanisms underlying the coordinated development of R cells and sub-retinal glia in the developing visual system. For example, it is reported that the R-cell-derived FGF8-like ligand Thisbe (Ths) induces the differentiation of PG into WG (Franzdottir et al., 2009). Recent studies from our lab also show that WG (a subtype of sub-retinal glia) express the transmembrane protein Borderless (Bdl), which interacts with another transmembrane protein, Turtle (Tutl), on R-cell axons to mediate axon-WG recognition for promoting WG extension and axon ensheathment (Cameron et al., 2016; Chen et al., 2017). However, two important questions remained unanswered when I initiated my Ph.D. study. The first question is, what is the surface recognition mechanism that mediates the exit of R-cell axons and WG membrane from the eye disc into the optic stalk? And the second question is, do sub-retinal glia produce signals to regulate the timing of R-cell differentiation?

To answer the first question, it is important to identify cell-surface receptors on R-cell axons and/or WG that act specifically to mediate the exit of R-cell axons and WG membrane from the eye disc. By performing a systematic RNAi screen, former trainees in our lab identified a number of genes whose knockdown in both R-cell axons and WG disrupted R-cell axonal projections and/or WG extension (Liu et al., 2020). Among them, *mys* encoding for βPS integrin was particularly interesting, as knockdown of *mys* in both R-cell axons and WG prevented R-cell axons and WG from exiting the eye disc without affecting the differentiation of R cells and WG (Liu et al., 2020). I hypothesized that integrins on R-cell axons and/or WG membrane, play important and specific roles in coordinating the extension of R-cell axons and WG membrane from the eye disc through the optic stalk into the developing optic lobe. To test this hypothesis,

the following aims were proposed: 1) Determining the cell-type-specific requirements of *mys*; 2) Examining the expression pattern of Mys; and 3) Determining the roles of other components of integrin pathway. In Chapter 2, I will describe my work to address the above aims, which has recently been published by *Molecular Brain*. In this study, I identify integrins as key cell-surface receptors on R-cell axons to mediate the exit of both R-cell axons and WG membrane from the eye disc into the optic stalk.

To answer the second question, it is necessary to examine if interfering with the function of sub-retinal glia affects the timing of R-cell differentiation, and subsequently determine the identity of glia-derived signals. Previous studies show that activation of InR signaling in eye precursor cells is required for temporal control of R-cell differentiation (Bateman & McNeill, 2004). However, the source and identity of ligands that activate InR in eye precursor cells were unknown at that time. I hypothesized that sub-retinal glial cells produce certain ILPs, which activate InR in eye precursor cells for temporal control of R-cell differentiation. Two aims were proposed to test this hypothesis: 1) Examining the effects of interfering with the function of sub-retinal glia on the temporal control of R-cell differentiation; and 2) Determining if the timing of R-cell differentiation requires the action of sub-retinal glia-derived ILPs. My work to address the above aims will be described in Chapter 3, which has been submitted to *Journal of Neuroscience* for publication. In this Chapter, I present evidence supporting the roles of sub-retinal glia-derived ILP3 and ILP6 in activating InR in eye precursor cells for temporal control of R-cell differentiation.

General discussions, conclusions and future directions will be included in Chapter 4.

Figure 1. 1

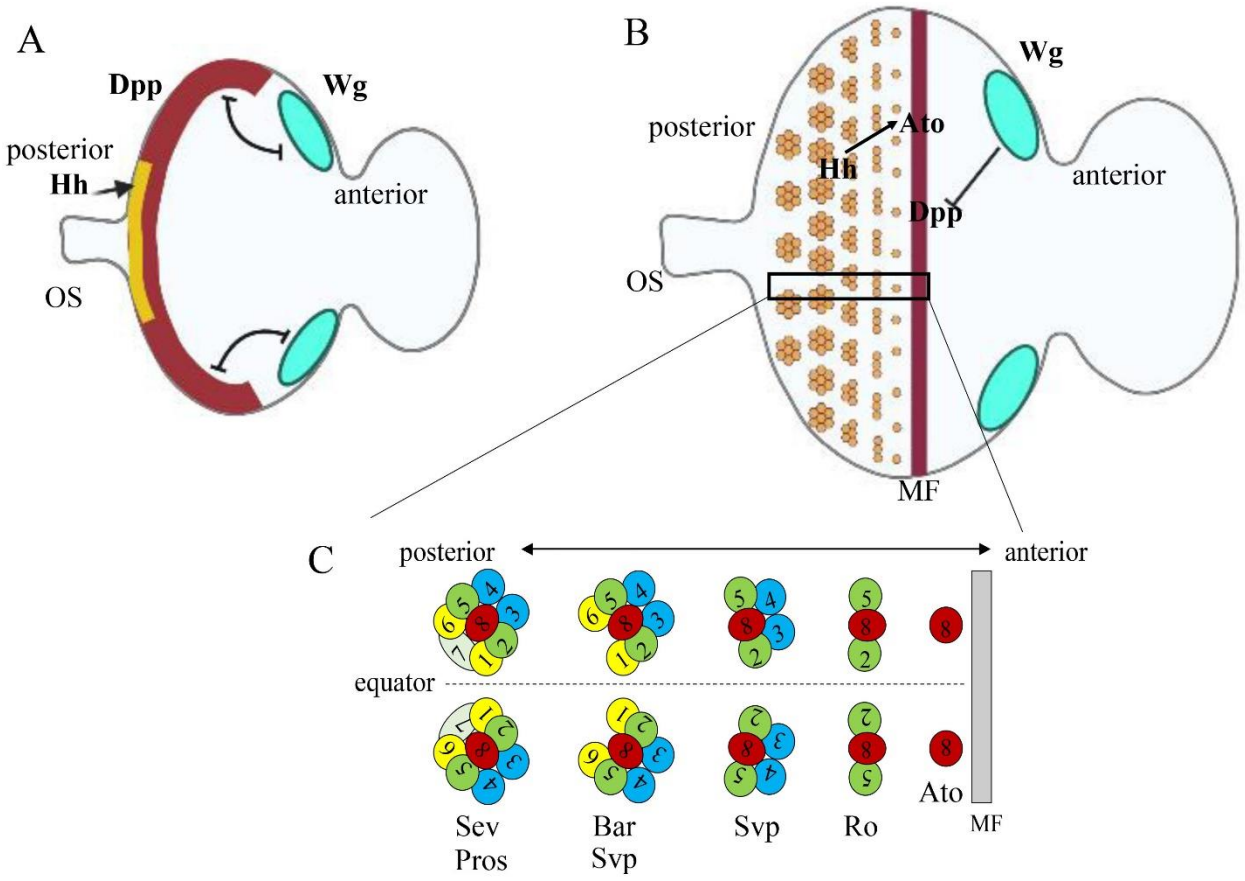

A
Dpp
Wg
posterior
Hh
anterior
OS
B
Wg
posterior
Ato
Hh
anterior
Dpp
OS
MF
C
posterior
anterior
equator
Ato
MF
Sev
Pros
Bar
Svp
Svp
Ro

Figure 1.1. Morphogens regulating eye disc development and the progression of ommatidial differentiation. A. Second-instar eye imaginal disc. *hedgehog* (*hh*, yellow) is expressed at the posterior margin. *wingless* (*wg*, blue) is expressed in the anterior dorsal and ventral margins of the eye disc. *decapentaplegic* (*dpp*, red) is expressed in a stripe at the posterior of the eye disc before the initiation of morphogenetic furrow (MF) and later in the MF. B. In the third-instar eye imaginal disc, the MF sweeps across the eye disc in the direction from posterior to anterior, and it is characterized by the expression of *dpp*. During the eye disc development, Wg inhibits Dpp expression, and Hh activates the expression of the proneuronal gene *atonal* (*ato*). Abbreviations: OS (Optic stalk). Adapted from *Silies, M., Yuva-Aydemir, Y., Franzdottir, S. R., & Klambt, C. (2010). The eye imaginal disc as a model to study the coordination of neuronal and glial development. Fly (Austin), 4(1), 71-79.* The R-cell subtypes are recruited in a precise temporal order, R8 differentiates first as the founder of each ommatidium, followed by R2/R5, R3/R4, R1/R6 and R7. R8 specification requires the function of Atonal (Ato). The Rough (Ro) protein and nuclear receptor Seven-up (Svp) are required for the specification of R2/R5 and R3/R4, respectively. Bar and Svp are required for the specification of R1/R6 cells, and the specification of R7 requires Sevenless (Sev). The activation of Sev increases the expression of Prospero (Pros) in R7 cells.

Figure 1. 2

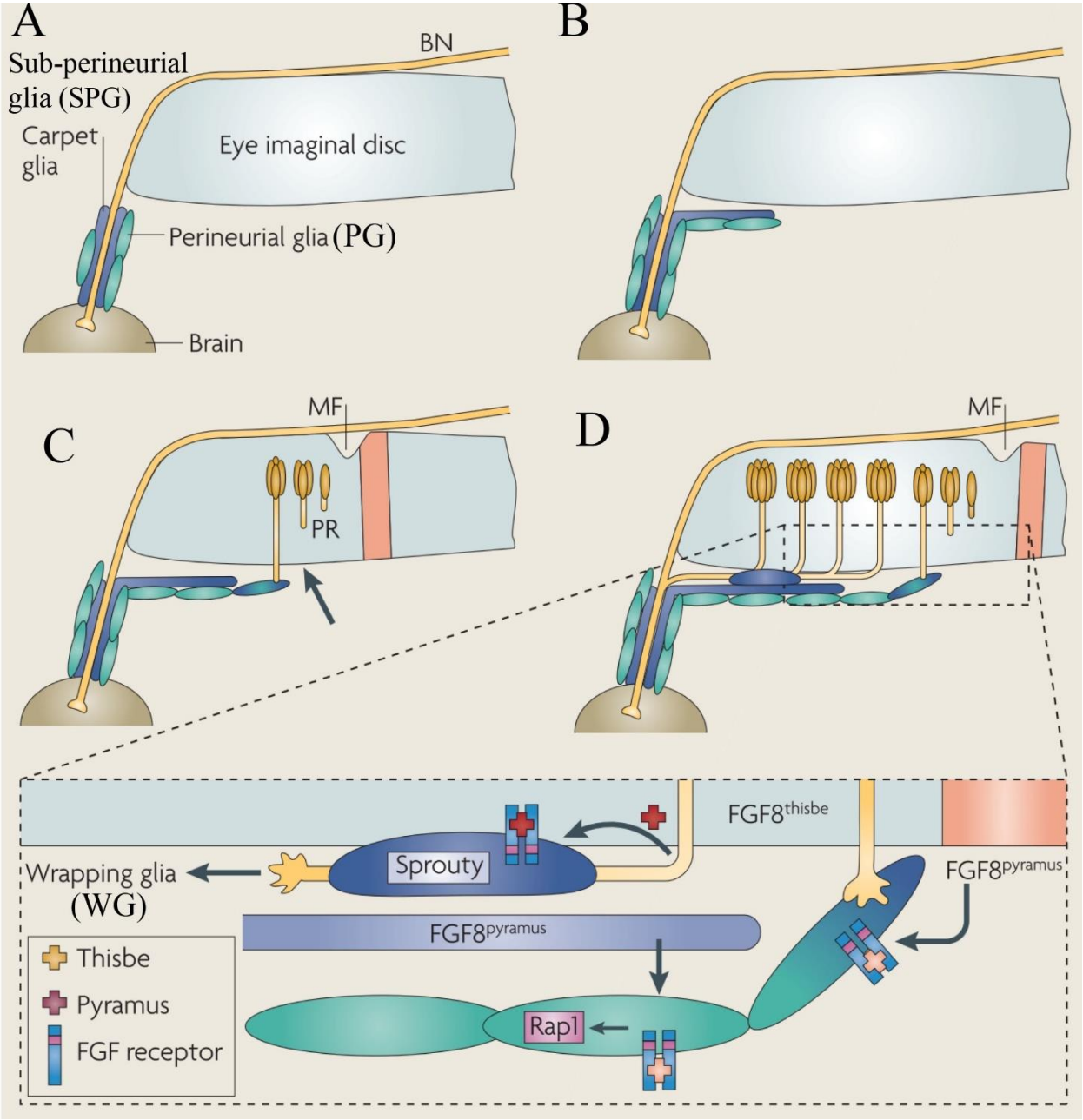

A
Sub-perineurial glia (SPG)
BN
Carpet glia
Eye imaginal disc
Perineurial glia (PG)
Brain
B
C
MF
PR
D
MF
FGF8thisbe
Wrapping glia (WG)
Sprouty
FGF8pyramus
FGF8pyramus
Rap1
Thisbe
Pyramus
FGF receptor

Figure 1.2. The "sequential differentiation model" for the migration and differentiation of sub-retinal glia from the optic stalk to the eye disc. A and B. At the third-instar larval stage, Bolwig's nerve (BN) goes through the eye imaginal disc, optic talk, and projects into the brain. Two large sub-perineurial glia (SPG; i.e. carpet cells) and perineurial glia (PG) at the basal layer of SPG surround BN. SPG and PG originated from the optic stalk and migrate into the eye disc. The number of PG increases during the growth of the eye disc. C and D. After contacting with FGF8-like protein Thisbe (Ths) from R-cell axons, PG are triggered to reprogram to differentiate into wrapping glia (WG) and enter the apical layer of SPG. Then, WG ensheath and follow the axons through the optic stalk toward the brain. Adapted from *Klambt, C. (2009). Modes and regulation of glial migration in vertebrates and invertebrates. Nat Rev Neurosci, 10(11), 769-779.*

Figure 1. 3

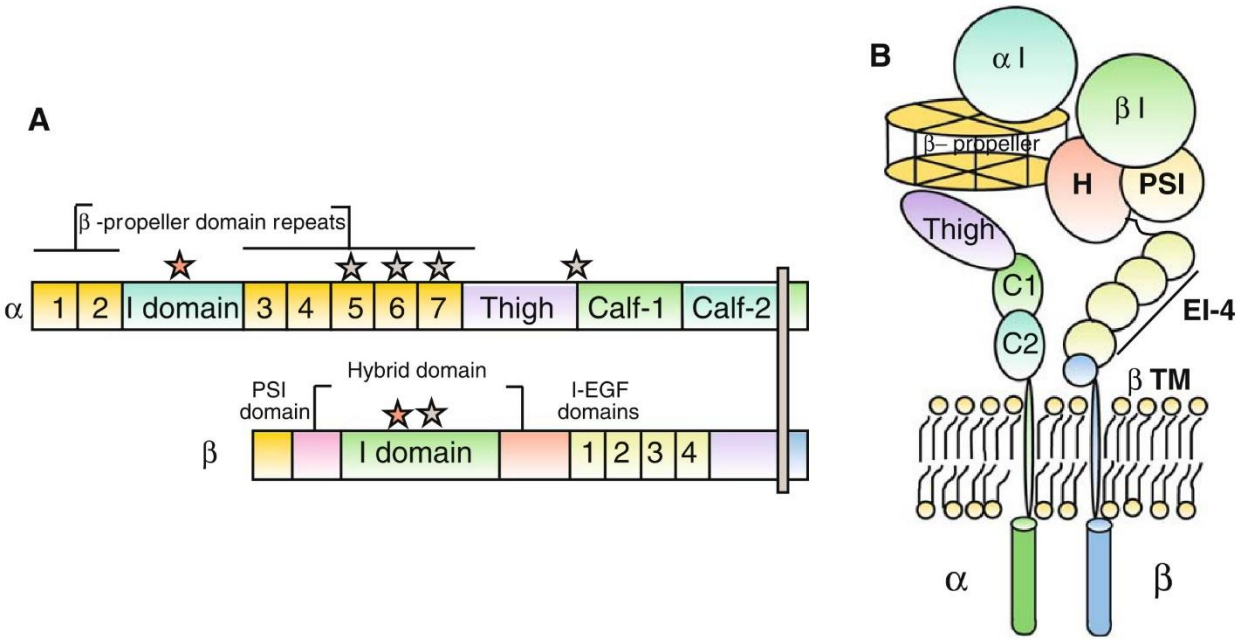


Figure 1.3 Diagram of nine prototypical integrin heterodimers containing αI-domain in mammals. A. All domains in αI-domain-containing integrins are indicated. B. The conformation and arrangement of different domains in integrin heterodimers containing αI-domain. Adapted from *Barczyk, M., Carracedo, S., & Gullberg, D. (2010). Integrins. Cell Tissue Res, 339(1), 269-280.*

Figure 1. 4

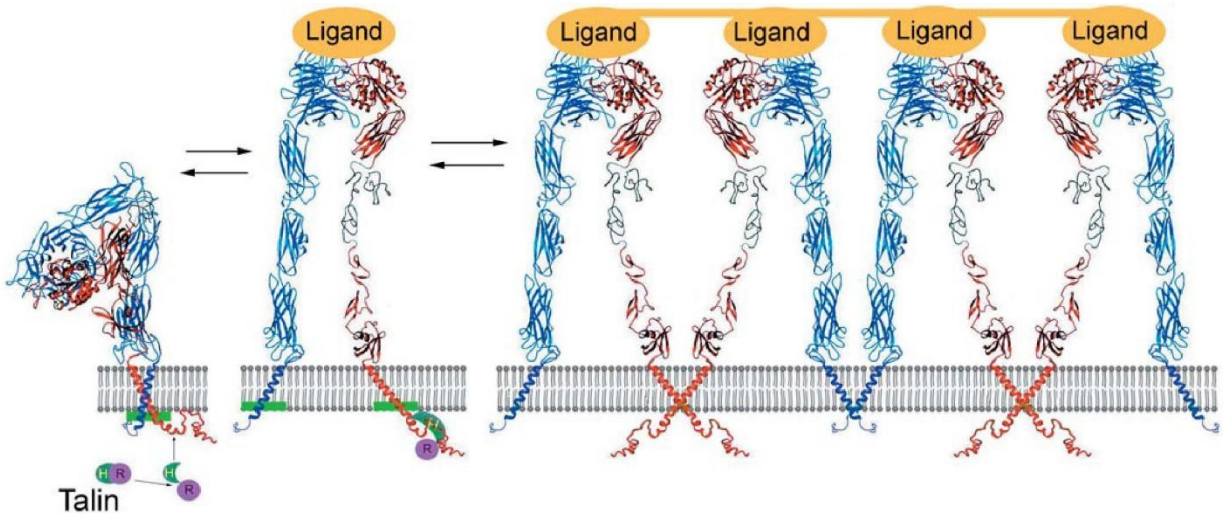


Figure 1.4. The model of integrin activation from the cytoplasmic face. The model is based on the crystal structure of integrins. The α subunit and β subunit are colored in blue and red, respectively. Talin is induced to expose the head domain and change its conformation by cellular signaling. The head domain of Talin binding to the β cytoplasmic tail of integrins unclasps the α tails, causing the structural change of integrin at the plasma membrane. The unclasping of β and α tails induces the opening of the integrin extracellular headpiece, which transforms from the bend to the extended form. The extended form of integrins has a high affinity to extracellular ligands. Adapted from *Qin, J., Vinogradova, O., & Plow, E. F. (2004). Integrin bidirectional signaling: a molecular view. PLoS Biol, 2(6), e169.*

Figure 1. 5

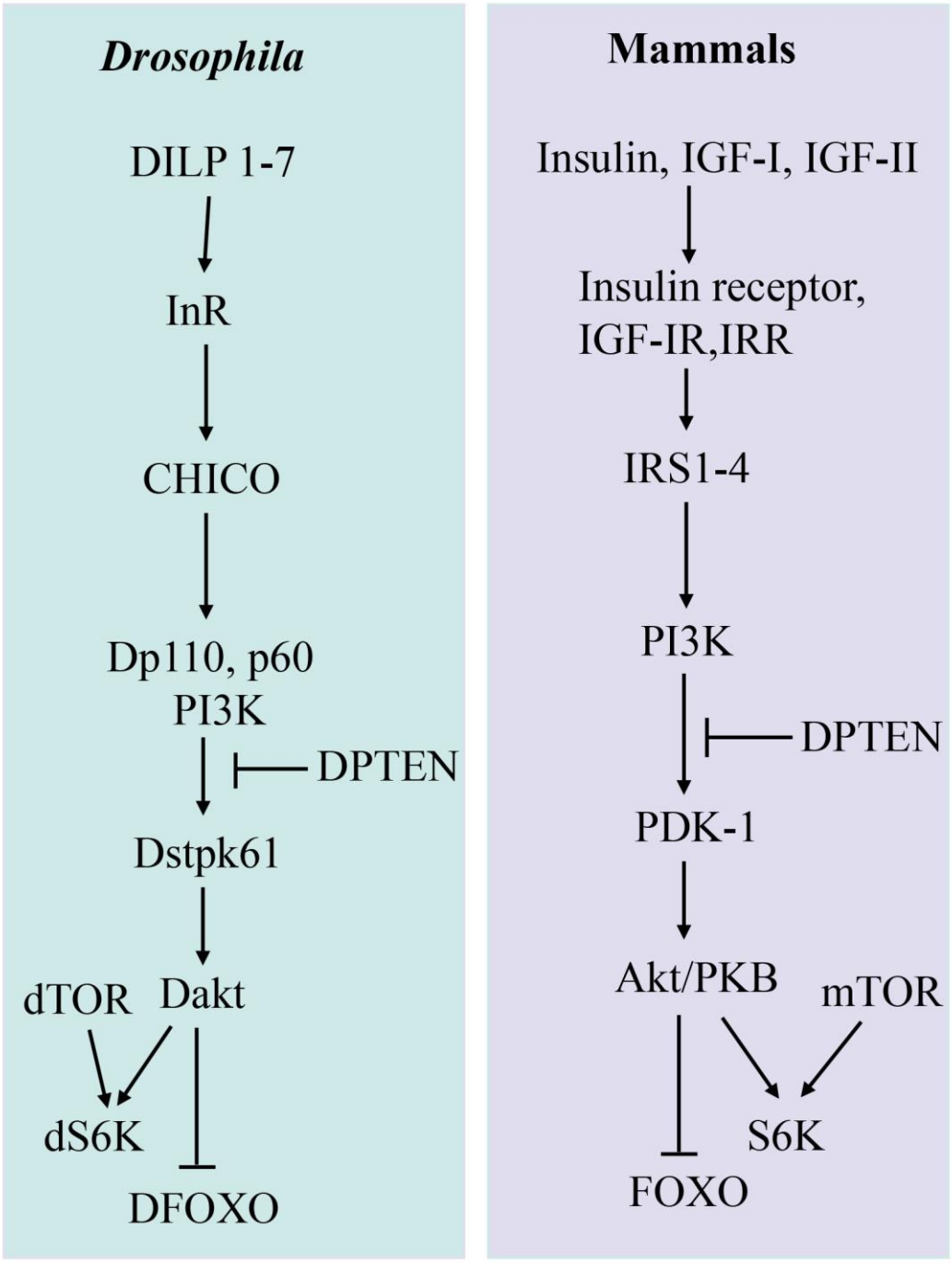

Drosophila
DILP 1-7
InR
CHICO
Dp110, p60
PI3K
DPTEN
Dstpk61
dTOR
Dakt
dS6K
DFOXO
Mammals
Insulin, IGF-I, IGF-II
Insulin receptor,
IGF-IR,IRR
IRS1-4
PI3K
DPTEN
PDK-1
Akt/PKB
mTOR
S6K
FOXO

Figure.1.5. The components of the conserved insulin signaling pathway (ISP) in *Drosophila* and mammals. ISP in both *Drosophila* and mammals acts through the conserved PI3K and Akt/PKB, which are negatively modulated by the phosphatase and tension homolog (PTEN). In *Drosophila*, the binding of insulin-like peptides 1-7 (DILP 1-7) to insulin receptors (InR) activates the insulin receptor substrate (IRS) Chico. Chico subsequently induces the phosphorylation of phosphatidylinositol 3-kinase (PI3K), which activates the fly Akt (Dakt). Fly PI3K contains the p60 subunit, while mammalian PI3K includes the p85 subunit. Abbreviations: IRR: insulin receptor-related receptor. Adapted from *Garofalo, Robert S., & Metabolism. (2002). Genetic analysis of insulin signaling in Drosophila. 13(4), 156-162.*

Chapter 2 : The Exit of Axons and Glial Membrane from the Developing *Drosophila* Retina Requires Integrins[1]

[1] This chapter has been published in the journal: *Molecular Brain.* 3 Jan 2022, 15(1):2; DOI: 10.1186/s13041-021-00888-2; Qian Ren and Yong Rao

2.1 Abstract

Coordinated development of neurons and glia is essential for the establishment of neuronal circuits during embryonic development. In the developing *Drosophila* visual system, photoreceptor (R cell) axons and wrapping glial (WG) membrane extend from the eye disc through the optic stalk into the optic lobe. Extensive studies have identified a number of genes that control the establishment of R-cell axonal projection pattern in the optic lobe. The molecular mechanisms directing the exit of R-cell axons and WG membrane from the eye disc, however, remain unknown. In this study, we show that integrins are required in R cells for the extension of R-cell axons and WG membrane from the eye disc into the optic stalk. Knockdown of integrins in R cells but not WG caused the stalling of both R-cell axons and WG membrane in the eye disc. Interfering with the function of Rhea (i.e. the *Drosophila* ortholog of vertebrate talin and a key player of integrin-mediated adhesion), caused an identical stalling phenotype. These results support a key role for integrins on R-cell axons in directing R-cell axons and WG membrane to exit the eye disc.

2.2 Introduction

The formation of neural networks during embryonic development requires proper neuron-glia interactions. Glial cells produce cues that guide the extension of growing axons. And neurons also produce signals to regulate glial proliferation and differentiation (He & Lu, 2013; Stipursky et al., 2011). The mechanisms underlying the coordinated development of neurons and glia for circuit development, however, are still not fully understood.

The establishment of photoreceptor neurons (R cells) to optic lobe connections in the *Drosophila* adult visual system is an excellent model for understanding the mechanisms controlling the coordinated development of neurons and glia for the establishment of neuronal connections. R-cell differentiation in the eye-imaginal disc begins at the third-instar larval stage and progresses in a posterior-to-anterior direction across the eye disc (Altshuler & Lillien, 1992; Sato et al., 2013). Within each ommatidium, R8 differentiates first, followed by R2/5, R3/4, R1/6, and R7. Differentiating R cells project axons that migrate towards the posterior end of the eye disc, where they exit the eye disc and into the optic stalk, a tubular structure connecting the eye disc to the optic lobe. After exiting the optic stalk, R-cell axons project into the first (i.e. lamina) and second (i.e. medulla) layers of the optic lobe, and establish a precise retinotopic map (Hadjieconomou et al., 2011).

Wrapping glia (WG), a subtype of sub-retinal glia, play an important role in the establishment of retinotopic map (Chang et al., 2018; Fernandes et al., 2017). WG are derived from perineurial glia (PG) in the optic stalk. At the third-instar larval stage, PG migrate from the optic stalk into the sub-retinal region of the eye disc (Yuva-Aydemir & Klambt, 2011). After exposure to FGF8-like ligand Thisbe from differentiating R cells, PG differentiate into WG (Franzdottir et al., 2009). R-cell axons are temporally enwrapped by WG membrane, which

extends from the eye disc through the optic stalk into the distal region of the lamina (Cameron et al., 2016; Chang et al., 2018; Chen et al., 2017). Our previous studies show that the ensheathment of R-cell axons by WG requires two cell-surface adhesion molecules Turtle (Tutl) and Borderless (Bdl) (Cameron et al., 2016; Chen et al., 2017), both of which belong to the conserved IgSF9 subfamily of the immunoglobulin superfamily (Hansen & Walmod, 2013). We show that the interactions between Tutl on R-cell axons and Bdl on WG are required for WG extension and the ensheathment of R-cell axons (Cameron et al., 2016; Chen et al., 2017). The extension of WG membrane from the eye disc into the optic lobe is required for the proper organization of R-cell axons within the optic stalk (Cameron et al., 2016; Chen et al., 2017), the topographic projection of R-cell axons in the optic lobe (Chang et al., 2018), and the differentiation of lamina neurons in the optic lobe (Fernandes et al., 2017).

The molecular mechanisms that direct R-cell axons and WG membrane to exit the eye disc remain unknown. Several studies suggest that sub-retinal glia may provide cues at the posterior end of the eye disc that allow the exit of R-cell axons from the eye disc (Hummel et al., 2002; Rangarajan et al., 1999; Xie et al., 2014). The molecular identity of glial-derived cues and their receptors on R-cell axons, however, remains elusive. While the temporal ensheathment of R-cell axons by WG during development leads to speculation that R-cell axons promote the extension of WG membrane, it remains unclear if the extension of WG membrane from the eye disc into the optic stalk is dependent on R-cell axons. In this study, we show that integrins are required in R cells but not WG for the exit of both R-cell axons and WG membrane from the eye disc.

2.3 Materials and Methods

2.3.1 Genetics

Fly stocks are reared at 25 ^{o}C with 50% humidity and 12/12h light-dark cycle. Following stocks were obtained from Bloomington *Drosophila* Stock Center (BDSC): *UAS-mys-RNAi-JF02819* (BDSC#27735), *UAS-mys-RNAi-HMS00043* (BDSC#33642), *UAS-scb-RNAi-JF02696* (BDSC#27545), *UAS-rhea-RNAi-HMS00799* (BDSC#32999), *UAS-rhea-RNAi-HMS00856* (BDSC#33913), *yw; P{white-un1}70C rhea*^{1} *P{neoFRT}80B/TM6B, P{iab-2(1.7)lacZ}6B, Tb* (BDSC#2296), and *yw; P{GAL4-ey.H}3-8, P{UAS-FLP.D}JD1; P{GMR-hid}SS3, l(3)CL-L1, P{Car20y}73C P{neoFRT}80B/TM2* (BDSC#43658). *Mz97*-GAL4; *ey*$^{3.5}$-GAL4/*Tb*, *ey*$^{3.5}$-GAL4/*Tb* and *Mz97*-GAL4; +/+ were either obtained from BDSC or generated in our previous studies (Cameron et al., 2016; Chen et al., 2017).

To knock down *mys* in both R cells and WG, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4/+; *ey*$^{3.5}$-GAL4/*UAS-mys-RNAi-JF02819*. To knock down *mys* in eye-disc epithelium but not WG, genetic crosses were performed to generate flies with the genotype *ey*$^{3.5}$-GAL4*/UAS-mys-RNAi-HMS00043*. To knock down *mys* in WG but not R cells, genetic crosses were performed to generate flies with the genotype *MZ97*-GAL4*/+; UAS-mys-RNAi-HMS00043/+*. To knock down *mys* specifically in R cells but not other cell types in the epithelial layer of the eye disc, genetic crosses were performed to generate flies with the genotype *C155*-GAL4*; UAS-mys-RNAi-HMS00043/+*.

To knock down *scb* in both R cells and WG, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4/+; *ey*$^{3.5}$-GAL4/*UAS-scb-RNAi-JF02696*. To knock down *mys* in eye-disc epithelium but not WG, genetic crosses were performed to generate flies with the genotype *ey*$^{3.5}$-GAL4/*UAS-scb-RNAi-JF02696*. To knock down *scb* specifically in WG but not R

cells, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4/+; +/*UAS-scb-RNAi-JF02696*.

To knock down *rhea* in eye-disc epithelium but not WG, genetic crosses were performed to generate flies with the genotype $ey^{3.5}$-GAL4/*UAS-rhea-RNAi-HMS00799* and $ey^{3.5}$-GAL4/*UAS-rhea-RNAi-HMS00856*. To knock down *scb* specifically in WG but not R cells, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4/+; +/ *UAS-rhea-RNAi-HMS00799*. To generate large clones of eye mutant tissues homozygous for the strong *rhea* loss-of-function mutant allele $rhea^{1}$, genetic crosses were performed to generate flies with the genotype *yw; P{GAL4-e3-8, P{UAS-FLP.D}JD1/+; P{white-un1}70C* $rhea^{1}$ *P{neoFRT} 80B/P{GMR-hid}SS3, l(3)CL-L1, P{Car20y}73C P{neoFRT}80B*.

2.3.2 Histology

Eye-brain complexes from third-instar larvae were dissected and fixed for immunostaining similarly as described previously (Cameron et al., 2016; Chen et al., 2017).

2.3.3 Immunostaining

Primary and secondary antibodies were used at following dilutions: mouse MAb24B10 (1:100; Developmental Studies Hybridoma Bank or DSHB Cat#24B10), mouse monoclonal anti-Mys (1:100; DSHB Cat#CF.6G11), mouse monoclonal anti-Repo (1:100; DSHB Cat#8D12), rabbit polyclonal anti-Bdl (1:500), rabbit polyclonal anti-HRP (1:500; Jackson ImmunoResearch Cat#323-005-021). Secondary antibodies including Alexa Fluor 488 goat anti-Mouse IgG (Invitrogen, Cat# A11001), Alexa Fluor 488 goat anti-Rabbit IgG (Invitrogen, Cat#A32731), Alexa Fluor 647 goat anti-Rabbit IgG (Invitrogen, Cat# A20991) and Alexa Fluor 647 goat anti-Mouse IgG (Invitrogen, Cat#A32728), were used at 1:500 dilution.

2.3.4 Confocal Microscopy

Epifluorescent images were captured and analyzed by confocal microscopy (Olympus FV1000). Photo stacks were performed using Z-stack projection by FluoView software.

2.3.5 Statistical Analysis

The number of sub-retinal glia in the eye disc of wild-type (n=8) and *mys* knockdown (n=8) third-instar larva was counted manually by using cell counter function in Image J. For *mys* knockdown, only individuals showing the stalling phenotypes were included. Two-tailed student's t-tests were used for statistical analysis. The difference is considered as significant when a p value is <0.05.

2.4 Results

2.4.1 Knockdown of *mys* in both eye-disc epithelium and WG prevented the exit of R-cell axons and WG membrane from the eye disc

In our previous study (Liu et al., 2020), we performed an RNA interference (RNAi) screen to search for cell-surface receptors and secreted factors that regulate the coordinated development of R cells and WG in the developing *Drosophila* visual system. We found that when a *UAS-RNAi* transgene (i.e. *UAS-mys-RNAi-HMS00043*) was expressed in both WG and eye-disc epithelium consisting of differentiating R cells and accessory cells, R-cell axons and WG membrane stalled in the eye disc (Liu et al., 2020). Since *UAS-mys-RNAi-HMS00043* targets the *myospheroid (mys)* gene that encodes for a beta integrin subunit (βPS), this result suggests a role for Mys in mediating the exit of R-cell axons and WG membrane from the eye disc. To further test this, we examined another independent RNAi line (i.e. *UAS-mys-RNAi-JF02819*) targeting a different region of *mys*.

UAS-mys-RNAi-JF02819 was simultaneously expressed in both R cells and WG under control of the eye-specific $ey^{3.5}$-GAL4 and WG-specific *Mz97*-GAL4. The R-cell axonal projection pattern was visualized using MAb 24B10, which recognizes the R-cell-specific cell adhesion molecule Chaoptin (Van Vactor et al., 1988). The extension of WG membrane was visualized with antibodies against Bdl that is specifically expressed on WG membrane at the third-instar larval stage (Cameron et al., 2016). In wild type (Fig. 2.1A and 2.1A”), R-cell axons migrate posteriorly and converge at the most posterior end where they leave the eye disc and into the optic stalk. After exiting the optic stalk, R1-R6 axons terminate at the intermediate target region in the lamina, and R7 and R8 axons pass through the lamina into the deeper medulla. The extension of WG membrane is closely associated with R-cell axons from the eye disc through the

optic stalk into the distal region of the lamina, where WG membrane ceases extension and dissociates from R-cell axons (Fig. 2.1A' and A").

In most animals expressing UAS-*mys-RNAi-JF02819* in both eye-disc epithelium and WG (Fig. 2.1B-B", Table 2.1), R-cell axons and WG membrane extended normally towards the posterior end of the eye disc but failed to exit the eye disc or stalled in the optic stalk. These phenotypes were identical to that in animals expressing *UAS-mys-RNAi-HMS00043* in both eye-disc epithelium and WG reported in our previous study (Liu et al., 2020).

2.4.2 Knockdown of *mys* in R cells but not WG caused the stalling of R-cell axons and WG membrane in the eye disc

The above results raise three possibilities for the role of *mys* in the exit of R-cell axons and WG membrane: 1) *mys* is only required in R cells; 2) *mys* is only required in WG; and 3) *mys* is required in both R cells and WG. To distinguish among these possibilities, we performed knockdown experiments by expressing *UAS-mys-RNAi* transgene under control of the eye-specific *ey*$^{3.5}$-GAL4 only or the WG-specific *Mz97*-GAL4 only. *ey*$^{3.5}$-GAL4 drives the expression of *UAS-mys-RNAi* transgene in eye-disc epithelium consisting of both R cells and accessory cells, but not in WG or other sub-retinal glia. Whereas *Mz*97-GAL4 drivers the expression of *UAS-mys-RNAi* transgene specifically in WG (Hummel et al., 2002).

Knockdown of *mys* under control of *ey*$^{3.5}$-GAL4 caused a stalling phenotype (Fig. 2.2B-B") identical to that in animals in which *mys* was knocked down in both eye-disc epithelium and WG (Fig. 2.1B-B", Table 2.1). In contrast, WG-specific knockdown of *mys* under control of *Mz97*-GAL4 did not affect the exit of R-cell axons or WG membrane (Fig. 2.2C-C"). These results indicate that *mys* is required in eye-disc epithelium but not WG for the exit of both R-cell axons and WG membrane from the eye disc.

Since eye-disc epithelium consists of both R cells and accessory cells, the above phenotypes may reflect a role for *mys* in R cells and/or accessory cells in mediating the exit of R-cell axons and WG. To further determine cell-type-specific requirements of *mys*, we performed knockdown experiments using the pan-neuronal-specific driver *C155*-GAL4, which turned on the expression of RNAi transgenes in R cells but not accessory cells in eye-disc epithelium. Consistently, we found that knocking down *mys* under control of *C155*-GAL4 caused the stalling of R-cell axons and WG membrane in the eye disc (Fig. 2.3B-B", Table 2.1). Since R cells are the only neuronal cell types in the eye disc, this result indicates that *mys* is required in R cells for the exit of R-cell axons and WG membrane from the eye disc.

2.4.3 Mys is expressed in R-cell axons

The above results suggest a role for Mys in R cells for the exit of R-cell axons and WG membrane from the eye disc. To further address this, we examined the expression pattern of Mys in the eye disc by staining third-instar eye disc with a mouse anti-Mys monoclonal antibody (Brower et al., 1984). Consistent with a previous report (Fernandes et al., 2014), we found that Mys was broadly expressed in the eye disc (Fig. 2.4A' and 2.4A"). At the posterior end of the eye disc and the optic stalk, strong Mys staining was detected in R-cell axons (Fig. 2.4B' and 2.4B"). The intensity of anti-Mys staining was greatly reduced when *mys* was specifically knocked down in the eye disc (Fig. 2.4C', 2.4C", 2.4D' and 2.4D"), supporting the specificity of this antibody.

2.4.4 Knockdown of *mys* in R-cell axons did not affect the migration of glial cells from the optic stalk into the eye disc

Previous studies suggest that the migration of glial cells from the optic stalk into the eye disc is required for the exit of R-cell axons from the eye disc (Hummel et al., 2002; Rangarajan

et al., 1999; Xie et al., 2014). This raises the possibility that the stalling defects in animals in which *mys* was knocked down in R cells (Fig. 2.2 and 2.3), were due to a failure of glial migration into the eye disc. To test this, we examined if knocking down *mys* in R cells affects glial migration. Glial cells in the sub-retinal region of the eye disc were stained with a mouse monoclonal antibody that recognizes the Repo expressed in the nuclei of all glia (Alfonso & Jones, 2002). Compared to that in wild type (Fig. 2.5A-A" and 2.5C), the number of sub-retinal glial cells was not significantly reduced in knockdown animals (Fig. 2.5B-B" and 2.5C). In *mys* knockdown eye discs showing stalling phenotypes, the number of sub-retinal glia varied from 160 to 229. Given that the presence of 3 to 20 sub-retinal glia in the eye disc was shown to be sufficient for the exit of R-cell axons from the eye disc into the optic stalk (Rangarajan et al., 1999), this result argues against that Mys mediates the exit of R-cell axons by promoting the migration of glial cells from the optic stalk into the eye disc.

2.4.5 Knockdown of *scab* (*scb*) encoding for αPS3 integrin subunit caused a mys-like R-cell axon and WG stalling phenotype

The integrin receptor is a heterodimer consisting of α and β subunits. In *Drosophila*, Mys (βPS) functions by forming a heterodimer with one of five α-integrin subunits (Takada et al., 2007). Among the five α-integrin subunits, Inflated (If) (i.e αPS2) and Scb (i.e. αPS3) are reported to be expressed in the eye disc and optic stalk (Xie et al., 2014). However, knockdown of *if* in both eye-disc epithelium and WG did not affect the exit of R-cell axons or WG membrane from the eye disc (Liu et al., 2020), arguing against that Mys functions together with If in R-cell axons. To determine the role of *scb* in R-cell axons, we examined the effects of *scb* knockdown on the extension of R-cell axons and WG.

Like *mys* knockdown, knockdown of *scb* in both eye-disc epithelium and WG caused the stalling of R-cell axons and WG membrane in the eye disc or in the optic stalk (Fig. 2.6B-B", Table 2.1). Similar defects were observed when *scb* was specifically knocked down in eye-disc epithelium (Fig. 2.6C-C", Table 2.1), while no defect was observed when *scb* was knocked down in WG only (Fig. 2.6D-D"). These results suggest strongly that Mys and Scb function together in R-cell axons to mediate the exit of R-cell axons and WG membrane.

2.4.6 Interfering with the function of Rhea (the fly ortholog of talin) in eye-disc epithelium prevented the exit of R-cell axons and WG membrane from the eye disc.

The requirements of Mys and Scb for the exit of R-cell axons and WG membrane support a key role for integrin-mediated adhesion in this process. To further address this, we examined if interfering with the function of Rhea (Brown et al., 2002), the fly ortholog of talin and a key player of the integrin-mediated adhesion (Klapholz & Brown, 2017), causes phenotypes similar to that observed in mys or scb knockdown animals.

Like that in *mys* and *scb* knockdown animals, R-cell axons and WG membrane failed to exit the eye disc or stalled in the optic stalk when *rhea* was knocked down in eye-disc epithelium but not WG (Fig. 2.7B-B" and 2.7C-C", Table 2.1). In contrast, no phenotype was observed when *rhea* was knocked down in WG only (0 out of 11 animals examined). Similar results were obtained using two independent *rhea-RNAi* lines (Fig. 2.7B-B" and 2.7C-C").

We also performed genetic mosaic analysis to examine if the removal of *rhea* specifically in eye-disc epithelium but not sub-retinal glia, disrupts R-cell axonal projections and WG extension. The *eyFLP-FRT* system was used to generate large clones of mutant eye tissue homozygous for $rhea^{1}$, a strong *rhea* loss-of-function mutant allele (Brown et al., 2002). Consistent with the results from eye-specific knockdown experiments, mosaic animals carrying

large clones of homozygous *rhea*^{1} eye tissues also displayed defects (Fig 2.7D-D") that were identical to that in eye-specific *mys* or *rhea* knockdown animals. These results support an essential role for integrin-mediated adhesion in mediating the exit of R-cell axons and WG membrane from the eye disc.

2.5 Discussion

In this study, we provide several lines of evidence that supports a specific role for integrins on R-cell axons to direct the exit of R-cell axons and WG membrane from the eye disc. First, knockdown of βPS integrin subunit Mys or αPS3 integrin subunit Scb in eye-disc epithelium but not WG caused the stalling of both R-cell axons and WG membrane in the eye disc. Second, knockdown of Mys specifically in R cells in eye-disc epithelium also prevented the exit of R-cell axons and WG membrane from the eye disc. Third, Mys is strongly expressed on R-cell axons at the posterior end of the eye disc where R-cell axons converge and extend into the optic stalk. And fourth, interfering with the function of the fly talin (i.e. Rhea) in eye-disc epithelium but not WG prevented the extension of R-cell axons and WG membrane from the eye disc into the optic stalk.

To our knowledge, this study identifies for the first time a cell-surface receptor on R-cell axons that plays an essential and specific role in mediating the exit of R-cell axons and WG membrane from the eye disc. In several previous studies (Garrity et al., 1996; Martin et al., 1995; Newsome et al., 2000), forward genetic screens were performed to search for key players that control R-cell axonal projections. Although these studies led to the identification of a number of important genes required for R-cell axonal guidance and layer-specific target selection in the optic lobe, no R-cell surface receptor that specifically controls the exit of R-cell axons from the eye disc was uncovered from these genetic screens.

It is reported that in the developing mouse retina, netrin-1 from neuroepithelial cells interacts with its receptor DCC on retinal ganglion axons to mediate the exit of retinal ganglion axons from the optic disc into the optic nerve (Deiner et al., 1997). However, the fly orthologs of netrins (i.e. Netrin A and B) and DCC (i.e. Frazzled) are not required for the exit of R-cell axons

from the eye disc into the optic stalk (Gong et al., 1999). We speculate that like the action of DCC on retinal ganglion axons, integrins on R-cell axons may recognize specific cues at the posterior end of the eye disc for the extension of R-cell axons and WG membrane into the optic stalk. And sub-retinal glia at the posterior end of the eye disc may present such cues that are recognized by integrins on R-cell axons. Consistent with this notion, it is reported that blocking the migration of glial cells into the sub-retinal region of the eye disc caused the failure of R-cell axons to exit the eye disc (Rangarajan et al., 1999; Xie et al., 2014). Integrins on R-cell axons may interact with certain cell-surface receptors on sub-retinal glia, which may serve as permissive substrates for the extension of R-cell axons. Alternatively or additionally, extracellular matrix (ECM) proteins associated with sub-retinal glia may be recognized by integrins on R-cell axons to promote the exit of R-cell axons. It is reported that glia-specific knockdown of integrins could cause the stalling of R-cell axons in the eye disc without affecting the migration of glial cells into the eye disc (Tavares et al., 2015). One likely explanation is that integrins on sub-retinal glia may regulate the organization of ECM proteins, and thus allow the ECM proteins to be properly recognized by integrins on R-cell axons to direct the exit of R-cell axons from the eye disc.

Our phenotypic analysis of mutants defective in the integrin pathway also showed that some R-cell axons and WG membrane were able to exit the eye disc and then stalled within the optic stalk (Table 2.1, Fig. 2.6C-C" and 2.7B-B"). One likely explanation is that knockdown efficiency may vary between animals, which contributed to phenotypic variability. That R-cell axons stalled in the optic stalk in some knockdown animals, further suggests that integrins on R-cell axons may also play a role in promoting the migration of R-cell axons through the optic stalk. Like that at the posterior end of the eye disc, cell-surface receptors and/or ECM proteins in the

optic stalk may be recognized by integrins, and thus serve as permissive substrates for the migration of R-cell axons through the optic stalk.

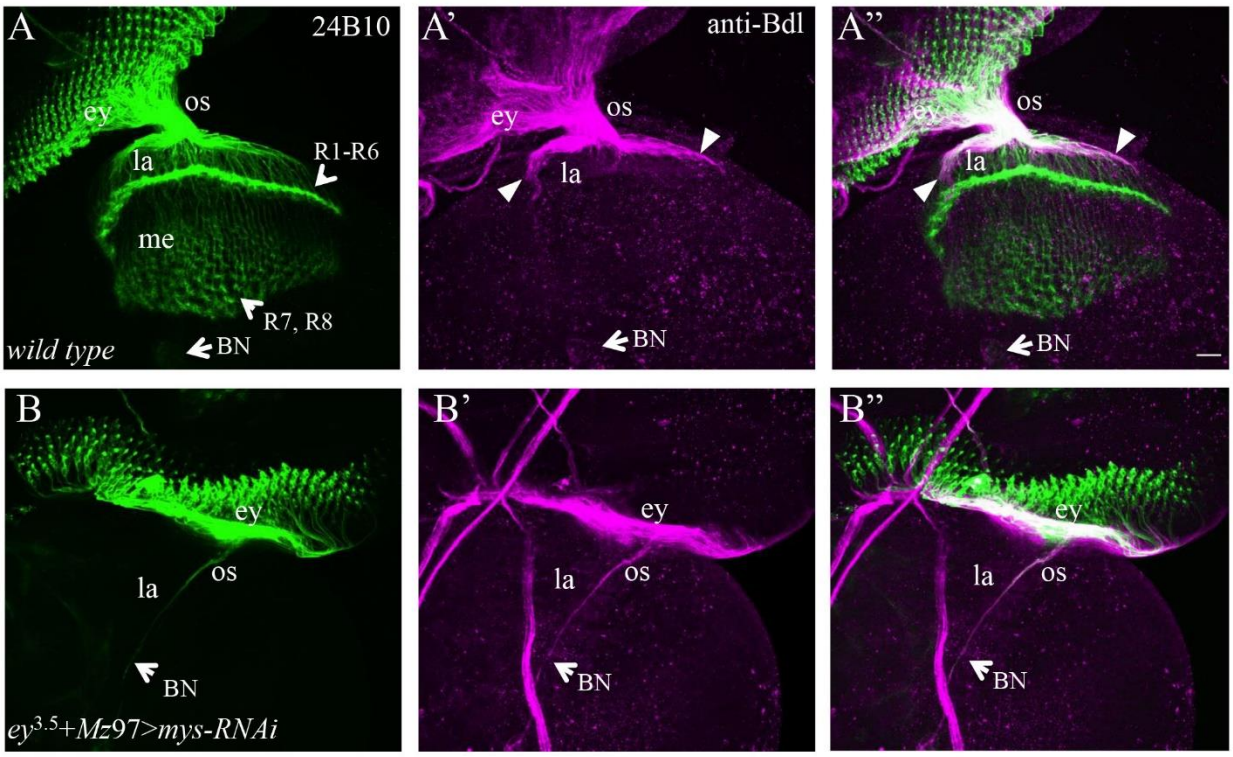
Fly integrins are highly homologous to their mammalian counterparts (Broadie et al., 2011). The closest homologs of Mys and Scb in mammals are β1 integrin and α4/9 integrin (Broadie et al., 2011), respectively. In mammals, β1 integrin and α4/9 integrin have been shown to be involved in mediating axonal projections (Hines et al., 2010; Pasterkamp et al., 2003; Vogelezang et al., 2001; Wingerd et al., 2002). For instance, it is reported that Semaphorin 7A binds to β1 integrin to promote axon growth in the mouse embryo (Pasterkamp et al., 2003), and α4 integrin interacts with VCAM-1 to mediate the innervation of the heart by sympathetic axons in rats (Wingerd et al., 2002). Whether β1 integrin and α4/9 integrin are also required for the exit of retinal ganglion axons from the optic disk in mammals, however, remains unclear. Interestingly, it is reported that the ECM protein laminin-1 interacts with netrin-1 to mediate the exit of retinal ganglion axons from the optic disk in Xenopus (Höpker et al., 1999). Given that β1 integrins are well-known laminin-1 receptors (Aumailley, 2013; Belkin & Stepp, 2000), it is highly likely that like the action of Mys in the fly eye disc, mammalian β1 integrins may also play a role in the exit of retinal ganglion axons from the optic disk.

Our results show that integrins are necessary for the exit of R-cell axons from the eye disc, but not required for the extension of R-cell axons towards the posterior end of the eye disc. The mechanisms that direct the posterior migration of R-cell axons in the eye disc remain unknown. Since R-cell axons migrate on the apical surface of carpet glia in the eye disc (Silies et al., 2007; Tsao et al., 2020), it is possible that the interactions between R-cell axons and carpet glia may be mediated by different cell surface receptors, which direct the posterior extension of

R-cell axons independent of integrins. Alternatively or additionally, R-cell axons may be directed towards the posterior end of the eye disc by some unknown chemoattractants.

Our results suggest strongly that the extension of WG membrane from the eye disc through the optic stalk into the optic lobe is dependent on R-cell axons. In mutants defective in integrin signalling in R-cell axons, the extension of both R-cell axons and WG from the eye disc into the optic stalk was disrupted. We never observed an individual in which WG membrane still extended into the optic stalk when R-cell axons stalled in the eye disc. Cell-surface molecules on R-cell axons may serve as permissive substrates for the extension of WG membrane. In our previous studies (Cameron et al., 2016; Chen et al., 2017), we show that the recognition between R-cell axons and WG mediated by Tutl on R-cell axons and its binding partner Bdl on WG membrane, is required for the extension of WG membrane in the optic lobe. However, loss of *tutl* or *bdl* did not prevent the exit of WG membrane from the eye disc (Cameron et al., 2016; Chen et al., 2017), suggesting the involvement of other cell-surface players. Future studies are needed to identify additional key players that mediate the recognition between R-cell axons and WG for the extension of WG membrane from the eye disc into the optic stalk.

Figure 2. 1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la
os
BN
B''
ey
la
os
BN

Figure 2.1. Knocking down *mys* in both WG and eye-disc epithelium caused a failure of R-cell axons and WG membrane to exit the eye disc. 3rd-instar larval eye-brain complexes were double-stained with MAb24B10 (green) and anti-Bdl (magenta). MAb24B10 (green) labels all R-cells, and anti-Bdl (magenta) labels WG. **A-A''**, the pattern of R-cell axonal projection and WG membrane extension in wild type. **A**, MAb24B10 staining. R-cell axons migrate towards the posterior end of the eye disc (ey) where they converge and enter the optic stalk (os). After exiting the optic stalk, R1-R6 axons terminate at the intermediate target region in the lamina (la), and R7 and R8 axons pass through the lamina into the deeper medulla layer (me). **A'**, the same section stained with anti-Bdl to visualize WG. WG membrane extends from the eye disc through the optic stalk into the distal region of the lamina (arrowheads). MAb24B10 and anti-Bdl staining also visualized Bolwig's Nerve (BN in A and A') that projects from Bolwig's organ in the larval anterior region into the optic lobe long before the birth of R cells and WG. **A"**, the section visualized with both MAb24B10 and anti-Bdl staining. Note that WG membrane associates with R-cell axons in the lamina (arrowheads). **B-B''**, a third-instar eye-brain complex in which *UAS-mys-RNAi-JF02819* was simultaneously expressed in both eye-disc epithelium and WG under control of the eye-specific $ey^{3.5}$-GAL4 and WG-specific *Mz97*-GAL4 drivers. *mys* knockdown caused the stalling of R-cell axons (**B** and **B"**) and WG membrane (**B'** and **B"**) in the eye disc. Note *mys* knockdown did not affect the projection pattern of pre-existing Bolwig's Nerve (BN). Scale bar: 20 μm.

Table 2. 1

Summary of R-cell axon and WG membrane stalling phenotypes observed in mutants defective in the integrin pathway.

Genotype	Penetrance of the stalling phenotype	Number of individuals in which most R-cell axons and WG stalled in the eye disc	Number of individuals in which many R-cell axons and WG stalled in the optic stalk
ey$^{3.5}$ + *Mz*97> *mys-RNAi-JF02819*	9/15	8	1
ey$^{3.5}$ > *mys-RNAi-HMS00043*	10/13	9	1
C155> mys-RNAi-HMS00043	9/16	3	6
ey$^{3.5}$ + *Mz*97> *scb-RNAi-JF02696*	6/10	5	1
ey$^{3.5}$ > *scb-RNAi-*	12/14	9	3

JF02696			
ey$^{3.5}$ > *rhea-RNAi-HMS00799*	11/12	9	2
ey$^{3.5}$ > *rhea-RNAi-HMS00856*	10/12	9	1
rhea^{1} eye-specific mosaic	15/25	15	0

Figure 2. 2

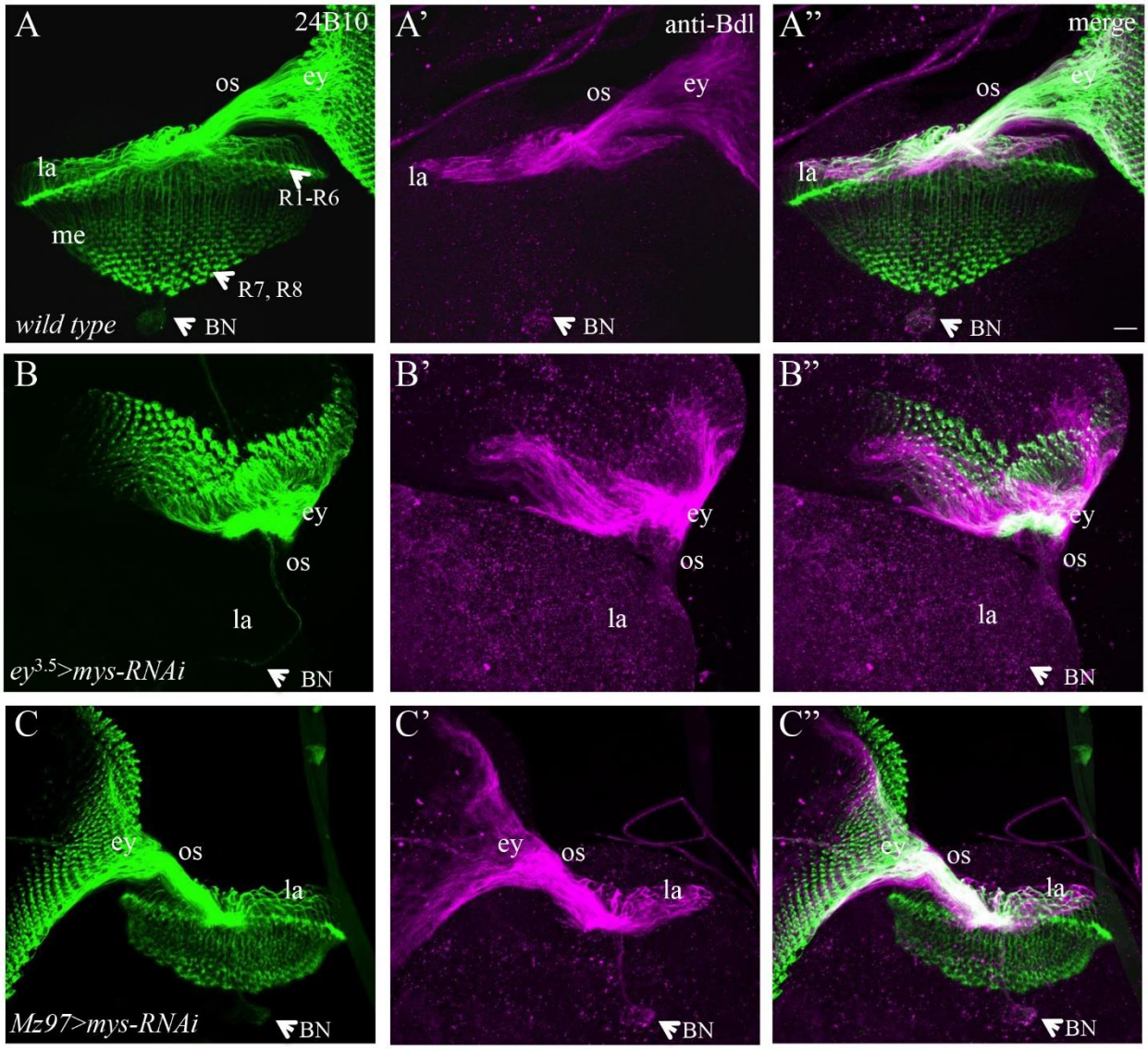

A
24B10
os
ey
la
R1-R6
me
R7, R8
wild type
BN
A'
anti-Bdl
os
ey
la
BN
A''
merge
os
ey
la
BN
B
ey
os
la
ey3.5>mys-RNAi
BN
B'
ey
os
la
B''
ey
os
la
BN
C
ey
os
la
Mz97>mys-RNAi
BN
C'
ey
os
la
BN
C''
ey
os
la
BN

Figure 2.2. Knockdown of *mys* in eye-disc epithelium but not WG prevented the exit of R-cell axons and WG membrane. 3rd-instar larval eye-brain complexes were double-stained with MAb24B10 (green) and anti-Bdl (magenta). **A-A''**, wild type. **B-B''**, knocking down *mys* in eye-disc epithelium only by expressing *UAS-mys-RNAi-HMS00043* under control of $ey^{3.5}$-GAL4 (i.e. $ey^{3.5}$>*mys-RNAi*) caused a stalling phenotype identical to that in animals with *mys* knockdown in both eye-disc epithelium and WG (Fig. 1**B**-**B**"). **C-C''**, the pattern of R-cell axonal projection and WG membrane extension appeared normal in animals in which *mys* was only knocked down in WG by expressing *UAS-mys-RNAi-HMS00043* under control of *Mz97*-GAL4 (i.e. Mz97>*mys-RNAi*) (n=16). Scale bar: 20 μm.

Figure 2. 3

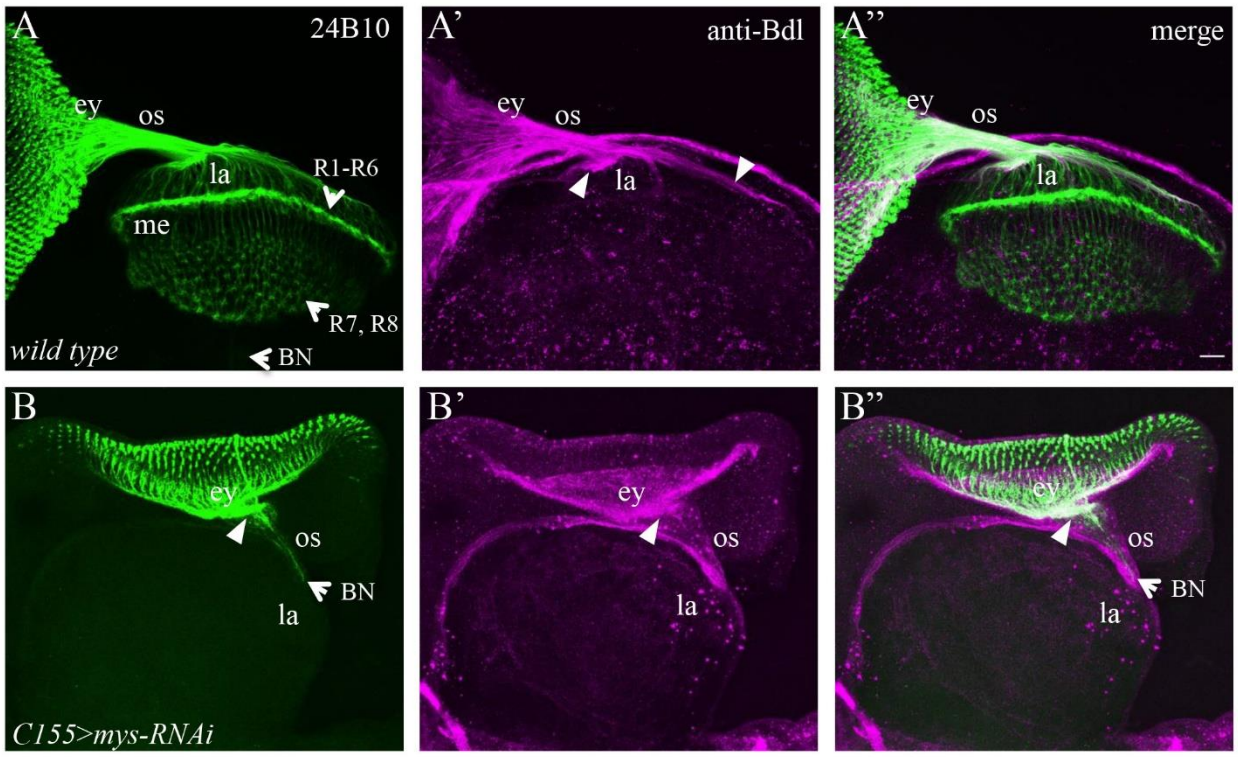


Figure 2.3. Neuronal-specific knockdown of *mys* caused a similar stalling phenotype. 3rd-instar larval eye-brain complexes were double-stained with MAb24B10 (green) and anti-Bdl (magenta). **A-A''**, wild type. Note that only Bdl-positive processes (arrowheads) that were associated with R-cell axons in the lamina were WG membrane. **B-B''**, *mys* was specifically knocked down in all differentiating R cells (the only neuronal cell types in the eye disc) by expressing *UAS-mys-RNAi-HMS00043* under control of the neuronal-specific *C155*-GAL4 (i.e. *C155>mys-RNAi*). Most R-cell axons and WG membrane stalled at the posterior end of the eye disc (arrowheads). Scale bar: 20 μm.

Figure 2. 4

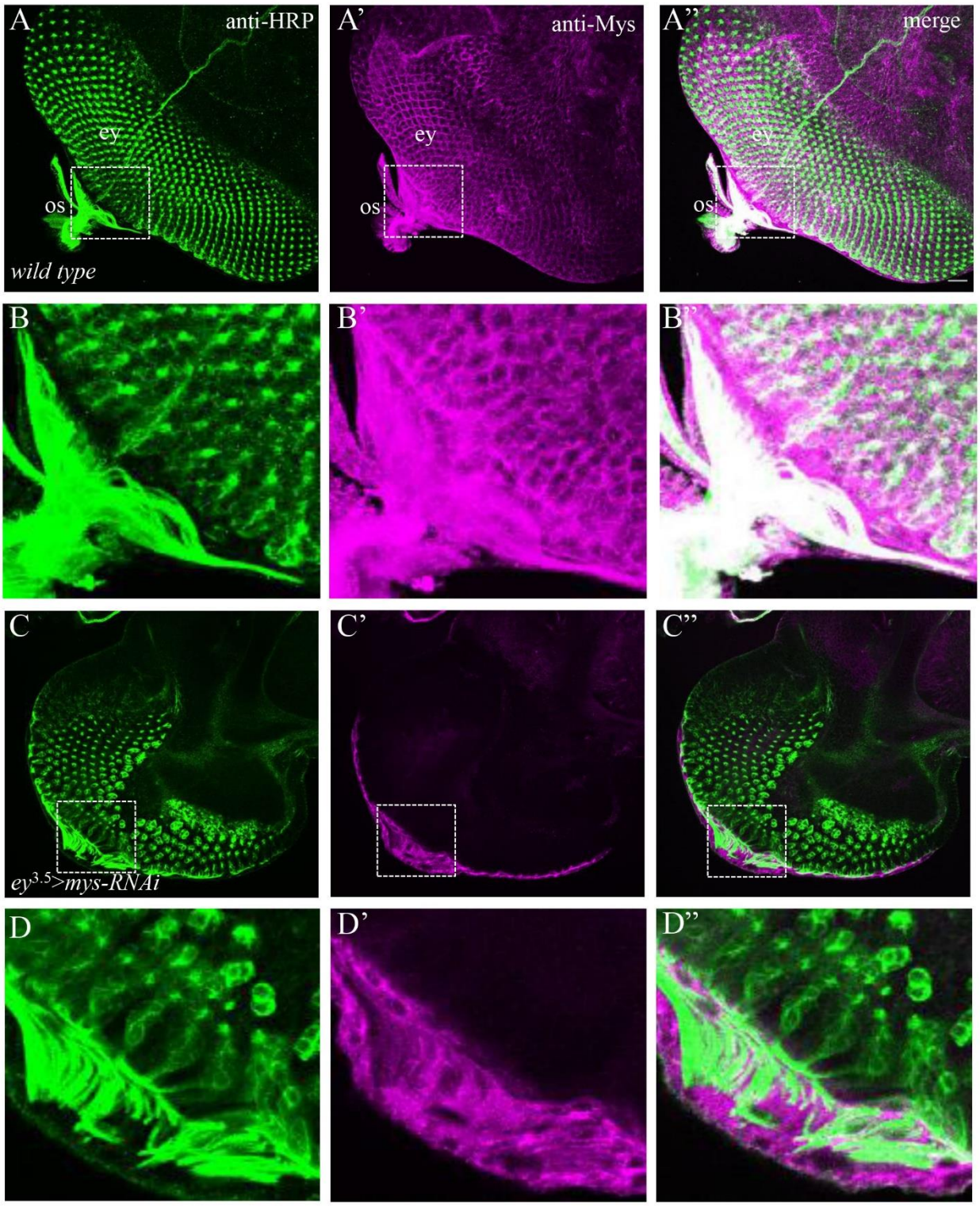
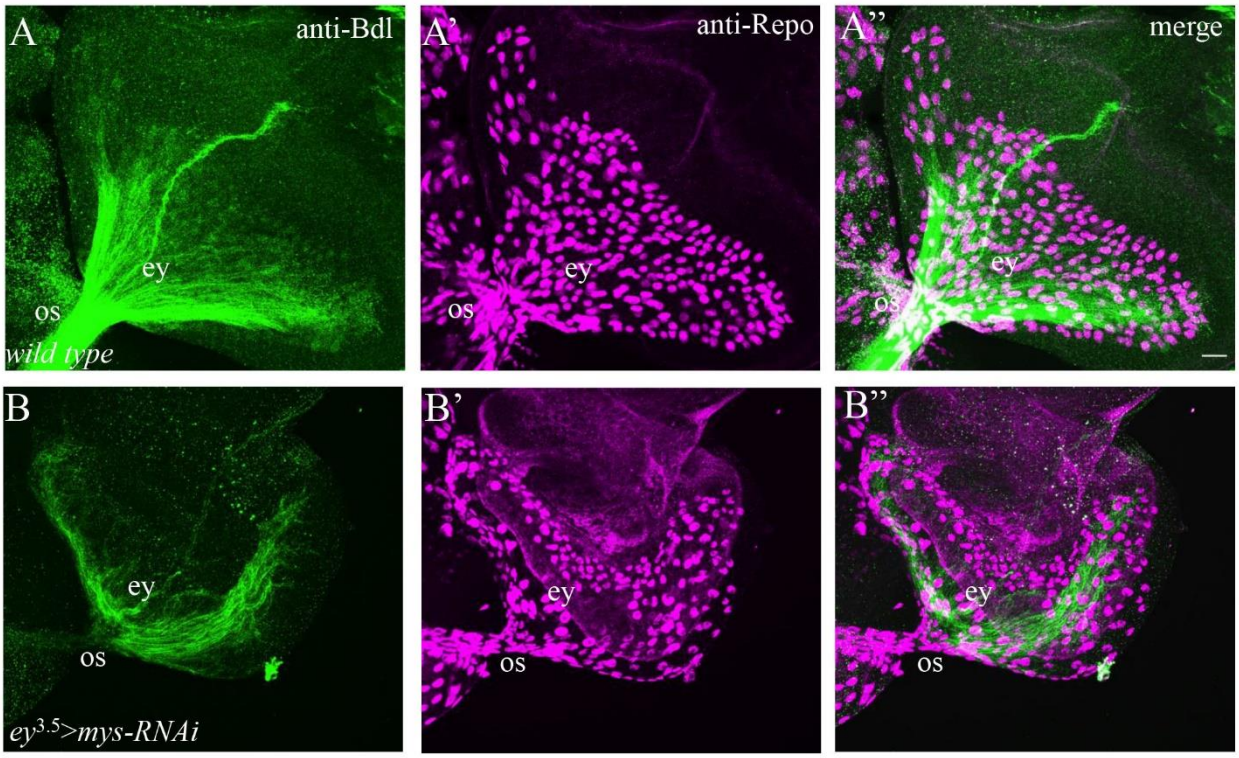

A
anti-HRP
ey
os
wild type
A'
anti-Mys
ey
os
A''
merge
ey
os
B
B'
B''
C
ey3.5>mys-RNAi
C'
C''
D
D'
D''

Figure 2.4. Mys is expressed in R-cell axons. 3rd-instar larval eye discs were double-stained with anti-HRP (green) and anti-Mys antibodies (magenta). **A**, wild type. anti-HRP staining visualized R-cell soma and axons. **A'**, the same section stained with anti-Mys antibody. **A"**, the section visualized with both anti-HRP and anti-Mys staining. Mys appeared to be expressed broadly in the eye disc. Strong Mys expression was detected in R-cell axons that migrate from the most posterior end of the eye disc into the optic stalk. **B-B''**, enlarged view of the boxed area in **A-A**". Note the strong Mys staining in R-cell axons. **C-C''**, when *mys* was knocked down in eye-disc epithelium but not WG by expressing *UAS-mys-RNAi-HMS00043* under control of $ey^{3.5}$-GAL4, the intensity of Mys staining was greatly reduced in both R-cell soma and accessory cells in the eye disc, confirming that Mys is expressed in both R cells and accessory cells. The staining in R-cell axons was also significantly reduced. **D-D''**, enlarged view of the boxed area in **C-C**". The intensity of Mys staining in R-cell axons was greatly reduced. Note anti-Mys staining at the most posterior region of the eye disc in knockdown animals were mostly associated with sub-retinal glia but not R-cell axons, which is consistent with that expression of *UAS-mys-RNAi-HMS00043* under control of $ey^{3.5}$-GAL4 knocked down *mys* in eye-disc epithelium but not sub-retinal glia. Scale bar: 20 μm.

Figure 2. 5


A
anti-Bdl
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anti-Repo
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ey3.5>mys-RNAi
B'
ey
os
B''
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C

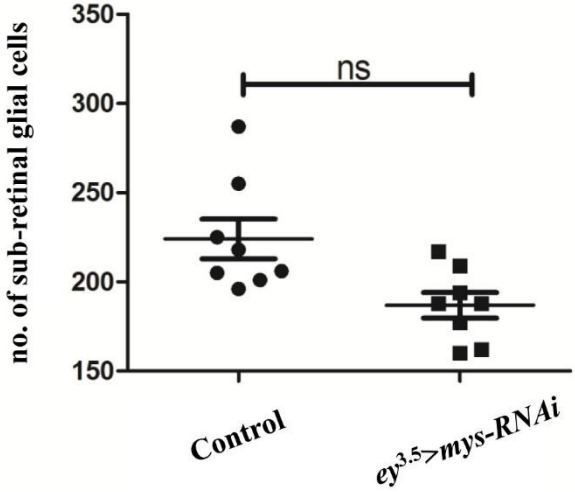
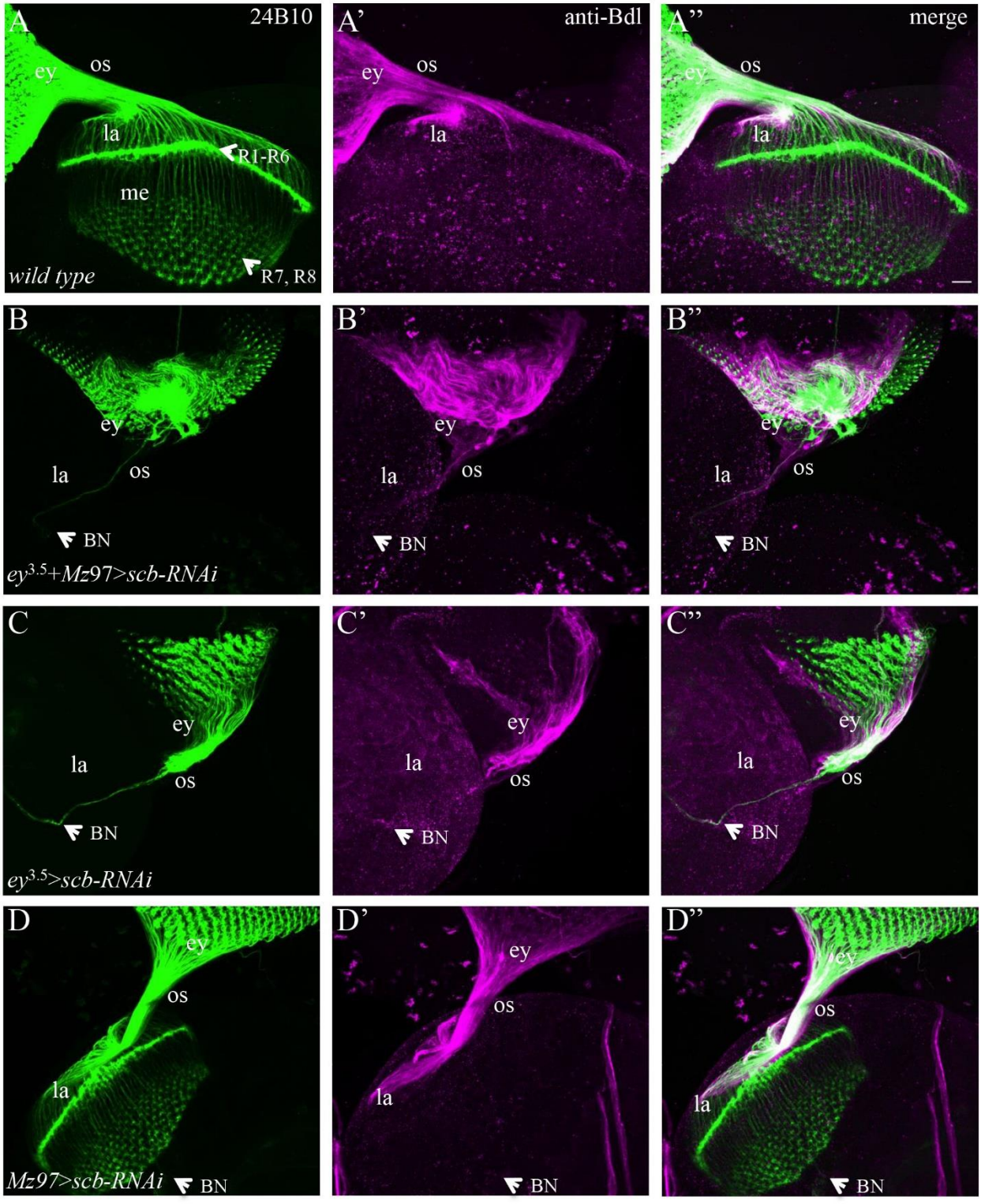

no. of sub-retinal glial cells
350
300
250
200
150
ns
Control
ey3.5>mys-RNAi

Figure 2.5. Knocking down *mys* in eye-disc epithelium did not affect the migration of glia from the optic stalk into the eye disc. 3rd-instar larval eye-brain complexes were double-stained with anti-Bdl (green) and anti-Repo (magenta). Anti-Bdl and anti-Repo label WG membrane and all glial nuclei, respectively. **A-A''**, in wild-type, glial cells in the optic stalk migrate into the sub-retinal region of the eye disc. **B-B''**, when *mys* was knocked down in eye-disc epithelium by expressing *UAS-mys-RNAi-HMS00043* under control of *ey*$^{3.5}$-GAL4, glial cells still migrated into the sub-retinal region of the eye disc. **C**, the number of glial cells in the eye disc was counted. The number of sub-retinal glial cells in *mys* knockdown eye disc is not significantly (ns, $p>0.05$) different from that in wild type. Scale bar: 20 μm.

Figure 2. 6


A
24B10
ey
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la
R1-R6
me
wild type
R7, R8
A'
anti-Bdl
ey
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A''
merge
ey
os
la
B
ey
la
os
BN
ey3.5+Mz97>scb-RNAi
B'
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ey3.5>scb-RNAi
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ey
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ey
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la
Mz97>scb-RNAi
BN
D'
ey
os
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BN
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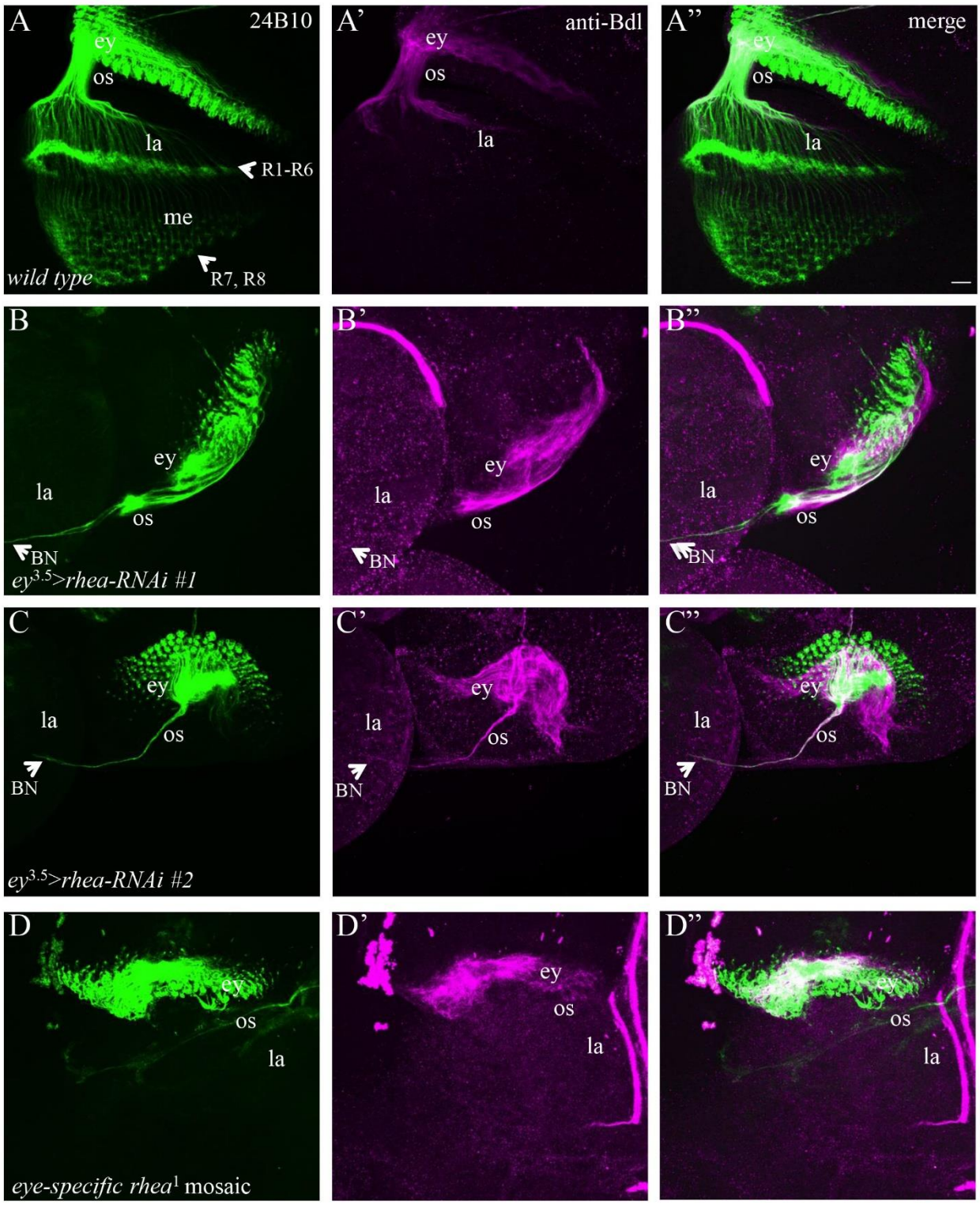
Figure 2.6. Knockdown of *scb* in eye-disc epithelium but not WG prevented the exit of R-cell axons and WG membrane. 3rd-instar larval eye-brain complexes were double-stained with MAb24B10 (green) and anti-Bdl (magenta). **A-A''**, wild type. **B-B''**, when *scb* was knocked down in both eye-disc epithelium and WG by expressing *UAS-scb-RNAi-JF02696* under control of *ey*$^{3.5}$-GAL4 and *Mz97*-GAL4, most R-cell axons and WG membrane stalled in the eye disc. **C-C''**, in an individual in which *scb* was knocked down only in eye-disc epithelium by expressing *UAS-scb-RNAi-JF02696* under control of *ey*$^{3.5}$-GAL4, some R-cell axons and WG membrane exited the eye disc and stalled in the optic stalk. **D-D''**, WG-specific knockdown of *scb* by expressing *UAS-scb-RNAi-JF02696* under control of *Mz97*-GAL4 did not affect R-cell axonal projection or WG membrane extension (n=13). Scale bar: 20 μm.

Figure 2. 7


A
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R1-R6
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wild type
R7, R8
A'
anti-Bdl
ey
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la
A''
merge
ey
os
la
B
ey
la
os
BN
ey3.5>rhea-RNAi #1
B'
ey
la
os
BN
B''
ey
la
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BN
C
ey
la
os
BN
ey3.5>rhea-RNAi #2
C'
ey
la
os
BN
C''
ey
la
os
BN
D
ey
os
la
eye-specific rhea1 mosaic
D'
ey
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la
D''
ey
os
la

Figure 2.7. Interfering with the function of Rhea in eye-disc epithelium but not WG also caused the stalling of R-cell axons and WG membrane in the eye disc. 3rd-instar larval eye-brain complexes were double-stained with MAb24B10 (green) and anti-Bdl (magenta). **A-A''**, wild type. **B-B''**, in an individual in which *rhea* was knocked down in the eye disc by expressing *UAS-rhea-RNAi-HMS00799* (i.e. *UAS-rhea-RNAi #1*) under control of *ey*$^{3.5}$-GAL4, the stalling of R-cell axons and WG membrane in the eye disc and optic stalk was observed. **C-C''**, in an individual in which *rhea* was knocked down only in eye-disc epithelium by expressing another independent *UAS-rhea-RNAi* line (i.e. *UAS-rhea-RNAi-HMS00856* or *UAS-rhea-RNAi #2*) under control of *ey*$^{3.5}$-GAL4, most R-cell axons and WG membrane stalled in the eye disc. **D-D''**, in many eye-specific mosaic animals in which large clones of homozygous *rhea*^{1} mutant eye tissues were generated, R-cell axons and WG membrane failed to exit the eye disc. Scale bar: 20 μm.

Chapter 3 : Glia-derived Insulin-like Peptides Regulate the Timing of Photoreceptor Differentiation in the *Drosophila* Visual System[2]

[2] This chapter has been submitted to *Journal of Neuroscience* for publication.
Qian Ren, Wen-Tzu Chang, and Yong Rao

3.1 Abstract

Recent studies suggest the involvement of insulin signaling in the timing of photoreceptor differentiation in *Drosophila* and mammals. The molecular and cellular mechanisms underlying temporal control of photoreceptor differentiation by insulin signaling, however, remain largely undefined. In this study, we reveal a novel role for sub-retinal glia in timing the differentiation of photoreceptor neurons (R cells) in the developing *Drosophila* eye imaginal disc. Decreasing the signaling of epidermal growth factor receptor (EGFR) in sub-retinal glia delayed R-cell differentiation. In contrast, hyperactivating the EGFR pathway in sub-retinal glia caused the precocious R-cell differentiation. Cell-type-specific knockdown, epistasis analysis and transgene rescue indicate that insulin-like peptides ILP3 and ILP6 are key downstream targets of the EGFR pathway in sub-retinal glia. We propose that the activation of the EGFR pathway in sub-retinal glia stimulates the release of ILP3 and ILP6, which in turn activate the insulin receptor (InR) in eye precursor cells to positively regulate the timing of photoreceptor differentiation.

3.2 Introduction

Proper timing of neurogenesis is essential for the establishment of complex neuronal circuits in the nervous system (Holguera & Desplan, 2018; Oberst et al., 2019). Both intrinsic and extrinsic factors are involved in temporal control of neurogenesis (Deska-Gauthier & Zhang, 2021; Holguera & Desplan, 2018; Oberst et al., 2019). For instance, downregulating the activity of the transcription factor STAT3 is required for initiating rod photoreceptor differentiation in mammals (Rhee et al., 2004; Zhang et al., 2004). And *in vitro* studies identify insulin-like growth factor 1 (IGF1) as an extrinsic factor that activates intracellular signaling events to inhibit STAT3 for initiating rod differentiation (Pinzon-Guzman et al., 2015; Pinzon-Guzman et al., 2011; Xing et al., 2018).

In *Drosophila*, insulin signaling has also been shown to be required for temporal control of photoreceptor (R cell) differentiation (Bateman & McNeill, 2004). The *Drosophila* compound eye is comprised of ~800 ommatidia. Each ommatidium contains eight R cells (Land & Fernald, 1992). The formation of ommatidia begins at the third-instar larval stage, which occurs progressively from posterior to anterior across the eye disc in a wave-like manner (Fig. 3.1A). The progression of ommatidial formation is marked by posterior-to-anterior movement of the morphogenetic furrow (MF), an indentation located in between anterior undifferentiated epithelium and posterior differentiating ommatidia (Ready et al., 1976). In the newly formed ommatidial row immediately posterior to the furrow, each ommatidium contains only R8 photoreceptor (Treisman, 2013). R8 secretes two antagonizing EGF-like molecules Spitz (Spi) and Argos (Aos), which iteratively activate EGF receptor (EGFR) to control the addition of other R-cell subtypes (Freeman, 1996; Freeman et al., 1992; Tio & Moses, 1997). As the furrow progresses more anteriorly, new rows of ommatidia are formed regularly at ~1.5-2 hour interval

(Adler, 2002; Choi & Benzer, 1994; Strutt & Mlodzik, 1995), and the development of each ommatidium in early-formed rows involves the sequential recruitment of R2/R5, R3/R4, R1/R6 and R7 photoreceptors into the cluster (Fig. 3.1A).

Previous studies show that activation of InR in eye precursor cells is required for timing R-cell differentiation (Bateman & McNeill, 2004). In *Drosophila*, there are eight insulin-like peptides (ILPs) (Brogiolo et al., 2001; Nässel et al., 2015). Among them, ILP8 is the ligand for a relaxin receptor homolog Lgr3 (Garelli et al., 2015; Liao & Nassel, 2020), while ILP1-7 are thought to act through the sole InR (Brogiolo et al., 2001; Nässel et al., 2015). The cellular source and the identity of ILPs that activate InR for temporal control of R-cell differentiation, however, remain unknown.

Recent studies reveal complex interactions between R cells and sub-retinal glia (Silies et al., 2010; Tsao et al., 2020). Sub-retinal glia include perineurial glia (PG), subperineurial glia (SPG) and wrapping glia (WG) (Fig. 3.1B). At third-instar larval stage, sub-retinal glia migrate from the optic stalk into the eye disc. In each eye disc, there are two SPGs whose membrane extends below the differentiating region of eye epithelium. The major subtype of sub-retinal glia is PG. After the activation of the receptor Heartless (Htl) by R-cell-derived FGF8-like ligand Thisbe, apical PG located above the membrane of SPGs differentiate into WG (Fig. 3.1B)(Franzdottir et al., 2009; Tsao et al., 2020). Our recent studies show that the recognition between R-cell axons and WG mediated by two Ig-like transmembrane proteins Turtle (Tutl) on R-cell axons and Borderless (Bdl) on WG, is required for WG extension and axonal ensheathment (Cameron et al., 2016; Chen et al., 2017).

In the course of analyzing the interactions between R cells and sub-retinal glia, we found that manipulating EGFR signaling in sub-retinal glia affected the timing of R-cell differentiation.

To identify EGFR-dependent signals from sub-retinal glia for temporal control of R-cell differentiation, we performed cell-type-specific knockdown, epistasis analysis and transgene rescue. Our results support that the activation of EGFR signaling in sub-retinal glia leads to the release ILP3 and ILP6, which activate InR in eye precursor cells for the timing of R-cell differentiation.

3.3 Materials and Methods

3.3.1 Genetics

Fly stocks are reared at 12/12h light-dark cycle and 25°C with 50% humidity. Following stocks were obtained from Bloomington *Drosophila* Stock Center (BDSC): *UAS-EGFR-RNAi-JF01368* (BDSC #25781), *moody*-GAL4 (BDSC#90883), *C527*-GAL4 (BDSC#90391*), UAS-ilp1-RNAi-JF01344* (BDSC#31491),*UAS-ilp2-RNAi-JF01518* (BDSC#31068), *UAS-ilp3-RNAi-JF01345* (BDSC#31492), *UAS-ilp4-RNAi-JF01346*(BDSC#31377), *UAS-ilp5-RNAi-JF01347* (BDSC#31378), *UAS-ilp6-RNAi-JF01348* (BDSC#31379), *UAS-ilp7-RNAi-JF01519* (BDSC#31069), *UAS-CD4-tdGFP (vk00033)* (BDSC#35836) and *UAS-Akt-RNAi-HMS00007* (BDSC#33615). *UAS-EGFR*^{DN} *and UAS-EGFR*^{Act} *(*i.e. *EGFR*$^{2.A887T}$) flies were provided by Dr. Claude Desplan.

To knock down *EGFR* in all sub-retinal glia, genetic crosses were performed to generate flies with the genotype *Repo*-GAL4*/UAS-EGFR-RNAi-JF01368.* To inactivate *EGFR* in all sub-retinal glia, WG, PG or SPG, genetic crosses were performed to generate flies with following genotypes: *UAS-EGFR*^{DN}*/+; Repo*-GAL4*/+*, *UAS- EGFR*^{DN}*/Mz97*-GAL4, *UAS- EGFR*^{DN}*/+; C527*-GAL4*/+*, and *moody*-GAL4*/UAS- EGFR*^{DN}. To knock down *Akt* in R cells, genetic crosses were performed to generate flies with the genotype *C155*-GAL4*; UAS-Akt-RNAi-HMS00007/+*. To hyperactivate the *EGFR* signaling in WG, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4*/+; UAS-EGFR*^{Act}*/+*. To knock down *ilp*1, 2, 3, 4, 5, 6 or 7 in sub-retinal glia, genetic crosses were performed to generate flies with the genotypes *UAS-ilp1-RNAi-JF01344/Repo*-GAL4, *UAS-ilp2-RNAi-JF01518/Repo*-GAL4, *UAS-ilp3-RNAi-JF01345/Repo*-GAL4, *UAS-ilp4-RNAi-JF01346/Repo*-GAL4, *UAS-ilp5-RNAi-JF01347/Repo*-GAL4, *UAS-ilp6-RNAi-JF01348/Repo-GAL4,* and *UAS-ilp7-RNAi-JF01519/Repo*-GAL4. To

hyperactivate EGFR signaling in WG, genetic crosses were performed to generate flies with the genotype *Mz97*-GAL4*/+; UAS-EGFR*^{Act}/+. To knock down *ilp3* or *ilp6* in flies that express the constitutively active *UAS-EGFR*^{Act}, genetic crosses were performed to generate flies with the genotypes *Mz97*-GAL4*/+; UAS-EGFR*^{Act}*/UAS-CD4-tdGFP, Mz97*-GAL4*/+; UAS-EGFR*^{Act}*/UAS-ilp3-RNAi-JF01345,* and *Mz97*-GAL4*/+; UAS-EGFR*^{Act}*/UAS-ilp6-RNAi-JF01348.* To overexpress *ilp3* or *ilp6* in flies that express *UAS-EGFR*^{DN} in sub-retinal glia, genetic crosses were performed to generate flies with the genotypes *+/UAS-EGFR*^{DN}*; Repo*-GAL4*/UAS-CD4-tdGFP*, *+/UAS-EGFR*^{DN}*; Repo*-GAL4*/UAS-ilp3*, and *+/ UAS-EGFR*^{DN}*; Repo*-GAL4*/UAS-ilp6.*

3.3.2 Molecular Biology

For generating the *ilp3* expression construct, the genomic fragment containing the entire *ilp3* coding sequence was amplified by PCR with the forward primer 5' ACTCTGAATAGATCTACTTGGCAGACACATACT 3' and the backward primer 5' CAAAGATCCTCTAGAGCAGTTTGAATACATAATTTATTTA 3'. For generating the *ilp6* expression construct, the genomic fragment containing the entire *ilp6* coding sequence was amplified by PCR with the forward primer 5' ACTCTGAATAGATCTAAACAACGAAGAACGTTCT 3' and the backward primer 5' CAAAGATCCTCTAGACTCACTTCAAATTTCTTTTATTGT 3'. The resulting PCR products were subcloned into EcoRI and BglII sites of pCaspeR-hs vector. The constructs were verified by DNA sequencing and sent to BestGene for generating transgenic lines.

3.3.3 Histology

Third-instar larval eye discs were dissected and fixed for immunostaining similarly as described previously (Cameron et al., 2016; Chen et al., 2017).

3.3.4 Immunostaining

Primary and secondary antibodies were used at following dilutions: rabbit polyclonal anti-HRP (1:500; Jackson ImmunoResearch Cat#323-005-021), mouse monoclonal anti-Prospero (1:300; Developmental Studies Hybridoma Bank or DSHB Cat#Prospero (MR1A)), mouse monoclonal anti-Seven-up (1:100;DSHB Cat#Seven-up 2D3), rabbit monoclonal anti-Phospho-Akt (Ser473) (1:100; Cell Signaling Technology Cat#4060S), mouse MAb24B10 (1:100; DSHB Cat#24B10), mouse monoclonal anti-Repo (1:100; DSHB Cat#8D12). Secondary antibodies including Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Cat# A11001), Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Cat#A32731), Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen, Cat# A20991) and Alexa Fluor 647 goat anti-mouse IgG (Invitrogen, Cat#A32728), were used at 1:500 dilution.

3.3.5 Confocal microscopy

Epifluorescent images were captured and analyzed by confocal microscopy (Olympus FV1000). Photo stacks were performed using Z-stack projection by FluoView software.

3.3.6 Statistical Analysis

The relative delay in R-cell differentiation was quantified by measuring the interval of rows between the 1st row of ommatidia (R8 immediately posterior to the morphogenetic furrow) and the row in which certain R-cell subtypes (R3/R4 or R7) initially appear. To quantify the relative intensity of phosphorylated Akt staining, the integrated density/area was measured. The results were analyzed by two tailed unpaired student's t-test or one-way ANOVA followed by *post hoc* Tukey's multiple comparisons test. The difference is considered as significant when a p value is <0.05.

3.4 Results

3.4.1 Downregulation of EGFR signaling in sub-retinal glia delayed R-cell differentiation

Previous studies show that R cells produce the EGF-like ligand Spi, which activates EGFR in sub-retinal glia (Fernandes et al., 2017; Firth & Baker, 2007). While it is reported that activation of EGFR in WG is required for the differentiation of lamina neurons (Fernandes et al., 2017), it remains unknown if activation of EGFR in WG and/or other sub-retinal glia by R-cell-derived Spi would modulate the progression of R-cell development in the developing eye disc.

To test this, we examined if interfering with the function of EGFR in sub-retinal glia affects R-cell differentiation. *UAS-EGFR*^{DN} encoding for a dominant-negative form of EGFR was expressed in all sub-retinal glia under control of the glial-specific *Repo*-GAL4 driver. In wild type, the initiation of R-cell differentiation occurs at the third-instar larval stage. The progression of ommatidial formation follows the movement of the MF that sweeps across the eye disc in a posterior-to-anterior direction (Fig. 3.1A). Precursor cells posterior to the MF differentiate into R cells, which are spaced evenly in a row in parallel to the MF (Fig. 3.1A and 3.1C). Following the movement of the MF, new rows of ommatidia are formed every 1.5-2 hours. Each ommatidium in the newly formed ommatidial row immediately posterior to the MF, contains only R8 photoreceptor that is the founding photoreceptor of each ommatidium (Fig. 3.1A and 3.1C). Ommatidia in rows that are formed earlier and thus located further away from the MF, recruit additional R-cell subtypes that differentiate in a precise temporal order with the R2/R5 pairs first, followed by R3/R4, R1/R6 and R7 (Fig. 3.1A and 3.1C).

Compared to that in wild type (Fig. 3.1C-C”), the progression of ommatidia differentiation in the eye disc appeared normal in individuals that expressed *EGFR*^{DN} in sub-

retinal glia (Fig. 3.1D-D"). Expression of *EGFR*^{DN} in sub-retinal glia also did not affect the overall organization of differentiating R-cell clusters posterior to the MF (Fig. 3.1D-D").

We then examined if interfering with EGFR signalling in sub-retinal glia affects the temporal order of R-cell subtype differentiation. Interestingly, we found that the differentiation of R7 photoreceptors was delayed for ~1-2 rows in individuals expressing *EGFR*^{DN} in sub-retinal glia (Fig. 3.1D-D", 3.1E). Similar results were obtained when *EGFR* was knocked down in sub-retinal glia (Fig. 3.1E). These results suggest a role for EGFR signaling in sub-retinal glia to positively regulate the timing of R7 differentiation.

To test if EGFR signalling in sub-retinal glia is also required for timing the differentiation of other R-cell subtypes, we examined the differentiation of R3/R4 pairs using a monoclonal antibody recognizing the nuclear protein Seven-up (Svp). In the third-instar eye disc, Svp is firstly expressed in R3/R4 pairs and later also expressed in R1/R6 pairs (Mlodzik et al., 1990). In wild type, the R3/R4 pair begin to appear ~2 rows behind the 1st row of R8-only ommatidia (Fig. 3.2A-A" and 3.2C). In flies in which *EGFR* was knocked down in sub-retinal glia, however, the differentiation of R3/R4 was delayed for ~1-2 rows (Fig. 3.2B-B" and 3.2C).

Together, these results suggest a novel role for EGFR signalling in sub-retinal glia for temporal control of R-cell differentiation.

3.4.2 Blockade of EGFR signaling in WG delayed R-cell differentiation

The above results suggest that the activation of EGFR signaling in sub-retinal glia is necessary for the timing of R-cell differentiation. Sub-retinal glia include SPG, PG and WG (Fig. 3.1B). To determine specific requirements of sub-retinal glial subtypes, we firstly examined if downregulation of EGFR signaling in WG affects the timing of R3/R4 and R7 differentiation.

To inhibit EGFR signaling specifically in WG, the *UAS-EGFR*^{DN} transgene was expressed in WG under control of *Mz97*-GAL4. *Mz97*-GAL4 is specifically expressed in WG that no longer undergo cell division (Tsao et al., 2020). Like that observed in flies in which EGFR signaling was inhibited in all sub-retinal glia (Fig. 3.1 and 3.2), the differentiation of R3/R4 and R7 was delayed when EGFR signaling was inhibited specifically in WG (Fig. 3.3B-B", 3.3D and 3.3E).

3.4.3 Hyperactivation of EGFR in WG caused precocious R-cell differentiation

To determine if WG play an active role in timing R-cell differentiation, we examined the effects of hyperactivation of EGFR in WG on R-cell differentiation.

To hyperactivate the EGFR signaling pathway in WG, a *UAS-EGFR*^{Act} transgene encoding for a constitutively active form of EGFR was expressed in WG under control of *Mz97*-GAL4. Interestingly, we found that expression of EGFR^{Act} in WG caused the precocious appearance of R3/R4 and R7 in rows of less mature ommatidia (Fig. 3.3C-C", 3.3D and 3.3E).

This result, together with that blockade of EGFR signaling delayed R-cell differentiation, suggest strongly that WG play an instructive role in positively regulating the timing of R-cell differentiation.

3.4.4 Inhibition of EGFR signaling in PG but not SPG also delayed R-cell differentiation

We then examined if EGFR signaling in PG and SPG is also required for temporal control of R-cell differentiation. EGFR signaling was inhibited in PG and SPG by expressing *UAS-EGFR*^{DN} under control of the PG-specific *C527*-GAL4 and SPG-specific *moody*-GAL4, respectively. We found that downregulation of EGFR signaling in PG also delayed the

differentiation of R7 (Fig. 3.4). In contrast, no effect was observed when EGFR signaling was inhibited in SPG (Fig. 3.4).

3.4.5 Knockdown of *EGFR* in sub-retinal glia prevented the activation of Akt, a key downstream target of InR signaling in the eye disc

Previous studies show that the activation of InR in precursor cells positively regulates the timing of R-cell differentiation (Bateman & McNeill, 2004). Loss of InR in precursor cells in the eye-disc epithelium delayed R-cell differentiation, while hyperactivation of InR signaling caused precocious R-cell differentiation (Bateman & McNeill, 2004). That manipulating EGFR signaling in sub-retinal glia caused phenotypes (Fig. 3.1-3.4) similar to that in flies with altered InR activity in the eye-disc epithelium (Bateman & McNeill, 2004), raises the interesting possibility that EGFR-activated sub-retinal glia upregulate the activity of InR in eye precursor cells for temporal control of R-cell differentiation. To test this, we examined if knockdown of *EGFR* in sub-retinal glia affects InR signaling in the eye disc.

The ser/thr kinase Akt is a major downstream target of InR signaling in both vertebrates and invertebrates (Nässel et al., 2015; Saltiel, 2021). Activation of InR triggers a cascade of signaling events leading to the activation of Akt by ser/thr phosphorylation (Alessi et al., 1996). If EGFR-activated sub-retinal glia positively regulate the timing of R-cell differentiation by upregulating the InR signaling pathway, the prediction is that downregulation of EGFR signaling in sub-retinal glia would decrease InR signaling in the eye disc and thus prevent the activation of Akt. To test this, we examined the effects of knocking down *EGFR* in sub-retinal glia on the levels of activated Akt in the eye-disc epithelium. Indeed, we found that inhibition of EGFR signaling in sub-retinal glia greatly decreased the levels of phosphorylated Akt in the R-cell-containing posterior region of the eye disc (Fig. 3.5).

This result suggests that sub-retinal glia control the timing of R-cell differentiation by positively regulating InR signaling in eye precursor cells.

3.4.6 Glial-specific knockdown of *ilp3* or *ilp6* delayed R-cell differentiation

That inhibition of EGFR signaling in sub-retinal glia delayed R-cell differentiation (Fig. 3.1-3.4) similarly as removal of *InR* in eye precursor cells (Bateman & McNeill, 2004), together with that knockdown of *EGFR* in sub-retinal glia caused a dramatic decrease in the levels of Akt phosphorylation in the eye disc (Fig. 3.5), suggest strongly that EGFR-activated sub-retinal glia produce certain ILPs that activate InR in the eye disc. Among eight ILPs in *Drosophila*, ILP1-7 are reported to act through the sole *Drosophila* InR (Brogiolo et al., 2001). Interestingly, a recent study shows that EGFR signaling is required for the release of ILP6 from WG to induce the differentiation of lamina neurons (Fernandes et al., 2017). Thus, ILP6 and/or other ILPs may be released by EGFR-activated sub-retinal glia to activate InR in eye precursor cells for temporal control of R-cell differentiation.

To test this, we performed knockdown experiments to examine if reducing the levels of ILP1-7 affects the timing of R-cell differentiation. *UAS-ilp-RNAi* transgenes were expressed in sub-retinal glia under control of *Repo*-GAL4. We found that like interfering with EGFR signalling in sub-retinal glia (Fig. 3.1-3.4), knocking down *ilp3* or *ilp6* in sub-retinal glia also significantly delayed R-cell differentiation (Fig. 3.6). This result suggests that ILP3 and ILP6 are the signals released by EGFR-activated sub-retinal glia to regulate the timing of R-cell differentiation.

3.4.7 Knockdown of *ilp3* or *ilp6* suppressed the EGFR^{Act}-induced precocious R-cell differentiation phenotype

One possible explanation for the *EGFR*^{Act}-induced precocious R-cell differentiation phenotype (Fig. 3.3), is that elevated EGFR signaling in sub-retinal glia increased the release of ILP3 and/or ILP6 that led to the hyperactivation of InR signaling in eye-disc precursor cells. To test this, we examined if reducing the levels of ILP3 and ILP6 in WG modifies the *EGFR*^{Act}-induced phenotype. Indeed, we found that knocking down *ilp3* or *ilp6* in WG suppressed the *EGFR*^{Act}-induced precocious R-cell differentiation phenotype (Fig. 3.7).

3.4.8 Expression of *ilp3* or *ilp6* transgene rescued R-cell differentiation phenotypes caused by inhibition of EGFR signaling in sub-retinal glia

To further test the regulation of ILPs by EGFR signalling, we examined if expression of *ilp3* or *ilp6* transgene in sub-retinal glia rescues the delayed R-cell differentiation phenotype caused by inhibition of EGFR signalling. Genomic fragments containing entire coding sequence of *ilp3* and *ilp6* were isolated and placed into the *UAS* expression vector. Multiple independent transgenic lines were generated and tested.

When *UAS-ilp3* transgene was expressed in sub-retinal glia under control of *Repo*-GAL4, the *EGFR*-knockdown-induced delay in R-cell differentiation was largely rescued (Fig. 3.8A). Similar results were obtained using four independent *ilp3* transgenic lines (Fig. 3.8A). We also found that the expression of *UAS-ilp6* transgene in sub-retinal glia with two independent transgenic lines, largely rescued the delayed R-cell differentiation phenotype in flies in which EGFR signalling was inhibited in sub-retinal glia (Fig. 3.8B).

Together, these results suggest strongly that EGFR-activated sub-retinal glia release ILP3 and ILP6 to regulate the timing of R-cell differentiation by activating InR in eye precursor cells.

3.5 Discussion

In this study, we present several lines of evidence that support a key role for sub-retinal glia to positively regulate the timing of R-cell differentiation in the eye-disc epithelium. First, manipulating the activity of EGFR signaling in sub-retinal glia affected the timing of R-cell differentiation. Second, inhibition of EGFR signaling in sub-retinal glia prevented the activation of Akt, a major downstream target of InR, in the eye-disc epithelium. Third, knockdown of *ilp3* or *ilp6* in sub-retinal glia delayed R-cell differentiation. And fourth, *EGFR* interacted genetically with *ilp3* and *ilp6* in sub-retinal glia to regulate the timing of R-cell differentiation in the eye-disc epithelium. These results support a model in which in response to R-cell-derived EGF-like ligand Spi, sub-retinal glia release ILP3 and ILP6 that activate InR in eye precursor cells to positively regulate the timing of R-cell differentiation.

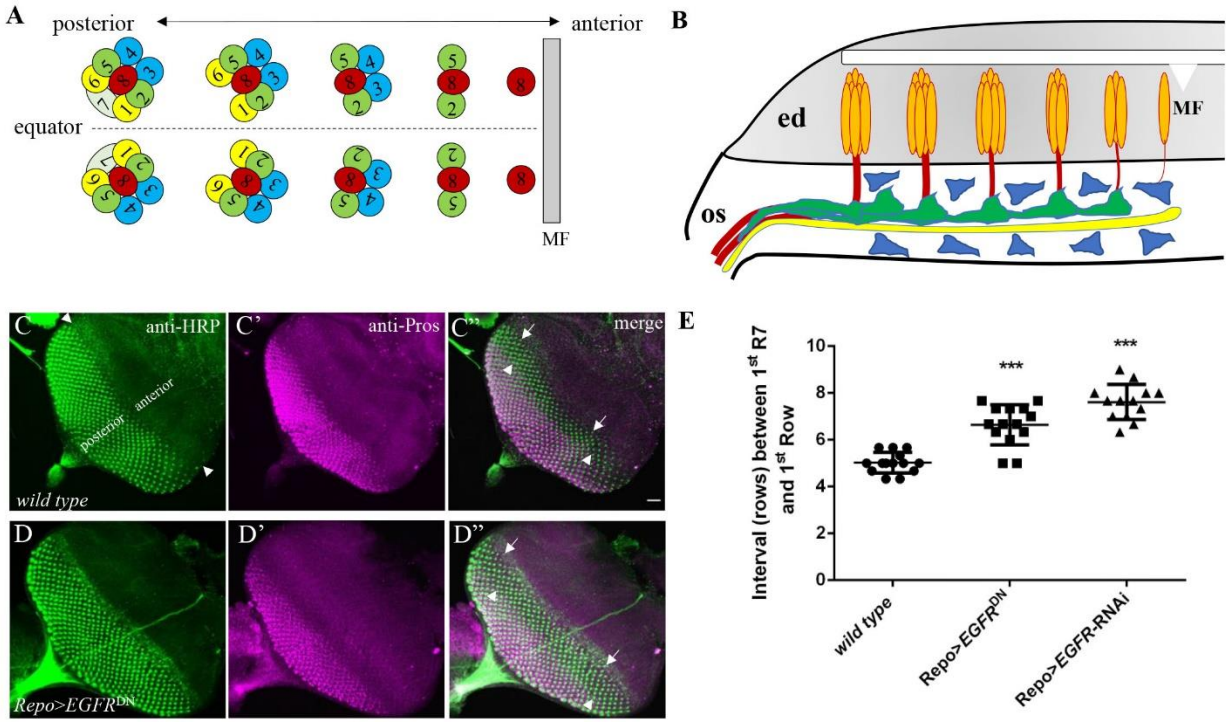
Our present study revealing the role of ILP3 and ILP6 from sub-retinal glia in the timing of R-cell differentiation, together with a previous report showing the action of ILP6 from WG of sub-retinal glia in inducing lamina neuronal differentiation (Fernandes et al., 2017), support that sub-retinal glia are major sources of ILPs that coordinate the development of neurons and glia in the fly visual system. In addition to sub-retinal glia, ILP3 and ILP6 are also reported to be produced in other cell types. For instance, ILP3 is shown to be produced and released by insulin-producing cells (IPCs) of the brain to the hemolymph (Nässel & Vanden Broeck, 2016). Similarly, ILP6 is also produced by adipocytes of the fat body (Nässel & Vanden Broeck, 2016). Thus, while our results show that ILP3 and ILP6 from sub-retinal glia are important for temporal control of R-cell differentiation, it remains to be determined if ILPs from the circulation also play a role in this process.

Given that ILPs are reported to act redundantly in many processes (Grönke et al., 2010), it is surprising that knocking down *ilp3* or *ilp6* only caused an obvious delay in R-cell differentiation. One possible explanation is that although ILP3 and ILP6 act similarly to activate InR, the limited production of ILP3 and ILP6 in sub-retinal glia may not allow the full activation of InR in eye precursor cells when only ILP3 or ILP6 is released. Alternatively or additionally, ILP3 and ILP6 may activate InR in eye precursor cells differentially, and proper timing of R-cell differentiation requires both ILP3- and ILP6-dependent InR activation. That different ILPs act through the same InR to induce distinct downstream signaling events is not unprecedented. For instance, it is reported that ILP2 induced transient phosphorylation of Akt, while ILP5 induced sustained phosphorylation of Akt (Post et al., 2018). Similarly, ILP3 and ILP6 may activate InR in eye precursor cells to trigger two distinct signaling effects, both of which may be required for the timing of R-cell differentiation.

in vitro studies suggest that insulin signaling also plays a role in the timing of rod photoreceptors in the mammalian visual system (Pinzon-Guzman et al., 2011). It is reported that the treatment of mouse retinal explant culture with IGF1 induced the differentiation of rod photoreceptors by modulating the activity of STAT3 (Pinzon-Guzman et al., 2011). Several studies reveal that STAT3 inactivation is required for initiating rod photoreceptor differentiation (Rhee et al., 2004; Zhang et al., 2004). That IGF1 induced intracellular signaling events leading to a decrease in STAT3 tyrosine phosphorylation and thus the inactivation of STAT3 in cultured cells (Pinzon-Guzman et al., 2015; Pinzon-Guzman et al., 2011; Xing et al., 2018), suggests strongly that IGF1 induces rod photoreceptor differentiation by inactivating STAT3. Whether and how insulin-like family members act *in vivo* to regulate the timing of rod photoreceptor differentiation, however, remain to be determined.

Like that in *Drosophila*, glial cells in the vertebrate visual system may provide inductive signals to regulate the timing of photoreceptor differentiation. Mammalian retina contains microglia and two types of macroglia, Müller cells and astrocytes (Reichenbach & Bringmann, 2020). Cone photoreceptors, but not rod photoreceptor, differentiate before the appearance of macroglia (Lago-Baldaia et al., 2020). A previous study shows that removal of microglia in the zebrafish retina severely delayed the differentiation of ganglion cells, cone and rod photoreceptors (Huang et al., 2012), suggesting that microglia produce inductive signals for timing the differentiation of retinal neurons. It is also reported that Müller cells could modulate the differentiation of rod photoreceptors in dissociated cell cultures of neonatal mouse retina (Neophytou et al., 1997), suggesting that macroglia such as Müller cells and astrocytes in the developing mammalian retina may also produce signals to regulate the timing of rod differentiation. In the future, it will be of interest to determine if like sub-retinal glia in *Drosophila*, microglia and macroglia in the developing mammalian retina also release insulin-like family members for temporal control of photoreceptor differentiation.

Figure 3. 1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posterior
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B
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merge
D
Repo>EGFR^DN
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Interval (rows) between 1st R7 and 1st Row
10
8
6
4
2
0
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***
wild type
Repo>EGFR^DN
Repo>EGFR-RNAi

Figure 3.1. Inhibition of EGFR signaling in sub-retinal glia delayed the differentiation of R7 photoreceptors. (**A**) Schematic diagram showing the progression of ommatidial differentiation in the 3rd-instar larval eye disc. Each ommatidium in the first ommatidial row (i.e. the newly formed ommatidial row immediately posterior to the MF) contains only R8 photoreceptor. As the MF moves more anteriorly, new rows of ommatidia are formed regularly. The sequential differentiation of other R-cell subtypes leads to the gradual appearance of R2/R5 pairs, R3/R4 pairs, R1/R6 pairs and R7 in ommatidial rows that are further away from the MF. Dorsal and ventral ommatidial clusters in the eye disc have opposite orientation above and below the equator. (**B**) Schematic diagram showing the longitudinal section of the 3rd-instar eye disc (ed). Glia in the sub-retinal space include SPG (yellow), PG (blue) and WG (green). After encountering nascent R-cell axons (red), apical PG differentiate into WG. WG associate closely with R-cell axons and extend the membrane from the eye disc through the optic stalk into the optic lobe. (**C**-**C**" and **D**-**D"**) 3rd-instar larval eye discs were double stained with anti-HRP (green) and anti-Pros (magenta). Anti-HRP specifically labeled the membrane of all differentiating R cells. Anti-Pros recognizes the transcription factor Prospero that is specifically expressed in R7 only in rows of less mature ommatidial clusters and is also expressed in cone cells in rows of more mature ommatidial clusters. **(C-C'')** In wild type (n=14), R7 photoreceptors initially appeared in ommatidia (arrowheads) that were ~5 rows from the 1st row behind the MF (arrows). (**D-D''**) In flies (i.e. *Repo>EGFR*^{DN}) (n=14) in which a dominant-negative form of EGFR (i.e. EGFR^{DN}) was expressed in all sub-retinal glia under control of the pan-glial-specific *Repo*-GAL4, the appearance of R7 was delayed for ~1-2 rows. (**E**) The relative delay in R7 differentiation was quantified by measuring the intervals (ommatidial rows) between the 1st row behind the MF and the row in which R7 initially appeared. Significant delay in R7 differentiation was observed

when $EGFR^{DN}$ was expressed in sub-retinal glia (n=14), or when EGFR was specifically knocked down in sub-retinal glia by expressing *UAS-EGFR-RNAi-JF01368* under control of *Repo*-GAL4 (i.e. Repo> *EGFR-RNAi*) (n=13). One-way ANOVA followed by post hoc Tukey's test, ***$P<0.001$. Error bars indicate SD. Scar bar: 20 μm.

Figure 3. 2

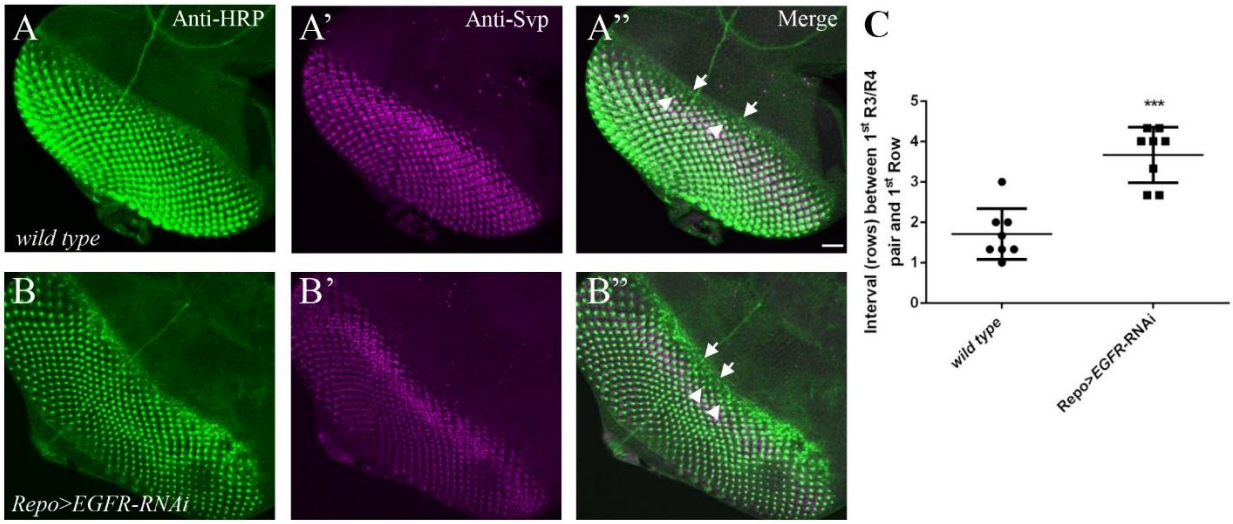
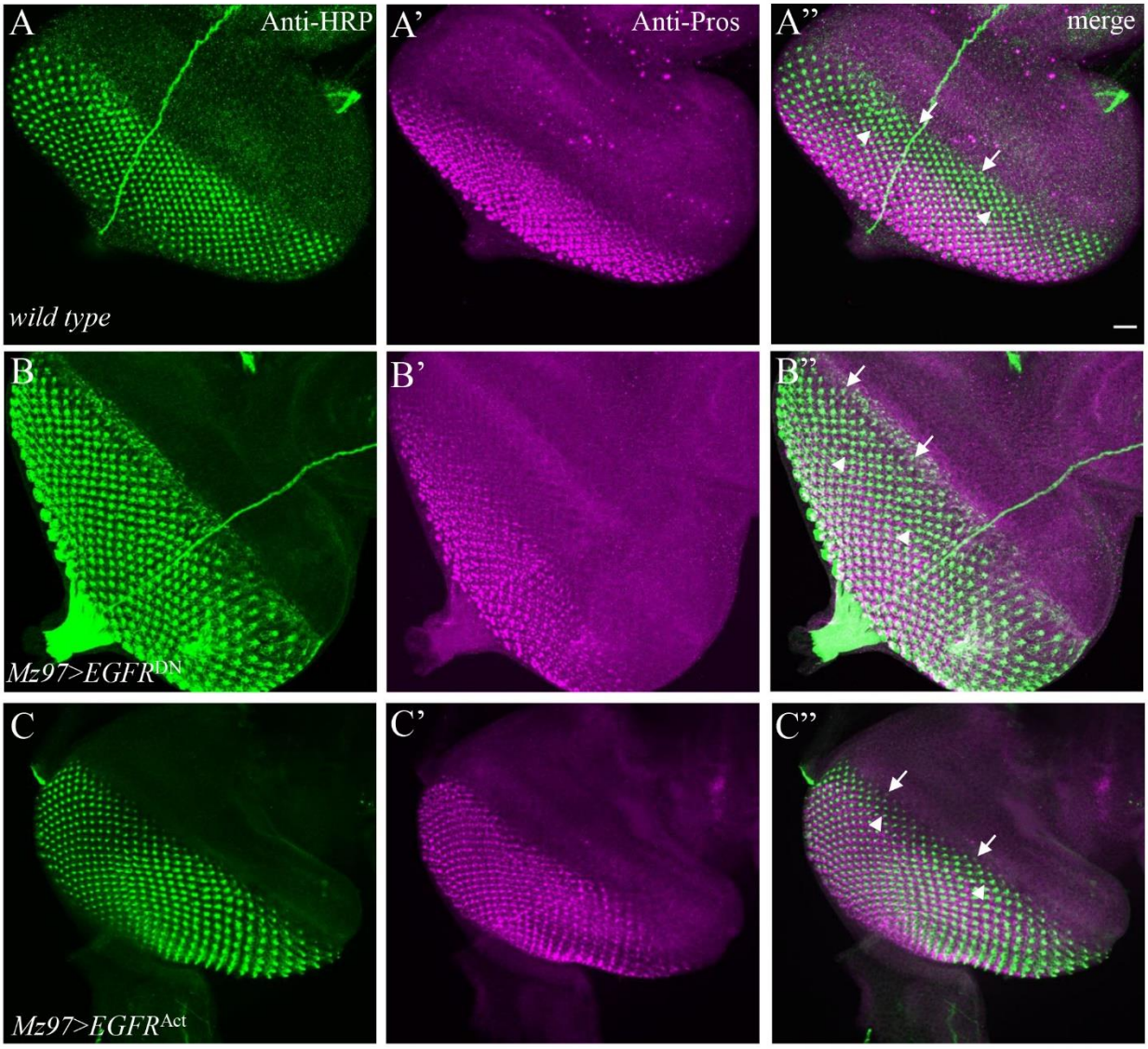


Figure 3.2. Downregulation of EGFR signaling in sub-retinal glia also delayed the differentiation of R3/R4 pairs. 3rd-instar larval eye discs were double stained with anti-HRP (green) and anti-Svp (magenta). Anti-Svp recognizes the transcription factor Svp that is expressed in R3/R4 pairs only in less mature ommatidia (arrowheads) and later expressed in both R3/R4 and R1/R6 pairs in more mature ommatidia. (**A-A'')** In wild type (n=8), R3/R4 pairs initially appeared in ommatidia (arrowheads) that were ~2 rows from the 1st row behind the MF (arrows). (**B-B''**) In flies (i.e. *Repo>EGFR-RNAi*) (n=8) in which *UAS-EGFR-RNAi* was expressed in sub-retinal glia under control of *Repo*-GAL4, the appearance of R3/R4 pairs was delayed. (**C**) The relative delay in R3/R4 differentiation was quantified by measuring the intervals (ommatidial rows) between the 1st row behind the MF and the row in which R3/R4 pairs initially appeared. Two tailed unpaired student's t-test, ***P<0.001. Error bars indicate SD. Scar bar: 20 μm.

Figure 3. 3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A'
Anti-Pros
A''
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B''
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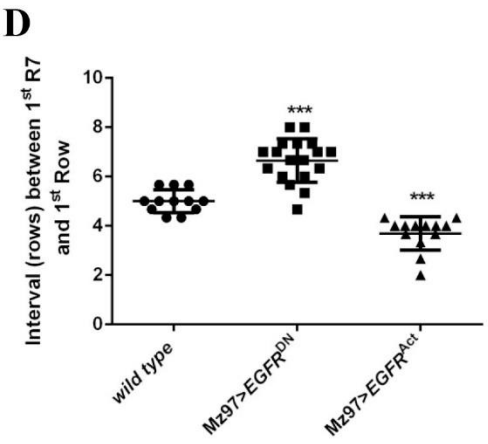
D
Interval (rows) between 1st R7 and 1st Row
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***
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wild type
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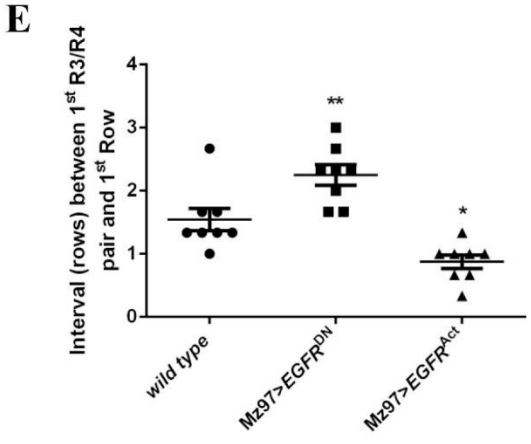
Mz97>EGFRAct


E
Interval (rows) between 1st R3/R4 pair and 1st Row
4
3
2
1
0
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*
wild type
Mz97>EGFRDN
Mz97>EGFRAct

Figure 3.3. Manipulating the activity of EGFR in WG affected the timing of R-cell differentiation. (**A**-**C**) 3rd-instar larval eye discs were double-stained with anti-HRP (green) and anti-Pros antibodies (magenta). (**A-A'')** Wild type (n=12). (**B-B''**) In flies (i.e. *Mz97>EGFR*^{DN}) (n=18) in which *UAS-EGFR*^{DN} was expressed in WG under control of the WG-specific driver *Mz97*-GAL4, R7 differentiation was delayed (arrowheads). (**C-C''**) In flies (i.e. *Mz97>EGFR*^{Act}) (n=13) in which a constitutively active form of EGFR was expressed in WG under control of *Mz97*-GAL4, R7 photoreceptors appeared precociously in rows of less mature ommatidia (arrowheads). (**D**) The relative rate of R7 differentiation was quantified by measuring the intervals (ommatidial rows) between the 1st row behind the MF and the row in which R7 initially appeared. (**E**) The relative rate of R3/4 differentiation was quantified by measuring the intervals (ommatidial rows) between the 1st row behind the MF and the row in which R3/R4 initially appeared. Number of eye discs double-stained with anti-HRP and anti-Svp antibodies: wild type, 8; *Mz97>EGFR*^{DN}, 8; *Mz97>EGFR*^{Act}, 8. One-way ANOVA followed by post hoc Tukey's test, ***P<0.001, **P<0.01, *P<0.05. Error bars indicate SD. Scar bar: 20 μm.

Figure 3. 4

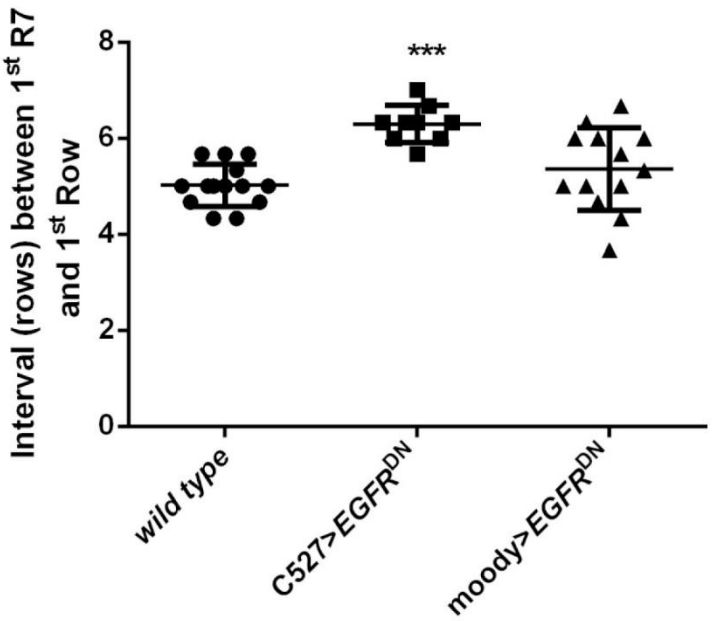
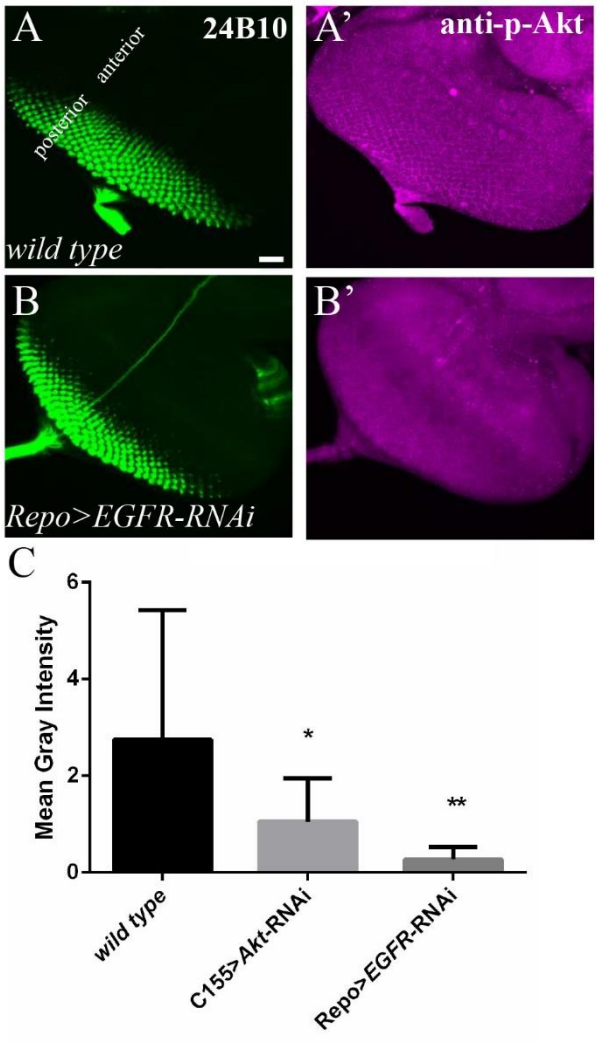


Figure 3.4. Inhibition of EGFR signaling in PG but not SPG delayed R7 differentiation. EGFR signaling was inhibited in PG and SPG by expressing *UAS-EGFR*^{DN} under control of PG-specific *C527*-GAL4 (n=9) and SPG-specific *moody*-GAL4 (n=13), respectively. Delay in R7 differentiation was observed when EGFR signaling was inhibited in PG. One-way ANOVA followed by post hoc Tukey's test, ***P<0.001. Error bars indicate SD.

Figure 3. 5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anterior
posterior
wild type
A'
anti-p-Akt
B
Repo>EGFR-RNAi
B'
C
Mean Gray Intensity
6
4
2
0
*
**
wild type
C155>Akt-RNAi
Repo>EGFR-RNAi

Figure 3.5. Knockdown of *EGFR* in sub-retinal glia decreased the levels of phosphorylated Akt in the eye disc. 3rd-instar larval eye discs were double-stained with MAb24B10 (green) and the antibody recognizing phosphorylated Akt (anti-p-Akt) (magenta). MAb24B10 staining visualized R-cell soma and axons. (**A**-**A'**) Wild-type eye disc (n=13) double-stained with MAb24B10 (**A**) and anti-p-Akt (**A'**). (**B**-**B')** Eye disc in which *EGFR* was knocked down in sub-retinal glia (n=7). The levels of phosphorylated Akt in the posterior region containing differentiating ommatidia were greatly decreased. (**C**) The levels of phosphorylated Akt were quantified by measuring the mean fluorescent intensity in the posterior region containing differentiating ommatidia. Knockdown of *EGFR* in sub-retinal glia significantly decreased the levels of phosphorylated Akt. That knockdown of *Akt* in R cells (i.e. *C155>Akt-RNAi*) by expressing a *UAS-Akt-RNAi* transgene under control of the neuronal-specific *C155*-GAL4 also decreased anti-p-Akt staining (n=20), supports the specificity of anti-p-Akt antibody. One-way ANOVA followed by post hoc Tukey's test, $^{**}P < 0.01$, $^{*}P < 0.05$. Error bars indicate SD. Scar bar: 20 μm.

Figure 3. 6

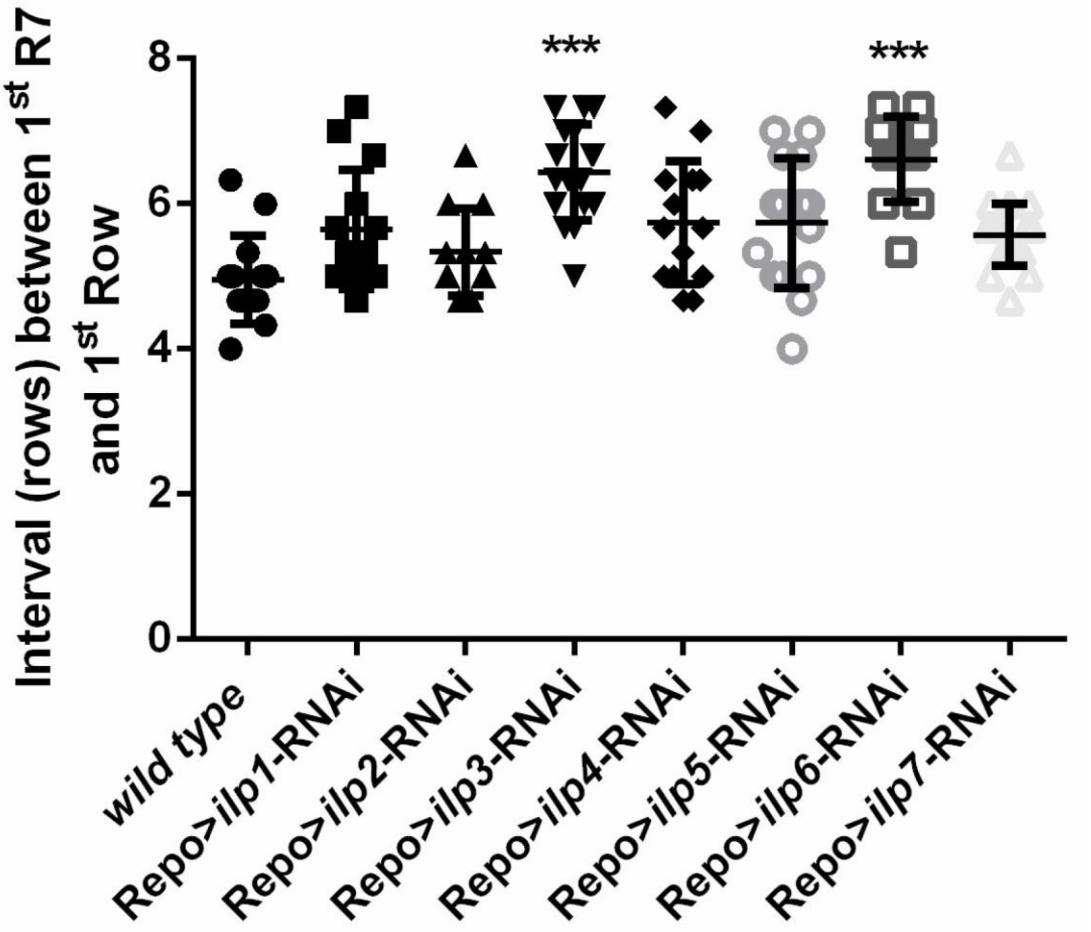

Figure 3.6. Knocking down *ilp3* or *ilp6* in sub-retinal glia delayed R7 differentiation. ILP1-7 that act through the sole *Drosophila* InR were tested by knockdown for potential roles in the timing of R7 differentiation. Knockdown of *ilp3* or *ilp6* in sub-retinal glia delayed the differentiation of R7 photoreceptors. Number of eye discs examined: wild type, 14; *Repo>ilp1-RNAi*, 14; *Repo>ilp2-RNAi*, 12; *Repo>ilp3-RNAi*, 17; *Repo>ilp4-RNAi*, 14; *Repo>ilp5-RNAi*, 16; *Repo>ilp6-RNAi*, 12; *Repo>ilp7-RNAi*, 21. One-way ANOVA followed by post hoc Tukey's test, ***P<0.001. Error bars indicate SD.

Figure 3. 7

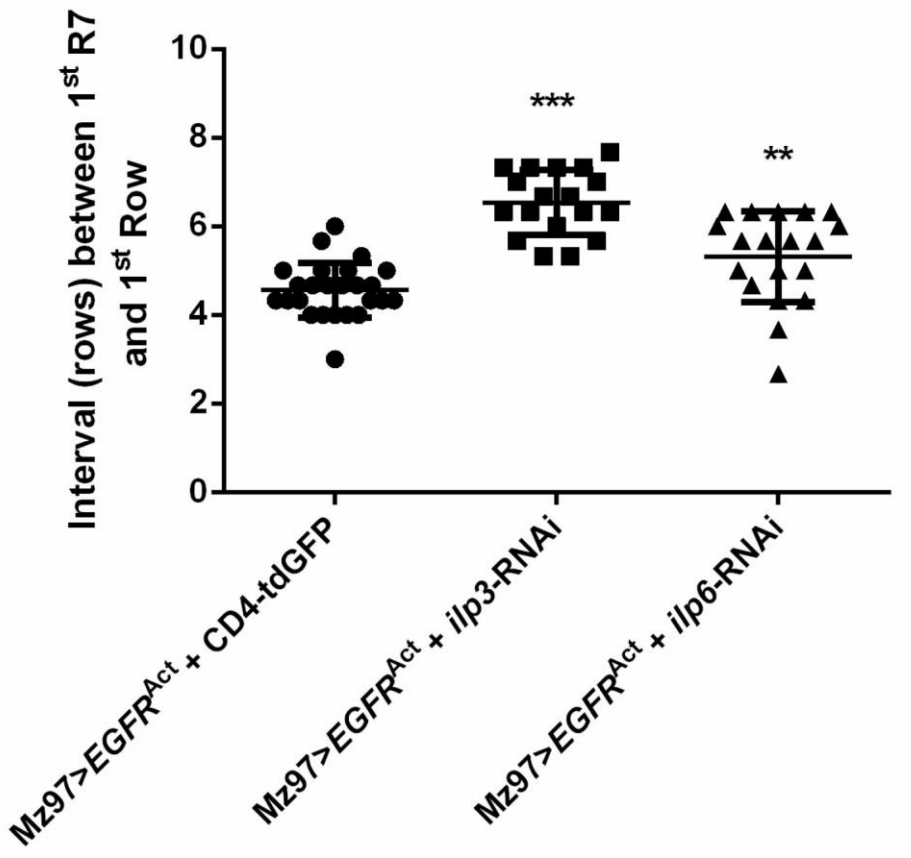

Figure 3.7. Reducing the levels of Ilp3 or Ilp6 suppressed the precocious R7 differentiation phenotype caused by hyperactivation of EGFR signaling in WG. *UAS-ilp3-RNAi*, *UAS-ilp6-RNAi* or *UAS-CD4-tdGFP* transgene was co-expressed with *UAS-EGFR*^{Act} in WG under control of *Mz97*-GAL4. Compared to flies in which EGFR was hyperactivated in WG (i.e. Mz97> *EGFR*^{Act} + *CD4-tdGFP*) (n=25), knockdown of *ilp3* (n=18) or *ilp6* (n=19) significantly suppressed the EGFR^{Act}-induced precocious R7 differentiation. One-way ANOVA followed by post hoc Tukey's test, ***P< 0.001, **P< 0.01. Error bars indicate SD.

Figure 3. 8

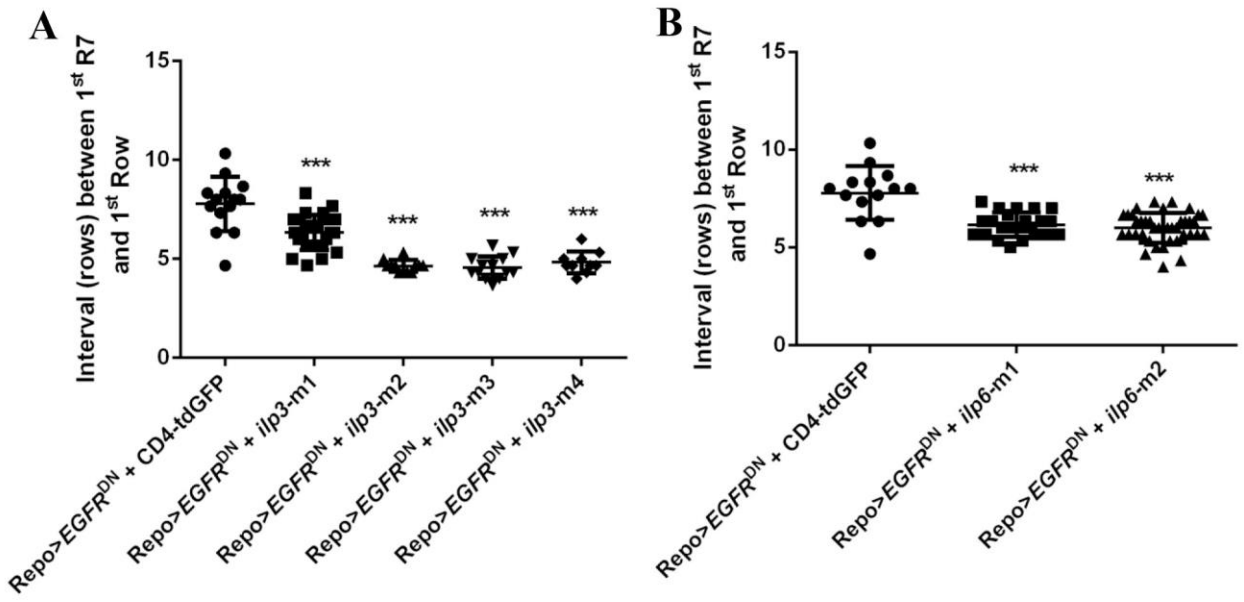

Figure 3.8. Expression of *ilp3* or *ilp6* transgene rescued the delayed R7 differentiation phenotype caused by inhibition of EGFR signaling in sub-retinal glia. *UAS-ilp3* or *UAS-ilp6* transgene was co-expressed with *UAS-EGFR*^{DN} in sub-retinal glia under control of *Repo*-GAL4. (**A**) For *ilp3* experiments, four independent *UAS-ilp3* transgenic lines (i.e. m1-m4) were tested. The expression of *ilp3* transgene in sub-retinal glia largely rescued the *EGFR*^{DN}-induced delay in R7 differentiation. Similar results were obtained with all four *ilp3* transgenic lines. Number of eye discs examined: *Repo> EGFR*^{DN} + *CD4-tdGFP*,14; *Repo> EGFR*^{DN} + *ilp3-m1*, 27; *Repo> EGFR*^{DN} + *ilp3-m2*, 12; *Repo> EGFR*^{DN} + *ilp3-m3*, 13; *Repo> EGFR*^{DN} + *ilp3-m4*, 10. (**B**) For *ilp6* experiments, two independent *UAS-ilp6* transgenic lines (i.e. m1 and m2) were tested. The expression of *ilp6* in sub-retinal glia also largely rescued the *EGFR*^{DN}-induced phenotype. Number of eye discs examined: *Repo> EGFR*^{DN} + *CD4-tdGFP*,14; *Repo> EGFR*^{DN} + *ilp6-m1*, 23; *Repo> EGFR*^{DN} + *ilp6-m2*, 40. One-way ANOVA followed by post hoc Tukey's test, ***P<0.001. Error bars indicate SD.

Chapter 4 : General Discussion, Conclusion and Future Directions

4.1 General Discussion

Growing evidence during the past decade supports the importance of the coordinated development of neurons and glia for the formation of complex neuronal circuits in the nervous system. However, our understanding of the molecular and cellular mechanisms coordinating the development of neurons and glia remains incomplete. My Ph.D. research leads to two important findings that illustrate the interactions between neurons and glial cells by using the developing *Drosophila* visual system as a model. In Chapter 2, the first cell-surface receptor (i.e. Mys/Scb integrins) on R-cell axons for mediating the exit of R-cell axons was identified by using immunohistological and genetic approaches. Additionally, our results also suggest that the extension of WG membrane from the eye disc into the optic stalk is dependent on its association with R-cell axons. In Chapter 3, our results support a model in which the activation of EGFR in sub-retinal glia positively regulates the timing of R-cell differentiation in the eye-disc epithelium by releasing ILP3 and ILP6. These results provide important clues to the understanding of the mechanisms controlling similar processes in mammals.

4.1.1 Integrins on R-cell Axons Mediate the Exit of R-cell Axons and WG Membrane from the Eye Disc.

At the third instar larval stage, R cells differentiate and project axons that navigate over a long distance from the eye disc through the optic stalk into the optic lobe, where they establish a precise retinotopic map (Hadjieconomou et al., 2011). WG, differentiated from PG (Silies et al., 2010; Yuva-Aydemir & Klambt, 2011), ensheath and migrate with R-cell axons through the optic stalk into the optic lobe (Franzdottir et al., 2009). Hence, WG is required for the establishment of the retinotopic map (Chang et al., 2018; Fernandes et al., 2017).

During the past two decades, studies by several groups have identified a number of key players involved in R-cell axonal guidance and the establishment of the retinotopic map (Garrity et al., 1996; Lee et al., 2003; Martin et al., 1995; Newsome et al., 2000; Ruan et al., 2002). For instance, previous studies by Zipursky and coworkers identify two transmembrane proteins N-cadherin and Flamingo as key cell-surface receptors in mediating R-cell axonal target selection in the developing optic lobe (Lee et al., 2001; Lee et al., 2003). Former trainees in our lab identified intracellular signaling proteins such as Misshapen (Msn) and Bifocal (Bif), key components of the Dreadlocks (Dock) signaling pathway, for regulating R-cell axonal guidance and target selection (Ruan et al., 2002).

Previous studies in our lab also identified two transmembrane proteins Tutl and Bdl as key surface players in mediating the recognition between R-cell axons and WG for the ensheathment of R-cell axons and WG extension (Cameron et al., 2016; Chen et al., 2017). Both Tutl and Bdl belong to the IgSF9 subfamily of the immunoglobulin (Ig) superfamily. At the third-instar larval stage, Bdl is specifically expressed in WG (Cameron et al., 2016), and Tutl is specifically expressed in R-cell axons (Chen et al., 2017). While *in vitro* studies revealed that Tutl binds to Bdl in trans on opposing cell surfaces, *in vivo* studies showed that *tutl* and *bdl* interact genetically to regulate R-cell axonal ensheathment and WG extension (Cameron et al., 2016; Chen et al., 2017). These studies support a model in which the binding between Tutl on R-cell axons and Bdl on WG membrane, mediates the recognition between R-cell axons and WG membrane for axonal ensheathment and WG extension. A later study by another group shows that the Tutl-Bdl-mediated recognition between R-cell axons and WG is also required for topographic projections of R-cell axons in the optic lobe (Chang et al., 2018; Fernandes et al., 2017).

At the time when I initiated my Ph.D. study, however, the identity of cell-surface receptors on R-cell axons and WG that mediate the exit of R-cell axons and WG membrane from the eye disc into the optic lobe, was unknown. It was suggested that cues on sub-retinal glia at the posterior end of the eye disc may provide guidance information to direct the exit of R-cell axons from the eye disc (Hummel et al., 2002; Rangarajan et al., 1999; Xie et al., 2014). However, whether such cues on sub-retinal glia indeed exist, and if so, what are their receptors on R-cell axons, remains unknown. It was also unknown if the recognition between R-cell axons and WG is required for the exit of R-cell axons and/or WG membrane from the eye disc into the optic stalk.

My findings in Chapter 2 identify the first cell-surface receptor on R-cell axons that plays an important and specific role in mediating the exit of R-cell axons and WG membrane from the eye disc. My results showed that βPS integrin Mys and αPS3 integrin Scb are required in R-cell axons, but not in WG, for the exit of R-cell axons and WG membrane from the eye disc (Fig. 2.1, 2.2, and 2.6). Among five α integrins in *Drosophila* (Broadie et al., 2011; Brown et al., 2000), Scb and If (αPS2) are expressed in the eye disc and optic stalk (Xie et al., 2014). A previous study by our lab showing that knockdown of *if* in both R cells and WG did not affect the exit of R-cell axons and WG membrane (Liu et al., 2020), suggests strongly that Scb, but not If, is the α integrin that associates with the βPS integrin Mys to form a heterodimeric receptor on R-cell axons to detect exit signals for extension into the optic stalk.

The molecular identity of exit signals recognized by integrins on R-cell axons remains unknown. As integrins are well-known receptors for ECM proteins (Broadie et al., 2011), it is possible that specific ECM proteins may function as such exit signals, which may be present at the posterior end of the eye disc where R-cell axons converge and enter the optic stalk. In

vertebrates, it is reported that the ECM protein laminin-1 is required for the exit of retinal ganglion axons from the optic disk (Höpker et al., 1999). Similarly, I speculate that *Drosophila* laminin or other ECM proteins may function as the guidance signal for the exit of R-cell axons from the eye disc.

Exit signals may also be membrane-associated proteins that are expressed by sub-retinal glia. Integrins interact with a number of membrane-associated ligands, such as VCAM-1 and Basigin (Bsg) (Curtin et al., 2005; Huang et al., 2000; Yang et al., 1995). Both VCAM-1 and Bsg are members of the Ig superfamily. And the interaction between α4 integrin and VCAM-1 is required for the innervation of the heart by sympathetic axons in rats (Wingerd et al., 2002). The ortholog of Bsg in *Drosophila* is expressed in glia (Curtin et al., 2005; Curtin et al., 2007; Hunter et al., 2020), raising the possibility that Bsg on sub-retinal glia acts as an exit signal recognized by integrins on R-cell axons. To test this, I performed knockdown experiments. However, I found that glial-specific knockdown of *bsg* did not prevent the exit of R-cell axons from the eye disc (data not shown). One possible explanation is that knockdown of *bsg* did not completely eliminate Bsg. Alternatively or additionally, Bsg may be functionally redundant with ECM proteins or some other membrane-associated integrin ligands in mediating the exit of R-cell axons.

My results argue against the role of integrins acting in WG for recognizing exit signals. Unlike R-cell-specific knockdown of *mys* or *scb*, WG-specific knockdown of *mys* or *scb* did not prevent the exit of R-cell axons and WG membrane (Fig. 2.2 C-C'' and 2.6 D-D''). And when *mys* or *scb* was knocked down in both R cells and WG, R-cell axons were still associated normally with WG membrane (Fig. 2.1 B-B'' and 2.6 B-B''). Thus, the presence of integrins in

WG is neither required for exiting the eye disc nor the association of WG membrane with R-cell axons.

My findings shed new light on the coordinated exit of R-cell axons and WG membrane from the eye disc. A previous study by Sun and coworkers showed that genetic ablation of WG affected R-cell projection patterns in the optic lobe, but did not prevent R-cell axons from leaving the eye disc into the optic stalk (Chang et al., 2018; Fernandes et al., 2017), suggesting that the exit of R-cell axons is independent of WG. In contrast, my results showed that when R-cell axons failed to exit the eye disc, all WG membrane stalled within the eye disc. Thus, it appears that the recognition of exit signals by R-cell axons plays a key role in the coordinated exit of R-cell axons and WG membrane from the eye disc.

Based on my findings described in Chapter 2 and previous studies by other researchers, I propose a model for the coordinated exit of R-cell axons and WG from the eye disc into the optic stalk. First, Mys and Scb form a heterodimeric receptor on R-cell axons, which detects local cues present at the posterior end of the eye disc to direct the extension of R-cell axons into the optic stalk. And second, the recognition between WG and R-cell axons allows WG membrane to associate with R-cell axons and thus follow R-cell axons into the optic stalk. Previous studies in our lab show that Bdl on WG membrane interacts with Tutl on R-cell axons to mediate the recognition between R-cell axons and WG for axonal ensheathment and WG extension in the optic lobe (Cameron et al., 2016; Chen et al., 2017). However, loss of *tutl* or *bdl* did not completely prevent the exit of WG membrane from the eye disc (Cameron et al., 2016; Chen et al., 2017), suggesting the involvement of additional cell-surface receptors in mediating the association between R-cell axons and WG. That fact that Mys, Scb, Tutl and Bdl all have close homologs in mammals, together with a previous study showing the requirement of laminin-1 (the

well-known ligand of β1 integrins) for the exit of retinal ganglion axons from the optic disk in Xenopus (Höpker et al., 1999), suggest strongly that similar mechanisms may act in the vertebrate eye for visual circuit development.

4.1.2 Sub-Retinal Glia-derived ILPs Regulate the Timing of Photoreceptor Differentiation

Previous studies in *Drosophila* and mammals suggest an important role for insulin signaling in retinal precursor cells for temporal control of photoreceptor differentiation (Bateman & McNeill, 2004; Pinzon-Guzman et al., 2011). The source and identity of insulin-family members that act *in vivo* to activate InR in retinal precursor cells, however, were unknown when I initiated my Ph.D. study. In Chapter 3, I provide evidence that supports the function of sub-retinal glia-derived ILP3 and ILP6 for the timing of R-cell differentiation in the eye-disc epithelium.

4.1.2.1 EGFR Signaling in Sub-Retinal Glia Is Involved in Temporal Control of R-cell Differentiation

My findings in Chapter 3 reveal a novel and important role for sub-retinal glia in the timing of R-cell differentiation. My results showed that while inactivating the EGFR signaling pathway in sub-retinal glia delayed R-cell differentiation (Fig. 3.1 C-C'', 3.1 D, and 3.2), hyperactivating EGFR signaling in sub-retinal glia induced precocious R-cell differentiation (Fig. 3.3). These results suggest that the activation of EGFR signaling in sub-retinal glia may stimulate the release of inductive signals from sub-retinal glia, which subsequently regulate the timing of R-cell differentiation. Among sub-retinal glia, WG and PG are likely sources of such inductive signals, as interfering with EGFR signaling in SPG did not delay R-cell differentiation (Fig. 3.4).

The identity of cell types and tissues that release EGF ligands to activate EGFR in sub-retinal glia for temporal control of R-cell differentiation is still unclear. One likely source of EGF ligands is the eye-disc epithelium. Previous studies show that differentiating R8, the founder of

each ommatidium, secretes the EGF-like molecule Spi that activates EGFR in precursor cells to control the recruitment of other R-cell subtypes (Freeman, 1994b, 1996; Tio & Moses, 1997). I speculate that R-cell-derived Spi simultaneously activates EGFR in sub-retinal glia, and thus allows the cooperation between R8 and sub-retinal glia for temporal control of the differentiation of other R-cell subtypes.

Another possible source of EGF ligands is the peripodial membrane. Within the eye-antennal disc, the eye-disc epithelial layer is overlaid by the peripodial membrane. It is reported that cells in the peripodial membrane produce secreted proteins such as Hh, Wg and Dpp to regulate pattern formation in the eye-disc epithelium (Cho et al., 2000). Similarly, the peripodial membrane may also produce Spi, which functions together with R-cell-derived Spi to activate EGFR in sub-retinal glia.

4.1.2.2 EGFR Signaling in Sub-retinal Glia Is Required for the Activation of Akt, A Key Downstream Target of InR in Eye Precursor Cells

My results in Chapter 3 showed that manipulating EGFR signaling in sub-retinal glia caused a dramatic effect on the timing of R-cell differentiation in the eye-disc epithelium (Fig. 3.1 and 3.2). These phenotypes caused by altered EGFR signaling in sub-retinal glia resemble that observed in mutants with altered InR signaling in eye precursor cells (Bateman & McNeill, 2004), suggesting that EGFR signaling in sub-retinal glia is required for the activation of InR in eye precursor cells. Consistently, my results showed that reducing EGFR signaling in sub-retinal glia caused a dramatic decrease in the levels of activated Akt (Fig. 3.5), which is a key downstream target of InR (Nässel et al., 2015; Saltiel, 2021).

In both vertebrates and invertebrates, activated InR recruits the adaptor scaffold protein IRS to activate PI3K, which in turn converts PIP_2 to PIP_3 leading to the phosphorylation and

activation of Akt by PDK1 (Leevers et al., 1996; Taniguchi et al., 2006). In the *Drosophila* eye disc, removing Dp110 (the fly homolog of the PI3K catalytic subunit) or InR, delayed R-cell differentiation (Bateman & McNeill, 2004). Together, these observations support a model in which sub-retinal glia regulate the timing of R-cell differentiation by activating InR signaling in eye precursor cells.

4.1.2.3 ILP3 and ILP6 are Sub-retinal Glia-derived Signals for Regulating the Timing of R-Cell Differentiation.

My results from cell-type-specific knockdown, epistasis analysis and transgene rescue suggest that in response to the EGF ligand Spi from R8 and/or other sources (e.g. peripodial membrane), sub-retinal glia produce ILP3 and ILP6, which activate InR in eye precursor cells to regulate the timing of R-cell differentiation (Fig. 3.6-3.8).

In *Drosophila*, ILP1-7 are reported to act through the sole *Drosophila* InR (Brogiolo et al., 2001). It is reported that there is a high level of functional redundancy between ILPs (Grönke et al., 2010). Unlike *ilp3* and *ilp6* knockdown flies, however, *ilp1, 2, 4, 5* and *7* knockdown flies did not display an obvious phenotype in R-cell differentiation (Fig. 3.6). One likely explanation is that ILP3 and ILP6, but not ILP1, 2, 4 ,5 and 7, are the downstream targets of EGFR in sub-retinal glia. That knocking down *ilp3* or *ilp6* only delayed R-cell differentiation, suggests the requirements of both ILP3 and ILP6 from sub-retinal glia for fully activating InR in eye precursor cells.

The activation of EGFR signaling may stimulate sub-retinal glia to produce ILP3 and ILP6 in two ways. First, in response to the EGF ligand Spi, EGFR activates the classical Ras/Raf/MAPK pathway, which turns on the transcription of *ilp3* and *ilp6* genes in sub-retinal glia. Consistently, a previous study shows that the inactivation of EGFR signaling reduced the

expression of *ilp6* in WG, a subtype of sub-retinal glia (Fernandes et al., 2017). And second, EGFR activation may stimulate the secretion of ILP3 and ILP6 peptides from sub-retinal glia. In mammals, it is reported that EGF stimulates insulin secretion in pancreatic β cells (Lee et al., 2008). Similarly, Spi may activate EGFR in sub-retinal glia to promote the release of ILP3 and ILP6.

The involvement of sub-retinal glia may provide another level of control to ensure the precise timing of R-cell differentiation in the eye disc. Sub-retinal glia such as WG and PG are located below differentiating ommatidia in the posterior region of the eye disc (Silies et al., 2010; Tsao et al., 2020). The exposure of PG and WG to Spi produced by older ommatidia may lead to the generation of an increasing anterior-to-posterior gradient of ILP3 and IL6. The quantitative difference in the levels of ILP3 and ILP6 along the anterior-posterior axis may specify the temporal pattern of InR activation in precursor cells to dictate the timing of R-cell differentiation.

4.2 Conclusion

My work described in Chapter 2 and Chapter 3 led to novel and exciting findings on the molecular and cellular mechanisms underlying the coordinated development of photoreceptor neurons and sub-retinal glia in the developing *Drosophila* visual system. Firstly, I identify the first cell-surface receptor (i.e. Mys/Scb integrins) on R-cell axons that plays a specific role in mediating the exit of R-cell axons from the eye disc into the optic stalk. My results also show that the exit of WG membrane from the eye disc is dependent on its association with R-cell axons, supporting an essential role for specific axon-glia interactions in directing the exit of glial processes. Secondly, I identify sub-retinal glia as an important source of inductive signals that regulate the temporal pattern of R-cell differentiation. By taking a combination of glial-specific knockdown, epistasis analysis and transgene rescue, I obtained evidence supporting the role of ILP3 and ILP6 from sub-retinal glia in activating InR in eye precursor cells for the timing of R-cell differentiation.

Given that both integrin and insulin pathways are conserved throughout evolution, it is highly likely that similar mechanisms are utilized to coordinate the development of neurons and glia in mammals. Thus, my work on integrins and ILPs in the *Drosophila* visual system provide important clues to the function of their mammalian counterparts in similar developmental processes and help the development of novel therapeutic strategies to treat brain diseases associated with alterations in these pathways.

4.3 Future Directions

4.3.1 What are the Exit Signals Recognized by Integrins on R-cell Axons?

While my findings in Chapter 2 identify integrins as key surface receptors on R-cell axons for the exit of R-cell axons and WG membrane from the eye disc, the molecular identity of the exit signals recognized by integrins remains unknown. That the presence of sub-retinal glia at the posterior end of the eye disc is required for the exit of R-cell axons (Rangarajan et al., 1999; Xie et al., 2014), raises at least two possibilities. First, the localization and organization of exit signals at the posterior end of the eye disc may be dependent on the presence of sub-retinal glia. And second, exit signals may be produced locally by sub-retinal glia at the posterior end of the eye disc. One way to identify exit signals is to determine the potential role of known integrin ligands whose localization and/or production are dependent on sub-retinal glia.

ECM proteins such as laminins are well-known ligands for integrins (Gotwals, Fessler, et al., 1994; Graner et al., 1998). Interestingly, it is reported that in the developing *Drosophila* visual system, the levels and organization of laminins such as LanA and LanB were perturbed when integrins were knocked down specifically in sub-retinal glia (Tavares et al., 2015; Xie et al., 2014), and glial-specific knockdown of integrins also caused R-cell axonal stalling (Tavares et al., 2015; Xie et al., 2014). These observations raise the interesting possibility that integrins on sub-retinal glia may regulate the levels and/or organization of laminins at the posterior end of the eye disc, which are recognized as exit signals by integrins on R-cell axons. Future studies will be needed to determine the *in vivo* requirements of LanA and/or LanB for the exit of R-cell axons from the eye disc.

Membrane-associated integrin ligands on sub-retinal glia may also function as exit signals that are recognized by Mys/Scb integrins R-cell axons. One membrane-associated

integrin ligand is the Ig-like transmembrane protein Bsg, which is reported to be expressed in glia (Curtin et al., 2005; Curtin et al., 2007; Hunter et al., 2020). However, I did not observe a *mys*-like R-cell axonal stalling phenotype when *bsg* was specifically knocked down in sub-retinal glia (data not shown). Since knockdown may not completely eliminate Bsg in sub-retinal glia, this result does not exclude the possibility that residual levels of Bsg in knockdown sub-retinal glia may be sufficient for directing the extension of R-cell axons. In the future, it will be necessary to obtain *bsg* null mutants for phenotypic analysis. It will also be important to perform double- and triple-mutant analysis to examine if Bsg, LanA and LanB function redundantly to mediate the exit of R-cell axons.

4.3.2 Integrins in Mammalian Retina May Function Similarly to Mediate the Exit of Retinal Ganglion Axons from the Optic Disk.

Integrins in *Drosophila* are highly homologous to their counterparts in mammals (Broadie et al., 2011). The closest homologs of Mys (βPS) and Scb (αPS3) in mammals are β1 and α4/9, respectively (Broadie et al., 2011), both of which have been shown to be involved in mediating axonal projections (Hines et al., 2010; Pasterkamp et al., 2003; Vogelezang et al., 2001; Wingerd et al., 2002). Interestingly, it is reported that α4β1 integrins are expressed in the developing chick retina (Cann et al., 1996; Sheppard et al., 1994) and the well-known β1-integrin ligand laminin-1 is required for the exit of retinal ganglion axons from the optic disk in Xenopus (Höpker et al., 1999). In the future, it would be of great interest to determine if α4β1 integrins, like fly Mys and Scb, play a role in mediating the exit of retinal ganglion axons from the optic disk in the developing mammalian retina.

4.3.3 What is the Mechanism by Which EGF Induces the Production of ILPs from Sub-Retinal Glia?

My findings in Chapter 3 support a model in which in response to EGFR activation, sub-retinal glia produce ILP3 and ILP6 to control the temporal pattern of InR activation in eye precursor cells for the timing of R-cell differentiation. The mechanisms underlying the control of ILP production by EGFR signaling in sub-retinal glia, however, remain unknown. In mammals, it is reported that EGF activates both MEK/ERK and PI3K/AKT pathways to control the production of insulin in beta cells (Yang et al., 2019). It will be interesting to determine if similar signaling pathways in sub-retinal glia are activated by the EGF ligand Spi.

EGFR signaling may also regulate the release of ILPs from sub-retinal glia. Previous studies show that ion channels, like K_{ATP} Channels, voltage-sensitive Ca^{2+} Channels, and glucose transporters regulate the release of ILPs from IPCs in adult flies for cell-autonomous glucose sensing (Fridell et al., 2009; Park et al., 2014). Similarly, EGFR signaling may modulate the activities of these proteins to promote the release of ILP3 and ILP6 from sub-retinal glia. Future studies may be necessary to examine if the expression and/or activity of these proteins are dependent on EGFR signaling, and if removing these proteins in sub-retinal glia affects InR activation in eye precursor cells and the timing of R-cell differentiation.

4.3.4 How Do ILP3 and ILP6 from Sub-Retinal Glia Control the Temporal Pattern of R-cell Differentiation in the Eye Disc?

My results from cell-type-specific knockdown, epistasis analysis and transgene rescue support that sub-retinal glia produce ILP3 and ILP6 to activate InR in eye precursor cells for the timing of R-cell differentiation. The exact mechanism by which ILP3 and ILP6 regulate the timing of R-cell differentiation, however, remains unknown. One attractive model is that

progressive exposure of sub-retinal glia to the EGF ligand Spi from older ommatidia leads to the formation of a gradient of ILP3/6 along the anterior-posterior axis, which specify the temporal pattern of InR activation in precursor cells for the timing of R-cell differentiation in younger ommatidia. One way to test this model in future studies is to raise antibodies that specifically recognize ILP3/6, and use the antibodies in immunostaining to determine if a gradient of ILP3/6 indeed exists in the developing eye disc. If so, it will be important to perform genetic analysis, such as genetic mosaic analysis and cell-type-specific knockdown, to determine if Spi produced by R cells is sufficient for the formation of the ILP3/6 gradient, or if the production of Spi from cells in the peripodial membrane is also required.

4.3.5 Do Glial Cells Produce IGF-I to Modulate the Timing of Photoreceptor Differentiation in the Developing Mammalian Retina?

Like that in the *Drosophila* eye disc, insulin signaling may play a role in controlling the timing of photoreceptor differentiation in the mammalian retina. It is reported that IGF-I was able to induce rod photoreceptor formation in cultured retinal progenitor cells by reducing the levels of tyrosine-phosphorylated STAT3 (pSTAT3) (Pinzon-Guzman et al., 2011). This *in vitro* study, together with *in vivo* evidence showing that down-regulation of STAT3 is required for the initiation of rod differentiation in the mouse retina (Rhee et al., 2004; Zhang et al., 2004), suggest a role for IGF-I in regulating the timing of rod differentiation. Consistently, it is reported that IGF-I is expressed in Müller glia (Fu et al., 2015). It will be interesting to examine if removing IGF-I from Müller glia by cell-type-specific knockdown or knockout affects the timing of rod differentiation. Genetic studies will also be necessary to determine if IGF-1R, the receptor for IGF-I, is required in retinal progenitor cells for the initiation of rod differentiation.

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