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migration in the developing *Drosophila* retina**

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Resumo

A interação precisa entre neurónios e células da glia é fundamental para a função do sistema nervoso. Contudo, como estas são, frequentemente, produzidas em locais distantes de onde desempenham a sua função, sinais molecular são importantes para direccionar a sua migração. A migração da glia está envolvida tanto no desenvolvimento do sistema nervoso como em doenças, pelo que é interesse do laboratório compreender mecanismos regulatórios subjacentes a este processo biológico. Com este propósito, o disco imaginal do olho de *Drosophila* tem sido usado como modelo.

Em resposta à diferenciação dos foto-receptores neuronais, as células da glia migram do sistema nervoso central para o disco do olho onde são responsáveis por organizar espacialmente os axónios. Para garantir o desenvolvimento correcto deste processo, sinais atractivos e repulsivos coordenam a migração da glia. Um destes sinais é Bnl, um ligando da via *Fibroblast Growth Factor* (FGF), expresso pelas células progenitoras dos foto-receptores. Foi sugerido que este ligando actua como um regulador não-autónomo negativo da migração de glia por se ligar e activar o receptor Btl expresso em glia.

Os nossos resultados experimentais representam um novo contributo no conhecimento da função de *bnl* no crescimento do disco do olho, no desenvolvimento dos foto-receptores e na migração da glia. Interessantemente, não foi descoberta uma relação directa entre defeitos nos foto-receptores e o fenótipo de exagerada migração da glia; contudo, o *knockdown* de *bnl* causou a morte por apoptose de células progenitoras do olho, o que se revelou fortemente relacionado com os defeitos na migração da glia. A exagerada migração da glia devido à depleção de Bnl nas células progenitoras do olho foi corrigida através da inibição tanto de genes pró-apoptóticos, como da actividade de caspases, assim como, através da depleção da função de JNK ou Dp53 nas células progenitoras de retina. Desta forma, nós sugerimos uma inter-comunicação coordenada entre estes sinais inter-celulares no controlo, à distancia, da migração da glia. Para além disso, estes resultados hipotetizam que Bnl não controla a migração da glia no disco do olho exclusivamente através da sua capacidade de se ligar e activar o receptor Btl nas células da glia. Não menos importante, nós também discutimos possíveis razões biológicas para o excesso de migração de glia quando Bnl é depletado.

Resultados obtidos anteriormente no laboratório mostraram uma interacção entre dMyc, uma regulador chave de crescimento tecidular, e Dpp, um *Transforming Growth*

Factor-β importante tanto na formação da retina como na exacta migração da glia no disco do olho. Assim sendo, surgiu interesse em compreender possíveis relações entre Bnl e dMyc. Aqui, nós mostramos que este sinais cooperam positivamente de forma a assegurar o correcto desenvolvimento do disco do olho.

Este trabalho realça a importância da sinalização activada por FGF no desenvolvimento do disco do olho e revela uma rede de sinalização onde uma variedade de sinais extra- e intra-celulares cooperam para, não autonomamente, controlarem a migração da glia. Esta inter-comunicação entre sinais biológicos pode ser importante noutros contextos celulares em *Drosophila*, assim como no desenvolvimento de tecidos em vertebrados.

Palavras-chave

Drosophila, retina, glia, migração, FGF, dMyc

Abstract

The function of a complex nervous system relies on an intricate interaction between neurons and glial cells. However, as glial cells are generally born distant from the place where they settle, molecular cues are important to direct their migration. Glial cell migration is important in both normal development and disease, thus current research in the laboratory has been focused on dissecting regulatory events underlying that crucial process. With this purpose, the *Drosophila* eye imaginal disc has been used as a model.

In response to neuronal photoreceptor differentiation, glial cells migrate from the CNS into the eye disc where they act to correctly wrap axons. To ensure proper development, attractive and repulsive signals must coordinate glial cell migration. Importantly, one of these signals is Bnl, a Fibroblast Growth Factor (FGF) ligand expressed by retinal progenitor cells that was suggested to act as a non-autonomous negative regulator of excessive glial cell migration (overmigration) by binding and activating the Btl receptor expressed by glial cells.

Through the experimental results described in chapter 3 we gained a detailed insight into the function of *bnl* in eye disc growth, photoreceptor development, and glia migration. Interestingly, we did not find a direct correlation between the defects on the ongoing photoreceptors and the glia overmigration phenotype; however, *bnl* knockdown caused apoptosis of eye progenitor cells what was strongly correlated with glia migration defects. Glia overmigration due to Bnl down-regulation in eye progenitor cells was rescued by inhibiting the pro-apoptotic genes or caspases activity, as well as, by depleting JNK or Dp53 function in retinal progenitor cells. Thus, we suggest a cross-talk between those developmental signals in the control of glia migration at a distance. Importantly, these results suggest that Bnl does not control glial migration in the eye disc exclusively through its ability to bind and activate its receptor Btl in glial cells. We also discuss possible biological roles for the glia overmigration in the *bnl* knockdown background.

Previous results in the lab showed an interaction between dMyc, a master regulator of tissue growth, and Dpp, a Transforming Growth Factor-β important for retinal patterning and for accurate glia migration into the eye disc. Thus, we became interested in understanding putative relationships between Bnl and dMyc. In chapter 4, we show that they positively cooperate in order to ensure proper development of the eye disc.

This work highlights the importance of the FGF signaling in eye disc development and reveals a signaling network where a range of extra- and intra-cellular signals cooperate to non-autonomously control glial cell migration. Therefore, such inter-relations could be important in other *Drosophila* cellular contexts, as well as in vertebrate tissue development.

Key-words

Drosophila, retina, glia, migration, FGF, dMyc

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List of abbreviations

Ago - Archipelago

BMP - Bone Morphogenetic Protein

Bnl – Branchless

Bsk - Basket

Btl - Breathless

Casp3 - Caspase 3

CNS - Central Nervous System

Dac – Dachshund

dMyc - *drosophila* Myc

Dpp - Decapentaplegic

EGF - Epidermal Growth Factor

Ey - Eyeless

Eya- Eyes absent

FGF - Fibroblast Growth Factor

FMW - First Mitotic Wave

Gcm - Glial cells missing

GFP - Green Fluorescent Protein

Hh - Hedgehog

Hid - Head involution defective

HRP - Horseradish Peroxidase

Hth - Homothorax

Htl – Heartless

JNK - Jun Kinase

ME - Main Epithelium

Mmp1 - Metalloproteinase 1

OS - Optic Stalk

PDGF - Platelet-Derived Growth Factor

PE - Peripodial Membrane

pH3 - phospho Histone 3

pMad - phosphorylated Mother Against Dpp

PNS - Peripheral Nervous System

Puc - Puckered

Pyr - Pyramus

RBG - Retinal Basal Glia

RD - Retinal Determination

Repo - Reversed polarity

RNAi - RNA interference

Rpr - Reaper

SMW - Second Mitotic Wave

So - Sine oculis

Ths - Thisbe

TGF-β - Transforming Growth Factor-β

Toy - Twin of eyeless

UAS - Upstream Activating Sequence

VEFG - Vascular Endothelial Growth Factor

Wg - Wingless

Chapter 1

Introduction

The intricate communication between the various cells that form the organism is key to a correct development. By transmitting and receiving information cells regulate their fate, leading to the formation of tissues and organs. As described by Gilbert et al. [1], cells are capable of changing the behavior of neighboring cells, inducing them to change their shape, proliferative status or patterning.

Cell-cell communication strongly depends on the production of extracellular signals that bind to transmembrane receptors on target cells. Ligand-receptor interaction activates signaling cascades that ultimately regulate gene expression, triggering cellular changes [2][3]. Among these ligands, some are transduced by cell-cell contact (juxtacrine signaling), while others are secreted such as diffusible growth and differentiation signals (paracrine signaling). Paracrine signaling represents the majority of the pathways involved in the organism development; these include, for example, the signal transducers Wnt/Wingless (Wg), Transforming Growth Factors-β (TGF-β)/Bone morphogenetic proteins (BMPs) and Fibroblast Growth Factors (FGFs) [3].

In the case of diffusible signals, one single ligand can induce different cellular responses due to distinct signaling profiles. This is mediated by the graded concentrations profile of the ligand (called morphogen) that results in different distributions along different distances. The final distribution of the ligand is modulated by a wide range of factors, including their diffusion capacity, mode of transport, and putative interactions with the extracellular molecules. These factors result in very different possible potentials of diffusion. Wnt/Wg and TGF-β/BMPs ligands are described to extend up to 30 cell diameters, while FGF-ligands seem to only diffuse through two to eight cell diameters [4].

Importantly, developmental processes strongly depend on cellular migration. When cells settle in their target locations, they establish cell-cell interactions that regulate cell behavior and, ultimately, organogenesis. Both spatial and temporal control of cells migration is crucial for proper development and this is often mediated by diffusible factors.

1. Glial cells: the key players on neuronal development, function and health

Glia and neurons are the main actors in a functional nervous system. Whereas neurons integrate and send information, glial cells perform distinct tasks to modulate neurons and ensure their correct function. Accordingly, the importance of glia is supported by their increase in number during evolution from simple nerve networks to more integrated devices [5].

1.1 Biological functions of glial cells

Glial cell function was initially thought to be restricted to nurturing neurons; however, now it's evident that they actively participate in all aspects of the nervous system development and function [6][7][8]. They associate with synapses, likely modulating their activity [9], and take up neurotransmitters after synaptic signaling [10]. Glia also helps to maintain the homeostasis of the blood-brain barrier (in CNS) and blood-nerve barrier (in PNS), which is important to protect neurons from pathogens and to avoid high concentrations of potassium ions in the hemolymph; appropriate biochemical environment is crucial to allow proper neuronal function [8][11]. During development, glia participates in the modulation of neural stem cell proliferation [12] and, occasionally, guide axons to their final destination [13][14]. Glia are also important to ensheath nerves and individual axons and to promote synapse formation and maturation [6][9]. Importantly, glial cells engulf degenerating axons during developmental axon pruning, showing the role of glia in the nervous system health [15]. Booth et al. [16] also showed that ablation of glial cells induces neuronal apoptosis.

To perform their roles in neuronal substrates, glial cells have to be correctly positioned in strategic spots [17]; however, as they are often born distant from these places, they need to actively migrate. Glia migration often depends on neuronal signals, thus requires intimate neurons-glia communication. Indeed, Hidalgo et al. [18] showed that the PVF ligand, the platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) mammalian homologue, expressed in neuronal

membranes are all involved in the regulation of the midline glia migration during *Drosophila* embryogenesis. In addition to their role in glia migration, diffusible gliotrophic signals produced by neurons are also important for glia survival and proliferation. For example, production of Vein (a ligand of the Epidermal Growth Factor (EGF) pathway) in pioneer neurons maintains the survival and proliferation of longitudinal glia during axon guidance [19].

Therefore, a highly plastic nervous system guarantees the precise rearrangement between neurons and glia allowing neuronal development, function and health. While neurotrophic cues sent by glial cells modulate the number and activity of neurons, neurons adjust glial number according to their needs. This tight communication led to the development of the neurotrophic model of the nervous system development [20], which is in agreement with the principle of Colon and Raff, 1999: "interacting cell populations adjust each other in number in ways that enable function" [21].

Sometimes, defects in glia migration occur; severe cases can trigger or enhance many human diseases, including neurodegenerative disorders and gliomas. Gliomas are among the most deadly types of cancer, being glioblastomas the most aggressive form [22]. They are characterized by extensive invasion and proliferation of glial tumor cells that creates an environment resistant to chemotherapy and radiotherapy, and therefore poor patient prognosis [22]. Thus, deciphering signaling cascades underlying glial migration can offer new opportunities to develop effective therapies for these diseases.

1.2 Glia in Vertebrates and *Drosophila melanogaster*

Despite the broad importance of glia in the nervous system, little is known about the underlying molecular pathways that mediate glial biology in vertebrates. Several studies describe the morphology of glia, in particular as they ensheath individual synapses; however, how glia and synapses acquire this intimate relationship, and the functional significance of these connections, remains to be addressed in detail. Furthermore, the molecular pathways that control axon guidance and wrapping by glia are poorly understood.

As we gain a better molecular and cellular characterization of glia-neurons interactions in *Drosophila*, it is becoming clear that glia shares significant molecular and functional features with their mammalian counterpart. Furthermore, many insights into glial functions were accomplished due to the accessibility of the *Drosophila* nervous system to single-cell genetic analysis [7][17]. Therefore, *Drosophila melanogaster* is a powerful organism to better interpret glial attributes in mammalian systems.

The three major glial cell types found in the *Drosophila* nervous system can be compared with vertebrate glia [7][23]. Astrocyte-like glia (*Drosophila*) and Astrocytes (vertebrates) associate closely with synapses in order to, for example, recycle neurotransmitter [19]; cortex glia (*Drosophila*) and satellite glia (vertebrates) surround neuronal cell bodies; and wrapping glia (*Drosophila*) and Oligodendrocytes (vertebrate CNS) and Schwann cells (vertebrate PNS) are responsible to wrap the neuronal axons. Interestingly, unlike most axons in vertebrates, axons in *Drosophila* are not myelinated. Furthermore, in vertebrates the blood brain barrier is formed by astrocytes and endothelial cells [24]; however, in *Drosophila*, two different types of glia are responsible for this structure, the subperineurial glia and perineurial glia [25].

Table 1: Glial cells types in *Drosophila melanogaster* and vertebrates. Adapted from [23].

Function	*D. melanogaster*	Vertebrates
Blood–brain barrier	Subperineurial glia and perineurial glia	Endothelial cells and astrocytes
Axon-wrapping	Ensheathing glia (CNS), wrapping glia (PNS)	Oligodendrocytes (CNS), Schwann cells (PNS)
Synapse association	Astrocyte-like glia	Astrocytes
Cell body association	Cortex glia	Satellite glia

1.3 Glial development

Although the function of the distinct glial cell types is regulated by similar mechanisms in *Drosophila* and vertebrates, the mechanisms underlying gliogenesis have important differences. In the mammalian nervous system, transcription factors such as oligodendrocyte lineage transcription factor 2 are responsible to direct glial

lineage [26]; however, in *Drosophila,* glial fate is established after activation of the unrelated *glial cells missing* (*gcm*) gene [27]. Gcm is a transcription factor that induces the expression of a large array of genes, including *reversed polarity* (*repo)*, which supports glial cell differentiation [27][28]. Importantly, *Drosophila* glial cells derive from glioblasts and neuro-glioblasts present in the neuroectoderm of the early embryo [29].

2. Drosophila melanogaster

The 20th century was, undoubtedly, a turning point in the field of classical genetics. In 1910, T.H. Morgan found the white gene mutation and its linkage to the X chromosome in *Drosophila melanogaster* [30]. This discovery triggered his curiosity on the potentials of this organism for genetic research. The subsequent development of sophisticated genetic tools and research during the second half of the century turned *Drosophila* a powerful model organism to link genetics to other biological disciplines, such as development, physiology and behavior [31][32]. Thus, *Drosophila melanogaster*, also generally known as fruit fly, emerged as a model organism well suited to understand the genetic and molecular mechanisms underlying biological systems.

In addition to its powerful genetic tools, *Drosophila* has many other advantages as a model organism for biological research. Fruit flies are easy and cheap to maintain, have a short generation time, and a low redundancy genome. Furthermore, their organs are easy to manipulate and observe due to their low complexity and size. More importantly, the major signaling pathways and development mechanisms between vertebrates and flies are evolutionary conserved [31][32][33]. Moreover, it was discovered that more than 70 per cent of the human disease-related genes have homologues in the *Drosophila* genome [34].

2.1 *Drosophila* life cycle

Drosophila adult females can generate several eggs in just a few days. Indeed, their sperm storage organ allows them to produce a significant amount of embryos

after a single mating. At 25ºC embryonic development lasts for about 21 hr, when the first instar (first stage, L1) larva hatches. This larva is specialized in feeding and grows dramatically during the second and third instar stages (L2 and L3). The transitions between larval stages are called molts, which help for an increase in body size. Each stage last for about one day. At the end of the third instar, larvae migrate away from their food source and pupariate. During pupariation, the larvae undergo metamorphosis: disintegration of most larval tissue to give rise to differentiated cells that will produce adult organs. At day ten, adult flies emerge from the pupal cases. Males require about 8 hours to mature sexually, allowing the collection of virgin females. The times referred are doubled when flies are raised at 18ºC [33] .
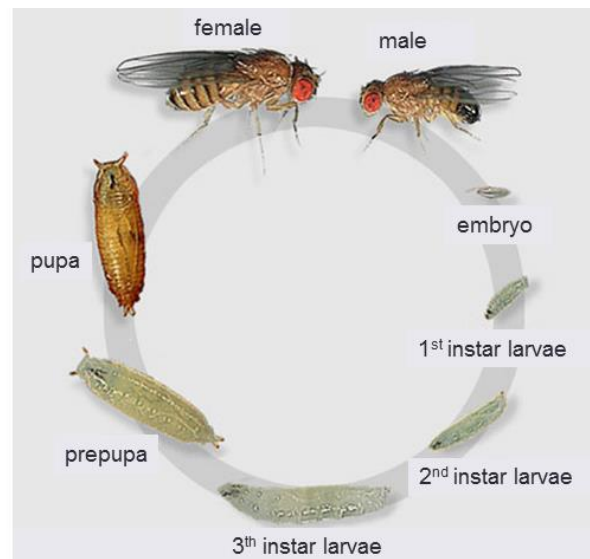
Figure 1: The Drosophila life cycle. After egg laying, the embryos develop subsequently in the first, second and third instar larva. In late L3 larvae crawl out of the food and pupariate. Around day ten the adult flies eclode. Adapted from [35].

3. Eye-antennal imaginal disc as a model to study glia migration

3.1 *Drosophila* imaginal discs

Adult compound eyes, like most other adult *Drosophila* structures, are derived from an sac-like epithelia of larval tissue called imaginal discs. Imaginal discs arise during embryogenesis in response to positional-specific signals that set cells to start

developing asynchronously from the rest of the organism [36]. During larval stages the different imaginal discs proliferate and differentiate according to specific growth control patterns, which will then dictate distinct final organ sizes and shapes (reviewed in [37]). Thus, a precise regulation of imaginal discs development is crucial to guarantee final functional adult *Drosophila* organs.

The imaginal discs are composed by two epithelial layers: the main epithelium (ME) formed by columnar cells, and the overlying peripodial membrane (PM) characterized by their squamous cells. The ME is responsible for giving rise to the final organ itself, and its survival and proliferation during the third instar stage is regulated by the PM (reviewed in [38]).

3.2 Eye-antennal imaginal disc development

The eye-antennal imaginal disc is a flat epithelium that gives rise to the adult eye, antenna, head cuticle, and other head structures such as the ocelli and maxillary palps (Figure 2).
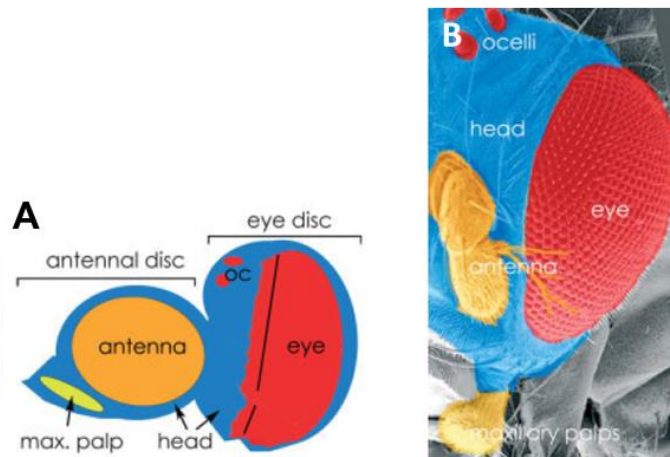
Figure 2: The eye-antennal imaginal discs develop into the fly head structures. The structures in the imaginal disc **(A)** have the same colour to the correspondent adult organ **(B)**. Adapted from [39].

Early in the first instar larval stage, the eye territory is already specified due to uniform expression of the selector genes *eyeless* (*ey*) and its paralogue *twin of eyeless* (*toy*), the mammal PAX6 *Drosophila* homologue [40]. Ectopic expression of these genes

induces the formation of ectopic eyes further proving the role of *ey* and *toy* in eye tissue commitment [41]. At this stage, the signaling morphogen Decapentaplegic (Dpp, BMP-4 family member) responsible for retinal differentiation is also expressed; however, it is inhibited by Wingless action (Wg, the Wnt-1 *Drosophila* homologue [42][43][44].

During second instar larval stage Wg signaling induces eye disc growth so that later in this stage the disc has grown enough to restrict Wg and Dpp signaling to the anterior and posterior margins respectively. As Dpp is, now, not inhibited by Wg, it induces retinal determination (RD) genes activation, like *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*), and leads to the beginning of the retina formation [44][45][39]. Therefore, Wg signaling is responsible for restricting photoreceptors differentiation to the posterior side of the disc, while simultaneously keeping anterior cells actively proliferating through the TALE-type homeodomain homothorax (Hth) protein expression [46][47]. Hth cooperates with Yorkie to enhance *bantam* expression, but it also interacts with RD genes repressing its activity [48][49].

At this stage, Hedgehog (Hh) signal is also expressed in the posterior margin of the eye disc and, together with Dpp, induces the formation of an apical dorso-ventral constriction of the disc epithelium called the morphogenetic furrow (MF). This indentation progresses anteriorly in a wave-like mode representing the boundary between anterior proliferative cells and posterior differentiated photoreceptors. As the morphogen Hh is also expressed in the recent differentiated photoreceptors it drives the progression of the MF by inducting Dpp expression in the furrow. The mutual agonist effect between Dpp and Hh maintains their protein levels such that MF keeps moving [39][50][51].

As the MF sweeps across the eye disc, the anterior cells adjacent to it receive the long range signal Dpp and are induced to undergo a few rounds of cell cycle divisions (first mitotic wave, FMW). Once cells are synchronized they arrest in the G1 phase of the cell cycle in the MF. Here, Dpp signaling inhibits *hth* leading to RD genes up-regulation, which sets cells into a pre-proneural state [49]. Sparingly, some of the cells will be selected to become the atonal-expressing founder cell (R8 photoreceptor) by Hh induction [52][53]. R8, which will be positioned in the middle of the R-axons bundle, will successively induce the R2, R3, R4 and R5 photoreceptors differentiation

[54]. Behind the MF, cells that do not differentiate after the FMW divide one last time (second mitotic wave, SMW) to produce the R1, R6, R7 neuronal cells, and the complement of non-neuronal accessory cone cells to form each ommatidial cluster. By the end of retina formation approximately 800 ommatidia are formed. Pigment cells are added to each cluster during the pupal stage, completing the ommatidia structure [49][55].
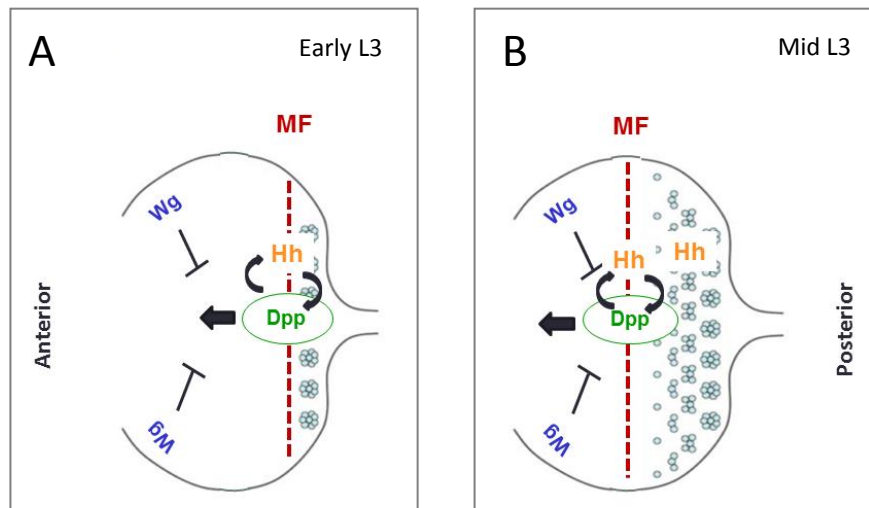
Figure 3: Eye imaginal disc development. Wingless (wg), Decapentaplegic (Dpp), Hedgehog (Hh), morphogenetic furrow (MF). (A) At the early 3rd instar stage the mutual antagonists Wg and Dpp are restricted to the anterior and posterior margins of the eye disc respectively, mainly due to the eye disc size. Dpp and Hh expression in the posterior edge induces the formation of the MF that sets anterior proliferating cells adjacent to it to arrest in G1 cell cycle phase. Then, retinal determination genes are up-regulated leading to the photoreceptors differentiation in the MF. Hh is also expressed in the recently formed photoreceptors inducing Dpp expression in the MF. At the anterior side Wg fine-tune the MF. (B) The MF sweeps from the posterior to the anterior side of the eye disc in a way that in the end of the larvae stages the retina is totally formed.

3.3 Glial cells types in the eye imaginal disc and optic stalk

The diversity of glial cells in the developing eye is apparently larger than it was predicted on glia initial studies [56][57]. Together, electron microcopy analysis and the generation of a Flipase recombinase specific to glial cells allowed a deeper understanding of the morphology (Figure 4) and functionality of the eye disc glia [57]: five morphologically distinct glial cells types were identified in the developing eye and

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optic stalk (OS), a tubular structure that connects the eye disc to the optic lobe in the brain; however, all glial cells types can be grouped in three major classes: the subperineurial glial cells, the perineurial glial cells and the wrapping glia.

3.3.1 Perineurial glia

When the optic stalk is formed in the second instar stage few perineurial glial cells are already populating the OS (Figure 4B, nº 1) [57]. They are surrounding the Bolwig´s nerve, a bundle of eight neurons that connects the eye field to the brain since the embryonic stage, and thus it prefigures the OS [58].

In the eye disc, perineurial glia is only found in the most basal layer of the epithelium. Here, they display migratory abilities and usually have a spindle-like appearance similar to the perineurial glia in the OS (Figure 4B, nº2) [57]. Close to the MF, the perineurial glial cells at the leading edge of the glial territory start to extend filopodia-like cell protrusions (Figure 4B, nº3,4). Current models propose that perineurial glial cells follow the ones at the leading edge migrating as a sheath ([57], and reviewed in [59]). At the lateral edges of the glial field, another type of RBG (edging glia) can be found, which, although more elongated, have a similar appearance to the migratory perineurial glia (Figure 4B, nº5). Yet, it is not clear if the edging glia originate from the migratory perineurial glia or, alternatively, they derive from distinct progenitor pools [57].

Perineurial glia are the only glial type capable of some proliferation potential [57], which suggests that they can generate more glial cells when required (injury/damage situations); however, there is still no clear evidence for that.

On the most basal side of the eye disc and, in the outer layer of the OS, the perineurial glia contacts with a collagen-rich extracellular matrix that may be important for its morphology and/or functionality. It was showed that mutations in focal adhesion Kinase (Fak) disrupts the OS structure, a phenotype that can be rescued by Fak56D expression in perineurial glia but not in photoreceptors [60].

3.3.2 Wrapping glia

In the eye disc, the perineurial glia with filopodia-like processes contacts the nascent photoreceptor axons and initiates the differentiation into wrapping glia cells. Accordingly, Silies et al. [57] showed that LacZ expression is still present in wrapping glia cells when induced in perineurial glia.

As the name suggests, these cell types are responsible to wrap the ommatidial axon bundles that are wrapped as units (Figure 4B, nº3). Therefore, wrapping glia are found in more apical regions to the perineurial glia cells, in the axonal layer ([57], and reviewed in [59]). Importantly, wrapping glial cells frequently extend long cellular processes until the brain following the R-axons. Occasionally, they also project their membranes along the photoreceptors into the apical side of the eye disc [57]. Wrapping glia can also be found in the OS where they associate with the R-axons [57].

3.3.3 Subperineurial glia

Similar to the perineurial glia, the subperineurial glia can be observed sooner in development in the OS. Later in the third instar, they are also present in the eye disc where they ensheath the entire differentiated part of the epithelium (Figure 4B, nº6). Although there are only two subperineurial glial cells, they are extremely large, probably a consequence of their remarkable nuclear size most likely due to endoreplication. As they have a very flat appearance they are also called carpet cells [57].

The subperineurial glia is always apical to the perineurial glia, separating the latter from the wrapping glia. Furthermore, close to the MF, the carpet cells reach about one cell posterior to the migrating glia and, in the posterior cell margin, carpet cells extend until the lamina in the brain. It is thought that subperineurial cells probably function as a platform to perineurial glia migration and have a role in the organization of all glial cells in the eye disc ([57], and reviewed in [59]).   Carpet   cells are characterized by their highly interdigitated septate junctions forming a tight epithelium that sets up the blood brain barrier [61].
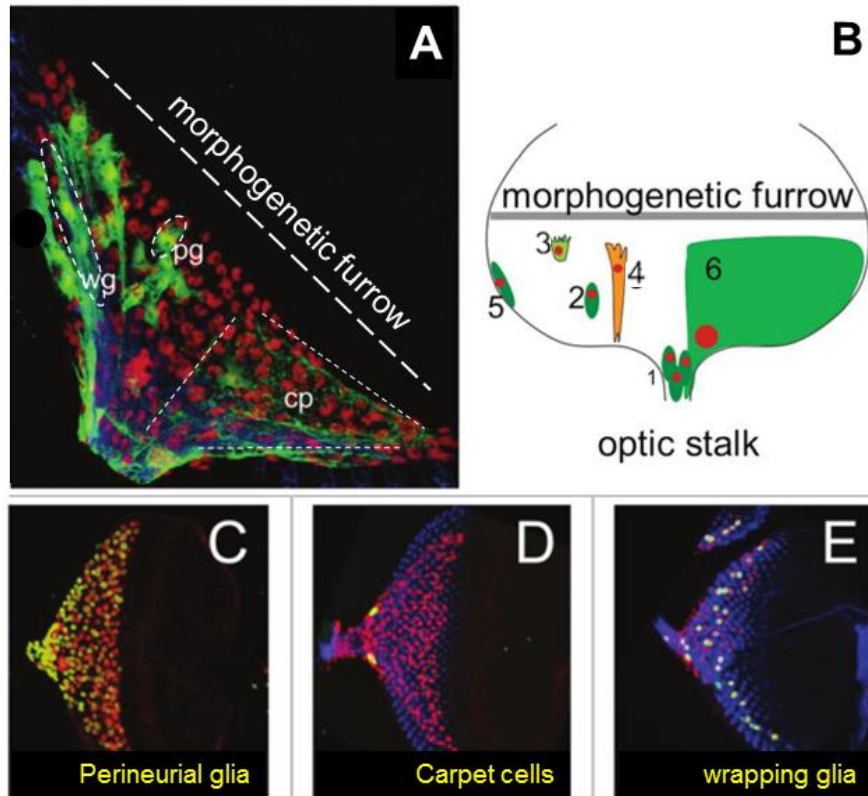
Figure 4: Glial cells types in the third instar eye imaginal disc and optic stalk. (A, D-E) Discs are stained for all glial cells types (red), neuronal membranes (blue) and glial cell membranes (green). Yellow staining represents the nuclei of all perineurial glia (C), carpet cells (D) and wrapping glia (E). **(A)** At the right side of the image it can be seen the membrane of one gigantic carpet cell (cp, note the schematic dash line surrounding it) and on the left side it is present the numerous processes of the wrapping glia (wg, note the schematic dash line surrounding it) that follow the photoreceptors axons. It is also recognized the membranes of perineurial glia (pg, note the schematic dash line surrounding it). **(B)** Schematic representation of the morphological distinct glial cell types. It is represented the perineurial glial cells in the optic stalk (1) with a similar morphology of the migratory glial cells in the eye disc (2, comparable with pg in A) and perineurial glia with filopodia (3), at the edge of the carpet cell (6, comparable with cp in A), which differentiates in wrapping glia (4, comparable with wg in A). It is also shown elongated glial cells at the margin of the eye imaginal disc (5). Adapted from [58] and [59].

3.4 Glial role and migration in the eye imaginal disc

During the second instar larval stage glial cells proliferate and accumulate in the OS. Glia migration into the eye disc only starts at the beginning of the retinal patterning where the differentiating apical photoreceptors start to extend their axons into the basal layer of the disc. During the third instar stage more and more glial cells

populate the disc and at the end of the larval stage about 350 glial cells are found in each eye imaginal disc [56][57].

As soon as neurogenesis starts, carpet cells extend basally its anterior cell margin behind the MF and the perineurial glia present in the OS start to migrate, following the carpet cells expansion (Figure 5). As the MF advances, carpet cells keep covering the entire differentiated portion of the eye disc and the perineurial glia continue to respond after carpet cells extension. A constant coverage of the outer surface of carpet cells by perineurial glia is then ensured. A few cell rows behind the MF, the migratory perineurial glia start to form extensive filopodia and contact with the recently formed axons. This interaction drags the perineurial glia from the carpet cells and triggers the differentiation of the perineurial glia from a migratory cell type to wrapping glia. They now ensheath the nascent ommatidia and follow the R-axons from the basal portion of the disc through the OS until the optic lobe ([57], reviewed in [58] and [59]).

Cell–cell communication is essential to establish neuronal connections in the developing eye disc. Growth cones, specialized sensori-motor structures at the leading edge of axons, are guided to their final destination by perceiving and interpreting different attractive and repulsive guidance cues in their environment [62]. Importantly, after reaching the brain, R-axons target specific innervated layers: while R1–R6 axons terminate in the lamina, R7 and R8 target deeper into the medulla [63]. A role for glial cells in axonal outgrowth has been demonstrated in the *Drosophila* larval brain [63]; however, in the eye imaginal disc it doesn´t seem that RBG cells are crucial for axons pathfinding [64]. Specifically, Poeck et al. [63] showed that when laminal glia present in the optic lobe are removed a large number of R1-R6 axons project through the lamina until the medulla, suggesting that glial cells in the brain are crucial to ensure proper targeting of R-axons into the optic lobe. In the eye imaginal disc it was, initially, hypothesized that RBG is important for axonal guidance since the creation of ectopic positions of RGB in the anterior region of the eye disc led to R-cell axons to follow ectopic glia rather than extension towards the OS [64]. However, recent results published by us [65] show that the absence of glial cells in the eye disc does not impairs axons outgrowth into the OS. In this report we show, in *Drosophila* retinal glia, a dual role for integrins. Integrins are cell membrane receptors that mediate cell-cell

and cell-extracellular matrix interactions and often associate with other membrane receptors to modulate signaling pathways within the cell. In *Drosophila*, only 3 heterodimers with vertebrate orthologues are commonly expressed: PS1 (αPS1/βPS), PS2 (αPS2/βPS) and PS3 (αPS3/βPS). While αPS3 expression is visible in the perineurial to subperineurial glia region, αPS2 is normally expressed in the outermost membrane of basal glial cells layer. Interestingly, by knocking-down αPS2 or βPS the number of glial cells in the eye disc decreased almost to zero as well as significantly less glia in the OS was observed when compared to the control. Importantly, in this experiment, no defects in photoreceptor axons targeting to the OS were detected; however, photoreceptor axon bundles were not wrapped and displayed a disorganized pattern. Therefore, we suggest that glial cells are important for R-axon wrapping and organization but not for axons targeting into the OS. Further research is needed to understand if less glial cells in the eye disc can lead to targeting defects in the brain. In contrast, αPS3 depletion in glia did not decrease glial cells number but R-axons became stalled in the OS. As αPS3 is expressed in perineurial glia, this result also supports a role for glial cells in axonal wrapping.

Photoreceptor neurons are born in the eye disc while all glial cells derive from progenitors present in the CNS or the OS [56]. Therefore, glial cells need to migrate into the eye imaginal disc to ensure correct R-axons bundles ensheathment. The temporal and spatial tight coordination between the initiation and progression of the photoreceptors differentiation and glial cells migration is crucial to ensure the proper development of the neuronal networks and, consequently, the formation of a functional eye. In agreement, the number of glial cells in the eye disc rises with the number of differentiated photoreceptors [57]. Importantly, such as the axons interpret attractive and repulsive signals from their surroundings, perineurial glial cells also receive both positive and negative cues to direct its migration.
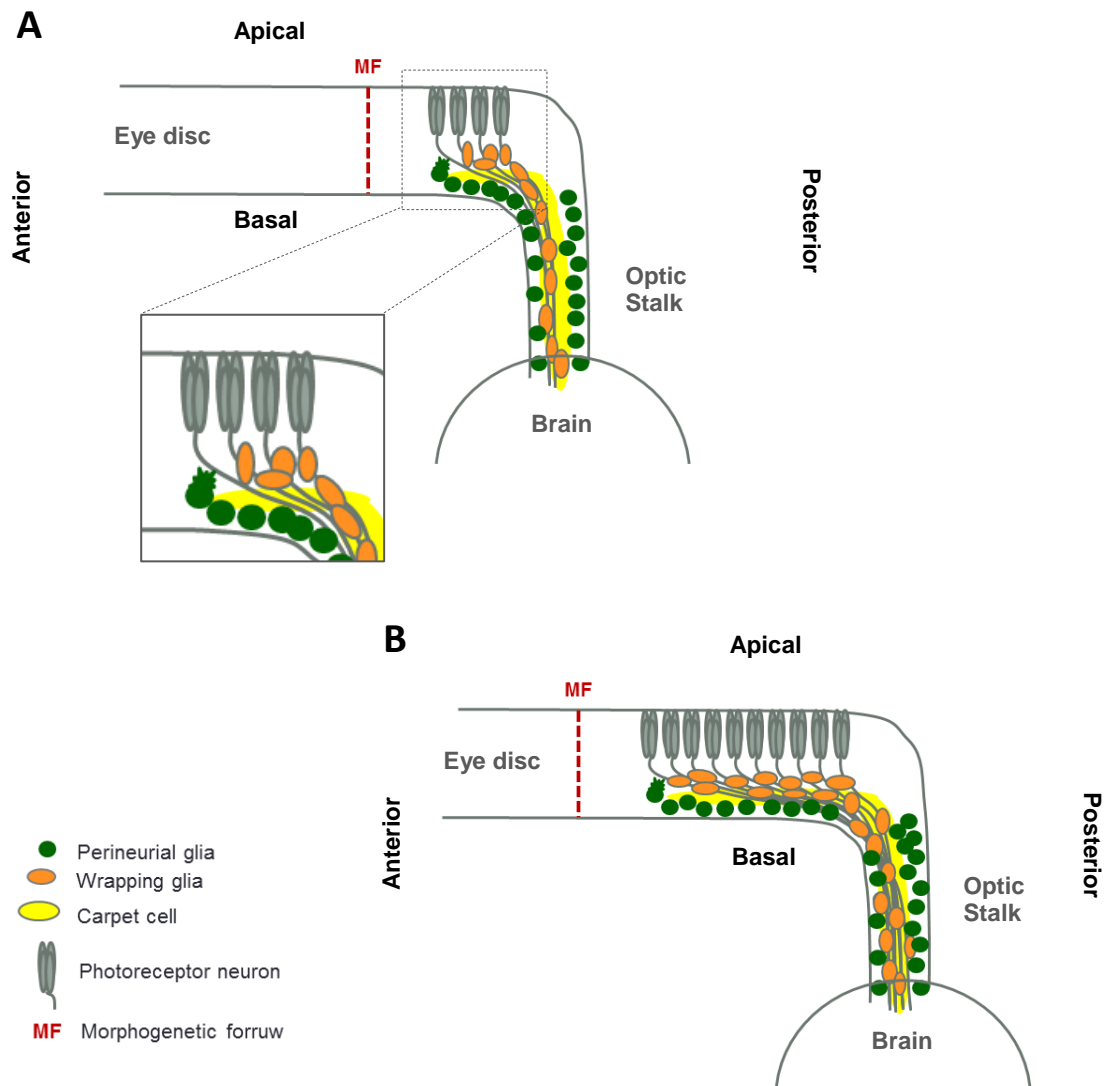
Figure 5: Glial cells migration into the eye imaginal disc. (A) As the retinal patterning begins, perineurial glia from the optic stalk start to migrate to the basal layer of eye disc, following the anterior cell margin of the carpet cells. Close to the MF, perineurial glia extend filopodia-like processes and contact with the nascent axons. Axons grow from the recent apical photoreceptors until the basal portion of the eye disc. This interaction triggers glial cells to differentiate in wrapping glia. **(B)** As the MF sweeps across the eye disc more glial cells populate the eye territory to wrap the axons of the new photoreceptors row.

3.4.1 What are the cues that trigger glial cell migration?

The mechanisms underlying glia migration into the eye imaginal disc are still not well understood. It is clear that some signals implicated in the eye disc development are also involved in the regulation of glial cells migration, as well as, it

seems that photoreceptors axons regulate glial migration directed to them [64][66]. However, more extensive research is needed to fully understand this process.

Rangarajan et al. [66] was the first in identifying a molecule involved in the control of glia migration in the eye disc. They showed that clones of Dpp-expressing cells induced in most anterior regions of the eye disc are capable of inducing glial cells migration beyond the MF. In contrast to what happen when ectopic patches of photoreceptors are created, clones without formed axons did not induced glia migration directed to them. This suggests that the Dpp protein have a role in synchronizing the initiation of the neurogenesis with the beginning of the glia migration into the eye disc; although not guiding glial cells. Importantly, this result also shows that photoreceptors were able to induce glia migration in their direction.

Later, Hummel et al. [64] observed that, a Kinase involved in the wingless signaling cascade, Gilgamesh (Gish), is also implicated in the control of glial cells migration. When eye progenitor cells was rendered homozygous mutant for *gish,* glia migrated into the eye disc in the second instar stage where no photoreceptors are present. This result suggests that glial cells don´t need photoreceptors signals to migrate into the eye disc and this protein acts as an inhibitory signal to prevent glia migration before the beginning of the neurogenesis. In the third instar stage, they also observed glia migration beyond the MF. At this stage, the two most important proteins in the regulation of the eye disc development, Wg and Dpp, were implicated in the control of glial cells migration into the eye disc. Hummel et al. [64] also showed that mutation in the RD genes *so* or *ey* induce glia migration into the eye disc in the second instar stage. Thus, *so* and *ey* are also important to prevent precocious glia migration.

Another signal from the eye disc that could influence glia movement is the Hh protein. It was showed that a mutation in this protein led to glia migration into the eye disc before the axonal patterning; however, a mutant for the receptor of Hh (*smoothened*) in glial cells did not induced defects in glia migration [64]. Thus, the role of Hh in glia migration it´s still not clear.

In conclusion, it seems that signals involved in the eye disc development work together to ensure proper migration of RBG over time. While some molecules function as attractants others behave as repulsive cues and the balanced between all of them is

crucial to guarantee precise glia migration. Moreover, these results suggest that axons are sufficient but not necessary to stimulate glia migration.

Finally, FGF signaling was also implicated in the regulation of glia migration (see below).

4. Fibroblast Growth Factors

4.1 Biological functions of Fibroblast Growth Factors

Fibroblast Growth Factors (FGFs) are an evolutionarily conserved family of secreted signaling molecules that perform a wide spectrum of roles in development. In vertebrates, FGF signaling is important in early stages of development to control mesoderm induction and formation [67] and to establish the dorso-ventral body axis [68]. Later, they are involved in brain patterning [69] and branching morphogenesis of tubular organs such as lungs [70]. Interestingly, in adult organisms, FGFs are implicated in tumorigenesis [71].

In contrast to mammalian organisms where 23 ligands and four receptors are found [72], in *Drosophila melanogaster* only three ligands (Pyramus, Pyr; Thisbe, Ths; Branchless, Bnl) and two receptors (Heartless, Htl; Breathless, Btl) exist (reviewed in [4]). This minor redundancy of FGFs in *Drosophila* makes the study of FGF signaling a simpler task.

As in vertebrates, FGF signaling in *Drosophila* is involved in the development of distinct tissues. Htl signaling is crucial in successive morphogenetic events during mesoderm layer formation and differentiation. Specifically, Htl receptors expressed in mesoderm cells drive mesoderm spreading and differentiation into specific lineages such as heart and somatic muscles. In *htl* null-mutants the mesoderm layer is not correctly positioned leading to heart cell differentiation ablation [73][74]. Furthermore, Htl signaling induces Myocyte enhancing factor 2 transcription, a key regulator of somatic muscle differentiation [75]. Htl signaling is also important to control nervous system development by providing neurons-glia communication.

Activation of this receptor in glial cells induces glia proliferation and differentiation, thus supporting the correct wrapping of neuronal axons by glial cells [76].

In contrast to Htl, Btl is involved in the tracheal system development. Btl signaling in tracheal and air sac cells is crucial for all the primary, secondary and terminal branches formation in the embryo and for air sac formation in the larva, respectively [77][78]. Additionally, Btl is also involved in some aspects of the nervous system development (discussed below in detail).

4.2 FGF signaling pathway in *Drosophila*

FGF signaling in *Drosophila melanogaster* shows significant similarities with the FGF pathway in mammals. Although some molecular features are still not addressed, downstream activation of the mitogen activated protein Kinase (MAPK) pathway and cytoskeleton rearrangement events are shared between the two systems. In both organisms, FGF signaling begins when extracellular FGFs bind to the respective FGFs receptor (FGFR) present in the target cell surface, activating an intracellular signaling cascade. Depending on the FGF-FGFR combination and cellular context, this signaling pathway may result in various cellular responses, including cell survival, proliferation, differentiation and/or migration [79][4].

FGFRs are members of the Receptor Tyrosine Kinase family, and therefore, they share many signaling components [79]. In *Drosophila*, ligand binding to FGFR promotes tyrosine phosphorylation of the receptor and of its adaptor protein Stumps/Downstream of FGF (Dof) [4]. Dof, the *Drosophila* FRS2-unrelated protein, contains multiple clusters of tyrosine residues that function as docking sites for signaling factors [4]. These include the tyrosine phosphatase Corkscrew (Crw) [80], (SHP2 in vertebrates), and the downstream of receptor Kinase (Drk) [81], the *Drosophila* homolog of the vertebrate Grb2 adaptor protein, both involved in the downstream activation of MAPKs. Upon Drk binding to Dof, Drk recruits Son of sevenless (Sos), the guanine nucleotide exchange factor, to activate the small GTPase Ras85D [82]. GTP-Ras85D activates the Draf protein kinase (*pole hole*, MAPKKK), which leads to the propagation of the signal trough the MAPK cascade [83]. Draf phosphorylates Dsor (Downstream of raf1, MAPKK), which phosphorylates ERK (*rolled*,

MAPK) resulting in the phosphorylation of transcription factors, such as Ap1 and Pnt [84]. Activation of these transcription factors leads to expression of target genes involved in cell proliferation and survival. Crw is implicated in the MAPK cascade activation by repressing Sprouty, an antagonist of the FGF signaling [85].

In addition to MAPK cascade activation, FGF signaling might also control regulators of the cytoskeleton and cell adhesion molecules. Indeed, the Rho-GEF Pebble (Pbl), the orthologous of the vertebrate proto-oncogene Ect2, is required for the Htl-dependent migration of mesoderm cells during gastrulation [80]. Rho-GEFs (nucleotide exchange factors) are important regulators of small GTPases, as Cdc42 and Rac proteins, that control actin cytoskeleton dynamics [86][87].
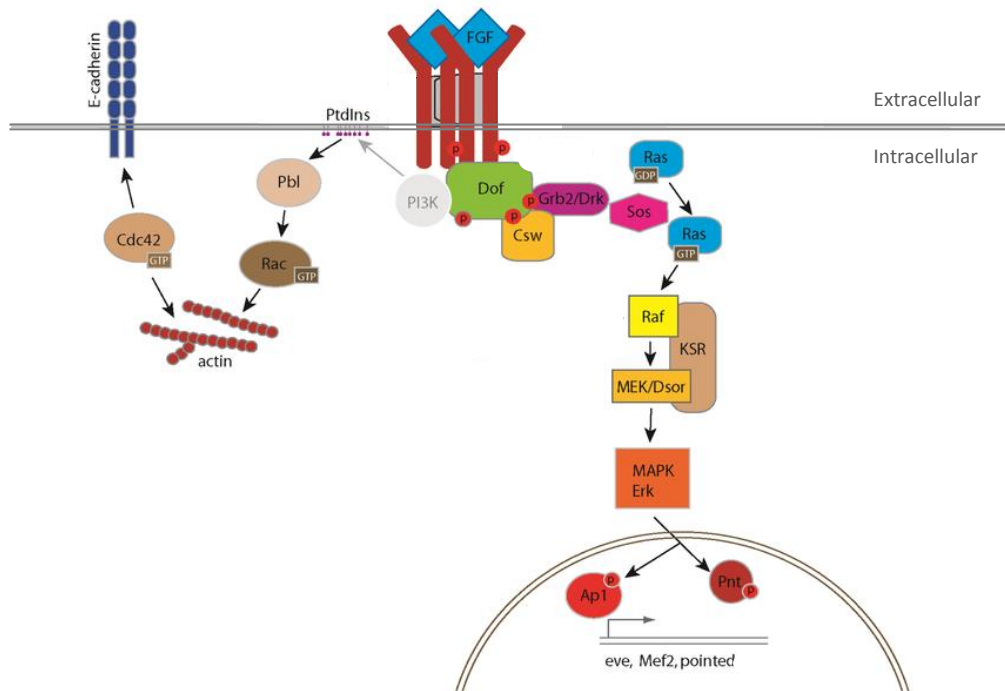
Figure 6: Schematic model of FGF signaling pathway in *Drosophila melanogaster*. FGFs bind to the respective FGFR, activating the FGF signaling cascade. This interaction triggers the auto- and tras-phosphorylation of the tyrosine kinase domains of the FGFR that phosphorylates its adaptor protein, Dof. Dof recruits both Csw and Drk proteins due to its docking sites enriched in tyrosine residues. Activation of Csw and Drk leads to MAPK activation that, ultimately, induces gene transcription. These gene targets encode proteins involved in proliferative and non-apoptotic responses. FGFRs activation also leads to RhoGEF Pbl activation, which are important for the formation of actin protrusions in the mesoderm. Actin protrusions are formed in a Rac and Cdc42 -dependent manner; however evidence for Cdc42 activation through FGF signaling is lacking. Pbl activation could result from modifications on the

phosphatidylinositol (PtdIns) composition of the plasma membrane induced by PI3K (Phosphatidylinositol-3-kinase). The grey arrow and grey circle represent a putative interaction and putative component of the FGF pathway, respectively. Adapted from [4].

Due to the significant amount of ligands and receptors, mammalians can generate around 70 FGF ligands-receptors combinations, while in *Drosophila* only three FGFs functional pairs are found: Pyr-Htl, Ths-Htl and Bnl-Btl. Domain swapping experiments demonstrate that Btl cannot be activated by Pyr or Ths, and that Htl cannot be activated by Bnl [88]. This specificity of ligand-receptor interaction probably results from structural differences between Btl and Htl, and between the three FGFs. Btl and Htl have different numbers of Imunoglobulin(Ig)-like domains at their extracellular domains [73], and the FGFs differ in the position of their FGF-core domain. The FGF-core domains of Pyr and Ths are highly homologous to the vertebrate FGF-8 [89], whereas the FGF-core domain of Bnl is not significant similar with any core domain of a vertebrate FGF [90]. Importantly, the *Drosophila* FGFs primary sequence contains an amino-terminal signal peptide, important for FGF secretion, and a long carboxy-terminal sequence with no significant homologies to other proteins [89].
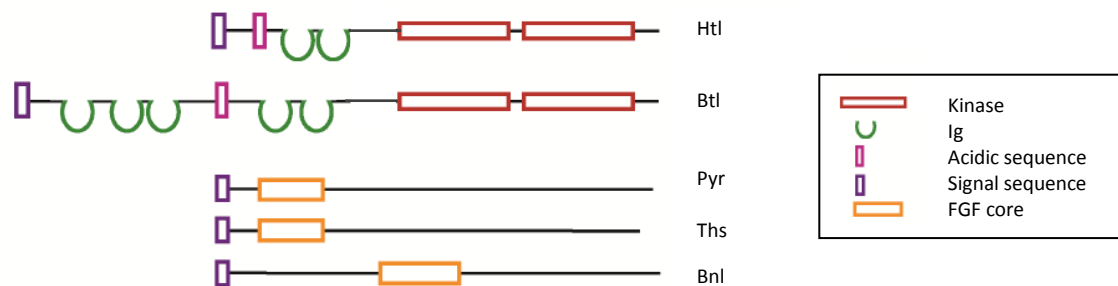
Figure 7: Domain structures of the *Drosophila melanogaster* FGFRs and FGFs. Adapted from [4].

Despite the structural differences and the distinct developmental functions of Htl and Btl, they both activate the same intracellular signaling pathway. Thus, interactions between the FGF pathway and components of additional signaling cascades may be required to induce specific cell responses. Interestingly, Myat et al. [91] showed that genetic interactions between *btl* and components of the Dpp signaling pathway control the migration of tracheal branches during embryogenesis.

4.3 FGF signaling in cellular proliferation, differentiation and survival

Downstream activation of transcription factors through FGF signaling induces expression of a wide range of coding genes. Evidence exists that activation of the FGF cascade leads to changes in the cellular *status* by inducing proliferation or differentiation.

In the developing larval brain and eye imaginal disc it was shown that, as the neurogenesis occurs, perineurial glia proliferation is stimulated by neuronal expression and secretion of Pyr [76]. Interestingly, after glial cells enter into the eye imaginal disc, they also autonomously control its own proliferation by expressing and secreting Pyr [76]. In the embryo, both Btl and Htl signaling seems to be dispensable for cell proliferation during mesoderm and trachea morphogenesis; however, they are important for the differentiation of these structures. During branch formation, Btl activates expression of genes, such as *pointed* and *blistered*, controlling secondary and terminal branches formation respectively [77]. In the developing mesoderm layer, Pyr, but not Ths, is required for the formation of Eve-positive mesodermal precursor cells, triggering these cells to differentiate into pericardial and dorsal muscle founder cells [92]. On the contrary, a function for Ths but not for Pyr in differentiation is known in the developing eye disc where expression of *ths* in the photoreceptors induces perineurial glia differentiation into wrapping glia [76].

Evidence for FGF signaling in cell survival also exists. Indeed, in *htl* mutant most caudal visceral mesoderm cells die [93].

4.4 FGF signaling in cellular migration

Probably the best well known process of cellular migration induced by FGF signaling in *Drosophila* is the mesoderm spreading, the final stage of its formation. The development of this structure starts with the invagination of the ventral furrow of the embryo forming an epithelial tube, which subsequently loses its epithelial structure to adopt a mesenchymal morphology - epithelial to mesenchymal transition (EMT). Finally, mesoderm cells spread leading to the formation of a transient monolayer. During these processes, both *ths* and *pyr* are expressed in the underlying

neuroectoderm and are responsible for the induction and control of the mesoderm migration. During EMT, FGFs induce changes in E-cadherin distribution along the cellular membrane in an Cdc42-dependent manner; during flattening, FGFs induce actin-dependent protrusions towards the ectoderm, promoting mesoderm-ectoderm attachment; and during spreading FGFs acts as attractants: Pyr is required for dorsal migration, while Pyr and Ths, together, induce radial migration [94]. Clark et al. and Van Impel et al. also showed that while Cdc42 is involved in the formation of radial protrusions, Rac specifies dorsal protrusions. [94][95]. Importantly, FGFs remodel E-cadherin adhesions probably to help promoting mesoderm-ectoderm attachment and guidance of mesoderm migration by inducing directional protrusive activity.

A role for Btl signaling in cells migration also exits. It was recently described by Mukherjee et al. [96] that, during eye development in the third instar stage, increased activity of Btl in glial cells disrupt their migration, thus leading to its accumulation in the OS. Furthermore, Bnl inhibition in eye progenitor cells stimulated glia migration. Specifically, they created clones of Bnl depleted cells and they observed that glial cells migrated beyond the MF until the anterior portion of the eye disc. Therefore, it is proposed that Bnl act as a non-autonomous negative regulator of excessive glial cells migration by binding and activating the Btl receptor expressed by glial cells. This finding represents another layer in the understanding of the temporal and spatial control of glial cells migration.
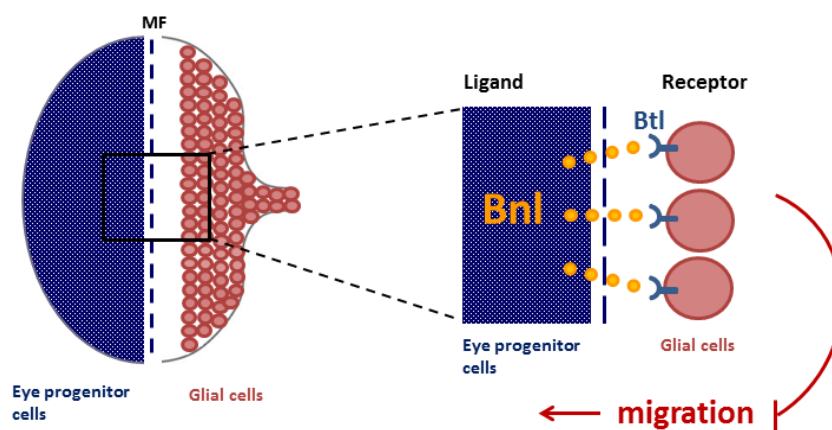
Figure 8: Bnl/Btl signaling in glial cells migration. MF: morphogenetic furrow.

Aims

Tissue development is a complex biological process that requires an interplay between a wide range of morphogenetic signals. The interpretation of these distinct signals is crucial for both cellular homeostasis and challenging situations, thus controlling each cell fate. Defects in growth and/or differentiation cues frequently lead to tissue pathologies.

The FGF pathway has a crucial role in the regulation of developmental mechanisms, including in glial cell migration: one of the main scientific interests of our laboratory. On the course of a previous study addressing the role of the Bnl protein in the eye disc, phenotypes in glia migration were observed but not addressed in detail. Therefore, we decided to re-address the function of Bnl in glial cell migration using a combination of genetic tools. After confirming that Bnl function is required for correct glia migration, we also aimed to identify signaling partners that could cooperate with Bnl in its function in this tissue.

As the FGF signaling is conserved from insects to mammals this work can be of general relevance to further understand other developmental processes in more complex organisms.

Chapter 2

Material and Methods

Experimental design

Most of the work present here was accomplished based on Gain-of-function (GOF) and Loss-of-function (LOF) approaches. GOF induces excessively or ectopically gene expression or stimulate its function and, contrary, LOF attempts to decrease or totally eliminate gene function. To perform these experiments, flies with mutant alleles and the GAL4-UAS binary system was used.

Gal4 is a transcription factor from yeast that controls the expression of genes downstream of UAS (upstream activation sequence) elements. As the Gal4 protein does not exist endogenously in flies it does not act on any endogenous loci. Importantly, the Gal4 regulator and the UAS construct are kept at independent transgenic lines and the system is only activated when the two fly lines are crossed. In the progeny, the produced Gal4 protein binds to the UAS element in the DNA, which activates the expression of it downstream target gene. The expression of the Gal4 gene is regulated by an endogenous regulatory sequence (driver) which mean that the offspring display the expression of the UAS-linked gene only in the cells with the chosen Gal4 driver [32][97].

The Gal4-UAS system can also regulate RNA of interference (RNAi) to knock down endogenous genes in a specific type of cells. The expression of dsRNA targeting the UAS-linked gene of interest is activated by the Gal4 driver and, consequently, the RNAi formed induces the degradation of the target gene mRNA [97].
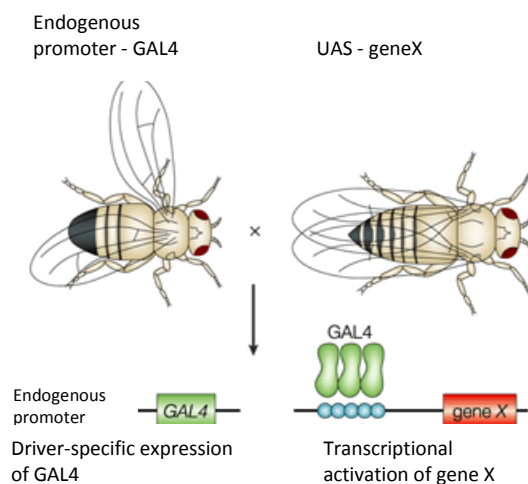
Figure 9: **Gal4-UAS system.** Two separate lines carry the Gal4 or the UAS regulator. When crossed, the Gal4 produced under the control of a specific endogenous driver binds to the UAS, activating the transcription of the UAS downstream gene. Adapted from [98].

Fly crosses and genotypes

Mating crosses were raised at 25 °C under standard conditions. The following stocks (described in FlyBase, http://flybase.org/reports/FBgn0262656.html, unless stated otherwise) were used: *w^1118^, ey-Gal4 (eyeless), UAS-lacZ (II), hth-Gal4 (homothorax), UAS6*mycGFP, repo-Gal4* [28]*, moody-Gal4;UAS-CD8GFP* (subperineurial glia [61][99]), *Mz97-Gal4, UAS Stinger* (wrapping glia [64]), 3'atonal1.2-pGWGFP, *UAS-bnlRNAi* (Vienna Drosophila RNAi Center (VDRC) #5730), *UAS-btl^λ^, UAS-bnl* [77]*, UAS-mycRNAi* (VDRC#2948), *UAS-dmyc, UAS-bsk^DN^, pucE69=pucLacZ* [100], *Df(3L)H99, tubulin CD8 small Drice Histone GFP, hid-5'F-GFP, rpr-11kb-lacZ, UAS-diap1-131.9, UAS-dp53RNAi (*VDRC#10692*), UAS-p53^R115H^, dm4/FM7,actGFP.*

Immunofluorescence of larval tissue

Third instar larvae discs were fixed in 4% formaldehyde in PBS for 20 min and washed 3×10 min in 0.1% Triton X-100 in PBS (PBT). Samples were then incubated in PBT with the primary antibody at room temperature (RT), in constant agitation, for 2h30min or 3 hours when mouse or rabbit anti-repo were used, respectively. After 3×10 min washes in PBT, samples were incubated in PBT with the secondary antibody and DAPI (1:1000) for 1h30 min at RT in agitation. Once again samples were washed 3×10 min in PBT and then mounted in 20 % glycerol in PBS. Images were taken with Leica SP5 confocal microscope at 40× magnifications and treated with the Adobe Photoshop CC software.

Primary antibodies used were: mouse anti-Repo at 1:10 dilution (DSHB 8D12), rabbit anti-Repo at 1:25000 (a gift from Benjamin Altenhein), goat anti-HRP Cy5 conjugated at 1:100 (Jackson ImmunoResearch 323-175-021), mouse anti-Elav (9F8A9) at 1:50 (DSHB), rabbit anti-pErk at 1:200 (Cell signaling 4370), mouse anti-Gliotactin at 1:250 (a gift from Vanessa Auld), rabbit anti p-Mad at 1:100 (Cell signaling 9516), mouse anti-Mmp1 at 1:25 (1:1:1 DSHB 14A3D2, 3A6B4, 5H7B11), rabbit anti-cleaved Caspase-3 (Asp175) at 1:1000 (Cell Signaling 9661), rabbit anti-pH3 at 1:1000 (Upstate), rabbit anti-Beta-galactosidase at 1:2000 (MP Biomedicals 55976), rabbit anti-GFP at 1:1000 (Molecular Probes A11122). The last two antibodies were used to

mark the expression pattern of a specific protein on which the correspondent gene was flanked by a *lacZ* or *gfp* insertion in reporter fly lines. Appropriate Alexa Fluor conjugated secondary antibodies were from Molecular Probes.

Lysotracker staining

For Lysotracker staining, samples were washed 2×10 min in PBS after dissection, incubated in a dilution of 1:500 in PBS for 10 min at RT in constant agitation, and washed again 2×10 min in PBS. After that, the imaginal discs were fixed and immunostained as described above.

Statistical analysis

To compare the percentages of eye discs displaying glia overmigration/no-overmigration in different genotypes we performed a two way-ANOVA taking in account three biological replicates in each genotype. On figure 15, statistical significance of different proliferation rates between overmigrating and no-overmigrating glial cells in Bnl depleted eye discs and relatively to control discs was tested by using the Welch´s t-test. Number of pH3 positive glial cells and the total number of glial cells was counted in all area of overmigrating and no-overmigrating glial cells and in all the glial field of control discs. Proliferation rates were calculated by using the following formula: $\frac{n^{\underline{o}}\ of\ pH3\ positive\ glial\ cells}{total\ n^{\underline{o}}\ of\ glial\ cells} \times 10$ (u.a.). In both analysis, p-values are shown with the following asterisk code: $*=p<0.05$; $**=p<0.01; ***=p<0.001; ****=p<0.0001$. Error bars present in all graphs represent the standard deviation.

Bnl down-regulation in eye progenitor cells

To down-regulate *bnl* in eye progenitor cells we used two different eye-specific Gal4 drivers, *homothorax* (*hth*) and *eyeless* (*ey*) (Figure 10). The Bnl depletion was accomplished by crossing one of these lines with and RNAi line targeting *bnl*. An schematic view of these crosses can be visualized in figure 11.

*hth>UAS6*mycGFP*   *ey>UAS6*mycGFP*
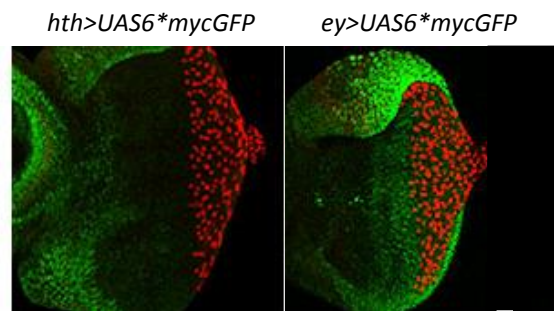
Figure 10: Expression pattern of *homothorax* (*hth*) and *eyeless (ey)*.
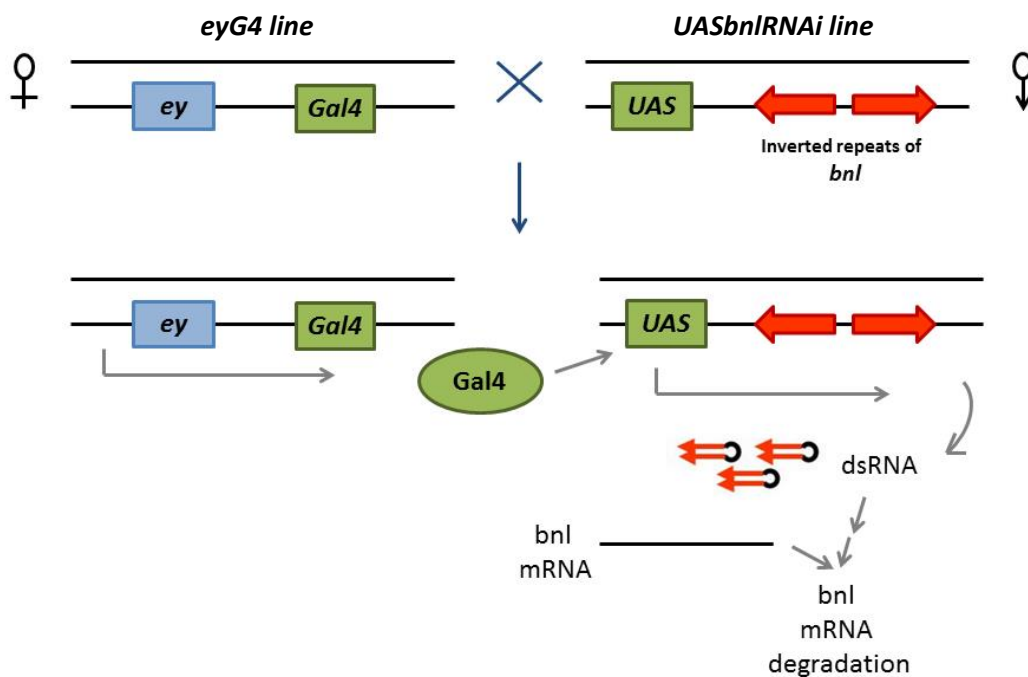
Figure 11: Scheme of the genetic cross performed to down-regulate Bnl in *ey*-expressing cells. A female with a *Gal4* gene downstream of the *ey* endogenous gene was crossed with a male, on which an UAS construct was inserted upstream of the endogenous *bnl* gene. In this line were also inserted inversed repeats downstream of UAS. The progeny of this cross produce Gal4 proteins exclusively in *ey*-expressing cells that binds to the UAS construct. Downstream transcription of inversed repeats give rise to dsRNA that, after some structural alterations, target the endogenous bnl mRNA for degradation.

Chapter 3

Understanding the role of Bnl in the eye imaginal disc

Results

Bnl/Btl signaling limits perineurial glia migration in an non-autonomous manner

As referred in the aim of the thesis, we are interested in understanding how *bnl* is able to regulate RBG migration in the eye disc. To answer this question we started by knocking down *bnl* in eye progenitor cells (EPCs), where this signal is produced and secreted [96]. For this purpose, we used different *Gal4* lines expressed in EPCs to drive expression of a *UAS-bnlRNAi* construct, which was also used by Mukherjee et al. [96].

Initially, *bnl* was down-regulated in EPCs under the control of the eye-specific *eyeless-Gal4* (*eyG4*) driver. This resulted in a glia phenotype; specifically, in most eye discs it was observed that glial cells migrated beyond their normal boundaries (hereafter called overmigration). Overmigration was never observed in controls but was found in 95% of the eye discs where Bnl was depleted (Figure 12 A, B and H). Different levels of overmigration were observed after Bnl depletion. Thus, to better classify and quantify phenotypes with different severities two phenotypic classes were defined. One where glial cells overmigrated but did not pass the MF, and a second one, more severe, where glial cells were found beyond the MF. Using this classification, 20% of the eye discs where Bnl was depleted presented the less severe phenotype, and the majority of the eye discs showed the most severe phenotype where glial cells overcomed the MF (Figure 12 H). Interestingly, transversal views of the eye discs showed that glia did not overmigrate as a mono-layer but instead they moved as a multi-layered single group (white arrow, Figure 12 E, F). Furthermore, we often found carpet cells extending more anteriorly in the eye when compared to a control condition (white arrows, Figure 12 A, B). As expected based on the Bnl expression pattern, when *bnlRNAi* was expressed in glial cells (using *repo-Gal4* as a driver) no phenotype was observed (Figure 12 C). Altogether, these results enhance the ones previously described [96] meaning that in the eye disc Bnl is important to limit glia migration in a non-autonomous manner.

To further understand if Bnl functions as a long range signal in the regulation of RGB migration, Bnl was depleted only in *homothorax(hth)*-expressing EPCs localized in the most anterior region of the eye disc (using *hthGal4* as a driver). As observed in *eyG4>UASbnlRNAi* experiments, *bnl* knock-down under the control of *hthGal4* also

induced glia overmigration, mostly beyond the MF (Figure 12 D, H). This result suggests that Bnl functions as a long range signal in the control of glia migration in the eye disc.

Importantly, we observed that in second instar stages of *eyG4>UASbnlRNAi* discs glial cells migrated into the eye imaginal disc, earlier than RGB migration in controls (Figure 12 G, G`). This result is in agreement with a previous report by Mukherjee et al. [96] indicating that Bnl controls glia migration even before the retinal patterning to limit precocious migration.
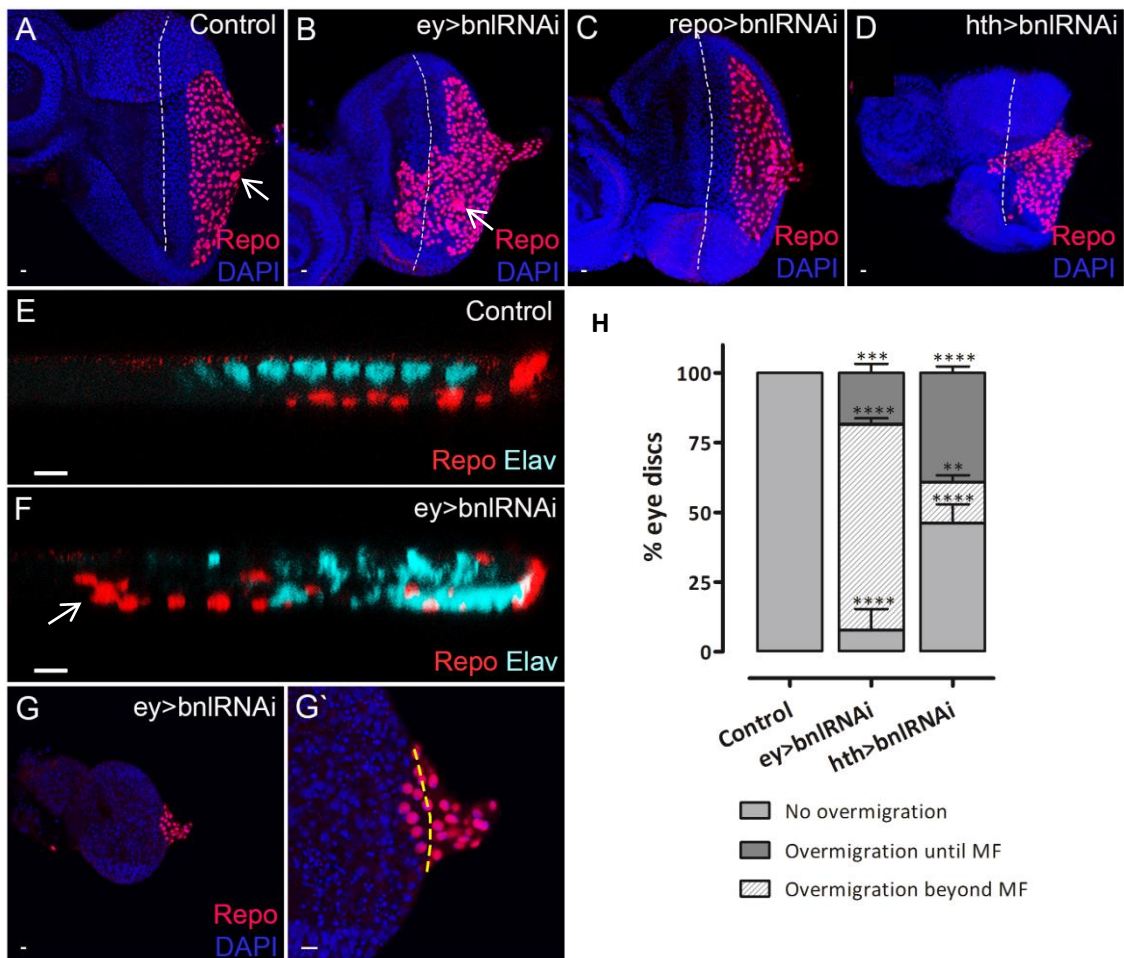
Figure 12: *bnl* **down-regulation in the eye disc induces glia overmigration non-autonomously.** Confocal images. Posterior is to the right. (A-D) Third instar larvae eye discs from (A) control (*eyG4/UASlacZ;+*) and (B-D) Bnl depletion experiments (B: *eyG4/+;UASbnlRNAi/+*), (C: *repoG4/UASbnlRNAi;+*), (D: *hthG4/UASbnlRNAi;+*). MFs are marked with a white dashed line. Glial cells were stained with repo (red) and DNA with DAPI (blue). Comparing with the **(A)** control, Bnl depletion in EPCs induced glia overmigration non-autonomously; two independent *Gal4* drivers were used: **(B)** *eyeless* (ey) and **(D)** *homothorax* (hth). In (B) carpet cells were often found (white arrow) more anteriorly comparing to the (A) control. **(C)** Bnl depletion in glial cells did not induce glia overmigration. **(E, F)** Glial cells were stained with repo (red) and photoreceptors with elav (cian). Transversal view of eye discs described in (A and B), where the characteristic multilayered overmigration of glial cells in (B) is shown (white arrow, F). **(G, G`)** Glial cells were stained with repo (red) and DNA with DAPI (blue). Yellow dashed

line marks the begining of the eye territory. *B*nl depletion in *ey*-expressing cells (*eyG4/+>UASbnlRNAi/+)* induced glia overmigration early in the second instar stage. Scale bars corresponds to 10μm. **(H)** Graph showing percentage of eye discs displaying glia overmigration until the MF or beyond the MF when Bnl was depleted in *ey* or *hth*-expressing cells. **p<0.01; ***p<0.001; ****p<0.0001. n=38.

The current model for the role of Bnl/Btl signaling in the eye disc claims that Bnl expressed by EPCs binds and activates Btl in glial cells, thus inhibiting its migration. Accordingly, when we used an UAS construct to express constitutively active Btl signaling (*UASBtlAct*) in glial cells, we observed a significant decrease of glia in the eye disc since they became stalled in the OS (white bracket, Figure 13 A, B). Despite this, we wanted to discard the possibility that Btl activation in EPCs could stimulate glia migration; thus, we stimulated Btl signaling in *ey*-expressing cells. Eye discs from this experiment didn´t show differences in glia migration when compared with the controls (Figure 13 C, D).

As described in the introduction of this thesis, FGF signaling can also induce cell proliferation, which made us hypothesize that Btl over-activation in EPCs could lead to increased eye discs size. However, we did not observe an evident increase in eye disc size when Btl was over-activated comparatively with the controls (Figure 13 C, D). These results indicate that Btl signaling regulates glia migration only when it is activated in glial cells and not stimulate eye disc growth.

Importantly, to confirm that the *UASBtlAct* construct was functional, we stained all eye discs with pERK (MAPK), a downstream protein of the FGF signaling. As expected, we observed pERK increased levels in the two over-expression experimental conditions (white arrows, Figure 13 B` and D`) when compared with the controls (Figure 13 A` and C`).
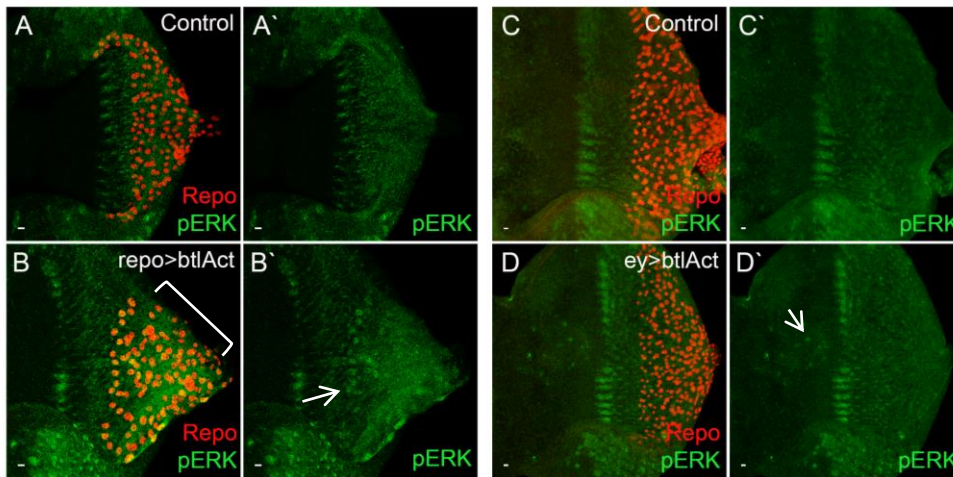
Figure 13: Btl signaling inhibits glia migration but does not stimulate eye disc growth. Confocal images from third instar eye discs. Posterior is to the right. A´ corresponds to the same disc present in A. The same is applicable for the following identification letters. Glial cells were stained with Repo (red) and all MAPK activated cell types were stained with pERK (green, note white arrowheads). **(A-B`)** (B, B`) Stimulated Btl signaling in glial cells induced glia accumulation in the optic stalk (white haircut, *UASbtl^λ/+; repoG4/+*); not seen in (A, A`) controls (*UASlacZ/+; repoG4/+*). **(C-D`)** Btl signaling in the eye disc epithelium did not induce either glia migration or eye disc growth (D and D´, *eyG4/UASbtl^λ*, compared with C and C`, *eyG4/UASlacZ*). Scale bars corresponds to 10µm.

After our results on Bnl/Btl signaling role in perineurial glia migration we became curious about possible effects of Bnl depletion in EPCs in other glial cell types. We asked if this signaling could also directly influence carpet cells extension or perineurial glia differentiation into wrapping glia. To address the first question we stimulated Btl signaling under the control of the carpet cells-specific driver *moodyGal4,CD8:GFP*, on which the CD8:GFP reporter allows the observation of the carpet cells membranes. Interestingly, we did not find defects in their spreading across the differentiation portion of the disc. As in controls, carpet cells of *ey>bnlRNAi* discs extended almost until the perineurial edging glia (white arrows, Figure 14 A-B`). After this approach, we used the wrapping glia-specific driver *mz97Gal4,stinger:GFP* to search for defects in wrapping glia numbers in *eyG4>UASbnlRNAi* discs; *stinger:GFP* is a reporter that marks the wrapping glia nuclei. In this experiment we also did not find differences when compared to the control (Figure 14 C-D`). Importantly, we also crossed *mz97Gal4,stinger:GFP* with *UASbnlRNAi* to make sure that Bnl depletion in wrapping glia does not result in a phenotype. As expected, it did not (data not shown); so, Bnl depletion in EPCs does not impair perineurial glia differentiation and this result

is not affected by Bnl depletion in wrapping glia. No wrapping differentiation is observed in overmigrating glial cells suggesting that Bnl inhibition in EPCs does not stimulate their differentiation. Specifically, Bnl depletion in EPCs does not up-regulate the glial differentiation marker *mz97* in overmigrating glial cells.

In conclusion, Bnl/Btl signaling regulates perineurial glia migration but not its differentiation or carpet cells extension in the eye disc.
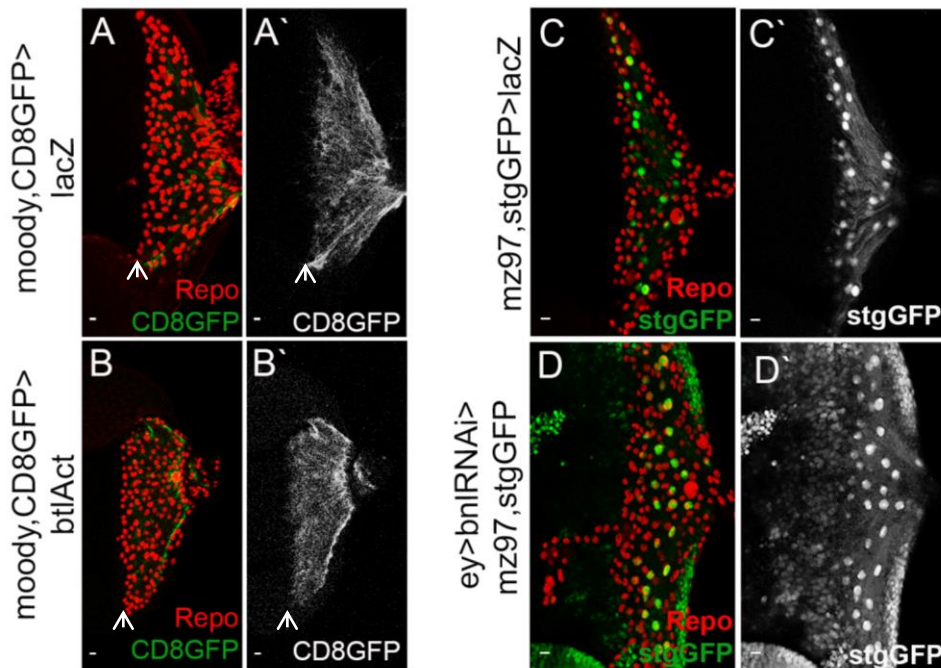
Figure 14: Bnl/Btl signaling does not regulate perineurial glia differentiation neither carpet cells extension. Confocal images from third instar eye discs. Posterior is to the right. A´ corresponds to the same disc present in A. The same is applicable for the following identification letters. Glial cells were stained with Repo (red). White arrows mark the limit of the perineurial edging glia. **(A, A`)** Control disc showing cytoplasmatic membranes of carpet cells (*CD8GFP*: green, *UASlacZ/+;moodyGal4,CD8:GFP/+*). **(B, B`)** Discs with Btl over-activation in carpet cells did not display defects in carpet cells extension (white arrow marks the carpet cells limit, *UASbtl$^\lambda$/+;moodyGal4,CD8:GFP/+*). **(C, C`)** Control disc showing wrapping glia nuclei (*stgGFP*: green, *mz97,UASstingerGFP/lacZ;+*). **(D, D`)** Discs with Bnl depletion in EPCs did not show evidences for differences in wrapping glia number (*ey/mz97,UASstingerGFP;bnlRNAi/+*).

Bnl regulates *Drosophila* eye disc development

In addition to its role in RGB migration, Bnl is involved in the control of the eye disc development. Previously, it was shown by Mukherjee et al. [96] that in *bnl* mutants the ommatidial organization is affected early in the third instar stage, giving rise to the adult "glossy-eye" phenotype, characterized by defects in the retinal

structure. Furthermore, also in the third instar stage, *bnl* mutant eye discs displayed lower Dpp signal in the MF when compared to control flies, indicating defects during the MF formation [96].

To gain insight into the regulation of photoreceptors development by *bnl*, the atonal reporter *3´atonal 1.2-pGWd:GFP* was used as a tool to characterize the expression pattern of this proneural protein. Atonal is a downstream target of Hh, a mutual agonist of Dpp, and it is expressed in the MF to induce R8-photoreceptors formation [52][53]. When compared with controls, Bnl depleted eyes discs (*eyG4>UASbnlRNAi*) showed less Atonal signal (Figure 15 A-B``). This result suggests that *bnl* affects the beginning of the photoreceptors formation and helps to explain the disorganized ommatidial phenotype described by Mukherjee et al. [96]. Note that we also observed disorganized ommatidia in *eyG4>UASbnlRNAi* discs when discs were stained with anti-Elav, a marker for neurons (Figure 12 E, F). These results also suggest that *dpp* down-regulation by Bnl depletion [96] might have an effect in the control of *atonal* expression, and consequently, in the photoreceptors formation.

By staining eye imaginal discs with HRP (horseradish peroxidase that marks polarized membranes) it was also possible to see defects in axonal extension. Comparing with controls, *eyG4>UASbnlRNAi* discs did not show defects in axons targeting into the OS; however, they displayed disorganized axons (Figure 15 C, D). To search for cues involved in axon extension defects, control and *eyG4>UASbnlRNAi* discs were stained with anti-Gliotactin, a marker for septate junctions found in wrapping glia/neuronal axons contacts and that has been suggested to be important for axonal ensheatment [101][102][103]. In control discs these junctions were easily visualized due to its bright spots (Figure 15 C`, C``); however, in *eyG4>UASbnlRNAi* experiments this characteristic staining was not found in any of the eye discs investigated (Figure 15 D`, D``). As previously shown, Bnl depletion in EPCs did not disrupt wrapping glia formation (Figure 14 C, D); yet, it is possible that production or membrane localization of the Gliotactin proteins in glial cells could be affected. Another explanation could be the presence of defects in Gliotactin proteins in photoreceptors and not in wrapping glia. These results suggest that the disorganization of the photoreceptors axons could be a consequence of axonal wrapping impairment due to septate junctions defects.

Altogether, these data imply a role for *bnl* in photoreceptors formation and axonal extension; yet, as the knock-down of *bnl* in the eye disc induces glia migration before the beginning of the neurogenesis (Figure G, G`), defects on the MF cannot be the sole reason behind the glia overmigration phenotype observed.

Importantly, *bnl* also controls eye disc growth. Generally, Bnl depleted eyes discs were smaller when compared with controls (Figure 12 A, B).
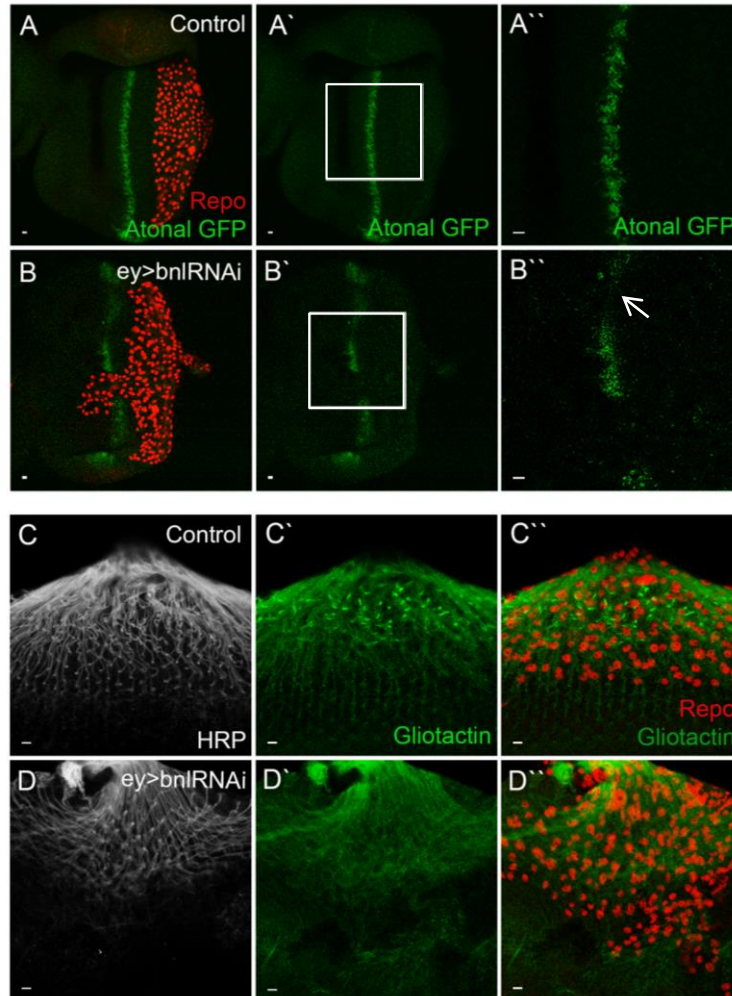
Figure 15: Bnl depletion in the eye disc induces photoreceptors defects. Confocal images from third instar larvae eye discs. **(A-B``)** Posterior is to the right. A`` and B`` are boxed areas from A` and B` respectively. Glial cells were stained with repo (red). (B-B``) Bnl depletion in EPCs induced less atonal expression (GFP:green; *eyG4/3`atonal1.2-pGWdGFP;UASbnlRNAi/+*) in the MF (white arrow), when compared to the (A-A``) control (*eyG4/3`atonal1.2-pGWdGFP;+*). **(C-D``)** Posterior is to up. C, C` and C`` correspond to the same eye imaginal disc (control: *eyG4/UASlacZ;+),* as well as D, D` and D`` are relative to the same eye imaginal disc (*eyG4/+;UASbnlRNAi/+*). Glial cells were stained with repo (red), photoreceptors with HRP (white) and septate junctions with gliotactin (green). Bnl depletion in EPCs cells showed photoreceptors disorganization (C compared with D) and a significant reduction in the gliotactin staining (C` compared with D`). Scale bars corresponds to 10µm.

To know if *bnl* up-regulation in EPCs also leads to photoreceptors development defects, an *UASbnl* line was crossed with the *eyG4* line previously used, and interestingly, axons pathfinding defects into the OS were often observed (Figure 16 A, B). Specifically, several R-axons targeted into the anterior region of the eye disc, instead of entering the OS. Thus, all together, these results suggest that accurate levels of Bnl in the eye disc are important to regulate photoreceptors development. Importantly, we also found less glial cells in the eye disc when *bnl* was up-regulated comparatively to the control (Figure 16 A, B). This result suggests an impairment of glial cells migration into the eye disc when high levels of Bnl are present in this tissue, thus supporting the current view of Bnl/Btl signaling in the eye disc. Occasionally, glial cells were found in the ectopic regions of neuronal axons (Figure 16 B), suggesting a directional migration of glia to ectopic axonal regions.
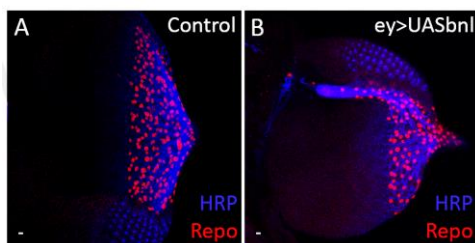
Figure 16: *bnl* up-regulation in the eye disc leads to non-autonomous defects in axons targeting and glia migration. Confocal images from third instar eye discs. Posterior is to the right. Glial cells were stained with Repo (red) and photoreceptor axons with HRP (blue). **(A)** Control disc (*eyG4/UASlacZ*). **(B, C)** *bnl* up-regulation (*UASbnl/+;eyG4/+*) in the eye discs led to less glial cells and axons targeting into the anterior region of the disc. Scale bars corresponds to 10µm.

Down-regulation of Bnl induces non autonomous glia proliferation

In order to address if the glia overmigration induced by Bnl depletion in EPCs is accompanied by glia proliferation we stained eye imaginal tissue with phospho-histone3 (pH3), an antibody marker for mitotic cells. In all experiments non-overmigrating glial cells displayed similar proliferation rates to those observed in the control glia. However, the proliferative rates of the overmigrating glial cells were significantly higher than those observed in the remaining glia (white arrows, Figure 17 B-B``, E).

Since it is already described that Dpp signaling induces glia proliferation [25], we next asked if overmigrating glial cells proliferation could be mediated by the Dpp pathway. With this purpose, eye imaginal discs were stained with phosphorylated Mad (pMad), the main downstream effector of the Dpp pathway [104]. In fact, up-regulation of pMad staining was found in overmigrating glial cells (white arrow, Figure 17 C-D``). Therefore, Bnl depletion in the eye disc stimulates non-autonomous proliferation of the overmigrating glia possibly through increased Dpp signaling activity. Importantly, glia over-proliferation by itself does not induce its overmigration, as showed by Rangarajan et al. [66] and reproduced previously in the lab (data not shown; the genotype used was *repoG4>UAStkvQD. UAStkvQD* is a fly line to constutively active the Tkv receptor of Dpp). This suggests that Bnl regulates glia migration independently of its effect on proliferation.

Figure 17: Overmigrating glia stimulated by Bnl inhibition in the eye disc proliferate through the Dpp pathway. Confocal images from third instar larvae eye discs from (A-A``, C-C``) controls

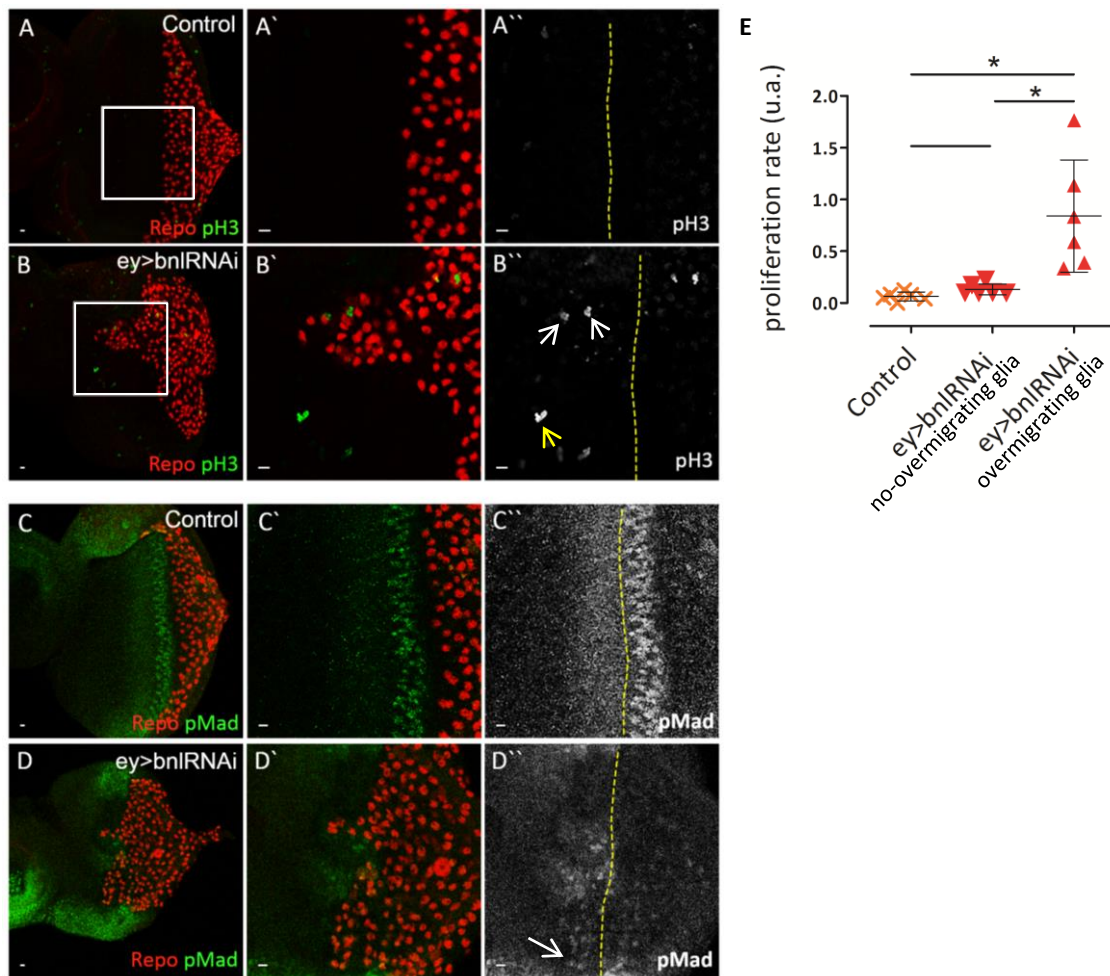(*eyG4/UASlacZ;+)* and (B-B``, D-D``) Bnl depletion experiments (*eyG4/+; UASbnlRNAi/+)*. Posterior is to the right. A`` and A` are boxed areas from A; the same is applicable for the following identification letters. Yellow dashed lines marks the MF. **(A-B``)** Glial cells were stained with repo (red) and mitotic cells with pH3 (green, or white in A`` and B``). (B-B``) Bnl depletion in EPCs stimulated overmigrating glia proliferation (white arrow), as well as EPCs proliferation (yellow arrow). **(C-D``)** Glial cells were stained with repo (red) and Dpp-positive proliferative cells with pMad (green, or white in A`` and B``). (D-D``) Overmigrating glia induced by Bnl depletion in EPCs showed pMad expression (white arrow). Scale bars corresponds to 10µm. **(E)** Quantification of glial cells proliferation rate in controls (*eyG4>UASlacZ*) and *eyG4>UASbnlRNAi* discs. Overmigrating and no-overmigrating glial cells were descriminated. Proliferation rate = $\frac{n^{\underline{o}}\ of\ pH3\ positive\ glial\ cells}{total\ n^{\underline{o}}\ of\ glial\ cells} \times 10$. * p<0.05. n=6.

Bnl regulates pro-apoptotic genes that stimulate *wg* expression

Intrigued by the smaller eye discs often found in Bnl depleted experiments we thought that low levels of Bnl could be activating cell death machinery and thus, decreasing the number of epithelial cells. To address this question we decided to search for cues related to apoptosis. Apoptosis is a form of cellular death in which damaged or potentially dangerous cells activate mechanisms for their own destruction. In *Drosophila*, DIAP1 (*Drosophila* Inhibitor of Apoptotic Proteins) keeps apoptosis in basal levels by inactivating both the initiator (Caspase-9-like Dronc) and effectors (Caspase-3-like Drice and Dcp-1) caspases. However, in stress conditions the pro-apoptotic genes, *head involution defective (hid), reaper (rpr)* and *grim* are up-regulated and their products bind and inactivate the Diap-1 protein (reviewed in [104]). Consequently, inactivation of Diap-1 leads to apoptosis. We stained *eyG4>UASbnlRNAi* and control discs with an antibody against activated cleaved Caspase 3 (a marker for Dronc activity/effector caspases [105]) and three different transcriptional reporters were used to address the expression pattern of the Dronc, Hid and Rpr proteins. As expected, we observed a significant increase of all apoptotic markers in *eyG4>UASbnlRNAi* experiments when compared with the correspondent control (Figure 18 A-H`). Thus, down-regulation of Bnl activated the pro-apoptotic genes *hid* and *rpr* that were able to induce the apoptotic process. These results may explain the reduced eye disc sizes found in *eyG4>UASbnlRNAi* or, at least, they can be part of the

explanation. Interestingly, we also found *rpr* expression in overmigrating glial cells (white arrow, Figure 18 I).

It has been shown that apoptotic cells express mitogenic secretory factors such as Wg, important for inducing over-proliferation of neighboring cells. This process allows to compensate for the dead cells and helps the formation of final structures with normal size and pattern (reviewed in [104]). Interestingly, in *eyG4>UASbnlRNAi* eye discs we found both proliferating (yellow arrow, Figure 17 A-B``) and Wg-expressing EPCs not found in controls (Figure 18 J-K`). Thus, it seems that apoptotic cells induced by *bnl* down-regulation in the eye discs stimulate proliferation of neighboring cells through secretion of Wg. However, as the *eyG4>UASbnlRNAi* discs are often smaller than the controls, apoptotic cells probably did not secret enough mitogenic factors to guarantee eye discs with normal size. A similar phenotype was already demonstrated suggesting that apoptotic cells die too soon to secrete a significant amount of mitogenic molecules [104]. Despite that, some eye discs visualized displayed overgrowth regions with abnormal tissue arrangements, probably due to apoptosis-induced proliferation (Figure 18 D).
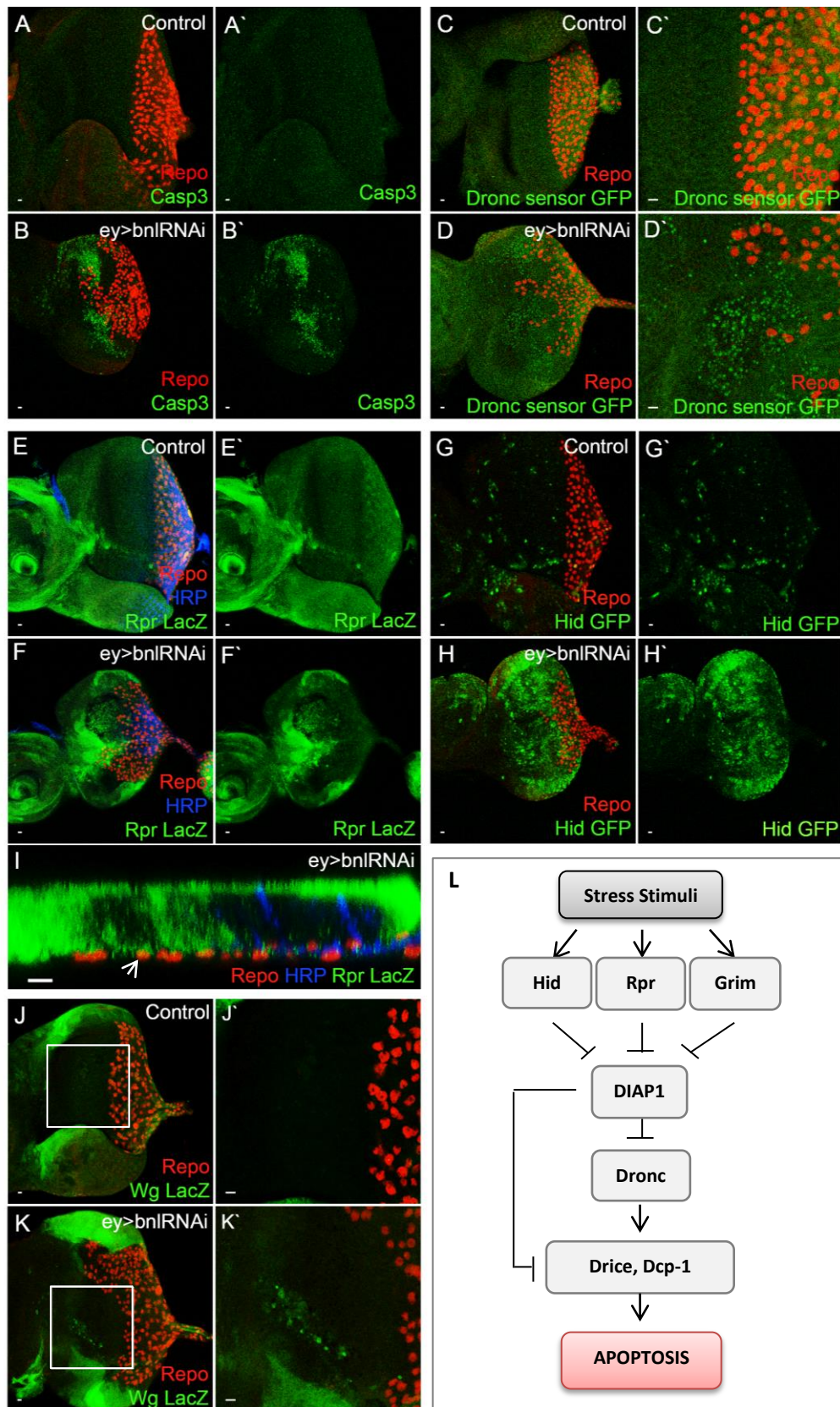
Figure 18: Activation of apoptosis-related proteins and *wg* expression were found in Bnl depleted eye discs. Confocal images from third instar larvae eye discs. Posterior is to the right. All discs were stained with Repo (red) to mark glial cells (A-G`) Assay for apoptotic activity. Bnl depleted ey e discs showed a

significant increase in effector caspases (see staining with cleaved Caspase 3, Caps3: green, in **B-B`**, *eyG4/+;UASbnlRNAi/+,* compared with **A-A`**, *eyG4/UASlacZ;+),* in the initiator Caspase Dronc (GFP: green; **D-D`**, *eyG4/+;UASbnlRNAi/ tubulin CD8 small Drice Histone GFP,* compared with **C-C`**, *eyG4/+; tubulin CD8 small Drice Histone GFP/+)* and in pro-apoptotic proteins Rpr (βgal: green; **F-F`**, *eyG4/+;UASbnlRNAi/rpr-11kb-LacZ,* compared with **E-E`**, *eyG4/+; rpr-11kb-LacZ*) and Hid (GFP: green; **H-H`**, *eyG4/+;UASbnlRNAi/ hid-5' F-GFP,* compared with **G-G`**, *eyG4/+; hid-5' F-GFP/+*). **(I)** Transversal view of the eye disc in F showing *rpr* expression in overmigrating glial cells (white arrow). (J-K`) *bnl* depleted eye discs stimulated *wg* expression in EPCs (βgal: green; **K-K`**, *eyG4/3'wg-lacZ 9.3.1;UASbnlRNAi/+,* compared with **J-J`**, *eyG4/3'wg-lacZ 9.3.1;+*). Scale bars corresponds to 10μm. **(L)** Schematic representation of the apoptotic pathway in *Drosophila*.

dJNK activity is necessary for glia overmigration

After assessing the main eye disc features induced by *bnl* down-regulation, we became particularly interested in dissecting the signaling pathways that could be interacting with *bnl* to regulate glial cells migration. As the Jun kinase (JNK) pathway has been often implicated in the migration of distinct cell types in both vertebrates and *Drosophila* [106][107][108], the expression level of two previously described JNK downstream targets were addressed [100][107]: using an antibody against secreted matrix metalloproteinases (Mmp1) and a transcriptional reporter for the Puckered (Puc) phosphatase (PucLacZ). Importantly, an increase in their expression level was found in the eye disc epithelium but not in glial cells of *eyG4>UASbnlRNAi*, which suggests that reduced levels of Bnl activate the JNK pathway only in an autonomous manner (Figure 19 A-D`). Furthermore, high levels of Mmp1 and PucLacZ were noted across the anterior region of the eye discs but, more importantly they were mostly found adjacent to the overmigrating glia. This suggested that a decrease in Bnl function stimulates JNK activity to further induce glia migration. To test this hypothesis Bnl depleted eye discs were targeted with a dominant negative form (DN) of the Basket (Bsk) protein (JNK homologue in *Drosophila*) to impair the JNK cascade activation. The results showed that in 23% of the discs glia overmigration was rescued, and the Mmp1 expression levels were comparable with the control (Figure 19 A, A`, E, E`, F). Altogether these results imply a role for JNK in inducing glia overmigration on the *bnl* knock-down background. Interestingly, the *UASbskDN* line rescued the overmigration phenotype but glial cells at the differentiation portion of the eye disc displayed a disorganized pattern.

Matrix metalloproteinases (Mmps) are proteolytic enzymes that hydrolyze both extracellular matrix (ECM) components and extracellular mediators of cell-ECM and cell-cell adhesion, thus helping cellular migration. Accordingly, they are involved in many cases of tissue repair, angiogenesis and metastasis [109]. Specifically, in mammals, high concentrations of a secreted type of Mmps were found in gliomas [110]. We hypothesized that, in the cell context described here, Mmp1 secreted by EPCs may interact with adherens junctions components, as E-cadherins and Integrins components, leading to glia overmigration. Besides, breakdown of ECM barriers by Mmp1 activity may create alterations in the eye disc epithelium that make easier the migration of glial cells. Importantly, additional intracellular components of the JNK pathway may be interacting with other signaling cascades to non-autonomously control glia migration.

Expression of BskDN in a Bnl depleted background (*eyG4>UASbnlRNAi>UASbskDN*) frequently resulted in larger eye imaginal discs than those in *eyG4>UASbnlRNAi*. Furthermore, the eye imaginal discs shape did not display similar patterns with those in the control; instead, overgrowth domains were found (white arrow, Figure 19 E`). Therefore, it seems that Bnl cooperate with JNK signaling to control eye disc development.

Importantly, previously in the lab, the *UASbskDN* line was crossed with the eyG4 driver to know if ectopic decreased levels of Bsk induce some phenotype in the eye disc. It did not; so, our results account for Bnl and JNK interactions.
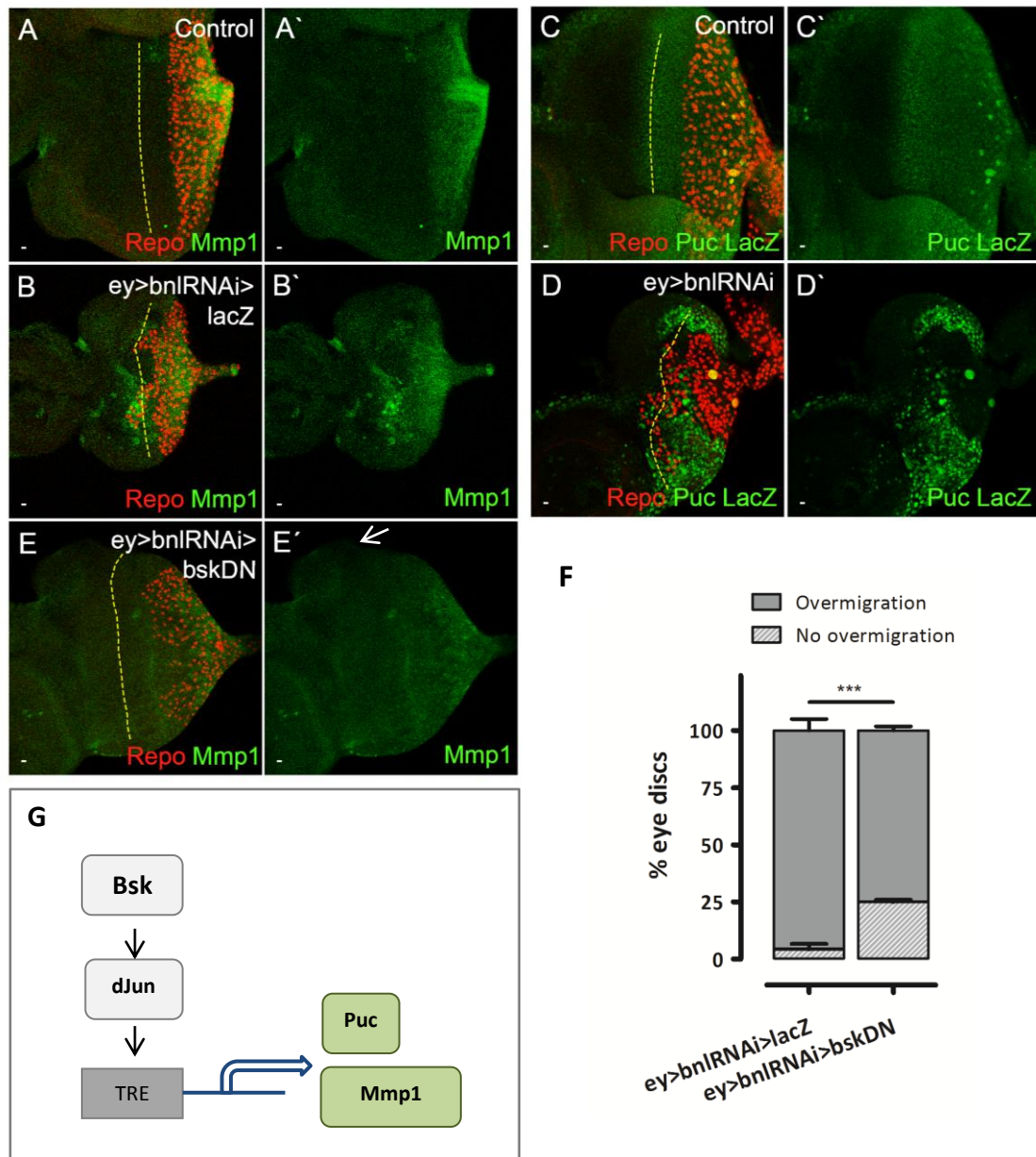
Figure 19: Bnl depletion in the eye disc induces autonomous activation of the JNK pathway that is necessary for glia overmigration. Confocal images from third instar larvae eye discs. Posterior is to the right. Yellow dashed lines mark the MF. All discs were stained with Repo (red) to mark glial cells. **(A-D`)** Bnl depletion in EPCs increased both levels of Mmp1 (green; A and A`, *eyG4/UASlacZ;+,* compared with B and B` *eyG4/UASlacZ;UASbnlRNAi/+*) and Puc (βgal: green; C, C`, *eyG4/+;PucE69-LacZ/+,* compared with D, D`, *eyG4/+;UASbnlRNAi/PucE69-LacZ*). **(E, E`)** Eye disc were stained as in A and B. Decreased activity of the JNK pathway (*UASbskDN/+; eyG4/+;UASbnlRNAi/+*) rescued glia overmigration and *mmp1* over-expression in the eye disc shown in B,B´, but diplayed overgrowth patterns (white arrow). Scale bars corresponds to 10µm. **(F)** Graph showing % of eye discs displaying glia overmigration when Bnl was depleted and when Bnl was depleted accompained with decreased activity of JNK. *** = p<0.001. 40<n<44. **(G)** Simple schematic representation of the JNK pathway.

Upon stress conditions JNK can induce cell death [111]. Thus, eye discs from *eyG4>UASbnlRNAi>UASbskDN* experiments were stained with cleaved Casp3 to know if decreased JNK activity was able to decrease apoptotic levels found in *eyG4>UASbnlRNAi* discs. In fact, this was true for all eye discs observed (Figure 20, n=18), which suggests that *bnl* down-regulation in eye discs stimulate apoptotic activity through the JNK cascade.

Figure 20: JNK activity regulates apoptosis found in Bnl depleted eye discs. Confocal images from third instar larvae eye discs. Posterior is to the right. All discs were stained with Repo (red) to mark glial cells and cleaved Casp3 (green) to mark apoptotic cells. **(A, A`)** Bnl depleted eye discs (*eyG4/UASlacZ;UASbnlRNAi/+*) showing high levels of apoptosis. **(B, B`)** Decreased activation of the JNK pathway (*UASbskDN/+; eyG4/+;UASbnlRNAi/+*) rescued apoptotic activity shown in A, A`. Scale bars corresponds to 10μm.

Hid/Rpr/Grim and Caspases activity is required for glia overmigration

Previous results from Morata et al. [111] showed that up-regulation of the JNK pathway enhance *rpr* and *hid* expression and that Hid is able to activate the JNK cascade, thus leading to a positive feedback loop between the pro-apoptotic genes and JNK. Therefore, as down-regulation of JNK activity in *eyG4>UASbnlRNAi* discs rescued glia overmigration we asked if defects on the *Drosophila* pro-apoptotic proteins activity could also rescue this phenotype. With that purpose we used a previous characterized line, *Df(L3)H99*, a deficiency that removes the major pro-apoptotic genes (*hid, rpr, grim*). Indeed, *eyG4>UASbnlRNAi* discs without these pro-apoptotic proteins activity showed a significant decrease in apoptosis (Figure 21, A compared with D) and, interestingly, in approximately 65% of all eye discs observed glia did not overmigrate (Figure 21 J). Accordingly, we found a significant decrease in apoptosis-induced proliferation (Figure 21, B compared with E) and the eye discs size

was similar to controls (*eyG4>UASlacZ*). Furthermore, in almost every discs observed we did not detect ectopic Mmp1 (Figure 21, C compared with F), suggesting that, in this cell context, the pro-apoptotic genes positively regulate JNK.

To know if glia overmigration is also dependent on caspases activity we co-expressed *eyG4>UASbnlRNAi* with *UAS-diap1* and found that in about 75% of the discs glia did not overmigrate (Figure 21 J). Moreover, we observed a significant decrease in apoptosis (Figure 21, A compared with G), apoptosis-induced proliferation (Figure 21, B compared with H) and Mmp1 levels (Figure 21, C compared with I), and the eye discs displayed normal sizes. This result suggests that glia migration is dependent on apoptotic signals sent from EPCs since caspases activity was exclusively inhibited in EPCs. The deficiency line that removes the major pro-apoptotic genes inhibits gene expression in all larvae, including glial cells, which did not inform about the contribution of ectopic apoptotic signals in EPCs to the glia overmigration.

All together these results suggest that glia overmigration, in an *eyG4>UASbnlRNAi* background, depends on Hid, Rpr, Grim and caspases activity in EPCs. Importantly, we found increased Rpr levels in overmigrating glia (white arrow, Figure 18 I) suggesting that apoptotic gene activation in EPCs can induce *rpr* expression in glial cells to stimulate its migration. Besides of the role in glia migration, activity of Hid, Rpr, Grim and caspases seem to regulate all features found in the anterior region of *eyG4>UASbnlRNAi* eye discs. In particular, it seems that the apoptotic process is able to up-regulate *mmp1* expression. These results, together with the fact that JNK down-regulation (Figure 20) or pro-apoptotic proteins inactivity (Figure 21) both decrease apoptosis, suggest that Hid, Rpr, Grim and JNK are mutual agonists in an *eyG4>UASbnlRNAi* background. Importantly, by depleting the initiator Dronc caspase (using the *UASdiap1* line) we also saw a significant decrease in Mmp1, which is in according to Morata et al. [111]: JNK activation by Hid depends on Dronc activity.

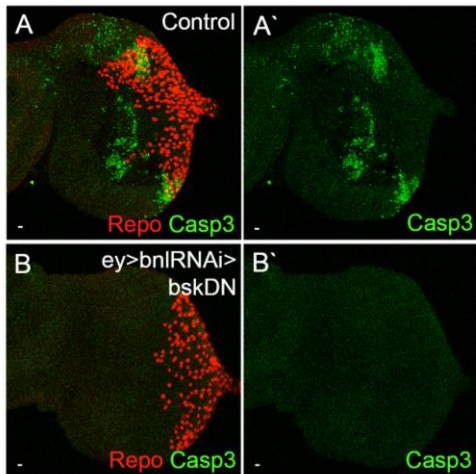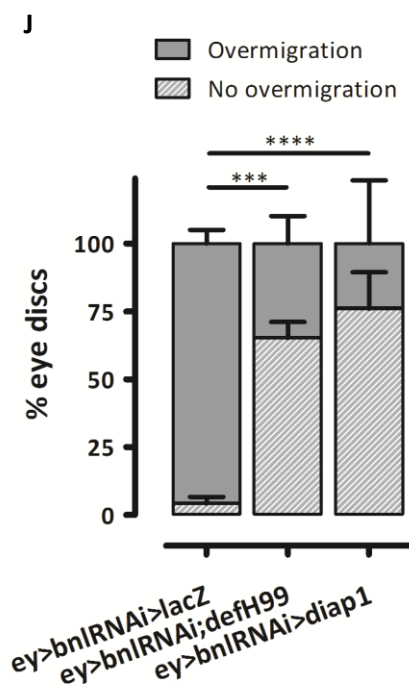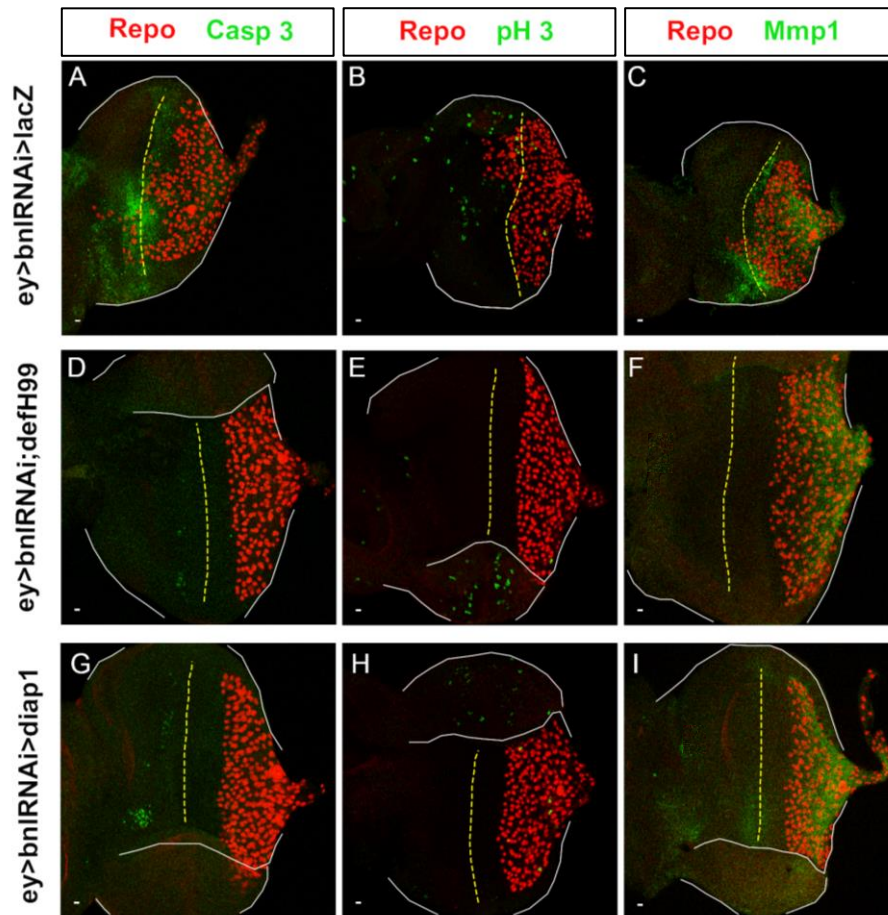**Figure 21: Df(L3)H99 or DIAP1 over-expression rescued glia overmigration and eye disc features found in Bnl depleted eye discs.** Confocal images from third instar larvae eye discs. Posterior is to the right. White lines outline the eye discs (note that some discs have lateral folds and thus the final disc size is bigger than it could seem). Yellow dashed lines marks the MF. **(A, B, C)** Bnl depleted eye discs (*eyG4/UASlacZ;UASbnlRNAi/+),* showing high levels of (A) apoptosis (Casp3:green), (B) EPCs proliferation (pH3:green) and (C) Mmp1 (green). (D, E, F) Removal of Hid, Rpr and Grim activity (*eyG4/+;UASbnlRNAi/Df(L3)H99)* or **(G, H, I)** inhibition of the inititator and effector caspases activity (*eyG4/UASdiap1-131,9;UASbnlRNAi/+)* rescued glia overmigration (Repo) and decreased apoptosis (Casp3), EPCs proliferation (pH3) and Mmp1 levels shown in A, B, C. Scale bars corresponds to 10μm. **(J)** Graph showing % of eye discs displaying glia overmigration when Bnl was depleted, when Bnl was depleted accompanied with removal of *hid/rpr/grim* (defH99) and when Bnl was depleted accompanied with down-regulation of caspases (*UASdiap1*). ***p<0.001; ****=p<0.0001. 40<n<45.

p53 activity is necessary for glia overmigration

p53 is a conserved protein from flies to humans that is highly involved in genotoxic stress responses. Upon extensive cellular damage, it drives cellular senescence or apoptosis to eliminate potentially tumorigenic cells (reviewed in [112]). In *Drosophila*, dp53 up-regulates the pro-apoptotic genes *hid* and *rpr* as well as JNK activity. In turn, Hid and Rpr are also able to activate *dp53* [111]. Thus, we hypothesized that dp53 could be involved in the regulatory mechanisms triggered by Bnl depletion in the eye disc. To answer this question we used to independent lines to down-regulate *p53* expression in *bnl* depleted EPCs: *UAS-p53R115H*, a dominant negative form, and *UAS-dp53RNAi*. In both experiments we found a significant decrease in apoptosis levels, accompanied by a decrease in apoptosis-induced proliferation. Moreover, we also found decreased levels of Mmp1 and the eye discs were bigger that in *eyG4>UASbnlRNAi experiments*. More importantly, glia overmigration was rescued to about 40% of the rate observed in the *eyG4>UASbnlRNAi* genotype (Figure 22).
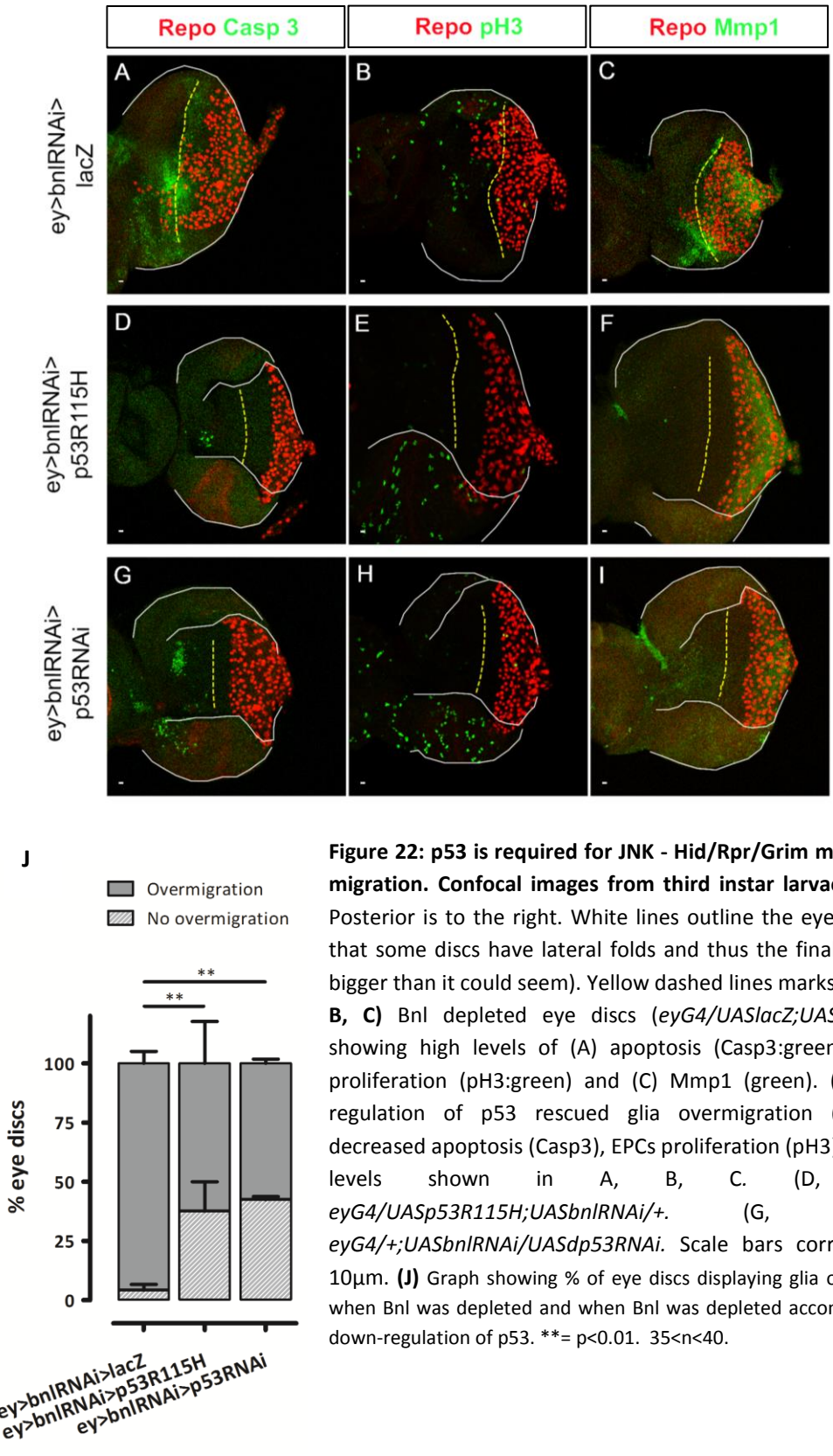
Figure 22: p53 is required for JNK - Hid/Rpr/Grim mediated glia migration. Confocal images from third instar larvae eye discs. Posterior is to the right. White lines outline the eye discs (note that some discs have lateral folds and thus the final disc size is bigger than it could seem). Yellow dashed lines marks the MF. **(A, B, C)** Bnl depleted eye discs (*eyG4/UASlacZ;UASbnlRNAi/+),* showing high levels of (A) apoptosis (Casp3:green), (B) EPCs proliferation (pH3:green) and (C) Mmp1 (green). **(D-I)** Down-regulation of p53 rescued glia overmigration (Repo) and decreased apoptosis (Casp3), EPCs proliferation (pH3) and Mmp1 levels shown in A, B, C. (D, E, F) *eyG4/UASp53R115H;UASbnlRNAi/+.* (G, H, I) *eyG4/+;UASbnlRNAi/UASdp53RNAi.* Scale bars corresponds to 10µm. **(J)** Graph showing % of eye discs displaying glia overmigration when Bnl was depleted and when Bnl was depleted accompanied with down-regulation of p53. **=p<0.01. 35<n<40.

Overmigrating glial cells induced by Bnl inhibition produce late endosomes

The presence of cellular debris or cell corpses often triggers phagocytic responses from neighboring cells, ultimately leading to corpse's engulfment and digestion. In the nervous system, degenerating neurons send injury signals to activate the phagocytic ability of glial cells thus promoting neuronal debris removal [113]. As, in the cell context described here, glia overmigration was rescued by decreasing the apoptotic activity found in Bnl depleted eye discs we asked if glial cells were migrating until the anterior portion of the disc to phagocytose dead EPCs. To gain insight in this question we used a previous characterized marker for late endosomes/lysosomes, lysotracker, expecting to observe high levels in the overmigrating glia. In fact, we found it and, furthermore, high levels of late endosomes were noted in EPCs; not seen in control discs (Figure 23 A-C). Thus, these results suggest that glia overmigrate to engulf dying and dead EPCs, which are also removed by healthy EPCs. In support to this finding we did not observe late endosomes in *eyG4>bnlRNAi;defH99* eye discs (Figure 23 D). Altogether these findings propose that Bnl depletion in the eye disc induces apoptosis thus leading to corpse's removal by neighboring cells including glial cells.
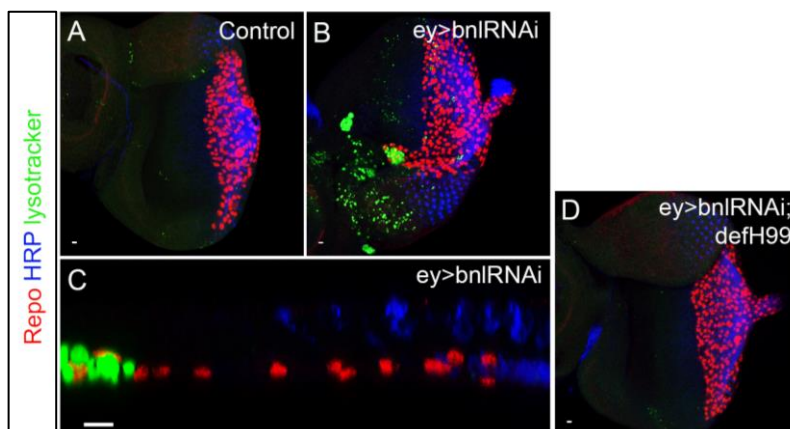
Figure 23: Bnl depletion in the eye disc induces late endosomes formation in EPCs and glial cells. Confocal images from third instar larvae eye discs. Posterior is to the right. Glial cells were stained with repo (red), photoreceptors with HRP (blue) and lysosomes with lysotracker (green). **(A)** Control discs (*eyG4/UASLacZ;+*) showed reduced levels of late endosomes. **(B)** Bnl depleted eye discs (*eyG4/+;UASbnlRNAi/+*) displayed high levels of late endosomes in both glial cells (white arrow) and EPCs (yellow arrow). **(C)** Tranversal view of the eye disc described in B showing overmigrating glial cells with high levels of late endosomes. **(D)** Inhibition of apoptotic activity (*eyG4/+;UASbnlRNAi/Df(L3)H99*) significantly decreased late endosomes formation in EPCs. Scale bars corresponds to 10µm.

Discussion and perspectives

In this chapter we dissected the role of the *bnl* gene in the eye disc growth, photoreceptors development and perineurial glia migration. Importantly, by using the Gal4-UAS system we depleted Bnl in a cell-type specific manner and showed that Bnl expression in EPCs as well as Btl function in glial cells both act to prevent glia overmigration in the eye disc. In addition, we depleted Bnl exclusively in photoreceptor cells, which did not cause glia migration defects (data not shown). Thus, Bnl controls glia migration exclusively when it is secreted by EPCs and not by its expression in differentiated cells. This capacity to influence cell migration in a non-autonomous manner was also observed by Chu et al. [114]; however, they observed that Bnl depletion in photoreceptors interferes with migration of retinal tracheal cells, a distinct cell type.

Glia migration into the anterior region of the eye disc in *eyG4>bnlRNAi* did not induce R-axons to follow these ectopic glia. This result is of significant importance in the context of the glial function, indicating that glial cells are not important to control early axonal pathfinding in the eye disc, as previously suggested by us [65].

The development of this project also clarified that Bnl/Btl signaling does not regulate perineurial glia differentiation into wrapping glia neither directly controls carpet cells extension. Although carpet cells moved anteriorly in the eye disc when Bnl was depleted in EPCs, its direct targeting by *UAS-BtlAct* did not show defects in its extension. We hypothesize that carpet cells movement found in *eyG4>UASbnlRNAi* eye discs is a consequence of the significant number of perineurial glia that moved anteriorly in the disc.

Cellular proliferation induced by Bnl inhibition in EPCs

In a previous report by Mukherjee et al. no differences in glial cells proliferation were observed when Bnl/Btl signaling was altered [96], which is not in agreement with our results. One putative reason to explain these differences might be the method used to quantify proliferation levels. While in the previous report, all the glial cells present in the eye disc were counted when Btl function was down-regulated in glia (which induces glia overmigration), we calculated the proliferation ratio of

overmigrating and no-overmigrating glial cells when *bnl* was down-regulated in EPCs. Thus, with this approach we gained resolution to detect putative differences in the proliferation ratio of specific domains of glial cells. Indeed, we observed that the overmigrating glia displayed higher proliferation rates than the remaining glia and the control (Figure 17 E). In addition, we propose that the overmigrating glial cells proliferate through the Dpp pathway (Figure 17 C,D).

Glia overmigration induced by Bnl inhibition in EPCs

Our overall results indicate that Bnl inhibition in EPCs induces a complex molecular network, constituted by Hid/Rpr/Grim, JNK and Dp53, that non-autonomously regulates glia overmigration. When we decreased Hid/Rpr/Grim, JNK or Dp53 activity in EPCs, glia lost their ability to overmigrate. Interestingly, down-regulation of JNK in EPCs rescued glia overmigration only in 23% of the discs observed (Figure 19 F), which might be explained by characteristics of the dominant negative line used to down-regulate the JNK pathway. This line did not completely eliminate JNK function since ectopic Mmp1 was found in most of the *eyG4>UASbnlRNAi>bskDN* discs, and these discs displayed glia overmigration (data not shown). It has been suggested that low levels of JNK is sufficient to induce cells migration [115].

The intricate relationship between Hid/Rpr/Grim, JNK and Dp53 proteins is reflected by the fact that decreased activity of each of them individually lead to a reduction of Mmp1, casp3 and pH3 levels found in Bnl depleted EPCs. Importantly, down-regulation of caspases activation or Dp53 decreased Mmp1 production but did not completely abolishe it, even when glia overmigration was rescued. This suggests that glial cells overmigration might be dependent on caspases and Dp53 even when reduced levels of Mmp1 are being produced. Alternatively, the levels of Mmp1 were not sufficient to induce glia overmigration. Therefore, further research is needed to clarify the role of each Hid/Rpr/Grim, JNK and Dp53 proteins in this biological system. It would be also important to address the extracellular signals secreted by Bnl depleted EPCs responsible for trigger glia overmigration. Understand the molecular cues responsible for EPCs-glial cells communication is crucial to better interpret this glial migration mechanism.

In addition, it would be important to unveil the molecular cues activated in overmigrating glial cells by ectopic expression of *hid/rpr/grim, jnk* and *dp53* signals in EPCs. JNK is a good candidate since it is described in many cell migration contexts. However, we did not find Puc-LacZ staining in overmigrating glial cells. JNK activity in overmigrating glial cells should be accessed using a more sensitive reporter line, as the TRE-GFP line [113]. On the other hand, we found *rpr* expression in this type of glial cells, what could also lead to increased JNK activity. It would be also interesting to understand if the expression of extracellular proteins involved in cell-cell and cell-ECM adhesions, as E-cadherin and/or integrins, are altered in overmigrating glial cells.

Dissection of the specific cues activated in glia crucial for their overmigration requires specific ablation of signals in glia when Bnl is depleted is EPCs. In order to perform these experiments it is important to develop new genetic tools. For example, by creating transgenic lines with two distinct genetic systems where gene expression can be independently modulated in EPCs and glia. LexA-LexOp is a similar system to the Gal4-UAS, which allows up or down-regulate genes downstream of LexOp under the control of the LexA driver. Therefore, we could, for example, generate a *eyG4>UASbnlRNAi;repoLexA* transgenic line and cross it with LexOp lines to modulate JNK, Dp53 and pro-apoptotic proteins activity in glia, while Bnl is depleted in EPCs. With this approach we could address if the activation of these signals in overmigrating glial cells is crucial for their exaggerated migration.

It would be also interesting to down-regulate JNK and pro-apoptotic proteins activity only in glial cells when Btl function is decreased (*repoG4>UASbtlRNAi*). This would clarify if JNK-Rpr/Hid/Grim activity in glia is required for glia overmigration induced by decreased function of Btl in glia.

Btl signaling controls glia migration into the eye disc as it is shown by the experiments where Btl is over-activated in glia. In this situations glia became blocked in the optic stalk (Figure 13 A,B). In contrast, decreased activity of JNK or Hid/Rpr/Grim in a Bnl depleted background blocked glia overmigration but did not impaired endogenous glia migration (Figures 19 A,E, and 21 A,D). Therefore, Hid/Rpr/Grim-JNK activity in EPCs is important to induce glia overmigration but not to stimulate their normal migration. Interestingly, these results also suggest that Bnl does not regulate glia migration exclusively by its binding to the Btl receptor present in glial cells. On the

other hand, up-regulation of *bnl* in EPCs led to a reduced number of glial cells in the eye disc (Figure 16), supporting a role for Bnl in glia migration through its binding to the Btl present in glial cells. Thus, the binding of Bnl to the Btl receptor in glia is important for glia migration. What would be the biological role of glial overmigration upon Bnl depletion in EPCs? The results shown at the end of this chapter suggest that glial cells seem to be overmigrating to regions with dead EPCs and where phagocytosis is detected perhaps contributing to the regeneration of the eye epithelium (Figure 23). Interestingly, Shklover et al. [113] showed that JNK activity in dying embryonic neurons leads to JNK increase in glial cells that are induced to migrate and eliminate the degenerating neurons. However, there are other explanations to the high levels of late endosomes found. Endocytosis and vesicle recycling are also involved both in the re-localization of signaling receptors at the cytoplasmatic membrane and in the internalization of exosomes, small vesicles that transport functional molecules [116][117]. Thus, the high levels of late endosomes found in glia in *eyG4>bnlRNAi* eye discs can be a response to extracellular signals that induce cellular membrane receptors rearrangements or to the internalization of exosomes, and not due to phagocytic activity. Yet, in both hypotheses it is possible that these cellular mechanisms may be involved in the induction of exaggerated glia migration.

In the model discussed here, Bnl function to limit cell migration; however, in other developmental contexts Bnl performs the opposite function. For example, during development of the traqueal system Bnl is crucial to guide cells until their correct target site [77]. Therefore, it would be interesting to dissect the signaling pathway downstream of Btl in both these cellular contexts to search for putative differences that could explain this functional change.

Finally, it is important to highlight the fact that we found another system where an FGF ligand, Bnl, cooperates with JNK for proper development. Furthermore, we provide the first description of a role for Bnl in cells survival, specifically by regulating pro-apoptotic proteins. Even among all *Drosophila* FGFs there are very few known contexts where they are important for survival.

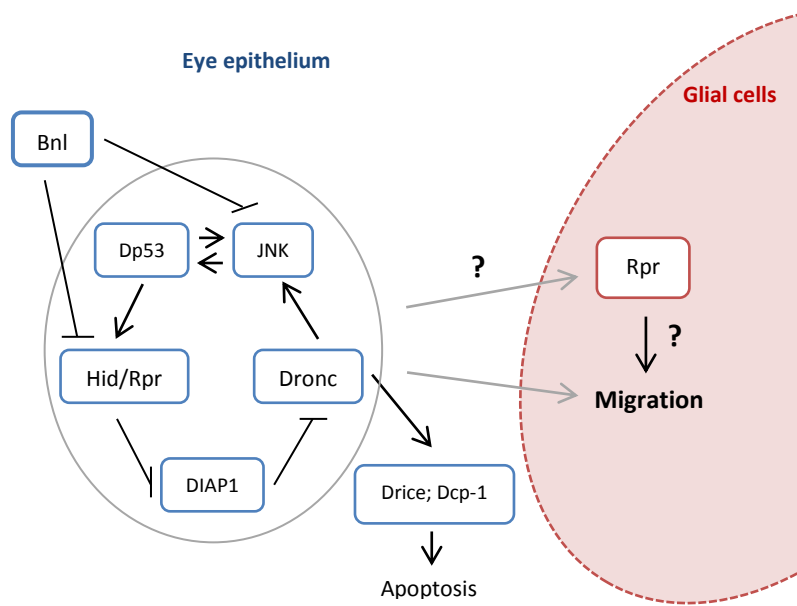**Figure 24: Schematic representation of the Hid/Rpr-JNK-Dp53 network regulated by Bnl.** Bnl depletion in EPCs induces Hid/Rpr-JNK-Dp53 activity in EPCs that triggers apoptosis and glial cell migration. One of these proteins directly or indirectly regulates glia migration, probably by over-expressing *rpr* in glia.

Chapter 4

Bnl and dMyc cooperate to control eye disc development

Results

In the last years, our laboratory has focused its attention in dissecting some of the several roles of dMyc, the Myc-homologue in *Drosophila*, during development. Myc is a transcription factor highly involved in tissue growth regulation, specifically by controlling cell cycle, protein synthesis and apoptosis [118]. Interestingly, it was shown that flies carrying hypomorphic Myc alleles exhibit delayed development and reach adulthood as small flies. Furthermore, Myc was associated with cell competition behaviors since it favors cells with higher dMyc expression comparing with surrounding cells that are induced to die [119]. Thus, it is clear that Myc is a crucial player in the correct development of tissues; however, we still lack detailed mechanistic information to understand all roles of Myc in growth and differentiation.

Recently in the laboratory, Viriato, the single *Drosophila* member of the Nol12 family, was identified as a novel dMyc target required for dMyc-induced growth by stimulating ribosomal RNA processing [120]. Furthermore, it was also showed that *viriato* is a genetically interactor of the Dpp/TGF-β signaling pathway during eye growth and differentiation [121]. At this time, a novel link between dMyc and differentiation signals was created. As Dpp is important to control spatial and temporal glia migration it was though that dMyc could also be involved in this process. Indeed, besides of inducing reduced eye discs size, knocking-down *dMyc* in EPCs, revealed an interesting phenotype: glia increases their migration, beyond the MF, even though not being targeted by dMyc interference. This finding reveals a novel non-autonomous role for dMyc in helping to establish the correct boundaries between neurons and glial cells.

Non-autonomous glia overmigration induced by dMyc down-regulation is mediated by JNK activity

As referred previously, down-regulation of *dMyc* only in EPCs induces non-autonomous glia overmigration, mostly beyond the MF. Therefore, both dMyc and Bnl are important to limit glia migration. Interestingly, we also observed that glia overmigration on the dMyc depleted background is accompanied by an increased in Mmp1 levels and it can be rescued by decreased activity of the JNK pathway (Figure

24). As in Bnl depleted eye discs, increased levels of Mmp1 were specifically observed adjacent to the overmigrating glia (white arrow, figure 25B)[1].
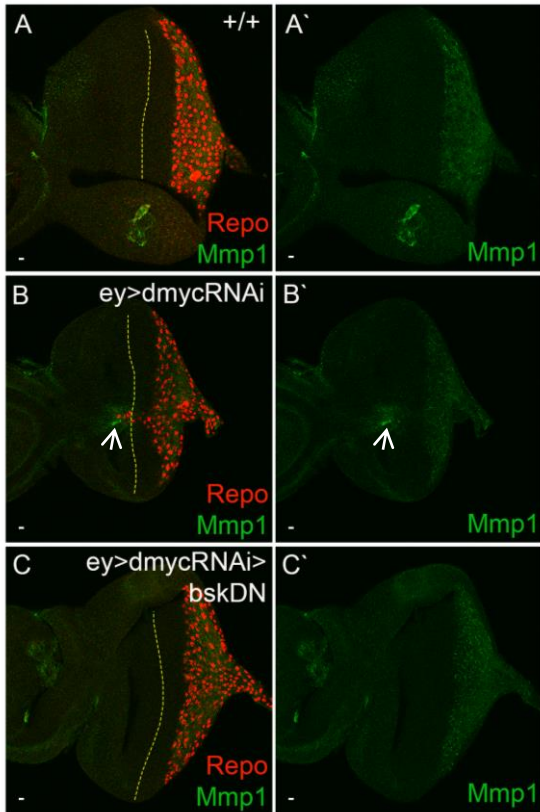
Figure 25: *dMyc* **down-regulation in the eye disc induces non-autonomous glia overmigration dependent on JNK activity.** Confocal images from third instar larvae eye discs (basal view). Posterior is to the right. Yellow dashed lines marks the MF. Glial cells were stained with Repo (red) and *mmp1*-expressing cells with Mmp1 (green). **(A-B`)** (A, A`) Compared to a wild-type disc (*W1118*), (B, B`) dMyc depletion (*eyG4/UASdMycRNAi;+*) in EPCs increased levels of Mmp1 adjacent to the overmigrating glia (white arrow). **(C, C`)** Decreased activation of the JNK pathway (*UASbskDN/+; eyG4/UASdMycRNAi;+*) rescued glia overmigration and *mmp1* overexpression in the eye disc (shown in B,B`). Scale bars corresponds to 10µm.

dMyc positively interact with Bnl

Due to the phenotypic similarities found between dMyc and Bnl depleted eye discs we next asked if these two proteins could interact in order to regulate glia migration. Therefore, it was decided to perform a series of experiments to address this question.

Initially, we decided to take advantage of the *dmyc^dm4^/FM7,actGFP* line, on which the *dmyc^dm4^* mutation abolish *dm* (diminutive) expression that encodes the dMyc protein. By crossing this line with an *eyG4>UASbnlRNAi* line, we could generate heterozygous flies for the *dmyc^dm4^* null allele with Bnl depleted in the eye discs. Thus, if Bnl and dMyc putatively interact, we were able to detect possible biological events

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[1] In preparation to submit. Article from Andreia Correia, Lígia Tavares, Marília A. Santos, Paulo S. Pereira.

resultant from misregulation of Bnl and dMyc. However, confocal microscopy images from this experiment did not show significant differences relatively to *eyG4>UASbnlRNAi* eye discs (Figure 26). Importantly, *dmyc^{dm4}* heterozygotes do not display a retinal glial migration phenotype.
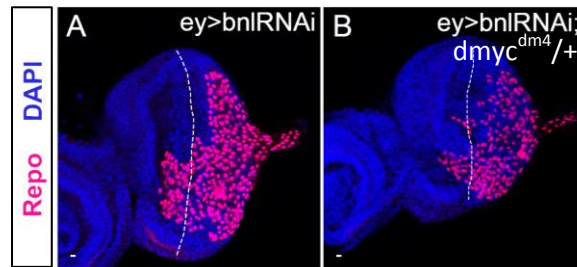
Figure 26: Removal of a *dm* allele in a Bnl depleted background does not induce phenotypic alterations. Confocal images from third instar larvae eye discs (basal view). Posterior is to the right. White dashed lines marks the MF. Glial cells were stained with Repo (red) and DNA with DAPI (blue). **(A)** Bnl depleted eye discs (*eyG4/+;UASbnlRNAi/+). **(B)** Bnl depleted eye discs heterozygous for the null *dmyc^{dm4}* allele (*dmyc^{dm4}* /+; eyG4/+;UASbnlRNAi/+*) did not show significant differences relatevely to A. Scale bars corresponds to 10µm.

After this approach, it was decided to co-express *eyG4>UASbnlRNAi* with a *dmycRNAi* line and, in contrast to what was previously observed, we observed a Bnl-dMyc interaction by detecting an aggressive phenotype in the third instar stage. When compared with *eyG4>UASbnlRNAi>UASlacZ*, eye discs were significantly smaller and displayed an irregular shape, mostly without photoreceptors differentiation. Importantly, even in the absence of photoreceptors, glial cells migrated into the eye disc (Figure 27 A, B).

Intrigued with these results, we next co-expressed *eyG4>UASbnlRNAi* with a *dmyc* overexpression (OE) line, *UASdmyc*, expecting to notice defects in the eye discs development. Indeed, when compared with *eyG4>UASbnlRNAi>UASlacZ,* we observed significant bigger eye discs and, even with such dimensions, glial cells were able to migrate beyond the MF almost until the antennal portion of the disc (Figure 27 A, C). Interestingly, we often found specific regions of the disc epithelium showing overproliferative patterns, contributing to overgrown irregular discs. (white arrow, Figure 27 C, see also Figure 30).

Furthermore, we always found another interesting feature resulting from abnormal levels of Bnl and dMyc: glial cells were able to migrate through the OS until the apical side of the disc in both conditions of *dmycOE* or *dmycRNAi* on the Bnl depleted background (Figure 27 D, E, F). Furthermore, in *eyG4>UASbnlRNAi>UASdmyc* discs we also observed defects in the ommatidial organization; specifically, in the apical side we found photoreceptors axons, not found in *eyG4>UASbnlRNAi>UASlacZ* discs (Figure 27 D, F). Importantly, targeting of only *dmycOE* or *dmycRNAi* by the *eyG4* driver showed very few glial cells migrating into the apical portion of the eye disc as well as they did not display such defects in the eye disc growth and photoreceptors rearrangement (Figure 27 I-L). Taken together, these results hypothesize that Bnl and Myc directly or indirectly communicate to control a wide range of developmental processes in the eye imaginal discs. They seem to cooperate to guarantee proper eye disc growth accompanied by the correct development of photoreceptors and both temporal and spatial precise migration of glial cells. Note that decreased but also increased levels of dMyc changed eye disc features in a Bnl depleted background meaning that balanced levels between Bnl and dMyc are crucial for proper development.
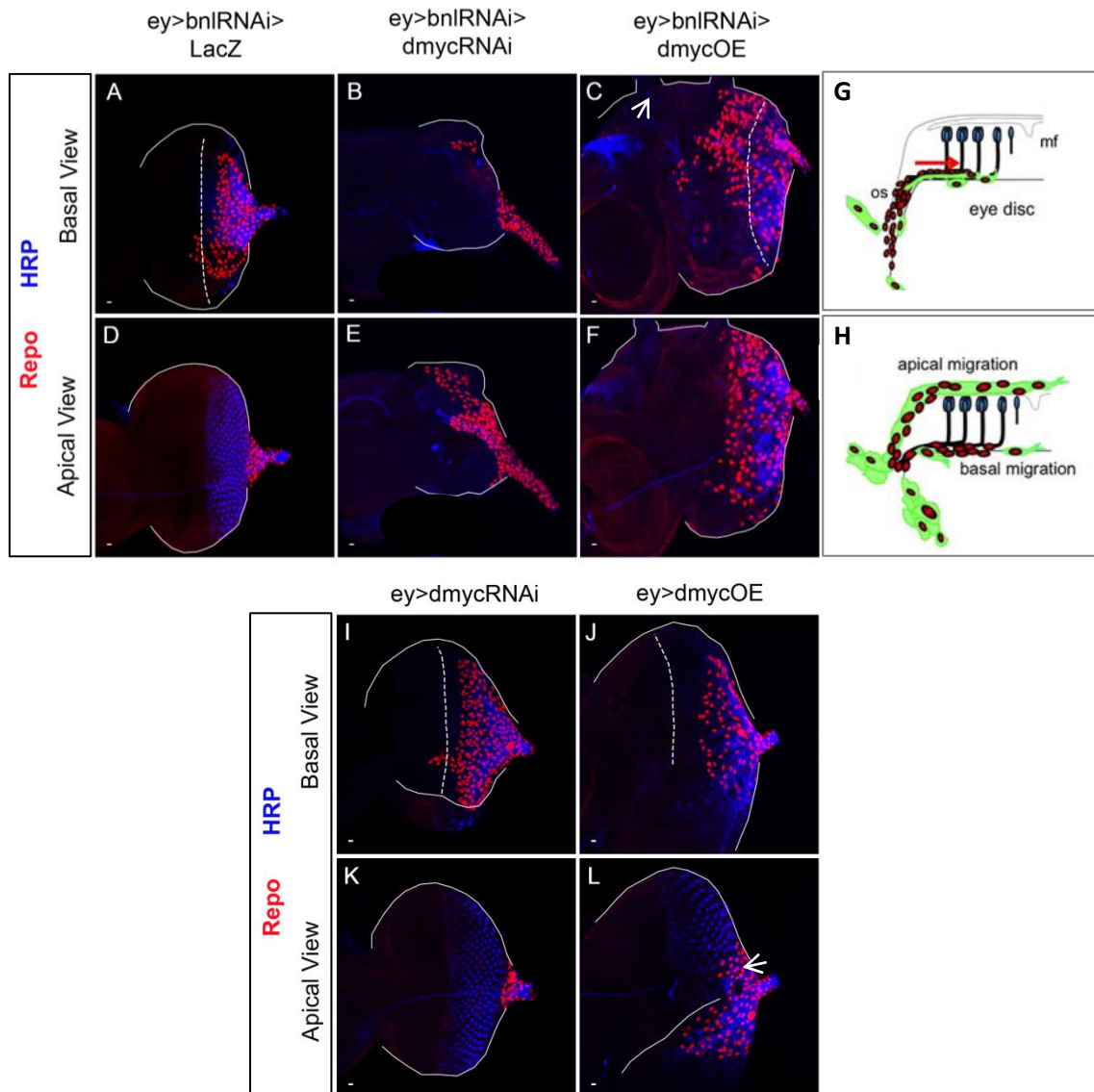
Figure 27: Bnl interact with dMyc to control a wide range of developmental processes in the eye disc. Confocal images from third instar larvae eye discs. Posterior is to the right. White lines outline the eye discs and white dashed lines marks the MF. Glial cells were stained with Repo (red) and photoreceptors with HRP (blue). (A, B, C) Basal view. **(A)** Bnl depleted eye disc (*eyG4/lacZ;UASbnlRNAi/+).* **(B)** Bnl and dMyc depleted eye disc (*eyG4/UASdmycRNAi;UASbnlRNAi/+)* showing reduced size (compare with A) and glia migration into the eye disc in the absence of photoreceptors differentiation. **(C)** Eye disc with Bnl depleted and dMyc overexpressed (*eyG4/+;UASbnlRNAi/UASdmyc)* showing overgrowth (white arrow). (D, E, F) Apical View. **(D)** Same eye disc of A. **(E)** Same eye disc of B showing apical glia migration. **(F)** Same eye disc of C showing apical glia migration. **(G)** Schematic representation of the basal glial migration into the eye disc. os: optic stalk. mf: morphogenetic forruw. **(H)** Schematic representation of apical and basal glial migration into the eye disc. (I, J) Basal view. **(I)** dMyc depleted eye disc (*eyG4/UASdmycRNAi/;+*) **(J)** Eye disc with increased levels of dMyc (*eyG4/+;UASdmyc/+).* (K, L) Apical view. **(K)** Same eye disc of I not showing apical glia migration. **(L)** Same eye disc of L showing few glial cells migrating apically (white arrow). Scale bars corresponds to 10μm.

Archipelago (Ago), the *Drosophila* ortholog of Fbw7 human tumor suppressor, negatively regulates dMyc levels and activity. It was shown that loss of *ago* leads to

elevated dMyc protein levels and increased expression of dMyc targets. Interestingly, it was also observed that Ago binds to dMyc, which correlates with the ability of Ago inhibit dMyc accumulation [122]. Thus, Ago helps in the dMyc turnover and in keeping dMyc at normal cellular levels. Having this in mind we decided to down-regulate Ago, using an RNAi line, in order to increase dMyc levels in EPCs in a Bnl depleted background. As expected, we observed overgrown irregular eye discs and apical glia migration not found in *eyG4>bnlRNAi>UASlacZ* and *eyG4>UASagoRNAi* discs (Figure 28), thus supporting the results previously described.
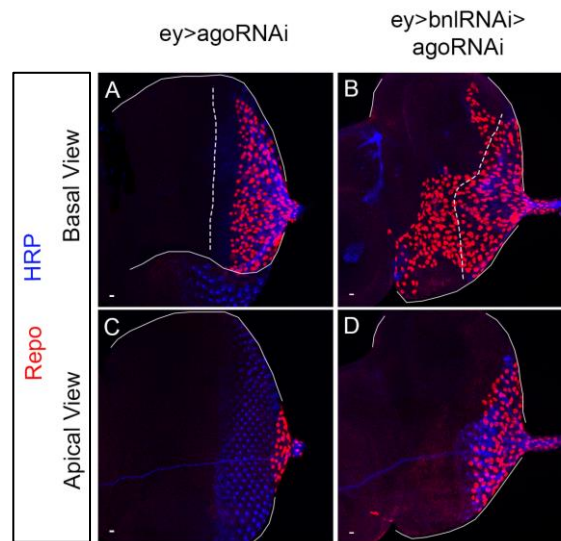
Figure 28: Low levels of Ago accompanied with low levels of Bnl induce abnormal eye disc development. Confocal images from third instar larvae eye discs. Posterior is to the right. White lines outline the eye discs and white dashed lines marks the MF. Glial cells were stained with Repo (red) and photoreceptors with HRP (blue). (A, B) Basal view. **(A)** Ago depleted eye disc (*eyG4/+;UASagoRNAi(III)/+)*. **(B)** Bnl and Ago depleted eye disc (*eyG4/+;UASbnlRNAi/UASagoRNAi(III))* showing shapeless size realtively to A. (C, D) Apical View. **(C)** Same eye disc of A. **(D)** Same eye disc of B showing apical glia migration. Scale bars corresponds to 10µm.

In addition to the phenotypes described above, increased levels of dMyc on a *bnl* down-regulation background often showed delay in the photoreceptors formation. Indeed, in both *eyG4>UASbnlRNAi>UASdmyc* and *eyG4>UASbnlRNAi>UASagoRNAi(III)* experiments we observed differentiating retina in the middle but not in the most posterior margin of the eye discs (Figures 29 and 30).
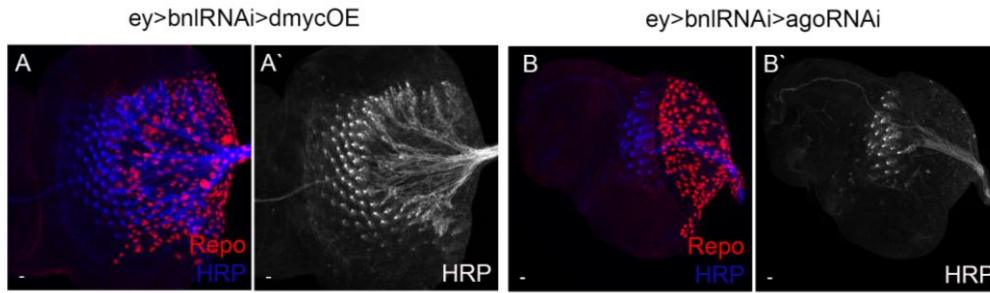
Figure 29: dMyc up-regulation in a Bnl depleted background induced photoreceptors formation delay. Confocal images from third instar larvae eye discs. Posterior is to the right. Glial cells were stained with Repo (red) and photoreceptors with HRP (blue). **(A-B`)** Apical view showing photoreceptors lens in the middle of the eye disc but not in the most anterior portion. Found in *eyG4/+;UASbnlRNAi/UASdmyc (*A and A`) *and* e*yG4/+;UASbnlRNAi/UASagoRNAi(III) (B and B`) eye discs.* Scale bars corresponds to 10μm.
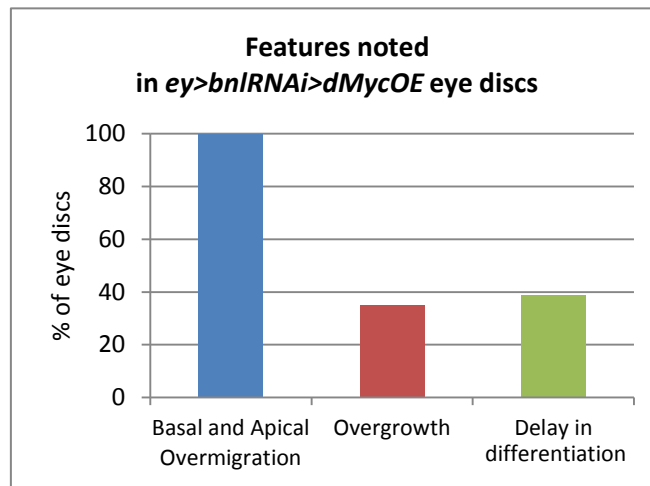
Figure 30: Eye disc features found when Bnl was depleted and dMyc was increased in EPCs. (*eyG4/+;UASbnlRNAi/UASdMyc)*. Basal and apical glial migration were always observed, eye disc overgrowth was found in 35% of the cases and in 39% of the discs observed it was noted differentiation delay (n=23).

After our analysis on dMyc levels modulation in a Bnl depleted background we asked if dMyc down-regulation in a Bnl up-regulated background also leads to eye disc defects. To answer this question we used an *UASbnl* line and the *UASdmycRNAi* line used in the previous experiments to drive over-expression of *bnl* and down-expression of *dmyc* in EPCs. Interestingly, when compared with *eyG4>UASbnl* and *eyG4>UASdmycRNAi* discs, *eyG4>UASbnl>UASdmycRNAi* discs displayed aggressive growth defects, including in eye discs shapes and photoreceptors development. Specifically, the eye epithelial cells displayed a disorganized rearrangement and a

photoreceptor differentiation delay was observed, both phenotypes not seen in *eyG4>UASbnl* and *eyG4>UASdmycRNAi* discs (Figure 31). This result supports the idea that balanced levels of Bnl and dMyc are important for proper eye development.
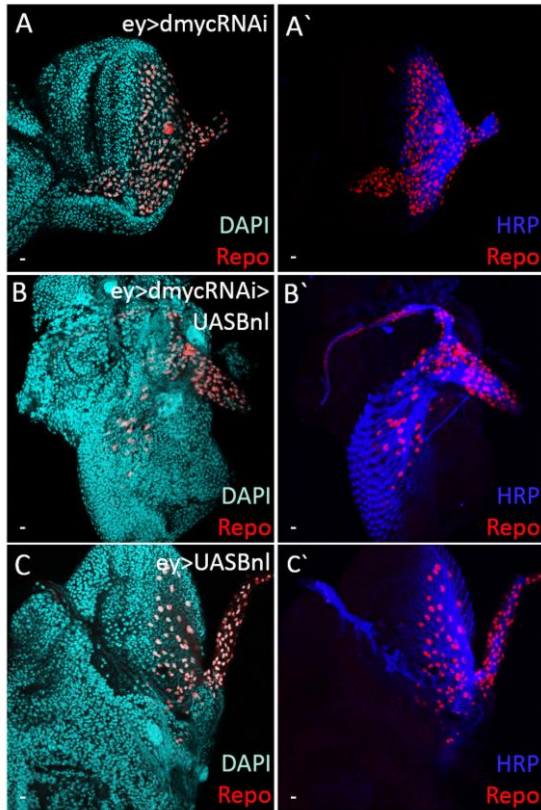
Figure 31: *bnl* **up-regulation induces eye disc defects, intensified by down-regulation of dMyc.** Confocal images from third instar larvae eye discs. Posterior is to the right. DNA was stained with DAPI (cian) glial cells with Repo (red) and photoreceptors with HRP (blue). **(A, A`)** *bnl* up-regulation in EPCs (*UASbnl/+;eyG4/+;+)* induced disc shape defects, less glial cells in the posterior domain when compared with normal conditions and targeting of photoreceptors into the anterior domain of the disc. **(B, B`)** *bnl* up-regulation in a *dMyc* down-regulated background (*UASbnl/+;eyG4/UASdmycRNAi;+)* induced aggressive defects in disc shape and photoreceptors differentiation delay. **(C, C`)** dMyc down-regulation in EPCs induced small eye discs (*eyG4/UASdmycRNAi;+*). Scale bars corresponds to 10µm.

Discussion and perspectives

The results presented in this chapter point to an unpublished interaction between Bnl and dMyc. Even among all *Drosophila* FGF ligands there are no reported evidences for this direct relationship. In mammals, Myc is a downstream target of the FGF pathway [123], so it would be expected that increased levels of dMyc in a Bnl depleted background reduced the eye disc defects found in *eyG4>UASbnlRNAi* discs; however, we found overgrown discs with significant increase of glial cells migration to both the basal and apical sides (Figure 27). Furthermore, up-regulation of Bnl in *eyG4>UASdmycRNAi* discs rescued the growth deficit found in these discs; however, it induced significant alterations on the disc morphology, including the formation of epithelial fold, accompanied with glia migration defects. Glial cells were often found in the apical side of the disc (Figure 31). Importantly, as experimental alterations in the relative expression levels of Bnl and dMyc in EPCs led to significant perturbations in the migration of glial cells, we propose that a fine balance between the levels of Bnl and dMyc in EPCs is crucial to maintain a regulated migration of glial cells. Thus, to further understand the inter-relationship between Bnl and dMyc it would be important to alter Bnl expression using mitotic clones in EPCs and address the expression levels of dMyc, and vice versa. These data might help to clarify the epistatic relationship between these proteins and the contribution of each to the complex phenotypes of eye disc growth and glia migration. All together, this data suggests that the relationship between Bnl and dMyc does not fit in a simple unidirectional signaling cascade; instead, they cooperate in a more complex manner.

As both dMyc and *drosophila* FGF proteins are crucial in a wide range of developmental processes such as cell survival, growth and migration it would be interesting continue the study of this interaction that may clarify some biological mechanisms in vertebrates.

Final remarks and open questions

Through the development of this project we dissected a complex cross-talk between developmental signals in the eye epithelium crucial in the regulation of glia migration; however, further research is needed to understand the glia migration mechanisms triggered by Hid/Rpr-JNK-Dp53 activity in EPCs. Furthermore, we shown an interaction between Bnl and dMyc in the control of several processes of the eye disc development, but we still don´t understand how they cooperate. Further research in the field is important to clarify all this questions and to understand the truly biological meaning of Bnl binding to Btl in the eye imaginal disc.

Btl autonomously inhibits glia migration.

Bnl autonomously inhibits Rpr/Hid/Grim-JNK activity, thus limiting glia migration.

Is there some relationship between Rpr/Hid/Grim-JNK activity in EPCs and Btl activation in glia?

What are the signals activated in glia to induce their migration when Bnl is depleted in EPCs?

Does Bnl activate dMyc to prevent Rpr/Hid/Grim-JNK activity?

What are the molecular signals involved in the Bnl-dMyc co-regulation of glial migration?

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