# Müller Cells and Retinal Regeneration : The Role of the Hippo/YAP Signaling Pathway

Diana García-García

université PARIS-SACLAY

# Müller cells and retinal regeneration: the role of the Hippo/YAP signaling pathway

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**Diana GARCIA GARCIA**

## Composition du Jury

| | |
|---|---|
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| **Olivier GOUREAU**<br>Directeur de recherche, Institut de la Vision | Examinateur |
| **Muriel PERRON**<br>Directrice de recherche, Neuro-PSI | Directrice de thèse |

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Las cosas podían haber sucedido de cualquier otra manera y, sin embargo, sucedieron así.

(Things could have happened in any other way, and yet they happened like this)

Miguel Delibes, *El Camino*

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# RESUME DETAILLE

Les maladies dégénératives de la rétine sont une des causes principales de cécité chez l'homme. Malgré les avancées de ces dernières années dans le développement de nouvelles stratégies thérapeutiques, à ce jour-là il n'existe pas de traitements curatifs pleinement efficaces. Parmi les différentes approches thérapeutiques actuellement étudiées, notre équipe s'intéresse au potentiel régénératif de la rétine. En effet, même si la rétine des mammifères, et donc des hommes, n'est pas capable de régénérer spontanément, contrairement à la rétine de certains vertébrés comme les poissons et les amphibiens, elle contient des cellules présentant des caractéristiques de cellules souches et donc possède une capacité latente de régénération.

Une source cellulaire d'intérêt sont les cellules de Müller. Il s'agit du principal type de cellules gliales de la rétine qui contribuent au maintien de l'homéostasie rétinienne et de la fonction visuelle. En cas de dégénérescence elles sont capables de se réactiver, un processus appelé gliose réactive, et notamment de rentrer dans le cycle cellulaire. Chez certaines espèces, cette réactivation s'accompagne d'un processus de reprogrammation en cellules souches et donc d'un potentiel régénératif. Ainsi, cette réactivation est hautement efficace chez les téléostéens ou le xénope, où la rentrée au cycle cellulaire s'accompagne d'une neurogenèse spontanée conduisant à la régénération de la rétine. Chez les poussins, les cellules de Müller sont aussi capables de proliférer efficacement en réponse au dommage, mais elles possèdent une compétence neurogénique limitée et elles génèrent seulement des neurones de la couche interne de la rétine. Chez les mammifères, les cellules de Müller ont une capacité prolifératives et neurogénique extrêmement limitée, ne conduisant qu'à un niveau de remplacement cellulaire négligeable en cas de dégénérescence. Par conséquent, avoir une meilleure connaissance des mécanismes moléculaires sous-jacents au comportement de ces cellules et comprendre pourquoi l'auto-réparation rétinienne varie énormément d'une espèce à l'autre est essentiel pour pouvoir transformer leur potentiel de régénération en nouvelles stratégies thérapeutiques.

Dans ce contexte, mon laboratoire s'est focalisé sur l'effecteur terminal de la voie Hippo, le cofacteur transcriptionnel YAP, dont il a été démontré qu'il stimule la régénération de plusieurs organes en cas de lésion, comme le cœur, le foie ou l'intestin, en régulant le comportement des cellules souches présentes dans les tissus de ces organes. Jusqu'en 2017, en ce qui concerne la rétine, le rôle de YAP n'avait été étudié qu'au cours du développement embryonnaire et aux stades postnatals, mais son rôle dans la rétine adulte était encore inconnu. Cette année-là, mon laboratoire a publié une étude rapportant la présence de YAP spécifiquement dans les cellules de Müller de la rétine. De plus, ils ont découvert que l'expression et

l'activité de YAP étaient augmentées en cas de dégénérescence rétinienne. Étant donné le rôle de YAP dans la régulation du comportement des cellules souches adultes dans d'autres tissus comme l'intestin, il était logique de se demander si YAP pouvait remplir une fonction similaire dans les cellules de Müller. La première partie de ma thèse visait donc à décrypter la fonction YAP dans les cellules de Müller de souris dans des conditions physiologiques et pathologiques. Sachant que le modèle de souris YAP KO est létal embryonnaire, nous avons développé deux modèles de souris présentant une perte de fonction YAP. Le premier modèle est une souris hétérozygote pour le gène *Yap*, qui est viable et fertile, et qui nous a permis d'étudier l'impact d'une réduction du niveau de YAP aux stades adultes en conditions physiologiques. Afin d'étudier le rôle de YAP spécifiquement dans les cellules de Müller, nous avons généré une lignée de souris dans laquelle *Yap* était conditionnellement délété spécifiquement dans les cellules de Müller grâce au système Cre-Loxp (*Yap cKO*).

Nous avons révélé que YAP joue un rôle central dans l'homéostasie des cellules de Müller et en tant que tel, est un acteur clé de la survie des cônes au cours du vieillissement. En cas de lésion rétinienne, nous avons montré que la régulation positive de YAP est essentielle pour la réactivation des gènes du cycle cellulaire qui accompagne normalement la gliose réactive. Dans ce contexte, nous avons également trouvé une interaction fonctionnelle entre YAP et la voie de signalisation EGFR, suggérant une fonction de YAP en tant qu'intégrateur des réseaux de signalisation mis en jeu dans le contexte régénératif. Sur la base de ces résultats, nous avons émis l'hypothèse que l'expression et l'activité de YAP pourraient réveiller le potentiel régénératif de ces cellules de Müller. J'ai donc ensuite développé un modèle de gain de fonction de YAP en utilisant un adeno-associated virus (AAV) comme vecteur pour exprimer une forme constitutivement active de YAP (YAP5SA) spécifiquement dans les cellules de Müller. Ainsi, j'ai pu constater que la suractivation de YAP *ex vivo* dans des explants rétiniens est suffisante pour induire la prolifération des cellules de Müller de souris, normalement dormantes. J'ai également montré *in vivo* que les cellules de Müller infectées par cet AAV-YAP5SA sont capables de se ré-entrer dans le cycle cellulaire et de se reprogrammer en progéniteurs rétiniens, même en absence de dommage. Par ailleurs, j'ai pu montrer que l'interaction YAP-EGFR est nécessaire pour cet effet mitogène de YAP sur les cellules de Müller. Dans l'ensemble, ce travail met en évidence le rôle clé de YAP dans la sortie de quiescence des cellules de Müller chez les mammifères et révèle ainsi une cible potentielle pour la médecine régénérative.

La deuxième partie de mon projet de doctorat naît des découvertes émergentes mettant en évidence les voies inflammatoires comme régulateurs du processus de régénération. Dans la rétine, différentes études ont reporté l'existence d'une interaction entre les cellules de Müller et les cellules immunitaires résidentes, les microglies, essentielles pour la régénération rétinienne. En effet, l'ablation de la microglie

supprime la prolifération des cellules de Müller en cas de dommage chez le poisson et le poussin. Pourtant, les mécanismes impliqués dans l'interaction microglie-Müller ne sont pas entièrement compris. Des études récentes ont montré que les cytokines de la famille TNFα et IL6 favorisent la reprogrammation et la prolifération des cellules de Müller chez le poussin ou le poisson en cas de dégénérescence rétinienne. Ces facteurs inflammatoires seraient nécessaires pour déclencher différentes voies de signalisation impliquées dans la réponse régénérative comme la voie mTOR ou la voie NF-kB. Dans ce contexte, la voie de signalisation Hippo/YAP pourrait également être impliquée dans la relation entre l'inflammation et la régénération rétinienne. En effet, dans d'autres organes comme le foie ou l'intestin, des études récentes ont suggéré que l'augmentation de l'expression et/ou de l'activité de YAP en cas de lésion, nécessaire pour la régénération de ces tissus, pourrait être médiée par la réponse inflammatoire. Ces découvertes récentes m'ont conduit à faire l'hypothèse que YAP pourrait jouer un rôle dans la relation entre l'inflammation et la régénération rétinienne.

Bien que l'inflammation soit considérée comme un obstacle à la régénération rétinienne chez les mammifères, il n'existe aucune étude concernant l'influence de l'inflammation sur le processus de régénération des cellules de Müller chez la souris. J'ai donc cherché, dans un premier moment, à étudier le rôle joué par l'inflammation sur le comportement des cellules de Müller de souris. Dans un deuxième temps, j'ai cherché à déterminer comment YAP s'inscrit dans cette interaction. A cette fin, j'ai tiré parti des explants de rétines comme modèle de dégénérescence rétinienne. En stimulant la réponse inflammatoire avec du LPS, un lipopolysaccharide bactérien reconnu par le récepteur Toll-like 4 (TLR4), et en éliminant la microglie avec un inhibiteur du récepteur CSF1R, PLX3397, j'ai découvert de manière inattendue qu'un contexte pro-inflammatoire chronique établi par les cellules microgliales hyperactives stimule la prolifération des cellules de Müller de souris. Mes données indiquent que IL-6 est l'une des cytokines inflammatoires qui pourrait être impliquées dans réactivation des cellules de Müller. Il est toutefois frappant de constater que cette prolifération s'accompagne d'une inhibition de la reprogrammation des cellules de Müller en progéniteurs neurogéniques. Dans l'ensemble, nos résultats soutiennent un modèle selon lequel les signaux inflammatoires émis par la microglie hyper-stimulée ont un impact positif sur la prolifération des cellules de Müller mais ne confèrent aucun potentiel neurogénique. Ces résultats mettent en exergue une différence des effets de l'inflammation rétinienne entre la souris et le poissons qui pourrait contribuer aux différences de capacités régénératives entre ces espèces.

J'ai ensuite utilisé les modèles de gain (AAV-YAP5SA) et perte de YAP (*Yap cKO*) développés pendant la première partie de ma thèse, pour étudier le rôle de YAP dans cette interaction microglie-Müller. De façon

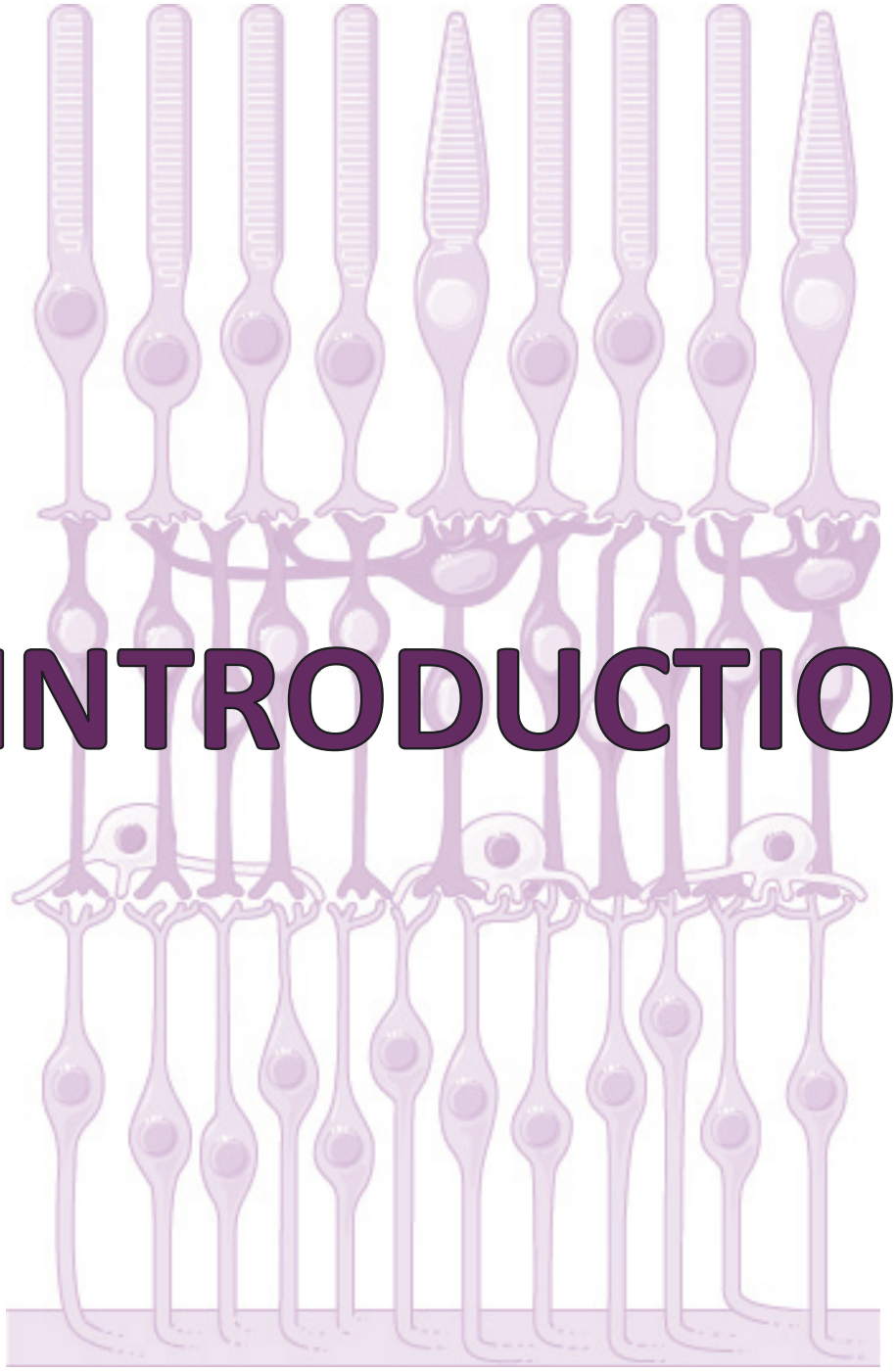
intéressante, j’ai découvert que YAP joue un rôle clé dans l'interaction réciproque entre ces cellules. En effet, en utilisant à nouveau les explants rétiniens comme modèle, j’ai mis en évidence des régulations croisées : la microglie hyperactive est capable de moduler l'expression de YAP tandis que YAP régule positivement l’expression de cytokines pro-inflammatoires. En outre, j’ai identifié des interactions fonctionnelles, les signaux inflammatoires et YAP étant dépendants les uns des autres pour favoriser la prolifération des cellules de Müller. Enfin, j’ai découvert des effets cumulatifs entre l’inflammation et YAP, permettant de réveiller presque toutes les cellules de Müller dans un explant rétinien de souris. Ce travail apporte donc de nouvelles connaissances sur l'interaction complexe entre le microenvironnement et les voies de signalisation intrinsèques qui sous-tendent la réactivation des cellules souches dormantes.

Dans l'ensemble, cette partie de mon projet permet non seulement d’approfondir nos connaissances concernant l'impact de l'inflammation sur le comportement des cellules de Müller de la souris, mais met également en évidence YAP en tant qu'acteur clé dans la connexion entre l'inflammation et la régénération rétinienne. Ce travail ouvre également la porte au développement de nouvelles thérapies basées sur la modulation de la réponse inflammatoire et de la voie de signalisation Hippo/YAP.

# ABBREVIATIONS

**AAA:** Alpha-Aminoadipate
**AAV:** Adeno- Associated Virus
**AMD:** Age-related Macular Degeneration
**AQP4:** Aquaporin-4
***Areg:*** amphireguline
***Ccnd1:*** Cyclin D1
**CMZ:** Ciliary Marginal zone
**CNS:** Central Nervous System
**CRALBP:** Cellular Retinaldehyde-Binding Protein
**CSF1R:** Colony Stimulating Factor 1 Receptor
***Ctgf:*** Connective Tissue Growth Factor
***Cyr61:*** Cystein-Rich angiogenic inducer 61
**DSS:** Dextran Sulfate Sodium
**EGF:** Epidermal Growth Factor
**EGFR:** Epidermal Growth Factor Receptor
**EGR-1:** Early Growth Response Protein 1
**ERBB4:** EGFR family member
**ESC:** Embryonic Stem Cell
**FGF2:** Fibroblast Growth Factor 2
**GABA:** Gamma-Aminobutyric Acid
**GCL:** Ganglion Cell Layer
**GDNF:** Glial cell line-Derived Neurotrophic Factor
**GFAP:** *Glial fibrillary acidic protein*
**GH:** Growth Hormone
**GLAST:** Glutamate Aspartate Transporter
**GPCR:** G Protein-Coupled Receptor
**GS:** Glutamine Synthase
**HBEGF:** Heparin Binding EGF Like Growth Factor
**HDR:** Homology Directed Repair
**HLA:** Human Leukocyte Antigen
**ICAM:** Intercellular Adhesion Molecule
**IGF-1:** Insulin-like Growth Factor 1
**IL:** Interleukin
**INL:** Inner Nuclear Layer
**IPL:** Inner Plexiform Layer
**iPSC:** Induced Pluripotent Stem Cell
**LATS1/2:** Large Tumor Suppressor ½
**LDL:** low Density Lipoprotein
**LPS:** lipopolysaccharide
**MATS:** Mobs-As-Tumor-Suppressor
**MHC:** Major Histocompatibility Complex
**miRNA:** micro RNA
**MNU:** N-methyl-N-nitrosourea
**MOB1:** (Mps One Binder kinase activator-like 1)
**MST1/2:** Mammalian Ste20-like ½
**NFI:** Nuclear Factor I
**NGF:** Nerve Growth Factor
**NIRG:** Non-astrocytic Inner Retinal Glial cells
**NMDA:** N-methyl-D-aspartic Acid
**NRG1:** Neuroregulin 1
**ONL:** Outer Nuclear Layer
**OPL:** Outer Plexiform Layer
**PDEF:** pigment Epithelium-Derived Factor
**PKA:** Protein Kinase A
**Ptbp1:** Polypyrimidine Tract-Binding Protein 1
**ROS:** Reactive Oxygen Species
**RP:** Retinitis Pigmentosa
**RPE:** Retina Pigment Epithelium
**SAV:** Salvador
**scRNA-seq:** single-cell RNA sequencing
**Six3b:** Sine oculis homeobox homologue 3b
**TAZ:** Transcriptional co-activator with PDZ-binding motif
**TGFβ:** Transforming growth factor-*beta*
**Tgif1:** TGFb-induced factor 1
**Th17:** T helper 17
**TLR4:** Toll-Like Receptor 4
**TNFα:** Tumor Necrosis Factor Alfa
**Treg:** T regulatory cell
**VEGF:** Vascular Endothelial Growth Factor
**WTS:** Warts
**YAP:** Yes-Associated Protein
**YKI:** Yorkie

# CONTENTS



# INTRODUCTION

# INTRODUCTION

Neurodegenerative diseases of the retina, such as age-related macular degeneration or retinitis pigmentosa, are characterized by retinal cell death that results in loss of vision. These diseases are widely present in the population, with millions of affected people around the world, and constitute a social, medical and economical problem. To date, there is no curative treatment. The only therapy available, and only for certain types of retinal degeneration, is centered on the delay in disease progression. Scientific progress in the field of stem cells have opened new lines of study based on endogenous stem cells stimulation to replace degenerated neurons; we are talking about regenerative medicine. An essential prerequisite is to better understand the molecular mechanisms that underlie the regenerative potential of the retina. My PhD project revolved around this subject, with a particular focus on Müller cells and the Hippo/YAP signaling pathway.

## 1. THE RETINA

### 1.1 STRUCTURE

In order to appreciate the upcoming printed letters in front of you, it is highly recommended to use the full capacity of your sense of vision in this adventure.

The vision begins with that ray of light that penetrates the eye, crosses the cornea and the lens and reaches the **retina**, the sensory tissue at the back of the eye that is organized into cell layers with microcircuits working in parallel and together to encode visual information (figure 1). With an extremely ordered structure, the fundamental plan of the retina, composed by two distinct structures (the retinal pigment epithelium (RPE) and the neural retina), is conserved across vertebrates.

Coding of visual information begins in the **neural retina**. As many central nervous systems regions, the neural retina is stratified and composed of six types of neuronal cells (two types of photoreceptors: cones and rods, horizontal, bipolar, amacrine and ganglion cells) and three types of glial cells (Müller glial cells, astrocytes and microglial cells). The cells bodies of the six types of neurons are distributed among three parallel layers of neuronal nuclei separated by two layers of synapses where connections are constrained (figure 2). In the outer nuclear layer (ONL) are the **photoreceptors**, which are light sensors that transduce the light stimuli into electric signals. Photoreceptors can be broadly categorized into rods and cones. The first ones are responsible for dim-light vision (scotopic vision) while the second ones are engaged in bright-light (photopic vision), high acuity and color vision. Both types of photoreceptors synapse onto bipolar cells at the outer plexiform layer (OPL). The **bipolar cells**

are located in the inner nuclear layer (INL), composed also by horizontal and amacrine cells, and transmit the information to the ganglion cells located on the ganglion cell layer (GCL) within the inner plexiform layer (IPL). **Horizontal and amacrine cells** will modulate the transmission of the electric signal from the photoreceptors to the ganglion cells. The **ganglion cells** are the sole output of the retina, projecting their axons to higher visual centers (figure 2) (Behan-cohen et al., 2009; Hoon et al., 2014; Miesfeld and Brown, 2019).

The other tissue of the retina, **the retinal pigment epithelium** (RPE), is a pigmented epithelial cell monolayer that absorbs the light and plays a fundamental role in maintaining the function and integrity of the retina. It occupies a strategic position with the apical membrane intertwined with the outer segments of the photoreceptors and the basolateral membrane towards the choroid, the vascular layer of the eye, playing an important role in supporting photoreceptor function, including two-way transport of nutrients and waste products and retinoid recycling (figure 1 and 2) (Behan-cohen et al., 2009; Veleri et al., 2015).

## GLIAL CELLS OF THE RETINA

The neuroretina is not only composed of neurons but also of three different types of glial cells. Retinal glia, like neuronal glia in the central nervous system, plays multiple roles in the neuronal homeostasis: transport of nutrients, hydro-ionic regulation, immunomodulation or production of neurotrophic factors. **Müller cells**, the main type of glial cells of the retina, span the entire thickness of the tissue and ensheath all retinal neurons (figure 2). Muller cells will be described in detail separately in section 3. **Microglia** are the nervous system resident tissue macrophages specialized in immune tasks (figure 2). Microglial cells with be described in detail separately in section 2. **Astrocytes** are located in the innermost retinal layer, around the retinal vessels, and are very numerous at the emergence of the nerve head (figure 2). In physiological conditions they perform beneficial roles including neurotrophic support, enhanced mechanical support for degenerating axons, and the maintain of the blood-retinal barrier. In response to injury or disease, astrocytes perform a neuroprotective effect by expressing neuroprotective trophic factors and regulating neurotransmitter, ion and water concentrations. However, the same astrocytes also express several proteins that compromise the integrity of blood-retinal barrier, up-regulating the expression of pro-inflammatory factors and promoting retinal degeneration (Hoz et al., 2016).

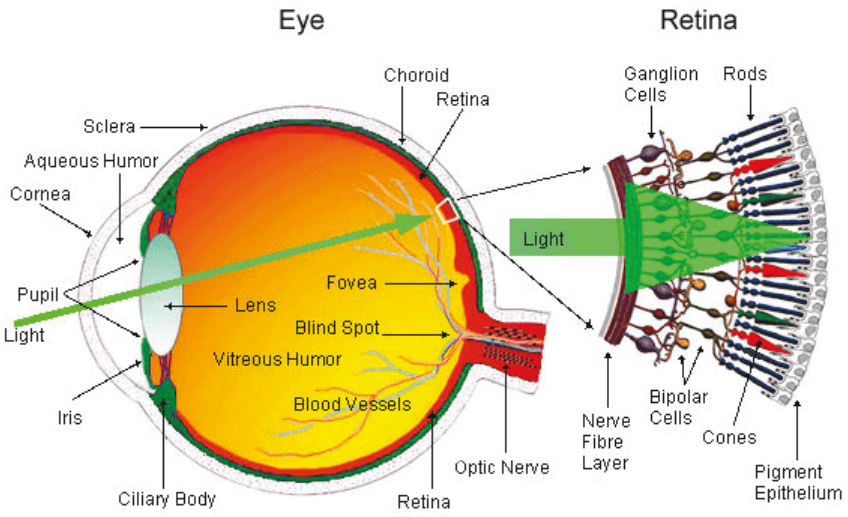



**Figure 1. Schematic sagittal section of the adult human eye**. The main tissues of the human eye are indicated in the picture. Light enters the eye through the cornea and the lens, pass through the vitreous humor and reaches the retina. In the retina, light is detected by the photoreceptor cells (rods and cones), that are light sensitive cells that converts the light rays into impulses that are sent through the optic nerve to the brain, where they are recognized as images.

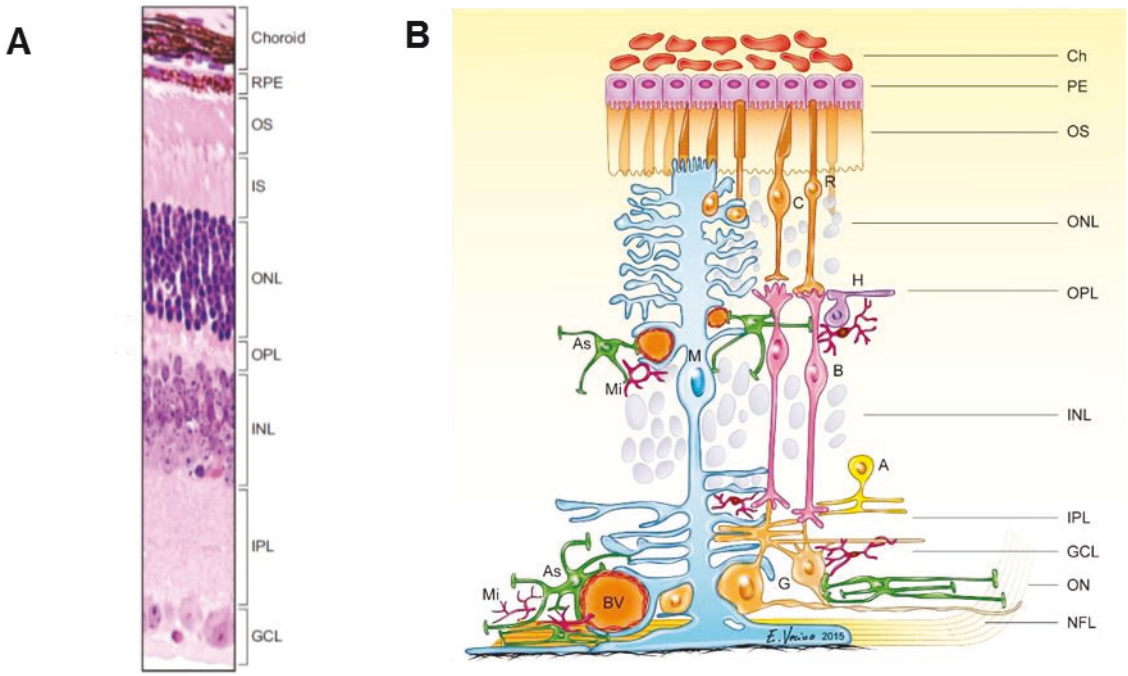



**Figure 2. Retinal structure.** (**A**) Mouse retinal section stained with hematoxylin and eosin. The different cellular layers are indicated in the picture. The outer nuclear layer (ONL) contains photoreceptor cell bodies and contact with the retinal pigment epithelium (RPE). The inner nuclear layer (INL) includes amacrine, bipolar and horizontal neurons, whereas the ganglion cell layer is formed by ganglion cells (GCL). Outer and inner plexiform layers (OPL and IPL, respectively) contain synaptic regions (Veleri et al., 2015). (**B**) Schematic drawing of a mammalian retina showing the cellular components: glia and neurons, as well as the interaction between them and with the blood vessels (BV). Amacrine cells (A), astrocytes (AS), bipolar cells (B), cones (C), ganglion cells (G), horizontal cells (H), Müller cells (M), microglia (Mi), rods (R), cones (C). The retinal cell layers are also indicated. From (Vecino et al., 2016).

## 1.2 DISEASES OF THE RETINA

The retina is highly sensible to the external or internal perturbations which can lead to an alteration of the visual circuit and the onset of a retinal disease. Retinal diseases are a major cause of low vision and affects millions of people in the world. They can affect all the retinal cell types, although the majority is related to photoreceptors or ganglion cell defects. Despite the fact that ganglion cell associated diseases as glaucoma are the second leading cause of blindness in the world, here I will focus on the retinal diseases involving photoreceptors cells. The origins of this photoreceptor associated disorders are diverse: they can be related to aging, such as age-related macular degeneration, be hereditary, such as retinitis pigmentosa, or derive from another pathology, such as complications of diabetes or uveitis. However, they are all characterized by the photoreceptor degeneration, either in a direct or an indirect way.

### PRIMARY PHOTORECEPTOR DEGENERATION

Among the retinal diseases producing a direct photoreceptor degeneration, we found the age-related macular degeneration and the retinitis pigmentosa. **Age-related macular degeneration** (AMD) is characterized by the deterioration of the center of the retina, generating symptoms as blurred central vision or a blind spot in the center of the visual field. It is the most frequent retinal disease (it has a global prevalence of ~170 million people) with a multifactorial etiology. The identified risk factors are age (the disease usually appears after the age of 50), genetic predisposition, smoking and possibly excessive sun exposure during youth. There are two types: dry macular degeneration and wet macular degeneration. The dry form of the disease has a slow evolution and is associated to the progressive disappearance of the cells of the macula, a small region in the center of the retina of diurnal species that's rich in cones. The wet form progresses rapidly, and it is due to the formation of abnormal blood vessels under the retina. In both cases this degenerative retinal disease causes a progressive loss of central vision (figure 3) (Armstrong and Mousavi, 2015; Evans, 2001; Ramsden et al., 2013). The **retinitis pigmentosa** refers to a group of inherited diseases causing retinal degeneration. It constitutes one of the most frequent monogenic diseases (1 in 3500-4000 people is affected). With a progressive development, it affects first the rod photoreceptors resulting in loss of peripheral and night vision. When the cones become affected, central and day vision diminished until total blindness. The rate of apparition, progression and degree of visual loss varies depending on the genetic mutation responsible of the disease. However, it is typically diagnosed in children, adolescents and young adults (figures 3 and 4) (Athanasiou et al., 2013; Kalloniatis et al., 2016; Veleri et al., 2015).

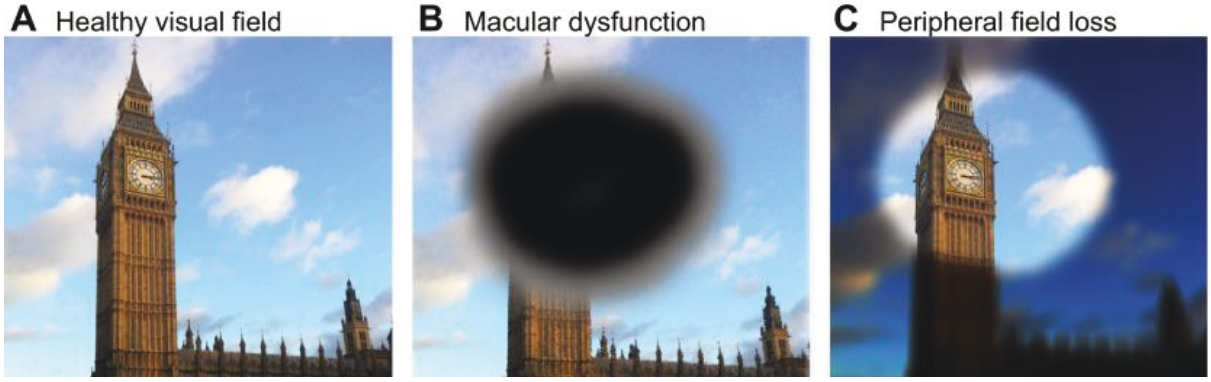



**Figure 3. Impact of different retinal degenerative diseases on visual field.** (**A**) Normal visual field. (**B**) Diseases affecting the macula, a small region in the center of the retina of diurnal species that is rich in cones. For example, age-related macular degeneration (AMD). (**C**) The tunnel visual field associated with peripheral photoreceptor loss, as in retinitis pigmentosa. From (Ramsden et al., 2013).

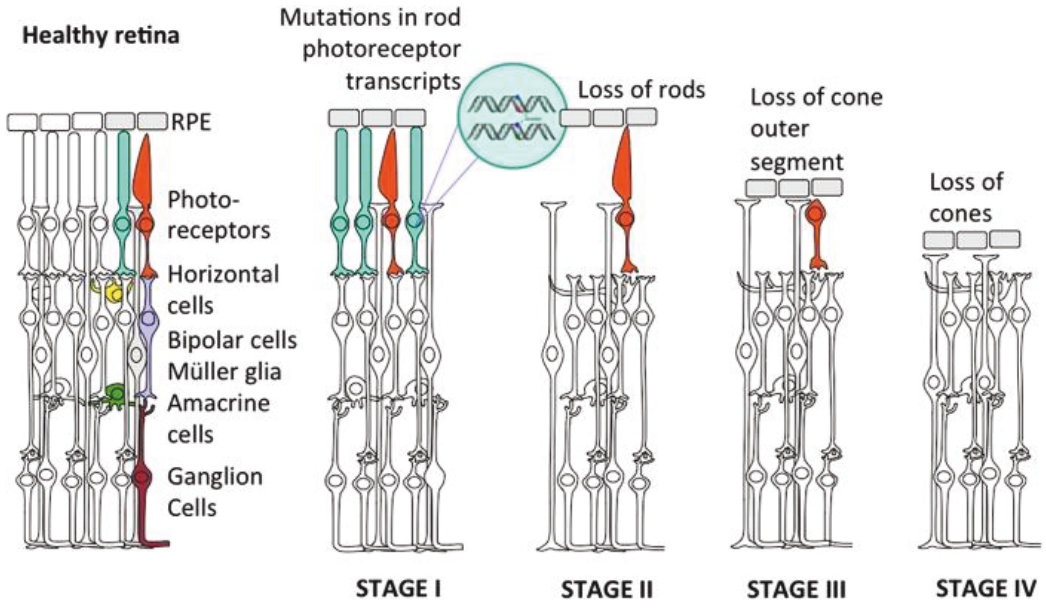



**Figure 4. Progressive development of retinal degenerative diseases.** Illustration of the effect of retinal cell death in autosomal dominant retinitis pigmentosa. The colored cells in the healthy retina indicates the five neuronal cell types that compose it. In this disease, mutation in rod photoreceptor transcripts (in blue) lead to rod degeneration (stage I). This is followed by the progressive loss of cones (in orange) (stage II and III). Finally, the complete absence of both types of photoreceptors lead to blindness (stage IV). From (Dalkara et al., 2016).

## SECONDARY PHOTORECEPTOR DEGENERATION

Photoreceptor degeneration can also be produced as a secondary effect. For example, because of the intimate relationship between photoreceptors and RPE cells, RPE dysfunction can have a deleterious effect on photoreceptor viability (Athanasiou et al., 2013). Moreover, photoreceptor degeneration can also be a consequence from a complication of another primary pathology. In the world, over 285 million individuals are estimated to have diabetes mellitus. Patients with diabetes mellitus can develop **diabetic retinopathy** as a complication. This type of disease is characterized by changes of the blood vessels in the back of the eye that can lead to an increase of the vascular permeability, and so a leakage of fluid and retinal swelling; or a neovascularization that finish by producing a hemorrhage and a retinal detachment. In both cases that blur and distort the vision (Wong et al., 2016).

## INFLAMMATION AND PHOTORECEPTOR DEGENERATION

In all this type of diseases, neuroinflammation is currently considered as an early hallmark, having both beneficial and detrimental effects. Indeed, photoreceptor cell death activate the inflammatory process in an attempt to protect the tissue during the early steps of the disease. However, a sustained proinflammatory stimulus eventually induces cellular damage and acceleration of retina degeneration. This is the case in retinitis pigmentosa and in other inherited retinal diseases, where the progressive photoreceptor cell death results in sustained inflammation that becomes detrimental. Similarly, vascular permeability of diabetic retinopathy activates leukocytes that in turn increase inflammation, aggravate retinal vascular damage, and accelerates retinal degeneration (Arroba et al., 2018; Murakami et al., 2019). The inflammatory process is thus a secondary consequence of retinal damage capable of promoting rod photoreceptor cell death. Yet, the inflammatory environment can also be a cause of retinal degeneration itself. **Uveitis** is an inflammation of the uvea, the middle layer of the eye between the retina and the sclera (the white of the eye), that can lead to vision loss if left untreated due primarily to retinal tissue damage, especially retinal photoreceptor degeneration, caused by the chronic release of pro-inflammatory factors. In most of the cases the etiology of uveitis is unknown; it could be caused by eye injury, infections, autoimmune diseases or systemic inflammatory disorders (Murakami et al., 2019). Due to the tight relationship between retinal degeneration and inflammation, not only photoreceptor conservation or regeneration, but also precise regulation of the inflammatory context, are likely to play significant roles in the future treatment of these diseases.

## 1.3 THERAPEUTIC STRATEGIES

Retinal injury or degeneration in species like zebrafish or some amphibians very rarely results in vision loss thanks to their natural capacity to regenerate the lost retinal cells. Unfortunately, mammalian retinas cannot regenerate spontaneously after damage. It is therefore compulsory to develop different therapies in order to avoid blindness. However, despite the advances on the knowledges of the pathological mechanism involved in these diseases, there are still no effective treatments nowadays.

The different therapeutic approaches that are actually been developed can be applied at different points of the disease progression, but their efficiency varies depending on the quantity of photoreceptors remaining in the retina. Thus, treatments focusing on rescuing the remaining photoreceptors must be applied at the early stages of the disease, while treatments based on photoreceptor replacement target late disease stages, when the majority of the photoreceptors have been lost (figure 5) (Santos-Ferreira et al., 2017).

### EARLY THERAPEUTIC APPROACHES

Among the initial therapeutic approaches that require the presence of intact photoreceptors, there are the neuroprotection approach, the gene therapy, and the antibody therapy. **Neuroprotection** is based on the administration of pro-survival, anti-apoptotic and anti-inflammatory molecules in an attempt to delay the progression of the retinal degeneration without treating the cause of the disease itself. This, together with the short effect of the treatment due to the half-life of the molecules administrated, leads to a controversial efficiency and a difficulty to the clinical applications. Nevertheless, this type of therapy has the advantage that it can be applied to a huge range of retinal diseases independently of the initial cause leading to degeneration (Cuenca et al., 2014; Dalkara et al., 2016; Wen et al., 2012). Similarly to neuroprotection, the **antibody therapy** is focused on the regression of the disease by targeting molecules associated with the degeneration of the photoreceptors, as for example the vascular endothelial growth factor (VEGF) responsible of the neovascularization of the wet form of AMD (Volz and Pauly, 2015). **Gene therapy**, on the contrary, treats the cause of the disease by restoring the mutant gene responsible of the production of a protein that either exhibits a decrease or absence of function, or takes on a new or detrimental role, leading to retinal degeneration. Briefly, the wild-type DNA sequence of a target gene is delivered to the affected cells where it generates a normal protein that improves or restore cellular function and address the underlying pathology. In the last years, different viral and non-viral vectors delivery strategies have been evaluated for their efficiency transducing RPE and photoreceptor cells, which are

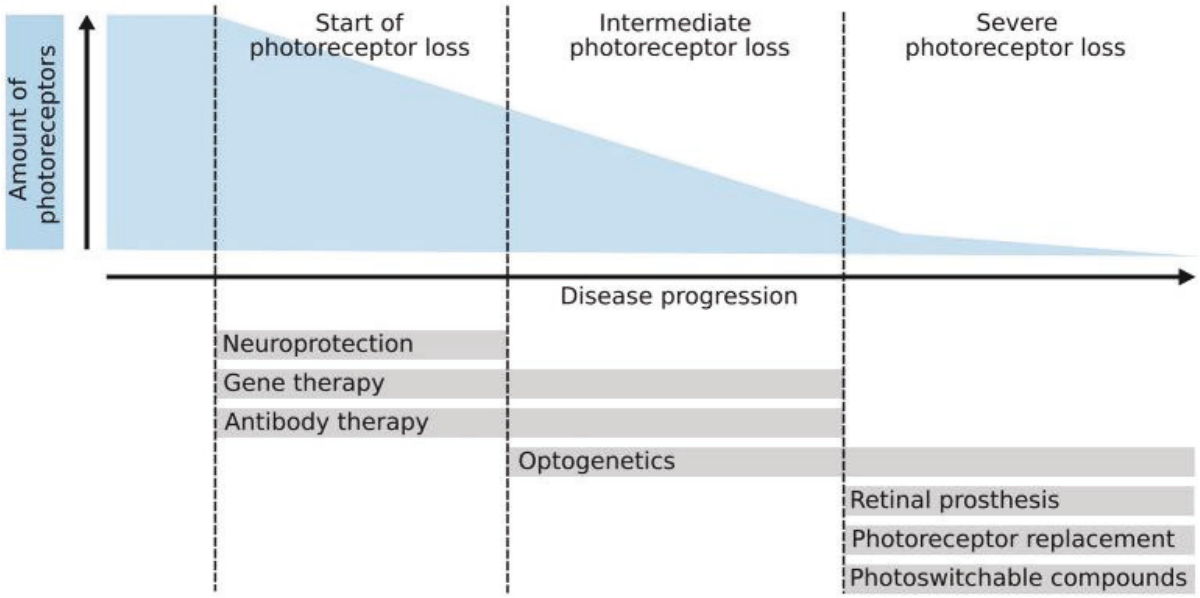



**Figure 5. Potential therapies against photoreceptor degeneration.** Schematic overview of several therapeutic approaches and their application based on the disease progression and number of photoreceptors left. Therapies focus on rescuing the remaining photoreceptors such as neuroprotection, gene therapy or antibody therapy, require the presence of an important number of photoreceptors and thus they are just applied at the early stages of the disease. Treatment based on photoreceptor replacement such as retinal prosthesis, stem cell-bases approaches or photo-switchable compounds target late disease stages, when the majority of the photoreceptors have been lost. Optogenetic approaches can also be used to reactivate remaining but dysfunctional cone photoreceptors. From (Santos-Ferreira et al., 2017).

generally the most affected by retinal degeneration, with promising results (reviewed in (Wood et al., 2019; Ziccardi et al., 2019)). However, this type of therapy is typically limited to autosomal recessively inherited genetic mutations, is very expensive and can just be applied at the beginning of those retinal diseases whose cause is genetically identified (Dalkara et al., 2016; Jayakody et al., 2015; Wood et al., 2019; Ziccardi et al., 2019). As an alternative to gene therapy, **gene editing** by CRISPR/Cas9 system has been recently shown to have a great promising use. Indeed, this type of system allows, by creating double-stranded DNA breaks at specific, predetermined sites, to delete, correct or insert a gene, being used to treat not only autosomal recessively inherited genetic mutations, but also autosomal dominantly inherited genetic mutations. Although as for the other types of gene therapies is expensive and with limited applications, it is a technology which is constantly and quickly evolving into a safer and more efficient treatment. For example, in 2019, *Cai et al.* developed a new Cas9/RecA system based on the homology-directed repair (HDR) mechanism of DNA, which allowed them to accurate correct the *Pde6b* mutation and saved the photoreceptor from degenerating in rd1 mice- a retinitis pigmentosa (RP) mutant model characterized by rapid photoreceptor degeneration and loss of vision- *in vivo* (Cai et al., 2019a). At the beginning of 2020, *Nature* reported a clinical trial where the CRISPR–Cas9 gene therapy had been for the first time applied directly to a person to treat it for a retinal disorder leading to congenital blindness (Ledford, 2020). Although some major concerns have still to be addressed, as the off-targeting effects or unintended mutations in other DNA sites, this technology can make a great impact in the early treatment of retinal diseases.

## LATE THERAPEUTIC APPROACHES

In the late stages of the disease where few or no photoreceptors are left, the therapies try to prevent the degeneration of the rest of the retinal neurons together with the restoration of the sensory inputs and/or the lost photoreceptors. We can find optogenetics, retinal prosthesis and photoreceptor replacement therapies. **Optogenetic** approaches try to bypass the loss of sensory inputs by transforming other retinal cells as bipolar or ganglion cells into light-sensitive cells thanks to the forced expression of specific genes coding for photo-activated proteins. Moreover, if the loss of photoreceptors is not severe, this approach can also be used to reactivate remaining dysfunctional photoreceptors (Cuenca et al., 2014; Wood et al., 2019). This type of therapy implies different limitations and considerations, as the requirement of an intense high level of light to activate the photo-activated proteins or the possibility of a distorted and/or an unusual visual experience due to the initiation of the visual pathway ahead the photoreceptor step. Nevertheless, significant progress is being made and several companies with optogenetic product candidates are already testing them in primates or even in patients affected with retinal degenerative diseases (Wood et al., 2019).

The **retinal prosthesis** as the Argus®II Retinal Prosthesis System, on the other hand, do not transform the rest of the retinal cells into photo-sensible cells, but they provide these neurons with electrical stimuli, being able to restore some basic visual functions (da Cruz et al., 2016; Luo and da Cruz, 2016; Mills et al., 2017). Although this type of therapy is still evolving to reduce the difference between natural and artificial vision and increase the performance, some retinal prosthetic systems are currently undergoing clinical trials with promising results (reviewed in (Bloch et al., 2019; Wood et al., 2019)).

**Stem cell-based therapies** aim at replacing damaged tissue with cells derived from stem cells. These therapies can be differentiated into exogenous stem cell-based therapies, and endogenous stem cell-based therapies. In the exogenous stem cells based therapies, thanks to their ability to proliferate and generate different cell types, stem cells are used to produce *in vitro* a large quantity of differentiated cells which will then be grafted into a patient, mainly photoreceptor precursors and retinal pigment epithelium cells, to replace the lost cells (Wood et al., 2019). There are two origins of the stem cells: the embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPSCs) with both advantages and disadvantages (Wood et al., 2019). Several human ESCs are well known and characterized. However, they possess the leukocyte antigen (HLA) responsible for antibody incompatible transplantation, requiring a systemic immunosuppression before transplantation of the ESC-derived retinal cells (Wood et al., 2019). The iPCS, on the contrary, allows the generation of autologous or allogeneic stem cell lines, but their generation process is still evolving to a better strategy to generate iPSCs avoiding the aleatory generation of mutations that could affect their properties and limit their applications (Liang and Zhang, 2013; Wood et al., 2019). Independently of the origin of the stem cell-derived retinal cells, great advances have been done in this domain. For example, in 2015 the first clinical approaches to replace the RPE injected a stem cell derived RPE cell suspension directly into the subretinal space. This technique showed encouraging results regarding the visual outcome but was associated with the development of subretinal pigmented clusters and several secondary postoperative complications (Wood et al., 2019). To overcome this poor cellular distribution within the subretinal space, basic and clinical studies used tissue-enginery to develop sheets of RPE, a product comprising stem cell–derived RPE cells that were grown as sheets on a biological scaffold. Transplantation of RPE sheet in an inherited rat model of retinal degeneration characterized by defective RPE function resulted in better cell engraftment and survival than the RPE cells transplanted as cell suspensions. In addition, an improved visual acuity was observed when the RPE sheet transplantation was used (Ben M'Barek et al., 2019; M'Barek and Monville, 2019; M'Barek et al., 2017). In humans, clinical trials using the RPE sheets reported a maintain of the sheet architecture and improved visual acuity one year after the transplantation in some patients, but also secondary effects

associated with clinical complications including a reduction on visual acuity in others (Wood et al., 2019). This heterogeneous response indicates that, although the transplantation of stem cell derived RPE is a promising strategy, more research is needed in this field to successfully overcome all the secondary clinical effects.

Regarding the transplantation of stem-cell derived photoreceptor progenitors, several promising studies in mice had been reported (Gasparini et al., 2019). Indeed, after photoreceptor transplantation several photoreceptors of the host retina displayed the donor fluorescent reported signal (Gasparini et al., 2019). Together with functional improvements in various mouse models of retinal degeneration, this led to conclude that the transplanted photoreceptors where able to migrate to the host ONL and functionally integrate into the retinal synaptic circuitry. However, with the discovery of the existence of cytoplasmic material transfer from donor to host photoreceptors, the efficiency of the integration of the grafted cells and the reconstruction of functional neuronal systems was questioned. Indeed, it seems that the considered new incorporate cells are just host cells that have integrated the proteins or RNA from the grafted cells in a process called “material transfer” (Gasparini et al., 2019). In addition to this material transfer concern, other major aspects affecting the efficiency of this therapy approach, as the cell source used to generate the donor cells, the delivery strategy or the immunological response, must be considered (Gasparini et al., 2019). For example, the photoreceptor cell replacement generally uses stem cell-derived photoreceptor precursors that once transplanted have to develop into functional photoreceptors with light sensitive outer segments. However, this is hardly the case. Most of the time, the generation of light-sensitive transplanted photoreceptor is not attained, highly reducing the efficiency of the therapy (Garita-Hernandez et al., 2019; Gasparini et al., 2019). Very interestingly, a recent study has combined the transplantation of stem-cell derived photoreceptors with optogenetics: optogenetically-transformed photoreceptors that are light responsive have been transplanted into blind mice, leading to visual function recovery (Garita-Hernandez et al., 2019). This strategy highlights how the combination of different approaches could help to overcome the actual problems of one or the other therapy.

One alternative strategy that would allow to avoid problems related to circuitry integration and immunological rejection of exogenous stem cells is the stimulation of the regenerative potential of endogenous stem cells of the retina. Indeed, even if the mammalian retina does not regenerate spontaneously, it contains three cellular sources harboring stem cell characteristics: the retinal pigment epithelium, that in some amphibians can dedifferentiate into retinal progenitors, proliferate and regenerate all neurons in the retina after retinectomy; cells of the ciliary marginal zone (CMZ), located in the peripheral region of the retina of fish and amphibians that contain stem cells for continuous growth in these species and are also capable of partially regenerating the retina in case of

retinectomy; and Müller glial cells, that are at the origin of regeneration in several species, such as zebrafis or *Xenopus*, and is the first target in the field of regeneration in mammals (Hamon et al., 2016a; Langhe and Pearson, 2019). In the recent years, fundamental research has been directed towards the understanding of the functioning of these cells to improve their stemness potential to use it as a new therapeutic approach. It is in this context that my research project has been developed, with a focus on Müller glial cells.

# 2. MICROGLIAL CELLS

## 2.1 MICROGLIAL CELLS DURING RETINAL DEVELOPMENT AND HOMEOSTASIS

**Microglia** are the nervous system resident macrophages specialized in immune tasks. They originate from primitive yolk sac progenitors, colonize the CNS during embryonic life and then self-renew throughout life (Ginhoux and Garel, 2018). In the retina, the colonization of microglia has been proposed to be done in two waves: 1. during early development prior to vascularization (figure 6), and 2. after blood vessels have formed through invasion from the optic disc or via blood vessels themselves (figure 6) (Li et al., 2019b). However, it has to be noted that much of this information has been obtained based on the spatiotemporal localization of microglial cells during development, and thus remains highly correlative (Li et al., 2019b). Lineage tracing experiments should be done to assess the timing and routes by which microglia enter the retina.

Microglia entry into the retina coincides with retinal neuron differentiation from retinal progenitor cells and have been shown to be required to regulate programmed cell death, neurogenesis, synaptic refinement and vascular development (figure 7) (Li et al., 2019b; Silverman and Wong, 2018a). Following the development phase, microglia distribute themselves to make up the stable and highly ordered network of ramified cells that can be observed in the healthy neural retinal tissue (Rashid et al., 2019). Microglia are located in the inner and outer plexiform layers, where they adopt a quiescent phenotype characterized by very small soma and extensively ramified filopodia-like processes (figure 6). In this situation, microglial cells express a high variety of cellular markers and receptors on their surface that allow them to interact with mature retinal cells and contribute to retinal homeostasis (figure 8). Among the different functions regulated completely or in part by microglial cells under physiological conditions, we find the remodeling of neuronal circuits, the elimination of cell debris and deranged synapses and the immunological surveillance of the entire retina (Rashid et al., 2019; Silverman and Wong, 2018a). However, the crosstalk between microglia and retinal neurons is not unidirectional. Indeed, some signals produced by retinal neurons regulate in turn microglial homeostasis and limit unnecessary microglial activation in the healthy retina. For example, the interaction between the membrane glycoprotein CD200, located in all the retinal cells, and the receptor CD200R, expressed predominantly in microglia, constitute a negative inhibitory signal required to maintain microglia in a quiescent state (Rashid et al., 2019). Another important retinal neuron-microglial interaction is the IL-34-CSFR1 signaling. Retinal neurons produce constitutively the cytokine IL-34. This cytokine interacts with the Colony stimulating factor 1 receptor (CSF1R) of microglia and promotes microglial survival (Greter et al., 2012). The maintenance of this interaction is

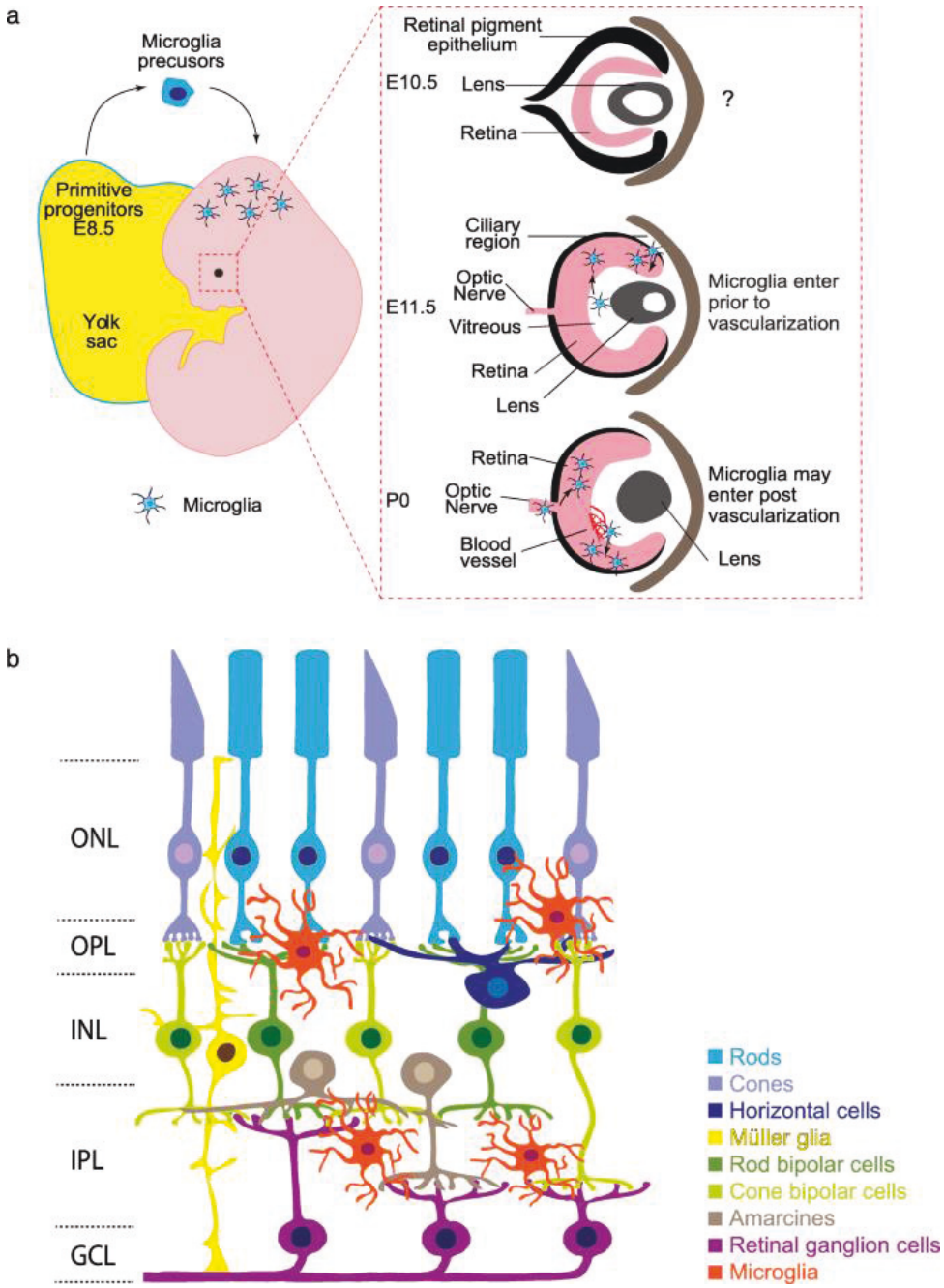



**Figure 6. Schematic of microglia development in mouse retina**. a. Timeline of microglia entry to the retina. Microglia are derived from primitive yolk sac progenitors and are thought to enter the CNS via the circulatory system. Two waves of retinal microglia infiltration have been proposed : a first one that occurs embryonically, and a second one that involved microglial infiltration from the optic disc or via blood vessels. **b**. Schematic of the adult retina showing the position of the microglial in the healthy adult retinal tissue. The retinal cell layers are also indicated. From (Li et al., 2019b)

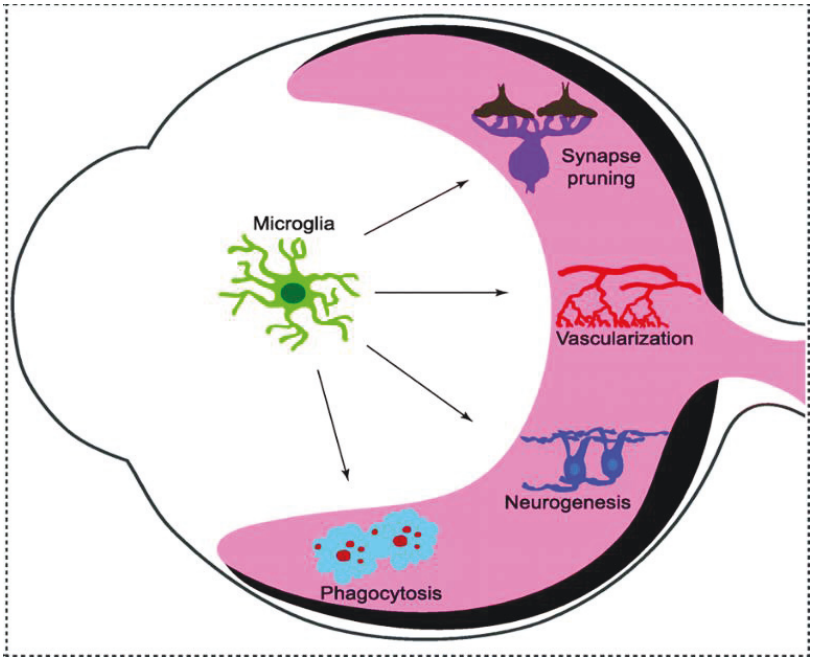



**Figure 7. Functional role of microglia during development.** Microglial cells are required to regulate programmed cell death, neurogenesis, synaptic refinement and vascular development (modified from (Li et al., 2019b)

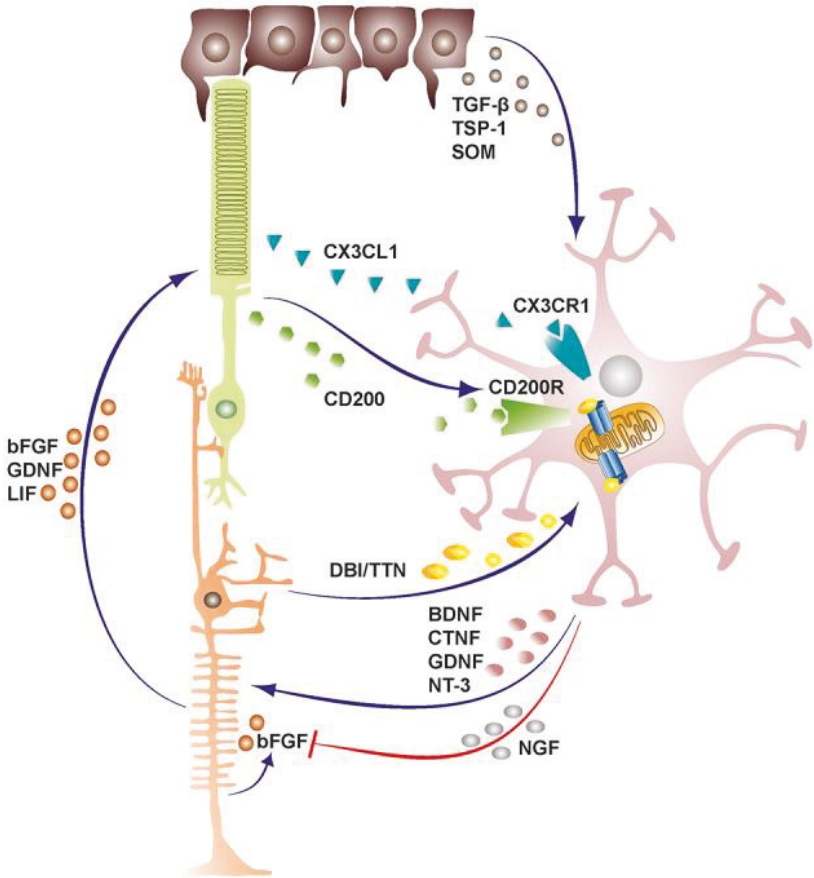



**Figure 8. Surveillant state of microglia.** In the healthy retina, microglial cells communicate in a bidirectional way with surrounding neurons and glial cells via various soluble factors and receptors that restrain microglia from tissue damaging activation and maintain them in a quiescent protective state (modified from (Rashid et al., 2019))

essential for microglial presence in the retina, as pharmacological inhibition of the CSF1R by PLX3397 results in widespread microglial depletion (Elmore et al., 2014).

## 2.2 MICROGLIAL CELLS DURING PHATOLOGICAL CONDITIONS

Alterations in retinal homeostasis can be detected by microglia by one of the several receptors that play a critical role in initiating their immunological response such as complement receptors, toll like receptors, scavenger receptors and purine receptors (Hanisch, 2002; Möller, 2002; Neumann et al., 2009; Rashid et al., 2019; Van Rossum and Hanisch, 2004). One well-known example is the toll-like receptor 4 (TLR4), which mediates the response of microglia to lipopolysaccharide (LPS). LPS is the major component of the outer membrane of Gram-negative bacteria and the viral envelope protein, and constitute the most used pro-inflammatory stimulus for microglia, both *in vitro* and *in vivo* (Chen et al., 2012). By its interaction with TLR4, it induces the secretion of several cytokines, chemokines, NO and proteases which help microglia communicate with astrocytes, Müller cells and peripheral immune cells (Chen et al., 2012; Hoshino et al., 1999; Takeda and Akira, 2003). The relationship between microglia and Müller cells will be detailed in section 3.3. The inflammatory factors secreted by microglia in case of retinal damage go from pro-inflammatory factors such as IL1β, IL18, IL6 and tumor necrosis factor α (TNFα) to anti-inflammatory factors and neurotrophic molecules such as IL-10 or TGFβ depending on the signals received from the others retinal cells (figure 9) (Hanisch, 2002; Lively and Schlichter, 2018; Rashid et al., 2019; Silverman and Wong, 2018a; Zhou et al., 2017a). Based on the main types of secreted inflammatory factors, microglia have been commonly classified into two phenotypes: the M1 phenotype, characterized by the production of pro-inflammatory mediators; and the M2 phenotype, characterized by triggering anti-inflammatory and immunosuppressive responses (Ransohoff, 2016; Zhou et al., 2017b). However, the reality is much more complicated and usually microglial cells display characteristics of both phenotypes (Ransohoff, 2016; Zhou et al., 2017b).

In addition to the secretion of multiple inflammatory factors, microglial activation is accompanied by morphological changes through an amoeboid form, increased proliferation, and migration to the sites of injury (Behan-cohen et al., 2009; Hoz et al., 2016; Rashid et al., 2019; Vecino et al., 2016). This later is mediated by microglial-secreted chemokines that activate chemokine receptors of nearby microglia and attract them to the site of damage (Garden and Möller, 2006). Moreover, it upregulates the expression of some markers while decreases the expression of others. Thus, as a kind of antigen-presenting cells, they upregulate the expression of the major histocompatibility complex (MHC) class

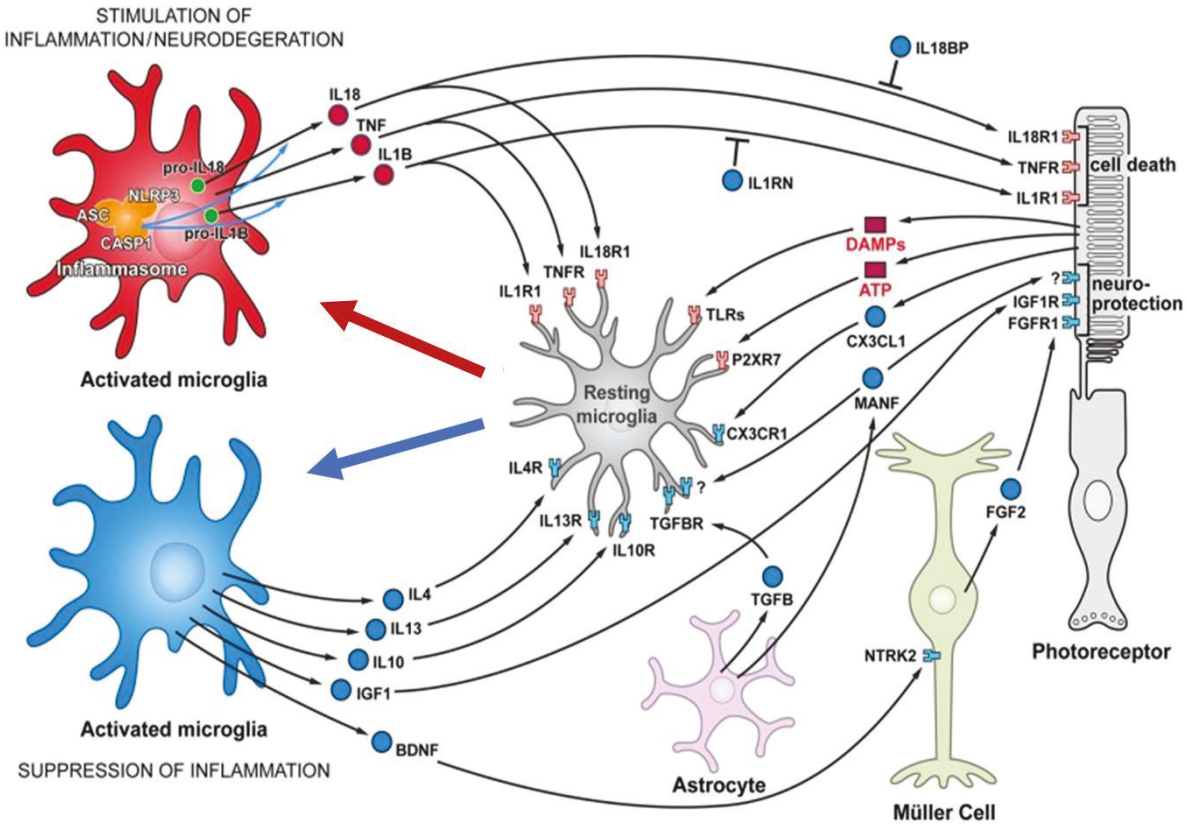



**Figure 9. Functional cell-cell interactions in retina responsible for stimulating and suppressing inflammation.** In case of damage, microglial cells activate and secret different inflammatory factors. Depending on the received signals, activate microglia will promotes a pro-inflammatory response (red) or an anti-inflammatory response (blue). The microglial response is control through autocrine and paracrine mechanism (modified from (Appelbaum et al., 2017)

I and class II (Rashid et al., 2019) but also other membrane molecules and receptors, such as F4/80, or the cluster of differentiation marker 86 (Cd68) and (Cd45), which have become markers of the activation state of microglia (Chen et al., 2002; Rashid et al., 2019). Once activated, microglial cells establish a neuroinflammatory context whose final result depends on the balance between pro- and anti-inflammatory cytokines (figure 9). In acute damage, the microglia-mediated inflammatory response promotes neuroprotection and facilitates a rapid return to tissue homeostasis (Bellver-Landete et al., 2019; Gupta et al., 2018; Rashid et al., 2019; Silverman and Wong, 2018b; Vecino et al., 2016). The role of microglia in the regenerative process will be described in detail in section 4.2. Nonetheless, if damage persists over time, the maintenance of the secretion of pro-inflammatory factors eventually leads to the induction of cellular damage and retinal degeneration (Behan-cohen et al., 2009; Hoz et al., 2016; Rashid et al., 2019; Vecino et al., 2016). The role of microglia is thus variable and can be either beneficial (Okunuki et al., 2018) or harmful (Peng et al., 2014) to the affected tissue according to the pathological condition.

# 3. THE MÜLLER CELLS

## 3.1 ORIGIN AND MORPHOLOGY OF MÜLLER CELLS

It was in 1851 that Müller cells were observed for the first time (Müller, 1851). Described at the beginning as radial fibers, Müller cells represent approximatively 4-5% of retinal cells and constitute the main type of glial cells of the vertebrate retina (Jeon et al., 1998; Strettoi and Masland, 1995).

They share the same embryonic origin as retinal neurons (Holt et al., 1988; Turner and Cepko, 1988; Wetts and Fraser, 1988). Birthdating experiments established that retinal cell types are generated in a determined order conserved along vertebrate evolution (Sidman, 1961; Vecino and Acera, 2015). Accordingly, Müller cells are among the latest cells to be born (figure 10) (Cepko et al., 1996). Of note, they have a significant transcriptome-overlap with that of multipotent late retinal progenitors (Blackshaw et al., 2004; Roesch et al., 2008), becoming specialized glial cells with a molecular signature of late stage progenitor cells (Jadhav et al., 2009). This shared feature with retinal progenitors could explain the astonishing capacity of these cells to acquire a stem-like state and serve as a source of retinal neurons in case of injury in certain species. This regenerative capacity will be described in detail in section 4.1.

In the retinal structure, Müller cells' bodies are localized in the INL, but their projections propagate throughout the thickness of the retina allowing them to interact with adjacent retinal cells at the somatic or process level (figure 11). At the distal part, Müller cells contact by adherent junctions with other Müller cells and with photoreceptors forming the outer limiting membrane, a barrier between the neural retina and the subretinal space (figure 11). In the proximal part, the conical endfeet of Müller cells interacts with astrocytes and blood vessels to form the inner limiting membrane, the boundary between the retina and the vitreous, and play a part in the structural organization of the blood-retinal barrier (figure 11) (Behan-cohen et al., 2009; Kolb, 2013; Vecino et al., 2016).

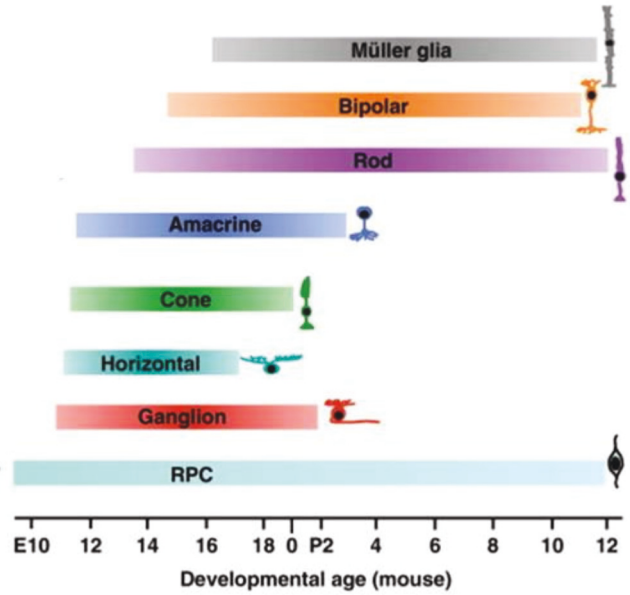



**Figure 10. Chronological order of generation of mouse retinal cell types.** During retinal development, retinal neurons differentiate from retinal progenitors in overlapping phases. Ganglion and horizontal cells are generated first, followed by cone-photoreceptors, amacrine cells, rod-photoreceptors, bipolar cells and, finally, Müller cells. The numbers relate to embryonic and postnatal days of murine development. Modified from (Bassett and Wallace, 2012).

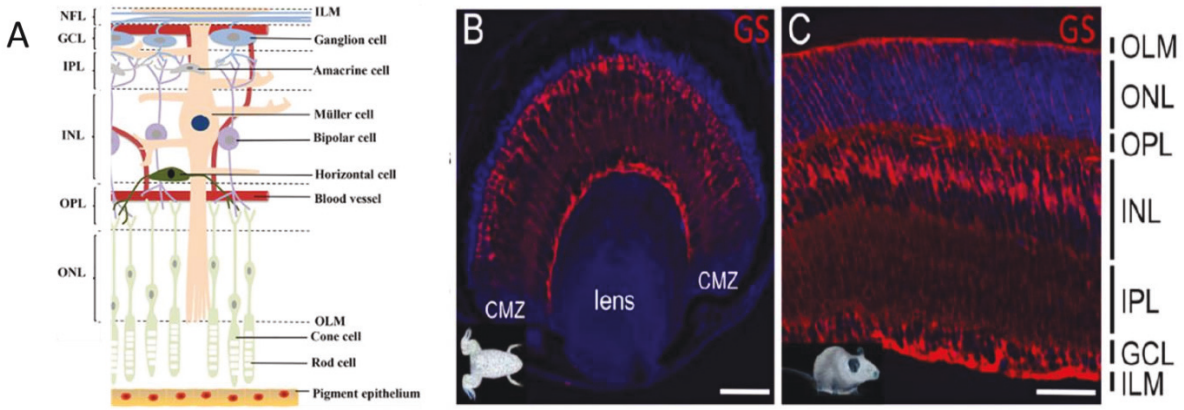



**Figure 11. Müller cells.** (**A**) Schematic representation of the Müller cell morphology. (**B** and **C**) Xenopus (stage 45, B) and mouse (P30, C) retinal sections immunolabeled for the Müller cell marker GS (glutamine-synthetase, red). Nuclei are DAPI counterstained (blue). In the retinal structure, Müller cells' bodies are localized in the INL, but their projections span all over the retina and interact with adjacent retinal cells. Their apical and basal prolongments (microvilli and endfoot respectively) contribute to the formation of the outer (OLM) and inner (ILM) limiting membrane respectively. CMZ: ciliary marginal zone; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar: 50 µm. From (Hamon et al., 2016a).

## 3.2 PHYSIOLOGICAL FUNCTIONS OF MÜLLER CELLS

Müller cells have an important expression of different ion channels, ligand receptors, transmembraneous transporter molecules and enzymes, many of them expressed specifically, or mainly, in Müller cells. All these molecules, together with the tight connections establish with the other retinal cells, allow Müller cells to perform different functions to maintain retinal homeostasis: structural and metabolic support, phagocytosis function, maintain of aqueous and ionic homeostasis and recycling of neurotransmitters and photopigments of cones (figure 12) (Baumann et al., 2017; Bringmann et al., 2006; Bringmann et al., 2009; Devoldere et al., 2019; Reichenbach and Bringmann, 2013). The different functions of Müller cells in healthy conditions are described below.

### STRUCTURAL, METABOLIC, HOMEOSTATIC AND TROPHIC SUPPORT

Structural characteristics of Müller cells make them essential in the maintenance of the neuroretinal architecture. Indeed, selective destruction of Müller cells leads to retinal disorganization photoreceptor apoptosis and, in the end, to retinal degeneration, revealing the importance of Müller cells as a **structural support** for retinal neurons (Baumann et al., 2017; Byrne et al., 2013; Shen et al., 2012). Ion channels implicating calcium-dependent mechanisms presents in the endfeet of Müller cells permit them to act as **mechanosensors** and to prevent retinal damage by regulating the expression of intermediate filaments as vimentin or GFAP, which defines then the viscoelastic properties of Müller cells, thus protecting retinal neurons from mechanical stress (Reichenbach and Bringmann, 2013; Vecino et al., 2016). Furthermore, Müller cells are implicated in the secretion of some extracellular factors such as laminin, collagen, proteoglycans or integrins (Vecino et al., 2016).

Related to this structural support, Müller cells also contribute to the **formation and maintenance of the blood-retinal barrier** and regulate the retinal blood flow. Indeed, the endfeet of Müller cells interacts with astrocytes and blood vessels to constitute the blood-retinal barrier (Vecino et al., 2016). Moreover, they participate to the regulation of the **permeability of the blood-retinal barrier** trough the expression of different factors. For example, release of pigment epithelium-derived factor (PDEF), thrombospondin or glial cell line-derived neurotrophic factor (GDNF) maintain the structure of the blood-retinal barrier, while the expression of VEGF or the tumor necrosis factor (TNF) leads to an increase of its permeability in case of stress (Devoldere et al., 2019; Eichler et al., 2004; Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013; Tout et al., 1993). This interaction Müller cells-blood vessels also allows the communication and metabolic exchanges between the blood flow and the neurons. For instance, thanks to their specialized energy metabolism, which relies mainly upon anaerobic glycolysis even in the presence of sufficient oxygen supply, Müller cells participate to retinal **glucose metabolism** providing retinal neurons with nutrients such as lactate/pyruvate for their

oxidative metabolism (figure 12) and removing neuronal metabolic waste products such as CO2 (Bringmann et al., 2006; Bringmann et al., 2009; Devoldere et al., 2019; Reichenbach and Bringmann, 2010; Tsacopoulos and Magistretti, 1996; Vecino et al., 2016). Moreover, Müller cells contribute to the **lipidic metabolism** of the neurons via their low density lipoprotein (LDL) receptors, which permit them to take the LDL to transfer it to the neurons, that will use the lipids to maintain their axons in the case of retinal ganglion cells or to form the external segments in the case of photoreceptors (Mauch et al., 2001; Tserentsoodol et al., 2006).

Another function of Müller cell is the regulation of **retinal water and ion homeostasis,** which have been associated with neuronal dysfunction in case of its deregulation. Müller cells contain ion channels and transmembrane water transporters which regulate the influx and efflux of water, potassium and sodium to restore extracellular space homoeostasis. For instance, aquaporin-4 water channels (AQP4) located at the membrane of Müller cells regulate water clearance and retinal osmoses (figure 12) (Bringmann et al., 2004; Bringmann et al., 2006; Devoldere et al., 2019; Nagelhus et al., 2004; Vecino et al., 2016). Similarly, the high density of K+ channels in the Müller cells membrane, specially Kir4.1, confer them a high K+ conductance of their plasma membrane and contribute to the regulation of the extracellular K+ concentration associated with neuronal activity by releasing it into the fluid-filled spaces outside of the neural retina (figure 12) (blood, vitreous and subretinal space) (Bringmann et al., 2006; Devoldere et al., 2019; Nagelhus et al., 2004).

Müller cells also play a role in the **regulation of the oxidative stress** generated by the normal activity of the retina. To protect the neurons from reactive oxygen species (ROS), Müller cells produce glutathione that will be release in response to oxidative stress and provided to neurons where it acts as cofactor of glutathione peroxidase to reduce the ROS. Furthermore, Müller cells can generate other antioxidant molecules such as ascorbic acid, pyruvate, α-ketoglutarate or metallothionines (Devoldere et al., 2019; Freitas and de Melo Reis, 2017; García and Vecino, 2003; Reichenbach and Bringmann, 2013). This neuroprotective function is completed by Müller cells thanks to the production of several neurotrophic and growth factors. This function will be explained in detail in section 3.3

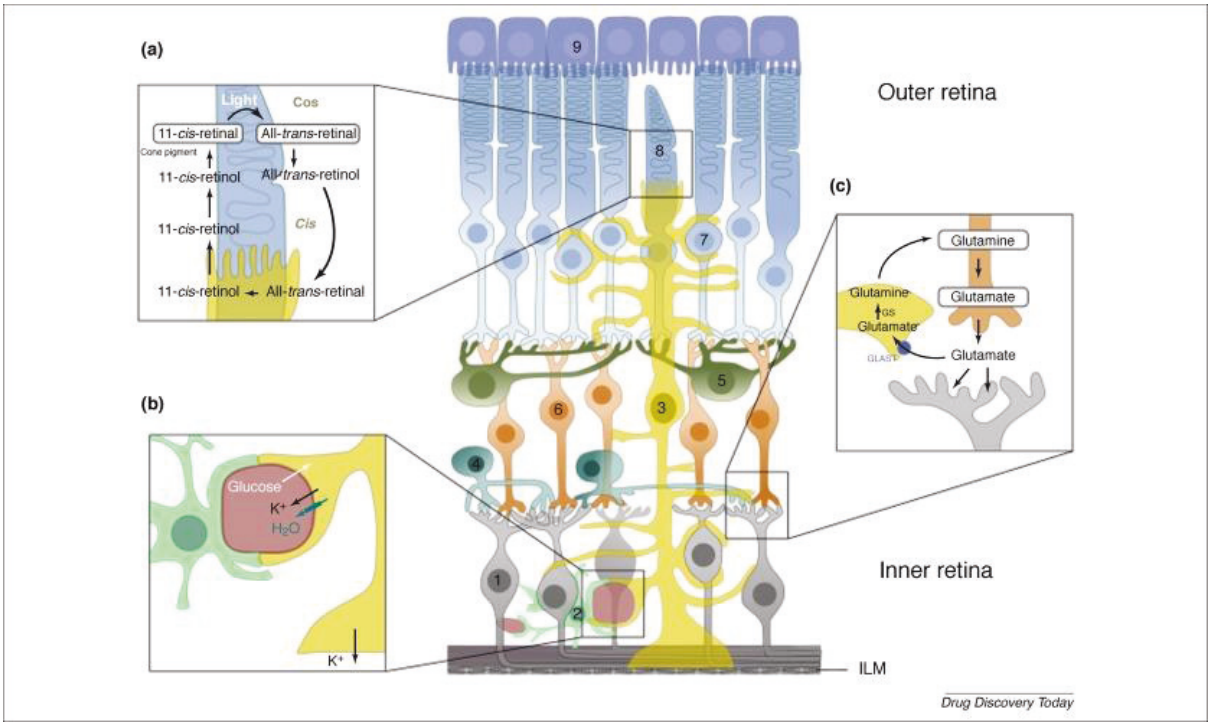



**Figure 12. Main functions of Müller cells.** Schema illustrating the cellular components of the retina, highlighting some important Müller cell functions in physiological conditions: (a) Müller cells have a key role in the cone-specific visual cycle. It is described further in the text. (b) retinal water and ion homeostasis and glucose metabolism (c) metabolic shuttle glutamate (Glu) to glutamine (Gln) implicated in the recycling of neurotransmitters. 1, ganglion cell; 2, astrocyte; 3, Muller cell; 4, amacrine cell; 5, horizontal cell; 6, bipolar cell; 7, rod; 8, cone; 9, retinal pigment epithelium cell. From (Devoldere et al., 2019).

## NEURONAL ACTIVITY REGULATION

Contrary to what is classically viewed, Müller cells are not passive participants in synaptic function. On one hand, they contribute to the neuronal signaling processes by the **clearance of neurotransmitters** from the extracellular space thanks to their membrane transporters for amino acid transmitters such as glutamate, γ-aminobutyric acid (GABA) and glycine. The clearance of these amino acids is required for the normal functioning of neuronal synapse and for the prevention of neurotoxicity, especially glutamate (Bringmann et al., 2009; De Melo Reis et al., 2008). On the other hand, they participate to the **neurotransmitter's synthesis** by recycling the uptaken neurotransmitters through their intracellular enzymes. Thus, after being taken up by Müller cells, glutamate is converted into glutamine by the Glutamine Synthase (GS), expressed specifically in these cells. Similarly, GABA is converted to glutamate and fed to the GS pathway. The final glutamine is transported back to neurons as precursor for their synthesis of glutamate and GABA (figure 12) (Bringmann et al., 2009; De Melo Reis et al., 2008). Moreover, Müller cells can modulate directly the neuronal activity through the synthesis of molecules called **gliotransmitters** (mainly glutamate, ATP and adenosine), that excite or inhibit the nearby neurons (Newman, 2015; Reichenbach and Bringmann, 2013). In addition to Müller cell-retinal neuron relationships, Müller cells can also interact with and regulate the function of other retinal glial cells, such as microglia. For example, the extracellular secretion of ATP from Müller cells mediates the activity-dependent regulation of microglial dynamic process motility under physiological conditions (Wang and Wong, 2014).

Besides, Müller cells are also involved in the **visual cycle**. First, to reduce the retinal light scattering that implies reduced visual sensitivity and acuity, Müller cells act as optical fibers guiding light all along the retina towards the photoreceptors (Franze et al., 2007; Reichenbach and Bringmann, 2013). Then, once the light has reached the photoreceptors, they are involved in the recycling of photopigments. The response to light is triggered by a visual pigment molecule present in rod and cone photoreceptors, formed by the 11-cis retinal and an opsin protein capable of activating signaling pathways, that once activated isomerize to all-trans retinal which is unable to detect a subsequent photon of light. The regeneration of the visual pigment to its initial state requires a series of steps involving specialized enzymes and retinoid binding proteins in a process known as the visual cycle. Briefly, the all-trans-retinal is transform into all-trans-retinol inside the outer segments, exported out the photoreceptor and transported to the RPE cells, where it is converted in three enzymatic steps into 11-cis-retinal. This newly molecule is then exported out and re-enters the photoreceptors, where it combines with a molecule of free opsin to regenerate the visual pigment (figure 13) (Saari et al., 2001; Travis et al., 2007; Xue et al., 2015). This classic visual cycle provides 11-cis-retinal to both rod and cones photoreceptors. However, it has recently been discovered that cones also have an

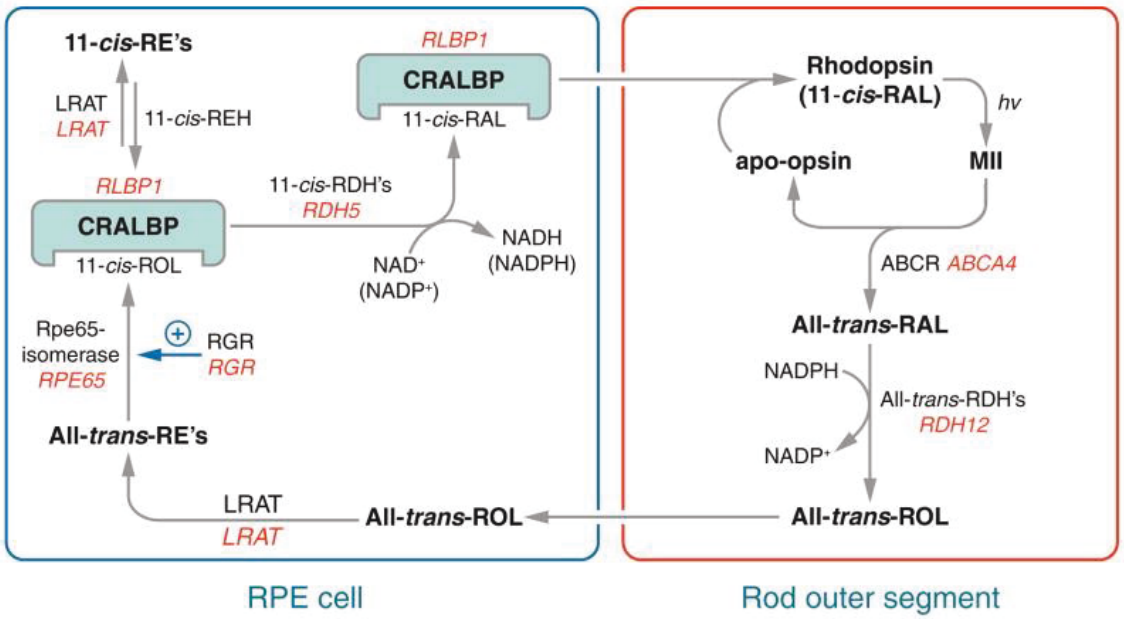



**Figure 13. Visual cycle in retinal pigment epithelium (RPE) cells.** Absorption of a photon by a rhodopsin pigment trigger its isomerization from 11-cis-retinal to all-trans retinal that is transform into all-trans-retinol inside the outer segments, exported out the photoreceptor and taken up by RPE cells, where it is converted into 11-cis-retinal in three enzymatic steps. This newly molecule is exported out and re-enters the photoreceptors, where it combines with a molecule of free opsin to regenerate the visual pigment. From (Travis et al., 2007)

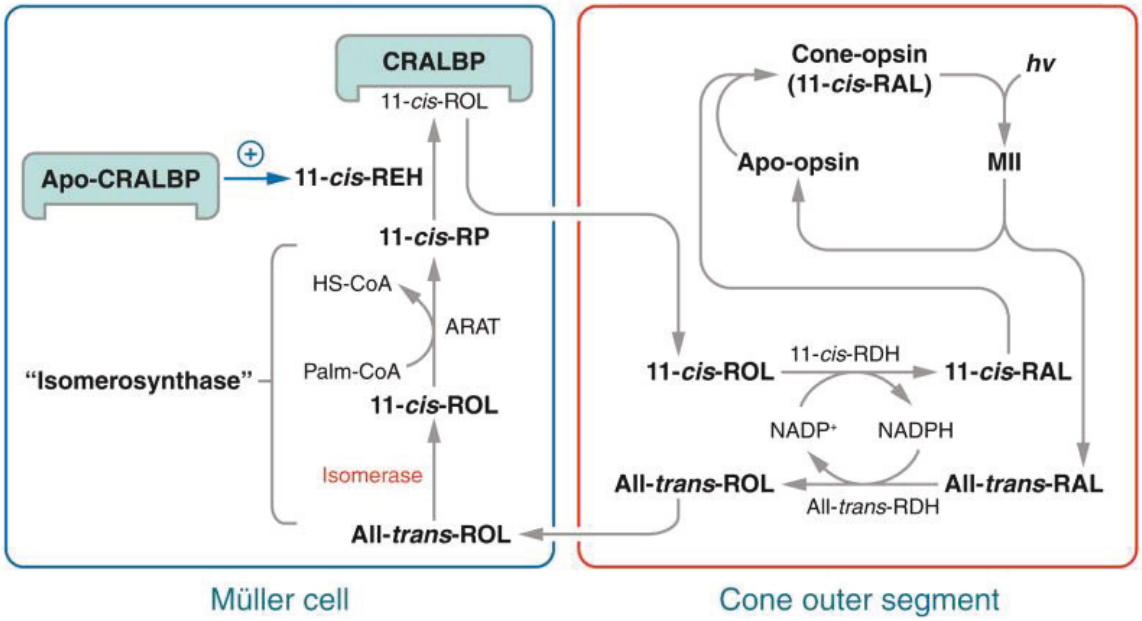



**Figure 14. Visual cycle in retinal Müller cells.** Absorption of a photon by a cone opsin pigment trigger its isomerization from 11-cis-retinal to all-trans retinal that is transform into all-trans-retinol inside the outer segments, exported out the photoreceptor and taken up by Müller cells, where it is converted into 11-cis-retinol. This newly molecule is exported out and re-enters the cones, where it is converted into 11-cis-retinal and combines with a molecule of free opsin to regenerate the visual pigment. From (Travis et al., 2007)

additional visual cycle pathways involving Müller glia. In this cone-specific visual cycle, all-trans-retinol from cones is transported to Müller cells and isomerized to 11-cis-retinol, which is then released into the extracellular space for uptake by cone photoreceptors. Cones have the unique ability to regenerate 11-cis-retinal from 11-cis-retinol to quickly recover from bright light exposure and to function over a wide range of light intensities (figure 14) (Saari et al., 2001; Travis et al., 2007; Xue et al., 2015). Among the enzymes implicated in the visual cycle, the cellular retinaldehyde-binding protein (CRALBP) deserves a special attention. CRALBP is expressed in both RPE and Müller cells and plays an important role in the RPE visual cycle by binding to the hydrophobic 11-cis retinoids and facilitating their intracellular transport (Kiser et al., 2014; Saari, 2012; Travis et al., 2007). In fact, mutations on the gene coding for this protein (RLBP1) in humans is associated with a delay in rod response and causes several autosomal recessive retinal diseases such as retinitis pigmentosa or Newfoundland rod-cone dystrophy (Eichers et al., 2002; Maw et al., 1997; Saari et al., 2001). Müller cell CRALBP, performing the same functions as in RPE, has also been identified as a crucial component for the cones visual cycle, specifically for maintaining normal cone function. Indeed, CRALBP deletion in both RPE and Müller cells impedes dark adaptation in cones and leads to cone degeneration. Importantly, restoring CRALBP expression only in Müller cells is sufficient to rescue the phenotype, showing the importance of Müller cell CRALBP for cone visual cycle (Xue et al., 2015).

## 3.3 PATHOLOGICAL FUNCTIONS OF MÜLLER CELLS

One of the key features of retinal Müller cells is their high resistance to the different forms of injury. Their secret resides in their specialized energy metabolism and capacity to perform anaerobic glycolysis, their high antioxidant content and their quick adaptation in response to an excess of glutamate (Bringmann et al., 2009). Due to these capacities, it is not surprising that Müller cells play an essential role in the response to pathogenic events.

### REACTIVE GLIOSIS: GENERALITIES

In case of retinal stress or damage, the affected neurons express different signals that activate Müller cells in a process called reactive gliosis. This reactivation is characterized by a series of morphological, biochemical and physiological changes implicating both cellular and molecular mechanisms that are not so well-known at this day. These changes can be separated into unspecific, if they are common to all types of pathogenic stimulus, or specific if they are dependent of the type of damage. Among the unspecific responses, used as early cellular marker for retinal injury, we can find the upregulation of the intermediate filaments, notably the glial fibrillary acidic protein (GFAP), cellular hypertrophy or

the re-expression of the cell cycle genes (Bringmann et al., 2006; Bringmann et al., 2009). Regarding specific changes, photoreceptor degeneration induces a specific gliotic response characterized by the decrease of the expression of GS, the enzyme involved in neurotransmitter recycling and ammonia detoxification. However, this decrease is not observed in other retinal pathologies, such as diabetic retinopathy (Bringmann et al., 2006; Bringmann et al., 2009; Mizutani et al., 1998). So, the gliotic response will depend on the pathology, more specifically on its severity and duration (Bringmann et al., 2006; Hippert et al., 2015), and on the type of Müller cells. Indeed, several studies on different species have shown an heterogeneity between Müller cells in the same region of the retina in response to the same stimulus, suggesting the existence of sub-populations of Müller cells (Bringmann et al., 2009; Roesch et al., 2012; Vecino et al., 2016).

With this gliotic changes Müller cells also acquire new capacities that allow them to protect neurons via the secretion of neurotrophic factors or by the activation of the inflammatory process, being beneficial and supporting the survival of retinal neurons. These two processes associated to the reactive gliosis, are described in detail below. However, they can also have a detrimental effect, accelerating the progress of neuronal degeneration. This is why reactive gliosis is considered as a Janus-face process.

At the beginning of the response, Müller cells reactive gliosis is neuroprotective in an attempt to limit retinal damages. It is characterized by buffering increased potassium or glutamate levels, the secretion of neuroprotective factors and antioxidative molecules, and the initiation of an inflammatory response by secreting cytokines. Some of the secreted factors, however, may have both neuroprotective and detrimental effects. This is the case of VEGF, which has a protective effect on retinal neurons at low concentrations, but whose prolonged expression can exacerbate the disease progression by inducing vascular leakage and neovascularization (Bringmann et al., 2006; Bringmann et al., 2009; Vecino et al., 2016). Proinflammatory cytokines can also have a double effect, calling for immune cells and contributing to the phagocytosis of retinal cells fragments, or increasing the cytotoxicity and the photoreceptors death (Bringmann et al., 2006; Bringmann et al., 2009). Similarly, the intermediate filaments involved in reactive gliosis also have both positives and negatives effects. The up-regulation of intermediate filaments such as GFAP has been shown to be necessary to increase Müller cell stiffness, likely to prevent mechanical lesions and assure a proper function of Müller cells in case of damage (Lu et al., 2011; Lundkvist et al., 2004; Verardo et al., 2008). However, it has also been associated with a reduced neuronal survival, as it has been shown that the absence of GFAP reduces the formation of the glial scar and increases photoreceptor survival in case of retinal detachment (Bringmann et al., 2006; Nakazawa et al., 2007).

At later stages of the gliotic response, Müller cells are capable to re-enter into the cell cycle and dedifferentiate into retinal progenitor cells to participate to retinal regeneration in some species but not in mammals (Devoldere et al., 2019; Hamon et al., 2016a; Langhe and Pearson, 2019). The regenerative potential of Müller cells is described in detail in section 4.1. Nevertheless, this dedifferentiation is associated with a decrease of some characteristic molecules of Müller cells such as GS, Kir4.1, AQP4 or CRALBP. As a consequence, the functional coupling between neurons and Müller cells is disrupted and the retinal ion and water homeostasis is altered, appearing edema, neuronal hyperexcitation and glutamate toxicity that induce neuronal degeneration (Bringmann et al., 2006; Bringmann et al., 2009; Lewis et al., 1994; Lieth et al., 1998; Tenckhoff et al., 2005).

Thus, the balance benefits/detriments of the reactive gliosis is a critical point regarding neuroprotective and regenerative functions of Müller cells.

### REACTIVE GLIOSIS: NEUROPROTECTION

Neuroprotection is one of the process associated with Müller cell reactivation. In fact, *in vitro* and *in vivo* studies have shown that Müller cells are capable of secreting neurotrophic factors, growth factors and cytokines to protect photoreceptors and retinal neurons from cell death. The main neuroprotective factors expressed by Müller cells are GDNF, CNTF, PEDF, bFGF, and BDNF (Bringmann et al., 2009; Devoldere et al., 2019; Eichler et al., 2004; Harada et al., 2003; Igarashi et al., 2000; Joly et al., 2007; Unterlauft et al., 2012; Walsh et al., 2001). These factors act directly on retinal neurons expressing the corresponding membrane receptors. However, some of them also have an autocrine effect on Müller cells allowing them to activate the expression of other factors that will have an effect on retinal neurons. This is the case of BDNF, released by Müller cells, that through a feed-forward loop will increase the production of CNTF and bFGF in Müller cells to enhance photoreceptor survival (Bringmann et al., 2009; Wexler et al., 1998). Similarly, CNTF protect photoreceptors via the activation of Müller cells and the buffering of glutamate excess (Peterson et al., 2000). GDNF acts both in a paracrine and an autocrine way. It protects photoreceptors from apoptosis either acting directly on them, either by increasing the expression of the glial glutamate transporter GLAST on Müller cells, favorizing the recapture of glutamate and decreasing the excitotoxicity (Bringmann et al., 2009; Delyfer et al., 2005; Harada et al., 2002; Koeberle and Bähr, 2008). PEDF secretion by Müller cells, on the other hand, do not protect photoreceptors but ganglion cells (Eichler et al., 2017; Unterlauft et al., 2012; Zhou et al., 2009a), while bFGF has neovascular effects (Hollborn et al., 2004; Yan et al., 2001). Thus, the neuroprotective response of Müller cells is diverse and essential to enhance the survival of different types of retinal cells in case of degeneration.

## REACTIVE GLIOSIS: IMMUNE AND INFLAMMATORY RESPONSES

Both acute damage and chronic degeneration of the retina result in inflammation, mediated by the accumulation of reactive microglia, alterations of the vasculature and immigration of blood-derived leukocytes into the retinal tissue (Bringmann et al., 2006; Karlstetter et al., 2010; Wang and Wong, 2014). In this context, Müller cells act as modulators of the immune and inflammatory response.

The factor that triggers Müller cell inflammatory response is still unknown. I*n vivo* studies showed an increase in Müller cells-microglia adhesion after LPS injection to induce an inflammatory context (Wang et al., 2011). Similarly, in an induced model of retinitis pigmentosa, microglia-Müller cell interactions have been observed, accompanied by an upregulation of the secretion of the chemotaxis factor CX3CL1 by Müller cells, that enhances microglial migration (Zhang et al., 2018). However, it remains still unknown whether it is microglia or Müller cells who initiate retinal inflammation.

It is widely accepted that molecules from activated inflammatory cells, mainly microglia, activate Müller cells that in turn express a diversity of inflammatory response-related factors to recruit and activate microglial cells in a positive feedback loop (Bringmann et al., 2009; Eastlake et al., 2015; Wang and Wong, 2014). For example, an *in vitro* study has shown that Müller cells cultured alone, or co-cultured with activated or inactivated retinal microglia exhibit marked differences in cell morphology and gene expression. Müller glia exposed to activated microglia upregulate their neuroprotective response by secreting growth factors such as GDNF, the pro-inflammatory factor production of cytokines like Il1β, TNFα or IL-6, and the chemokine and adhesion protein expression, which allow Müller cells to attract and adhere to microglia (Wang and Wong, 2014; Wang et al., 2011). This increased of the proinflammatory factors are likely mediated in response to the inflammatory mediators from activated microglia such as IFN-g, IL-1b, and TNF-a, that induce the secretion of IL-8 or MCP-1 by Müller cells, participating to the recruitment of inflammatory cells like neutrophils to sites of inflammation (Cotinet et al., 1997; Yoshida et al., 2001; Yoshida et al., 2004). IL1-β also stimulate the expression of pro-inflammatory chemokines like CCL2 or CXCL1/2 by Müller cells, implicated in microglial recruitment and activation (Natoli et al., 2017). Surprisingly, culture Müller cells did not display markers of reactive gliosis response in the presence of activated microglia. Indeed, these cells decreased the expression of gliosis markers such as glutamate aspartate transporter (GLAST) and vimentin, and their proliferation was also reduced (Wang and Wong, 2014; Wang et al., 2011). The regulation of the neuroprotective response had been described before in a model of light injury, where it has been shown that microglial activation due to the photoreceptor death leads to a secondary trophic factor expression in Müller cells creating a microglia–Müller cells–photoreceptor network that serves as a trophic factor-controlling system during retinal degeneration (figure 15) (Harada et al., 2002). Moreover, some of the pro-inflammatory cytokines expressed by Müller cells

also have a role in the neuroprotection as IL-6, which protect photoreceptors and ganglion cells from death (Chong et al., 2008; Inomata et al., 2003).

On the contrary, a recent study suggests that Müller cells, because of their structural characteristics, can sense the damage much before microglial cells (Abcouwer, 2017a; Portillo et al., 2017). Indeed, it appears that retinal inflammation in diabetic retinopathy starts by the activation of the cluster of differentiation gene 40 (CD40) receptor present in Müller cells. This activation induces Müller cells to release ATP, leading to activation of P2X7 purinergic receptors on retinal microglia and their subsequent expression of inflammatory cytokines (figure 16) (Abcouwer, 2017a; Portillo et al., 2017). This mechanism would be similar to the ATP signaling observed between Müller cells and microglia under physiological conditions, which is related to microglial function in neuronal activity (Li et al., 2012b; Wang and Wong, 2014). In any case, all these data reveal the existence of a bi-directional microglia-Müller cell signaling which results, in case of damage, in a positive feedback cycle that amplifies local inflammation, induces adaptive neuroprotection and increases physical interaction between them probably to help mobilize migratory microglia within the retina and to promote Müller cell gliosis (Wang and Wong, 2014).

The existence of immune receptors in Müller cells that would be implicated in the activation of the inflammatory context brings to light another function of Müller cell as immunocompetent cells. In fact, it has been shown that in response to the Interferon-γ secreted by active T-lymphocytes, Müller cells express class I and II major histocompatibility (MCH) molecules and become antigen-presenting cells (Bringmann et al., 2009). Furthermore, Müller cells are capable of phagocytosing fragments of retinal cells and foreign substances even before microglial cells (Bejarano-Escobar et al., 2017). In turn, Müller cells regulate the immune response and inhibit the proliferation of T-lymphocytes (Bringmann et al., 2009; Kumar et al., 2013; Roberge et al., 1991). Besides, activated Müller cells express the intercellular adhesion molecule (ICAM)-I which regulates the infiltration of macrophages and activated T cells (Richardson et al., 1996). This immune activity can also be applied to innate response. Indeed, Müller cells express functional toll-like receptors (TLR) 2, 3, 4 and 5, that promote Müller cells to produce TNF-α, IL-1β, IL-6, IL-8IL-6 and CXCL2 in response to the agonists for TLRs (Kumar and Shamsuddin, 2012; Lin et al., 2013; Liu and Steinle, 2017).

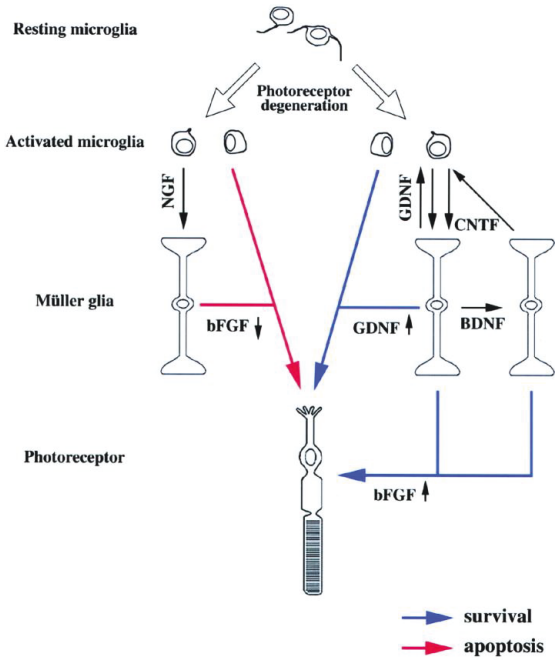



**Figure 15. Microglia-Müller cell interactions to control the production of neurotrophic factors.** In case of light degeneration, microglial cells induce the expression of several factors. Reduced bFGF induce photoreceptor apoptosis, but increased GDNF rescue photoreceptors from cell death (middle). The production of one or the other factor is regulated by the microglia-derived factors NGF (left), GDNF and CNTF (right), that acts on Müller cells to induce the production of bFGF or GDNF. From (Harada et al., 2002)

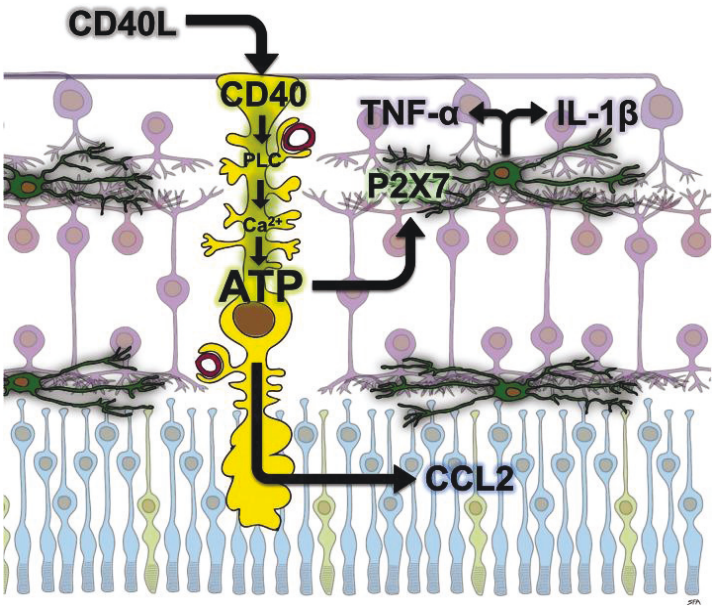



**Figure 16. Proposed role of CD40-dependent activation of Müller cells in diabetic retinal neuroinflammation.** The activation of the CD40 receptor on Müller cells trigger the release of ATP and the chemokine CCL2. Extracellular ATP activates the P2X7 receptor on microglial cells and induce the production of inflammatory factors as TNFα or IL-1β. From (Abcouwer, 2017a)

# 4. RETINAL REGENERATION

As mentioned before, the reactivation of Müller cells in pathological conditions can also be associated with the re-entry into the cell cycle and to a regenerative potential. However, this capacity is highly variable and dependent of the vertebrate species. Given the importance of Müller cell retinal regeneration in my research project, in this section I will explain in detail the implication of Müller cells into this process in different vertebrate species. Moreover, an update of the literature of the field has been the subject of an invited review where I have contributed as a first author and published in *Current Opinion in Genetics & Development* for a special issue on *Cell Reprogramming, Regeneration and Repair*. This review (García-García et al., 2020) is presented in the appendix.

## 4.1 MÜLLER CELLS IMPLICATION IN RETINAL REGENERATION

### IN TELEOST FISH

Müller cells have been recognized as the major source of endogenous stem cells for retinal regeneration in zebrafish upon different types of damages (Bernardos et al., 2007; Fausett and Goldman, 2006; Fausett et al., 2008; Fimbel et al., 2007; Montgomery et al., 2010; Ogai et al., 2016; Powell et al., 2016; Raymond et al., 2006; Thummel et al., 2008; Vihtelic and Hyde, 2000; Yurco and Cameron, 2005). In case of injury, zebrafish Müller cells respond rapidly to damage by initiating a reactive gliosis response (Hoang et al., 2019; Thomas et al., 2016) before entering into a regenerative program that implies several cellular actions: de-differentiation to a stem/progenitor-like state, proliferation, migration, neural differentiation and functional integration into retinal circuit (Gallina et al., 2014). Gene profiling analysis have shown that more than thousand genes are differentially regulated In their way to acquire stem cell properties (Qin et al., 2009; Sifuentes et al., 2016). Among genes that are upregulated, three deserve to be highlighted: *Ascl1, Lin-28* and *Stat3*, cell reprogramming factors that interact to form a regulatory cascade that controls the dedifferentiation and proliferation of Müller cells (Fausett et al., 2008; Hamon et al., 2016b; Nelson et al., 2012; Ramachandran et al., 2010).

Cell cycle re-entry involves the migration of the nuclei along the apicobasal axis of the retina in phase with the cell cycle, a process known as interkinetic nuclear migration (Lahne and Hyde, 2016; Nagashima et al., 2013). At the apical part, mitosis takes place and Müller cells undergo a single asymmetric division to self-renew and produce a proliferating retinal progenitor (Lenkowski and Raymond, 2014; Nagashima et al., 2013). This retinal progenitor will proliferate until the formation of a cluster of progenitor cells, that migrate to the retinal cell layer where cells that need to be replaced

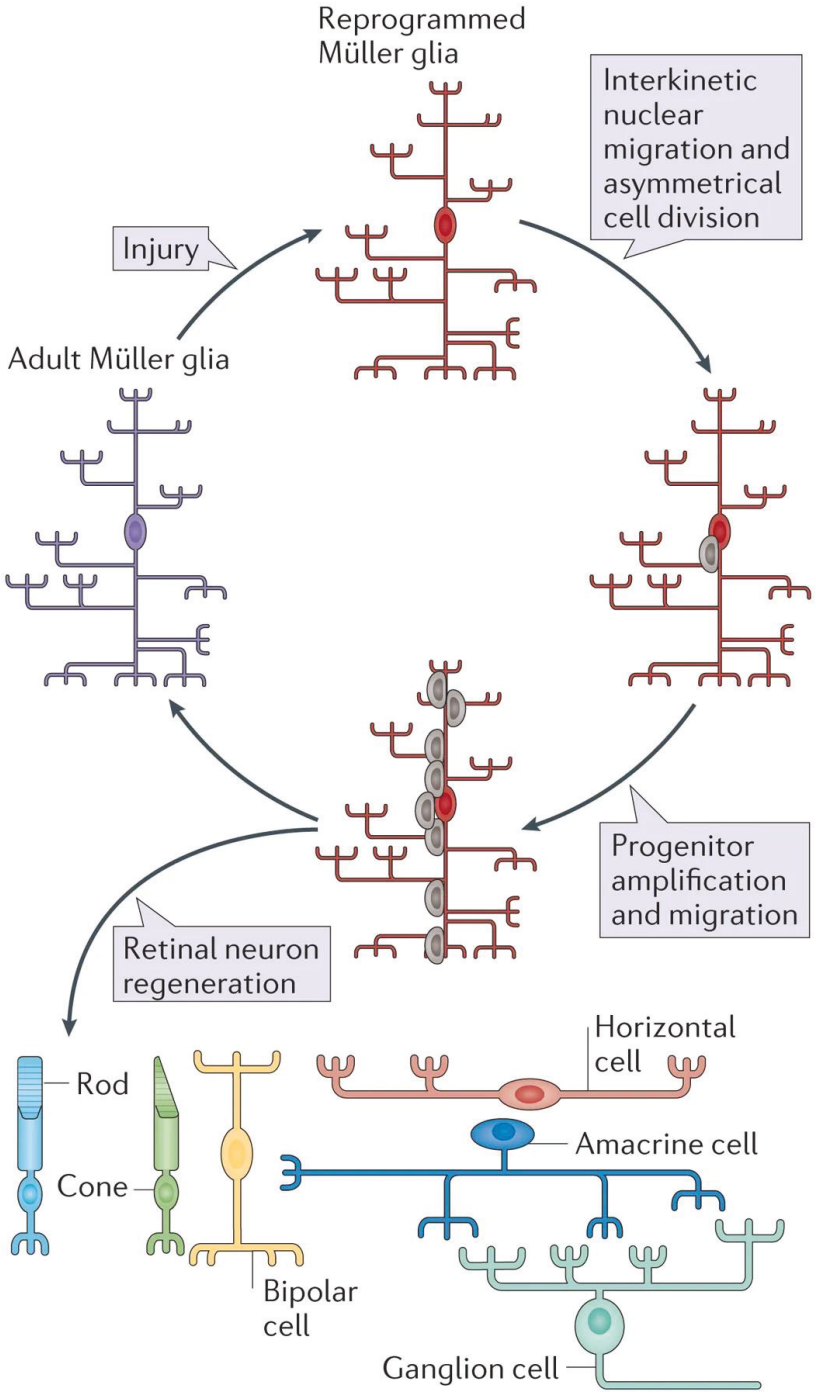



**Figure 17. Generation of multipotent Müller glial cell-derived progenitors for retinal repair.** In zebrafish, the pathogenic response leads to the reprogramming of Müller cells to acquire stem cells properties including nuclear interkinetic migrations to the outer cell layer which are associated with the re-entry to the cell cycle. This latter leads to an asymmetric division that generates multipotent progenitors that proliferate and differentiate to regenerate the retinal neurons. From (Goldman, 2014)

are located (Bernardos et al., 2007; Fausett and Goldman, 2006; Nagashima et al., 2013; Powell et al., 2016; Stenkamp, 2011). Finally, these retinal progenitor cells differentiate and generate new neurons that establish pre- and post-synaptic connections to integrate into the retinal circuitry (figure 17) (Hitchcock and Cirenza, 1994; Nagashima et al., 2013). Interestingly, although some studies suggest that Müller cell-derived progenitors may sense the cell lost and drive the regeneration towards that particular cell type (Fimbel et al., 2007; Fraser et al., 2013; Hochmann et al., 2012), another study suggest that Müller cell-derived progenitors migrate to all the retinal layers and generate all the retinal neuronal types independently of the damaged cell type. The presence of healthy neurons would then perform a feedback inhibition on the proliferation of this multipotent progenitors focusing the regeneration to the injured retinal layer (Powell et al., 2016).

### EXTRINSIC SIGNALS AND SIGNALING PATHWAYS IMPLICATED IN RETINAL REGENERATION

Pathological response of Müller cells is not homogeneous. Depending on the lesion paradigm, the amount of cell proliferation varies in an attempt to adapt it to the neuronal death (Thomas et al., 2012; Vihtelic et al., 2006). However, if damage is too important this regulation is altered leading to an overproduction of new neurons by Müller cells that leads to histological defects (Sherpa et al., 2014). Thus, Müller cells are highly sensitive to the type and quantity of the signals derived from dying cells, adapting their behavior to the retinal insult.

The regenerative process present in Zebrafish, together with its easy genetic manipulation and the variety of possible injury paradigms, make this fish an excellent model to study the molecular mechanism involved in retinal regeneration. Thus, although the molecular mechanisms implicated in Müller cells regeneration are not well-known so far, an important quantity of secreted factors and signaling pathways have been identified in this model. Among the main sources of signals triggering Müller cells reprogramming, we should highlight the apoptosis of photoreceptors. Indeed, neuronal cell death leads to the secretion of different diffusible molecules as for example the tumor necrosis factor alpha (TNFα), that triggers Müller cell dedifferentiation and proliferation through the upregulation of *Stat3* and *Ascl1* gene expression (figure 18) (Nelson et al., 2013). This apoptotic process also results in a loss of glutamatergic stimulation from photoreceptors to horizontal cells, decreasing the release of γ-aminobutyric acid (GABA) from horizontal cells. As an homeostatic mechanism to regulate Müller cell proliferation, this disruption on GABA signaling contributes to retinal regeneration (Rao et al., 2017).

Müller cells also constitute a source of signals themselves. As described above, during reactive gliosis Müller cells secrete different growth factors and cytokines that can regulate their own regenerative response in a positive autocrine/paracrine manner (Wan et al., 2014; Zhao et al., 2014a). As examples, we can cite the interleukin 6-family cytokines or growth factors such as insulin, insulin-like growth

factor-1 or fibroblast growth factor (Wan et al., 2014; Zhao et al., 2014a). Those factors activate several regeneration-associated signaling pathways implicated in Müller cell proliferation like MAPK-Erk, PI3k/Akt and Jak-Stat signaling. Similarly to the TNFα, these protein kinase cascades converge on *Ascl1* and *Stat3* gene regulation to trigger Müller cell reprogramming (Kassen et al., 2009; Lenkowski and Raymond, 2014; Wan et al., 2012a; Wan et al., 2014; Zhao et al., 2014a). Furthermore, they can converge with the canonical Wnt/β-catenin signaling pathway, shown to be necessary for the dedifferentiation and proliferation of Müller cells via *Ascl1* regulation (Meyers et al., 2012; Ramachandran et al., 2011; Wan et al., 2014) . Due to the importance of the epidermal growth factor (EGF) signaling pathway in my PhD project, the role of HB-EGF on retinal regeneration deserves a special attention. HB-EGF is a membrane-bound glycoprotein that can be proteolytically cleaved to a soluble form. This latter has the capacity to bind to, and activate, the EGFR family receptors and MAPK-signaling (Prenzel et al., 1999). In zebrafish retinas, the HB-EGF present in the Müller cell membrane acts in an autocrine way, activating the EGFR/MAPK-signaling pathway and promoting Müller cell dedifferentiation via the upregulation of the expression of reprogramming factors, such as *Ascl1a* (Wan et al., 2012b). ASCL1A would then activate the Wnt/β-catenin-signaling pathway to lead to the generation of a proliferating population of Müller cell-derived multipotent progenitors (figure 19) (Wan et al., 2012b). Importantly, this regulation of Müller cell reprogramming and proliferation by HB-EGF occurs in both the injured and uninjured retina, showing that HB-EGF is necessary and sufficient to trigger the Müller cell regenerative program in zebrafish and suggesting that Müller cells can influence their regenerative capacity by their own (Wan et al., 2012b). In addition to Wnt/β-catenin-signaling pathway activation, *Ascl1a* also acts in a negative regulatory feedback loop and inhibits HB-EGF expression via Notch signaling pathway (figure 19) (Wan et al., 2012b).

Notch signaling pathway belongs to the group of signaling pathways that instead of promoting Müller cell reprogramming and proliferation, inhibit Müller cell proliferation to maintain Müller cells in a quiescence state. In the undamaged adult zebrafish retina, Notch signaling is present at high levels in quiescent Müller cells. This activation of Notch signaling in the mature retina serves to maintain differentiated Müller cells in a quiescent state by inhibiting *Stat3*, *Ascl1* and *hb-egf* expression and preventing injury-dependent Müller cell reprogramming and progenitor proliferation (figure 19) (Elsaeidi et al., 2018; Wan et al., 2012b). Consistent with that, inhibition of Notch signaling results in Müller cell proliferation even in the absence of injury (Conner et al., 2014), whereas inhibition of Notch combined with Ascl1 overexpression results in an enhanced Müller cell proliferation (Elsaeidi et al., 2018). This negative regulation of Müller cell-dependent regeneration in zebrafish could have as a finality to regulate the number of Müller cells going into proliferation and therefore to adapt their

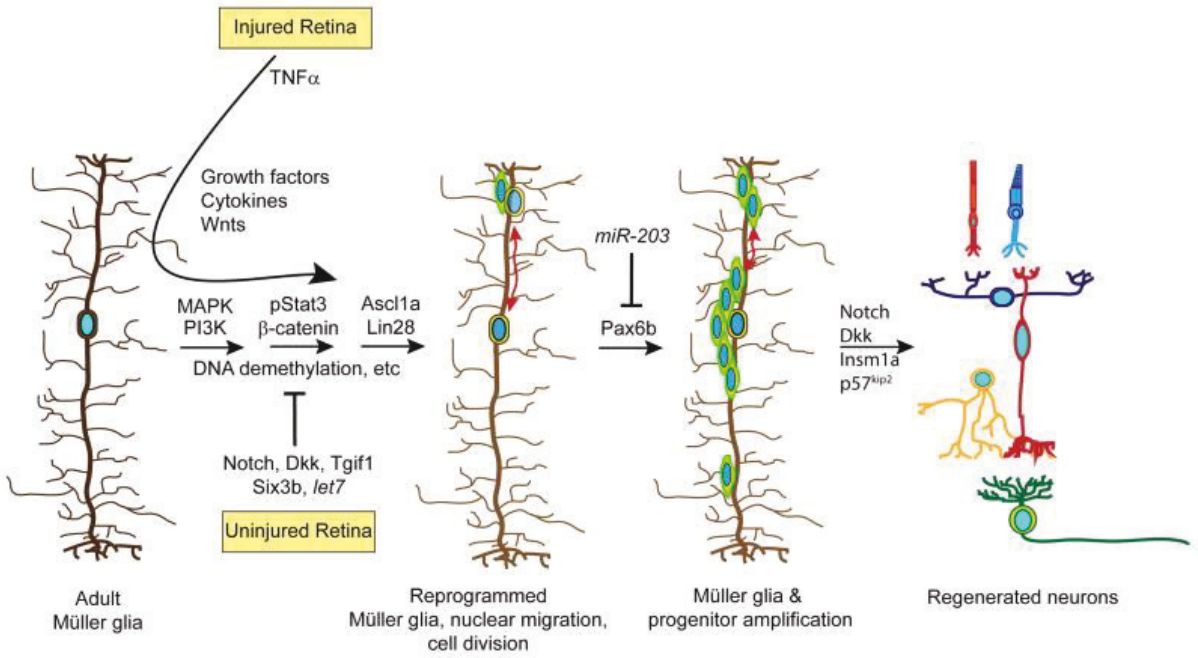



**Figure 18. Signaling pathways regulating Müller cell reprogramming and retinal regeneration in zebrafish.** Representation of the main signaling pathways implicated in the regulation of the reprogramming and division of Müller cells, the proliferation of the generated progenitors and their differentiation into retinal neurons. From (Wan and Goldman, 2016).

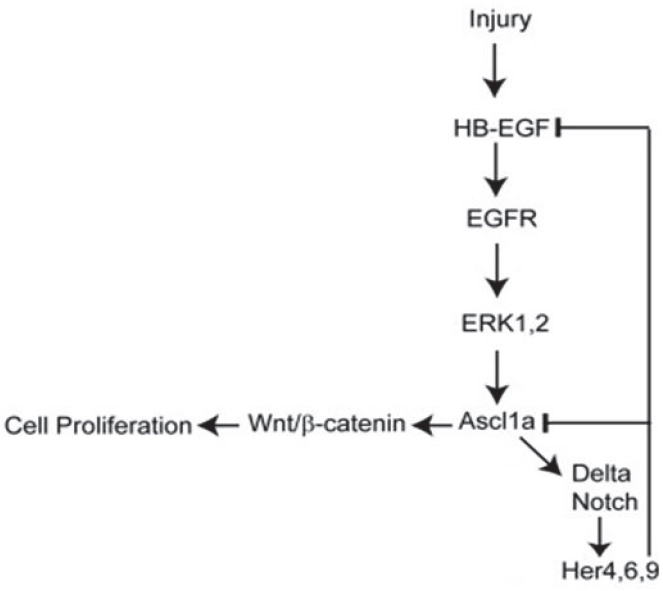



**Figure 19. Signaling cascades and genes regulated by HB-EGF in zebrafish retina upon damage.** HB-EGF activates MAPK-signaling pathway that regulates expression of regeneration-associated genes like *Ascl1a*. *Ascl1a* trigger Wnt/β-catenin-signaling cascade resulting in Müller cells proliferation. In addition, *Ascl1a* feeds back and inhibits *Hb-egf* expression via a Notch-signaling pathway to regulate Müller cell reprogramming, proliferation and the differentiation of Müller cell-derived progenitor to photoreceptor (Modified from (Wan et al., 2012b)).

response to the extent of the injury (figure 18) (Conner et al., 2014; Wan et al., 2012a). Interestingly, the function of Notch signaling in Müller cells is not only restraint to a negative regulation of Müller cell dedifferentiation and progenitor proliferation. Indeed, it also seems to have a role at late stages of the retina regeneration, specifically regulating the differentiation process of Müller cell-derived progenitors by stimulating photoreceptor formation (Wan et al., 2012b). Of note, Notch signaling could be differently regulated depending on Müller cell location/subpopulation (Wan and Goldman, 2017). Indeed, in newly formed peripheral Müller cells an increase of Fgf8a, a fibroblast growth factor acting upstream of Notch, triggers Notch activity, whereas in older central Müller cells this increase of Fgf8a inhibits Notch activity (Wan and Goldman, 2017).

Another signaling pathway that negatively regulates injury-dependent Müller cells reactivation is TGFβ, that activates Smad2/3 to inhibit Müller cells proliferation (Lenkowski et al., 2013; Tappeiner et al., 2016). Similarly to Notch signaling, TGFβ regulates Müller cell regeneration acting at different steps of the regenerative process. Indeed, it seems that TGFβ promotes Müller cell activation at the beginning of the regenerative process. Then, the TGFβ-induced factor 1 (Tgif1) and sine oculis homeobox homologue 3b (Six3b), co-repressors of TGFβ, would inhibit this signaling pathway in order to promote Müller cell proliferation. Finally, the TGFβ signaling pathway would activate again to inhibit the proliferative process and trigger Müller cell-derived progenitor cells differentiation (Lenkowski et al., 2013; Sharma et al., 2020; Tappeiner et al., 2016). One of the factors that could be responsible of the regulation of TGFβ activity is the reprogramming factor Oct4. Indeed, it has recently being found that Oct4 inhibits TGFβ signaling pathway during Müller cell reprogramming and progenitor proliferation, but stimulates it at the differentiation step to induce the cell cycle exit of Müller cell-derived retinal progenitors and the generation of new (Sharma et al., 2019).

In addition to this signaling pathways, several microRNAs (miRNAs) proved to play important roles during retinal regeneration (Quintero and Lamas, 2018). Highlighting this fact is the finding that suppression of Dicer, a critical regulator of miRNA biogenesis, impairs zebrafish Müller cell cycle re-entry in response to injury (Rajaram et al., 2014). High-throughput sequencing allowed identifying miRNA that exhibit differential expression in intact *versus* regenerating retinas. Some of them, miR-142b, 146a, 7a, 27c, and 31 turned out to be required for the proliferation of MGPCs (Rajaram et al., 2014). Conversely, other miRNAs are downregulated following injury, and are therefore potentially involved in the maintenance of Müller cell quiescence and/or in the inhibition of their reprogramming. A well-known example is let-7miRNA, which needs to be inhibited to allow derepression of crucial dedifferentiation genes. Blockade of its maturation occurs through the action of the RNA binding protein Lin28, which is activated downstream Ascl1 (Ramachandran et al., 2010), Wnt signaling (Yao

et al., 2016) or Shh signaling (Kaur et al., 2018). Of note, let-7miRNA in turn regulates Shh pathway components, thereby participating to a complex regulatory loop (Kaur et al., 2018).

### IN AMPHIBIANS

It has long been known that urodele amphibians, such as the newt or the salamander, possess eye regenerative properties following ablation (Mitashov, 1996). Regarding anuran amphibians like *Xenopus laevis*, during years it was though that their retinal regenerative capacity was restricted to larval stage (Hitchcock et al., 2004). However, in 2007 a study showed that in case of retinectomy, adult *Xenopus* could also regenerate the retina, indicating that the retinal regeneration capacity was retained after metamorphosis (Yoshii et al., 2007). Unfortunately, even though these studies allowed to describe the contribution of the RPE and of CMZ stems cells in *Xenopus* retinal regeneration, because retinectomy surgeries remove the entire retina, the response of Müller cells to the damage in this species could not be investigated (Hidalgo et al., 2014; Hitchcock et al., 2004; Vergara and Del Rio-Tsonis, 2009; Yoshii et al., 2007). In 2011, Martinez-De Luna et al. reported the finding of retinal precursor cells at the wound site after partial retinal resection in premetamorphic *Xenopus laevis* tadpoles suggesting that *Xenopus* Müller could re-enter to the cell-cycle, but the exact origin of this retinal precursors was not investigated and thus whether Müller cells have a regenerative potential in this species remained unknown (Martinez-De Luna et al., 2011). In a *Xenopus* model of conditional rod cell ablation, Müller cell hypertrophy was observed suggesting the occurrence of reactive gliosis, but no cell cycle re-entry was detected (Choi et al., 2011). Recently, however, my laboratory has reported that *Xenopus* Müller cells can re-enter the cell cycle upon retinal damage (Langhe et al., 2017). Using a mechanical retinal injury and a degenerative model of nitroreductase-mediated photoreceptor cell ablation, they showed in *Xenopus laevis* premetamorphic tadpoles that a subset of Müller cells was able to re-enter into the cell cycle and replace lost cells following damage/degeneration in the retina (Langhe et al., 2017) . Interestingly, the response intensity regarding the cell cycle re-entry of Müller cells differs between the two injury models, suggesting that, as in teleost fish, Müller cell response in amphibians is dependent on the type of damage or the extent of the damage (Langhe et al., 2017) . But more importantly, they also found that the reactivation of Müller cells was dependent on the age of the animal, since Müller cell response was very limited in young tadpoles compared to late stage tadpoles (pre or post-metaphorphic), or adult *Xenopus* (Langhe et al., 2017) . This counterintuitively finding is similar to what was observed during *Xenopus* tail regeneration: it occurs from development to adulthood except from a refractory period between stages 45 to 47, when the tail heals over without regenerating (Beck et al., 2003). Strikingly, inhibition of the immune response is sufficient to restore the regenerative capacity during the refractory period (Fukazawa et al., 2009). The situation in *Xenopus* retina is however likely very different since preliminary data from my host laboratory

suggest the opposite: promoting inflammation in early tadpoles seems to favor Müller cells proliferation. So, the differences found in *Xenopus* Müller cell reactivation at different stages might be due to different inflammatory responses. The role of the inflammatory process in retinal regeneration will be discussed in detail in section 4.2.

### IN BIRDS

In chicken, acute retinal damage induces an important proliferative response of Müller cells in the central retina at least during the first two weeks post-hatching leading to the regeneration of some retinal neurons (Fischer and Reh, 2001; Fischer and Reh, 2002). However, after this period this ability disappears and adult chicken cannot regenerate their retina following injury (Fischer and Reh, 2001; Fischer and Reh, 2002).

The initial steps of Müller cells regenerative response in post-hatched chickens are common to zebrafish regeneration. In case of damage, the majority of Müller cells re-enter into the cell cycle, express reprogramming genes such as *Ascl1* and differentiate into new neurons according to the type of cells that have been lost (Fischer and Reh, 2002). Thus, neurotoxic damage by N-methyl-D-aspartic acid (NMDA) affecting amacrine and bipolar cells leads to differentiation of Müller cell-derived progenitors into both types of neurons, while toxins like colchicine or kainic acid trigger their differentiation into ganglion cells (Fischer and Reh, 2002). However, no photoreceptor regeneration has been observed in a retinal detachment model even after the re-entry of Müller cells in the cell cycle (Cebulla et al., 2012). Of note though, due to the limited number of photoreceptor degenerative models, the possibility that chicken Müller cells retains the capacity to generate photoreceptors cannot be excluded. The main difference between teleost fish and chick Müller cell response is found on their neurogenic capacity. Indeed, although numerous proliferating Müller cell-derived progenitors are generated, just a little fraction differentiates into the lost neurons, limiting the regenerative response and being thus not sufficient to restore visual function (Fischer, 2005; Fischer and Reh, 2001; Fischer and Reh, 2002; Fischer and Reh, 2003; Gallina et al., 2014; Wilken and Reh, 2016).

### EXTRINSIC SIGNALS AND SIGNALING PATHWAYS IMPLICATED IN RETINAL REGENERATION

Similarly to teleost fish, dying neurons in chick retinas release some factors that trigger Müller cell response. Besides, secreted growth factors have been shown to stimulate Müller cells dedifferentiation and proliferation (Fischer and Reh, 2002; Fischer et al., 2002). For example, sustained administration of Growth hormone (GH) improved Müller cell reactivation, reprogramming and proliferation after kainic acid excitotoxic damage (Martinez-Moreno et al., 2019). Likewise, other exogenous growth factors as the fibroblast growth factor 2 (FGF2), insulin and insulin-like growth factor-1 (IGF-1) stimulate Müller cell reprogramming and proliferation (Fischer and Reh, 2002; Fischer

et al., 2002; Todd et al., 2015). Moreover, as for the zebrafish, HB-EGF also influences Müller cell proliferation (Todd et al., 2015). Indeed, in case of damage, the addition of exogenous HB-EGF in the chicken retina stimulates the proliferation of Müller cell-derived progenitors, whereas the inhibition of the EGF-receptor (EGFR) results in the opposite effect, with a decrease in the proliferation of Müller cell-derived progenitors (Todd et al., 2015). Previous studies have shown that the combination of IGF1 and FGF2 stimulates synergistically the proliferation of Müller cell-derived progenitors (Fischer and Reh, 2002; Fischer et al., 2002; Ritchey et al., 2012). Interestingly, the combination of HB-EGF with FGF2 has no more effect on Müller cell-derived progenitors than after the addition of FGF2 alone (Fischer et al., 2009; Todd et al., 2015). One possible explanation is that, as both growth factors activate the MPAK-signaling, this pathway would be already saturated by one of them, making the addition of the other useless. However, it could also be related to the effect of insulin on microglial cells, as IGF1, but not HB-EGF or FGF2, increases microglial reactivity in retinal explants (Fischer et al., 2009; Fischer et al., 2010; Gallina et al., 2014; Todd et al., 2015). So, it would be easy to hypothesize that, in addition to signaling factors, Müller cells response in chick may be influenced by other types of glial cells such as microglia or infiltrating macrophages (Fischer et al., 2014). The influence of microglial cells on Müller cell retinal regeneration is described in detail in section 4.2.

In addition to the MAPK signaling, another signaling pathway implicated in the avian retina regeneration is the Notch pathway. As in the zebrafish retina, Notch activity has two distinct roles in retinal regeneration regarding Müller cell reactivation and Müller cell-derived progenitor differentiation (Hayes 2007). Strikingly, contrary to zebrafish, Notch signaling in chicken appears to be necessary for Müller cell dedifferentiation and proliferation (Ghai et al., 2010; Hayes et al., 2007), whereas its inhibition is necessary to induce the neuronal differentiation of the progenitors cells (Hayes et al., 2007). The reason why Notch pathway has an inverted role between the two species and the consequences of this difference are still unknown, but it could be an explanation to the variations observed in their regenerative potentials.

Other signaling pathways implicated in the stimulation of Müller cells dedifferentiation and proliferation in teleost fish are also implicated in avian retinas. This is the case of Wnt/β-catenin, JAK/STAT, Hedgehog, mTOR, retinoic acid and BMP/SMAD (Gallina et al., 2016; Todd and Fischer, 2015; Todd et al., 2016; Todd et al., 2017; Todd et al., 2018; Zelinka et al., 2016). Some other signaling pathways as TGFβ would be responsible for the suppression of Müller proliferation and differentiation into progenitors via SMAD2/3 signaling (Todd et al., 2017). Interestingly, these signaling pathways would form a complex network in which MAPK activation by FGF2 crosstalk with Notch signaling

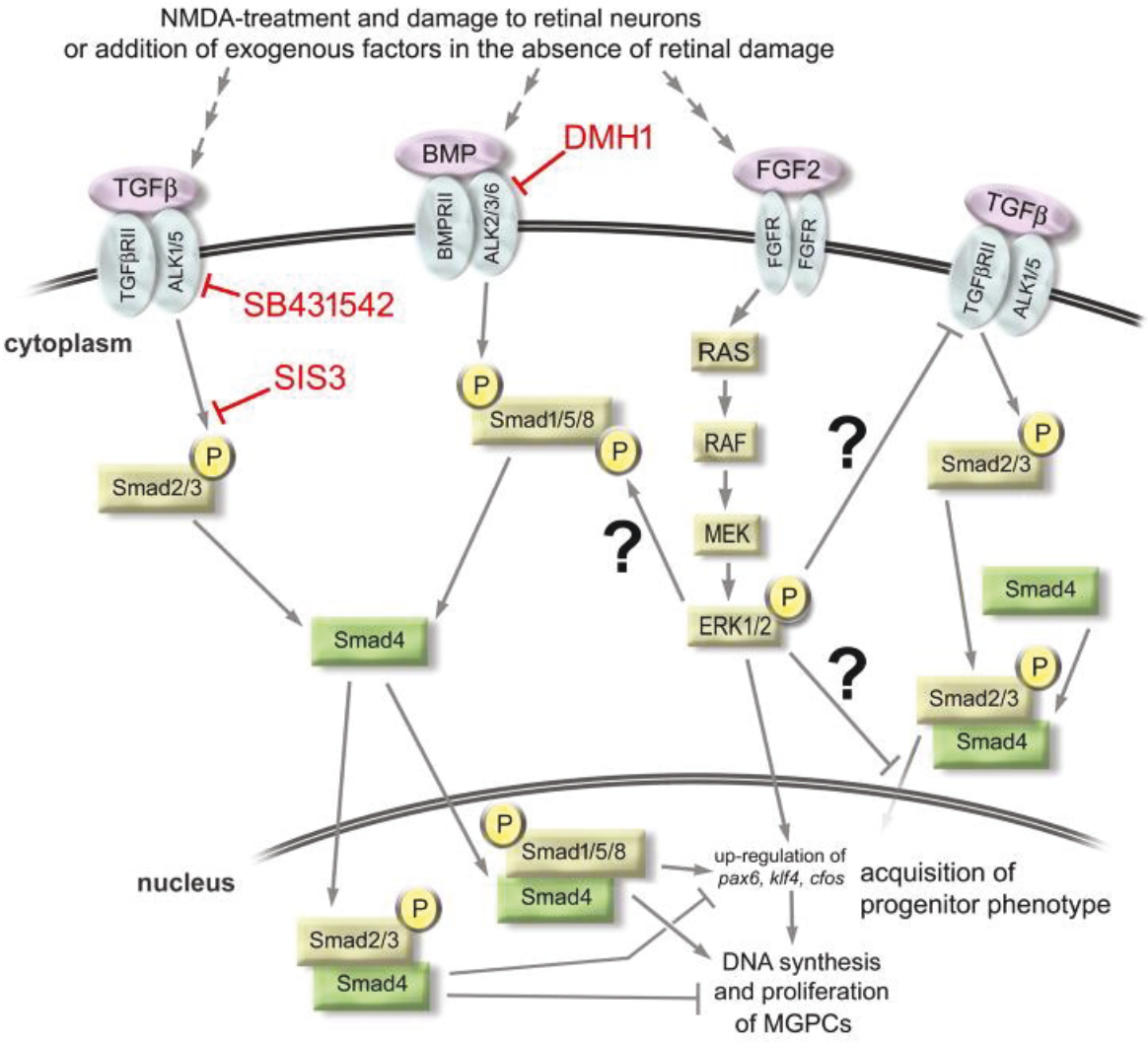



**Figure 20. Signaling pathways regulating the formation of Müller cell-derived progenitor in chicken.** Representation of the main signaling pathways implicated in the formation of proliferative Müller cell-derived progenitors. The different possible cross-talks among TGFβ/Smad2, BMP/Smad1/5/8 and FGF2/MAPK are indicated. Question marks indicate unconfirmed interactions. From (Todd et al., 2017).

pathway and increase susceptibility of Müller cells to Hedgehog signaling (Ghai et al., 2010; Todd and Fischer, 2015). Similarly, MAPK-signaling activates Wnt/β-catenin response (Gallina et al., 2016), while mTOR, BMP et TGFβ act upstream of FGF2/MAPK (figure 20) (Todd et al., 2017; Zelinka et al., 2016).

### IN MAMMALS

In case of damage, mammalian Müller cells reactivate in the same way as in the fish or chick retina and undergo reactive gliosis, changing their morphology and up-regulating various markers (Bringmann et al., 2009). However, this reactivation does not result in spontaneous neuron replacement and self-repair, leading to the belief during several years that the mammalian Müller cells lack any regenerative potential. This concept changed in 2004 after the publication of a study showing that a small percentage of rat Müller cells were also able to de-differentiate and proliferate following neurotoxic retinal injury (Ooto et al., 2004). Following this finding, other studies reported some rat Müller cell proliferation upon different forms of retinal damage (N-methyl-N-nitrosourea (MNU), NaIO3, ouabain, excessive light or retinal explants, or spontaneous model of retinal degeneration) (Ooto et al., 2004; Osakada and Takahashi, 2007; Wan et al., 2007; Wan et al., 2008). In mice, Müller cell proliferation has also been observed, although not as consistent as in rat retinas. First, the number of Müller cells detected in mouse retinas following damage is much lower than that observed in the rat retina. Second, although Müller cell proliferation has been detected in mouse retinal explants or in mouse degenerative models induced by alpha-aminoadipate (AAA), glutamate, NMDA, MNU or light injury (Harada et al., 2011; Karl et al., 2008; Osakada and Takahashi, 2007; Takeda et al., 2008), some other studies have not detected any Müller cell proliferation following damage (Joly et al., 2011; Karl et al., 2008; Kugler et al., 2015; Takeda et al., 2008). Together, these studies showed that rodent Müller cells can respond to injury with a proliferative response and that this mitogenic activity of Müller cells in rats is more efficient than in mice. However, in both species, the percentage of proliferative Müller cells was highly limited compared to non-mammalian vertebrates. In addition, some of these studies also showed that rodent Müller cells possess a neurogenic potential, as new bipolar and rod photoreceptors have been detected following NMDA-induced damage (Ooto et al., 2004), N-methyl-N-nitrosourea (MNU) administration (Wan et al., 2008) or in retinal explants (Osakada and Takahashi, 2007).However, the number of new neurons generated from Müller cells is very little and this remains inefficient to compensate for retinal damage. Nevertheless, these studies have permitted to bring into the light the regenerative potential of Müller cells, a potential that could be stimulated for therapeutic purposes.

Following the establishment of the existence of a regenerative potential in mammalian Müller cells, several studies have analyzed in detail the Müller cell regenerative behavior upon damage. It has been determined that the regenerative process of mammalian Müller cells follows the major phases described in fish and chick: quiescent mammalian Müller cells reactivate, dedifferentiate, proliferate and generate Müller cell-derived progenitors that differentiate into retinal neurons (Löffler et al., 2015). Among the different signals that trigger Müller cell reactivation, it has been shown that the release of subtoxic levels of glutamate from dying neurons may serve as a signal to stimulate the mammalian Müller cell cycle re-entry and neurogenesis (Takeda et al., 2008). Going a little bit further, another study has shown that, as in other species, Müller cell cycle re-entry in mammals depends on the extension of the retinal damage (Sardar Pasha et al., 2017). Using retinal explants, it has been shown that the pharmacological inhibition of different cell death signaling pathways or the maintenance of the retinas under hypothermic conditions until putting them in culture, minimized neuronal cell death (Sardar Pasha et al., 2017). Under these conditions, the number of proliferative Müller cells was decreased compared to controls (Sardar Pasha et al., 2017). Compared to the *in vivo* models, where usually only a specific cell type is targeted and degenerates, in retinal explants the damage is more important, as after 4 days of culture there is a wide decrease of the number of both photoreceptors and ganglion cells (Johnson et al., 2016; Müller et al., 2017). Considering that the proliferation of Müller cells is dependent on retinal damage, this could explain the differences in the number of proliferative rodent Müller cells observed in retinal explants compared to the *in vivo* models of retinal degeneration.

## PRONEURAL TRANSCRIPTION FACTORS AND MÜLLER CELL REPROGRAMMING

In an effort to identify factors underlying Müller cell regenerative potential in mammals, several signaling molecules that have been shown to be necessary and/or sufficient for zebrafish Müller cell regeneration have been tested. Thus, the neurogenic factor ASCL1 has been quickly considered as a potential candidate to trigger Müller cells reprogramming as it does in fish. Strikingly, contrary to zebrafish, no *Ascl1* upregulation has been found in the adult mice retina following damage or growth factor stimulation (Karl et al., 2008). This failure to activate *Ascl1a* could explain the limited regenerative capacity of mammalian Müller cells compared to zebrafish. Indeed, although ASCL1 overexpression does not have an effect in Müller cells behavior in physiological conditions (Jorstad et al., 2017; Ueki et al., 2015), it is sufficient to reprogram mouse Müller cells and activate a neurogenic program both *in vitro* and *in vivo* under degenerative conditions (Pollak et al., 2013; Ueki et al., 2015). Interestingly, the effect of *Ascl1* overexpression was restrained to young mice up to two weeks after

birth (Jorstad et al., 2017; Ueki et al., 2015). Thus, although these results support the hypothesis that a deficit in *Ascl1* up-regulation in mammalian Müller cells might restrict their ability to reprogram, other mechanisms could be implicated in the limited regenerative capacity in adult mice. For instance, *Ascl1* overexpression-dependent reprogramming involves chromatin remodeling of ASCL1 targets from a repressive to an active configuration (Pollak et al., 2013), while the maturation of Müller cells is characterized by a reduced chromatin accessibility (Jorstad et al., 2017).

So, in addition to the deficit in *Ascl1* up-regulation, epigenetic factors could also restrict the reprogramming capacity of adult mammalian Müller cells. This hypothesis was supported by Jordstat and colleagues, who showed that the combination of both Müller cell-specific overexpression of *Ascl1* and trichostatin-A, a histone deacetylase inhibitor, was effective to trigger retinal regeneration from adult Müller cells after injury (Jorstad et al., 2017). The role of epigenetic remodeling as another mechanism involved in Müller cells regeneration is reviewed in more details in (García-García et al., 2020). Moreover, other factors could limit the Ascl1-mediated Müller cell reprogramming. For instance, a recent publication has shown that Müller cell-dependent retinal regeneration was more efficient when the overexpression of ASCL1 was combined with the inhibition of STAT signaling (Jorstad et al., 2020). More specifically, activation of STAT signaling hampers the ability of Ascl1 to reprogram Müller cells into retinal neurons by redirecting Ascl1 to inappropriate regulatory sites in the genome (Jorstad et al., 2020). Thus, identify the factors that limit the activity of Ascl1 in Müller cell reprogramming could be used to increase the efficiency of the process.

Lin-28 is another reprogramming factor whose up-regulation is essential for retinal regeneration in zebrafish (Kassen et al., 2007). Strikingly, its expression is not only not increase, but even down-regulated in mice in degenerative conditions (Tao et al., 2016). Overexpression of *Lin-28* in mice led to Müller cell proliferation even in the absence of injury (Yao et al., 2016) and improved vision function in RCS rats, a genetic model of retinitis pigmentosa (Tao et al., 2016). So, this decrease of *Lin-28* expression could be also related to the limited dedifferentiation of mammalian Müller cells. Overall, these studies suggest that an insufficient quantity of proneural transcription factors may be the cause of the limited proliferative response to injury of Müller glia in mammals compare to other regenerative species.

## GROWTH FACTORS, SIGNALING PATHWAYS AND MÜLLER CELL-MEDIATED REGENERATION

As in non-mammalian species, the proliferation of rodent Müller cells can be enhanced in case of damage by the addition of exogenous growth factors, such as EGF, FGF, IGF (insulin growth factor) or NGF (nerve growth factor) (Close et al., 2006; Fischer and Bongini, 2010; Karl et al., 2008; Ooto et al., 2004; Sardar Pasha et al., 2017; Wang et al., 2016; Wilken and Reh, 2016). For instance, the

combination of NMDA-retinal damage with EGF or FGF and insulin treatment resulted in an increase of mouse Müller cell proliferation and differentiation into amacrine cells (Karl et al., 2008). FGF2 and insulin had a similar effect of enhancing the proliferation and the generation of bipolar and rod cells in the 2-weeks post-natal rat retina damaged by NMDA (Das et al., 2006a). Strikingly, this combination did not increase the regenerative capacity of Müller cells in the adult rat (Ooto et al., 2004), demonstrating again that the effect of mitogen stimulation is dependent on the age of the animal. Indeed, another study has shown that in physiological conditions, the intraocular injection of EGF induced a robust proliferative response of Müller cells *in vivo* at postnatal day 10, whereas no proliferative Müller cells was observed at P14, probably due to a down-regulation of the EGF-receptor at this age (Close JL 2006). A similar age-dependent decline in Müller cell proliferation has been observed *ex vivo* in cultured retinas. Indeed, when retinal damage is induced at P8, the EGF stimulation is highly effective, being able to stimulate the re-entry into the cell cycle of more than half of Müller cells, whereas the number of proliferative Müller cells is greatly reduced in response to EGF when damage began at P12 (Löffler et al., 2015; Ueki et al., 2012). This reduced response to EGF could be associated with the downregulation of the receptors after Müller cell maturation in an attempt to maintain Müller cells in a quiescent state (Close et al., 2006; Löffler et al., 2015; Ueki et al., 2012). Of note, some studies have shown that the regenerative process in mammals is also dependent on the strain of the mice. For example, mouse line 129×1/SvJ (129) has a larger proliferative Müller cell response compared with that observed in C57BL/6 (B6) after photoreceptor cell death (Suga et al., 2014). Transcriptomic analysis comparing both mice strains have associated this different proliferative efficiency to a more restrictive environment for regeneration in the B6 strain (Suga et al., 2014). More specifically, they found lower expression levels of Cyclin D1 and Nestin, increased expression of immunitary genes, or differential expression of two chromatin remodeling factors involved in the regulation of the stemness potential between B6 and 129 mice (Suga et al., 2014). Thus, further analysis should be done in order to find new potential candidates involved in the regulation of Müller cell proliferation.

Downstream signaling pathways and genetic changes involved in mammalian Müller cell proliferation after stimulation with growth factors are still not so well known. Recent studies have shown that the activation of both MEK/ERK1/2 and PI3K/AKT pathways are required for Müller cell proliferation in response to EGF (Beach et al., 2017; Ueki and Reh, 2013). Bone morphogenetic protein (BMP) signaling is also activated by the EGF pathway via PI3K/AKT kinase cascade and is essential for Müller cell proliferation (Ueki and Reh, 2013). Similarly, NGF also activates MAPK/ERK and PI3K/AKT signaling pathways by stimulating VEGF production (Wang et al., 2016). Thus, as in fish, different signaling factors converge to MAPK/ERK and PI3K/AKT activation in Müller cells to upregulate the expression of

some proliferative genes, such as *Ccnd1*, coding for Cycline D1, or even reprogramming genes, such as *Ascl1* or *Neurog2* (figure 21) (Löffler et al., 2015; Schäfer and Karl, 2017).

Other studies have also revealed that key signaling pathways, such as Wnt (Angbohang et al., 2016; Das et al., 2006b; del Debbio et al., 2010; Liu et al., 2013; Osakada et al., 2007; Yao et al., 2016), Notch (Das et al., 2006a; del Debbio et al., 2010) and SHH (Gu et al., 2017; Wan et al., 2007) also stimulate the proliferative and neurogenic properties of mammalian Müller cells after injury. For example*, in vitro* treatment of rat Müller cells with purmorphamine, an agonist of SHH signaling pathway, stimulates Müller cell reprogramming and proliferation by increasing the expression of cell cycle regulators, such as Cyclin D1 and Cyclin D3, and of some progenitors markers, such as PAX6 (Gu et al., 2017).

Notch signaling in mammalian Müller cells is, as in fish and birds, dependent on the phase of the regenerative process. At the beginning of the regenerative process, the activation of Notch is necessary to inhibit the Cyclin-dependent kinase inhibitor 1B (p27Kip1), which is required for maintain Müller cells in a quiescence state, in order to stimulate Müller glia cell cycle re-entry (Del Debbio et al., 2016; Jian et al., 2015). On the contrary, the inhibition of Notch signaling is required to induce neuronal differentiation of progenitor cells (Jian et al., 2015). Thus, contrary to zebrafish but similar to chicken regeneration, Notch signaling is necessary for triggering Müller cell dedifferentiation and proliferation but exert a negative effect on Müller cell-derived progenitors impeding their differentiation into retinal neurons. Therefore, as in birds, this inverted effect of Notch signaling on Müller cell behavior compared to zebrafish could be also related to the limited regenerative potential of mammals Müller cells.

Wnt/β-catenin signaling pathway has been shown to be upregulated and involved in the limited proliferative response of Müller cells upon injury, for instance *Wnt3a* stimulation or GSK-3β inhibition results in an increase of the number of proliferative Müller cells after retinal damage (Liu et al., 2013; Osakada et al., 2007). Strikingly, Wnt/β-catenin signaling overactivation is the most powerful stimulator of Müller cell proliferation compared to other signaling pathways, as it is capable of inducing the proliferative potential of Müller cells in adult mice even in the absence of damage (Yao et al., 2016). Indeed, gene transfer of β-catenin by AAV vectors was sufficient to induce important Müller cell proliferative activity in the absence of injury in mouse retinas by regulating the transcription of *Lin-28* (Yao et al., 2016).

Taken together, these studies have revealed the existence of an important repertoire of signaling pathways involved in mammalian Müller cell-dependent regeneration, stimulating the proliferation of Müller cells and the generation of neurons. However, none of these signaling pathways lead to an

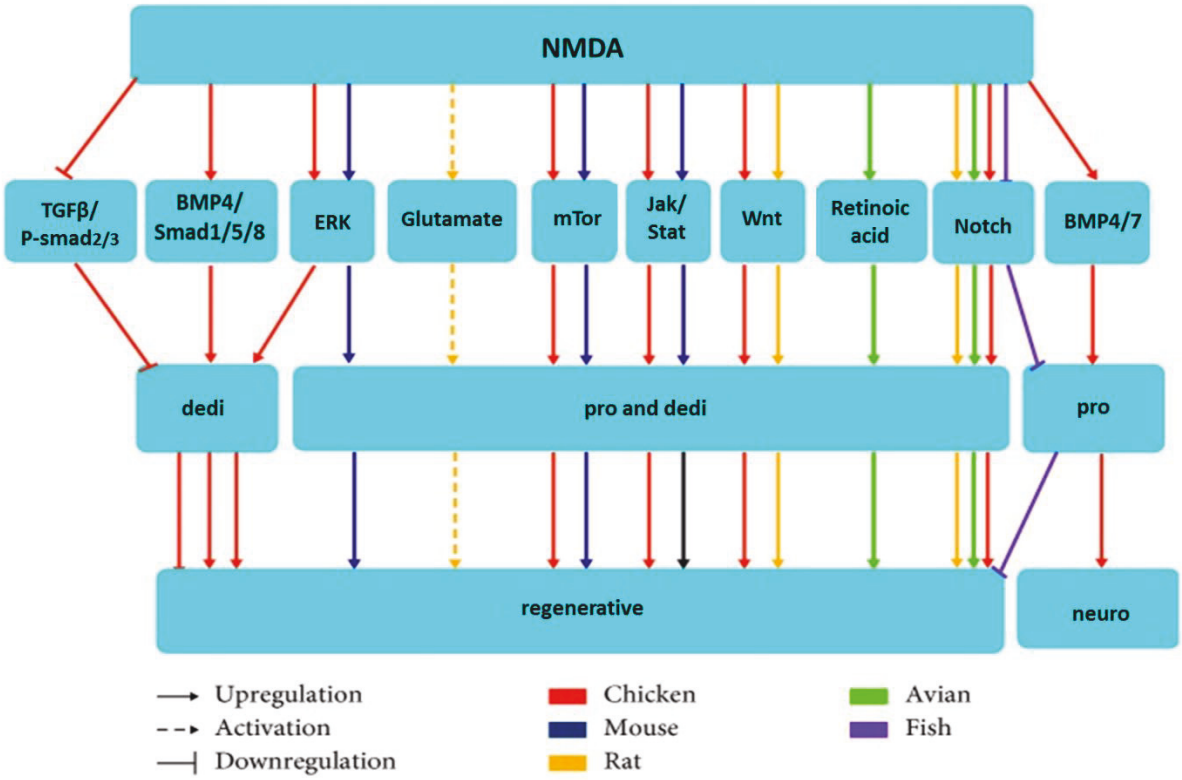



**Figure 21. Signaling pathways regulating Müller cell reprogramming and proliferation in different species.** Representation of the main signaling pathways implicated in the differentiation and proliferation of Müller cell after retinal degeneration induced by NMDA. Pro: proliferation, dedi: dedifferentiation, neuro: neuroprotective (Modified from Liu et al., 2019)

efficient regeneration, due to a limitation in the number of proliferative Müller cells and/or limited neurogenesis. Indeed, in case of NMDA-induced damage a subset of Müller cells enters the cell-cycle and arrives to the S-phase. However, over 12-24 additional hours the expression of Cyclin D1 is turned off and the proliferation stops, suggesting the existence of a molecular mechanism that prevents Müller cells from expanding clonally into a proliferative, progenitor-like population (figure 22) (Cepko and Dyer, 2000; Martin and Poché, 2019). In addition, the majority of the generated Müller cell-derived progenitors do not survive long after their generation (Ueki et al., 2015; Yao et al., 2016), and those that differentiate seem to prefer to adopt an inner retinal identity, while the generation of new photoreceptors occurs rarely (Langhe and Pearson, 2019). In an effort to boost the differentiation process and thus mammalian retinal regeneration, Yao and colleagues have developed a two-step approach that allows to address the differentiation of the Müller cell-derived progenitor cells into rod photoreceptors (Yao et al., 2018). For that, following gene transfer of β-catenin, they forced the differentiation of proliferative Müller cells to differentiate into rod photoreceptors by a subsequent injection of AAV coding for the transcriptional factors Otx2, Crx, and Nrl, known to be implicated in rod cell fate specification (figure 23) (Yao et al., 2018). The authors then nicely demonstrated that this was sufficient to partially restore the visual function in a mouse model of congenital blindness (Yao et al., 2018). Of note, this two-step method could not be necessary, as a recent study has shown that the knockdown of Ptbp1 (polypyrimidine tract-binding protein 1) using an *in vivo* viral delivery of a new RNA-targeting CRISPR system CasRx is sufficient to trigger Müller cell conversion into retinal ganglion cells and partially restore the visual function in NMDA-injured retinas (Zhou et al., 2020). Interestingly, this downregulation of PTBP1 is also sufficient to convert mouse and human astrocytes to functional neurons (Qian et al., 2020). Of note, a complete different interpretation of the data has been raised (Wang et al., 2020b). It could be possible indeed that the existing photoreceptors in the blind mice were repaired either because they took up the virus carrying the corrective gene (none-specific cell infection could indeed occur), or because of gene transfer from Müller cells (this has recently been observed from transplanted photoreceptors to resident photoreceptors). In either case, the visual restauration would not result from Müller-derived photoreceptors or ganglion cells, but from existing photoreceptors or ganglion cells with restored function. It is thus absolutely crucial to perform lineage labeling to fully prove that functioning photoreceptors and ganglion cells really derive from reactive Müller cells.

In addition to signaling pathways, miRNA can also regulate Müller cell regeneration in mammals as it does in fish. For example, in case of damage, miRNA let-7 controls Müller cell reactivation via activation of several signaling pathways such as Wnt (Yao et al., 2016), while miR-124, -9, -9*, -124 and -25 participate in the reprogramming of Müller cells (Wohl and Reh, 2016a; Wohl et al., 2019).

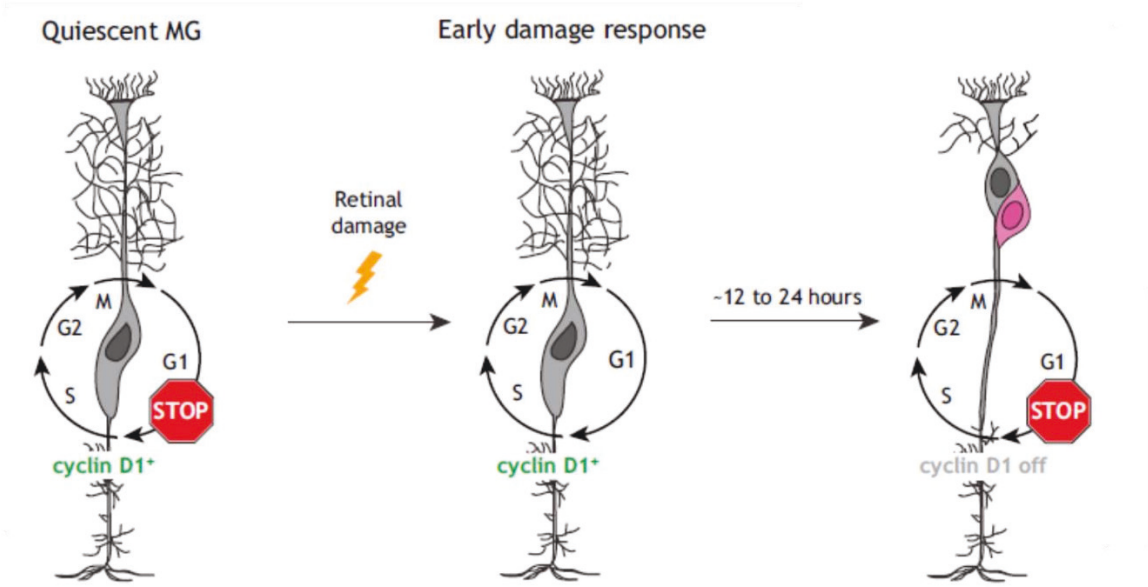



**Figure 22. Schematic representation of the inhibition of Müller cell cycle progression.** MG normally express low levels of the S phase-promoting protein cyclin D1. However, the activity of cyclin D1 is inhibited by cyclin kinase inhibitors during quiescent state and so the proliferation of Müller cells is repressed. Upon retinal damage, the expression of the cylin kinase inhibitors is lost coincident with upregulation of cyclin D1 expression and activity, which triggers MG to enter the S phase. However, over an additional 12-24 h, the expression of cyclin D1 is reduced, MG exit the cell cycle, and are prevented from expanding clonally into a proliferative, progenitor-like population. Modified from (Martin and Poché, 2019).

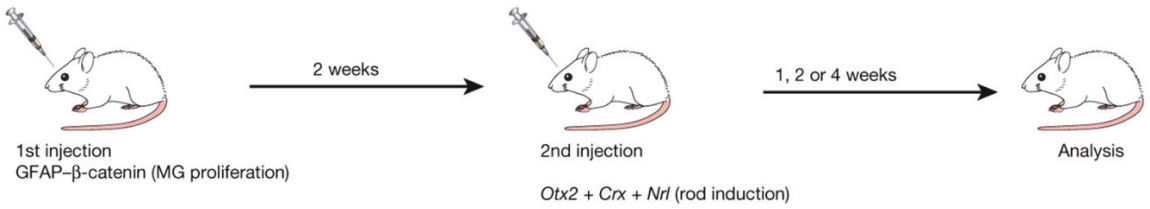



**Figure 23. Schematic representation of the Schematic of the two-step reprogramming method to generate rod photoreceptors.** After the stimulation of Müller cell proliferation by intravitreal injection of ShH10-GFAP-β-catenin, the differentiation of the proliferative Müller cell-derived progenitors is triggered by a second injection of ShH10-GFAP-mediated gene transfer of Otx2, Crx and Nrl. From (Yao et al., 2018).

### SIGNALING PATHWAYS AND MÜLLER CELL QUIESCENCE

Similar to the fish situation, other factors and signaling pathways could inhibit Müller cell reprogramming and proliferation in order to maintain rodent Müller cells in a quiescent state or to favor the differentiation of Müller cell-derived progenitor cells into retinal neurons. For example, the TGFβ signaling pathway plays a role on Müller cell quiescence maintenance by stimulating the expression of the cyclin-dependent kinase inhibitor 1B (p27Kip1) and avoiding cell cycle re-entry of rodent Müller cells at post-natal stages (Close et al., 2005). However, the influence of the TFGβ pathway in Müller cell-dependent regeneration is not so clear. Insulin stimulation of cultured human Müller cells induces the activation of the canonical Wnt signaling pathway and their differentiation into photoreceptors (Angbohang et al., 2016). Under these conditions, the activation of the TFGβ pathway inhibits the Wnt signaling pathway and impairs neuronal differentiation (Angbohang et al., 2016), suggesting that the effect of TGFβ could counterbalance the pro-regenerative activity of other signaling pathways. However, the moderate inhibition of TGFβ pathway *in vivo* does not lead to Müller cell proliferation after NMDA degeneration (Kugler et al., 2015). Yet, as there is no data showing the effect of the complete inhibition or the overactivation of TGFβ on Müller cell behavior in case of damage, the possible implication of TGFβ as an essential factor regulating Müller glia cell proliferation and subsequent differentiation into retinal neurons cannot be excluded.

Another signaling pathway that could limit the neurogenic potential of Müller cells is BMP/Smad. As mentioned, it has been shown to stimulate Müller cell activation and proliferation (Ueki and Reh, 2013). However, given the known gliogenic function of BMP, we cannot exclude that this signaling pathway could interfere with the neurogenic potential of mammalian Müller cells. Of note, other factors that could restrict Müller cell regenerative potential are p53 (Nomura-Komoike et al., 2016; Ueki et al., 2012; Zhao et al., 2014b) and the RE1-silencing transcription factor, known to repress the expression of neuronal genes in non-neuronal cells (Nesti et al., 2014; Wohl and Reh, 2016b).

## MECHANISMS UNDERLYING THE REGENERATIVE ABILITY

Understanding why retinal self-repair varies tremendously across different species is essential to explore therapeutic strategies based on the stimulation of Müller cell regenerative capacity. Thus, to identify the mechanisms underlying the variability of Müller cell regenerative behavior in different species has been the subject of intense investigation. As described, one of the species-specific differences that have been identified is the expression of the proneural transcription factor *Ascl1*, which is upregulated in fish and bird Müller cells upon injury but not in mammalian ones (Ueki et al., 2015). Another one is the different impact of Notch signaling on Müller glia proliferation between fish and mammals, as its inhibition was reported to potentialize the pro-proliferative effects of reprogramming factors such as *Ascl1* and *lin28a* in zebrafish, but not in mice (Elsaeidi et al., 2018). In

addition, a recent study has reported that the ligand Tgfb3, which controls Müller cells quiescence via non-canonical Tgfb signaling pathway in zebrafish, might also contribute to the different regenerative capabilities of Müller cells as this pathway is active in zebrafish Müller cells, but not in mice (Lee et al., 2020).

In the last years, the huge technological advances in the field of single-cell RNA sequencing (scRNA-seq) made it possible to investigate transcriptomic dynamic changes that occur specifically in Müller cells in response to injury in different species. With this in mind, bulk and single-cell RNA sequencing (scRNA-seq) were recently applied (Hoang et al., 2019). RNA levels were profiled in zebrafish, chick and mouse, in undamaged retinas or at different time points following damage (Hoang et al., 2019). This study allowed to identify species-specific Müller cell transcriptional responses to retinal injury. For instance, NFI (Nuclear Factor I) transcription factors are expressed in resting Müller cells and downregulated after acute retinal injury in all the species. However, they are rapidly re-activated in mouse Müller cells just after the upregulation of cell cycle genes (Hoang et al., 2019). NFI-deficient mouse Müller cells upregulate cell cycle regulators, along with the neurogenic factor Ascl1 (Hoang et al., 2019). This suggests that, upon acute retinal injury, NFI factors in mammals eventually repress proliferative and neurogenic competence, forcing reactive Müller cells to revert back to a resting state. This large-scale and cross-species study supports the idea that distinct regenerative capacities in animals may be due, at least in part, to the differential activation of intrinsic regulatory networks that force quiescence of activated Müller cells. However, the differences in the ability to regenerate may also be related to environmental signals. Indeed, different studies have linked the regenerative potential with changes in the immune response across evolution. Actually, it seems that it exits an inverse correlation between the immune system reaction and the regenerative process: the more the immune system is developed, the less regeneration is found (figure 24) (Godwin and Brockes, 2006; Godwin and Rosenthal, 2014; Mescher and Neff, 2005). Along this line, a recent study has suggested that the differences observed between mice and zebrafish in Müller cell cycle progression may be due to a differential cytokine/chemokine contribution of the immune cells during the inflammatory response and point the Midkine cytokine as a core component of the mechanisms that regulate proliferation of Müller cells (figure 25) (Nagashima et al., 2019). Interestingly indeed, its loss-of-function in zebrafish mutants triggers reactive Müller glia to behave as in the mouse, initiating a reprogramming response, entering the G1 phase of the cell cycle following injury, but failing to efficiently progress further into the S phase (figure 25) (Nagashima et al., 2019) . Thus, analyzing the precise contribution of each immune cell types to the regeneration process and comparing the cytokine repertoire between fish and mouse may help bringing new insights into the mechanisms that regulates Müller cell-dependent retinal regeneration. Giving the importance of the innate immune system response in my PhD project, I will describe in detail in the next section the influence of the inflammation on the regenerative process with a special focus on the retina.

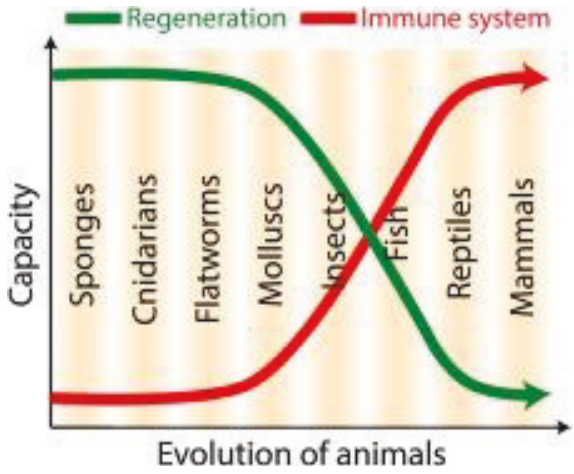



**Figure 24. The Influence of evolution on the Immune System and the regenerative response.** The ability of regeneration seems to be linked to the development of the immune system. The more it is developed, the less regenerative capacity exists. From (Abnave and Ghigo, 2019).

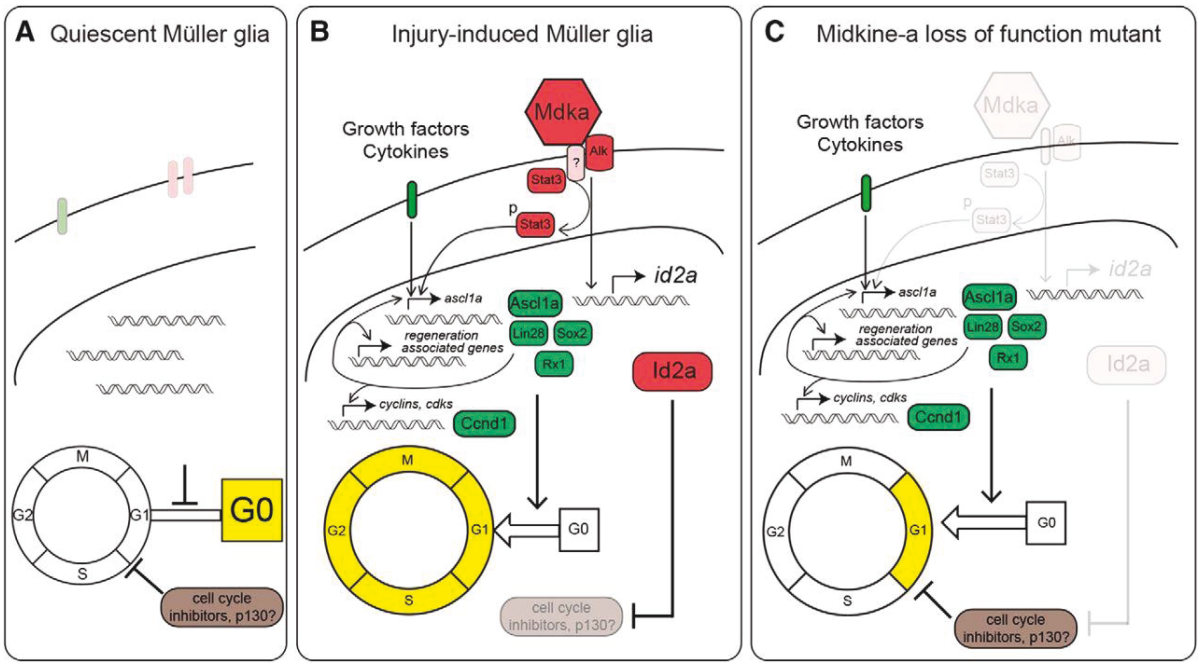



**Figure 25. Schematic model of the regulation of Müller cell cycle progression by Midknine cytokine.** **A**, In quiescent Müller glia, cell cycle inhibitors keep cells in $G_0$ phase. **B**, Injury-associated signals induce the reprogramming of Müller cells and trigger the cell cycle re-entry. Midkine cytokine signaling participates in the Müller cell reprogramming by inducing the expression of Ascl1a. In addition, it induces the expression of the cell cycle regulator, *id2a*, that inhibits cell cycle inhibitors and allows cell cycle progression. ***C***, In the absence of Midkine-a, Müller glia fail to suppress cell cycle inhibition, resulting in compromised progression of the cell cycle. From (Nagashima et al., 2019).

## 4.2 INFLAMMATORY PROCESS AND MÜLLER CELL RETINAL REGENERATION

### INFLAMMATION AND REGENERATION: GENERAL SITUATION

As mentioned before, in case of injury, different damage signals are produced. These signals are detected by resident immune cells, that activate and acquire new morphological and functional characteristics, mainly the secretion of several pro-inflammatory cytokines, initiating acute inflammation and recruiting different peripheral immune cells that subsequently help orchestrate the tissue repair response. Depending on the degree and duration of the inflammatory process, the final effect will be either beneficial, leading to tissue regeneration, or detrimental, leading to fibrotic disorders (Kizil et al., 2015; Mescher, 2017).

The inflammatory response is generally divided in three distinct phases that act sequentially to promote tissue restoration (Eming et al., 2017). The first phase is an early proinflammatory step where the innate immune elements mobilize and recruit key inflammatory cells such as pro-inflammatory (M1-like) macrophages that potentiate the inflammation by secreting more pro-inflammatory factors (figure 26) (Eming et al., 2017). This step has been shown to be essential for stem cell activation and tissue regeneration. For instance, in regenerative models such as Axolotl, activation of the inflammatory response and macrophage recruitment after amputation is critical for limb regeneration, as systemic macrophage depletion results in impeded limb regeneration (Godwin et al., 2013). In adult zebrafish, macrophages are required for normal regeneration of the caudal fins (Li et al., 2012a; Petrie et al., 2014). Similarly, in *Xenopus* tadpoles, interleukins such as IL-11 have been shown to be implicated in tail regeneration (Tsujioka et al., 2017). On the same line, inhibition of the inflammatory response in *Xenopus* tadpoles inhibits the regeneration of the hindlimb (King et al., 2012). The presence of macrophages, or proinflammatory factors such as TNFα or IL-6, is also necessary to trigger the response to injury and the regeneration of several mammalian organs such as the skin (Lucas et al., 2010), kidney (Lin et al., 2010), liver (Boulter et al., 2012; Cressman et al., 1996; Duffield et al., 2005; Tirnitz-Parker et al., 2010; Yamada et al., 1997), heart (Aurora et al., 2014; Horckmans et al., 2017; Lavine et al., 2014), pancreas (Xiao and Gittes, 2015; Xiao et al., 2014), intestine (Pull et al., 2005) or skeletal muscle (Arnold et al., 2007; Dort et al., 2019; Lesault et al., 2012; Yang and Hu, 2018). The positive effect of this early inflammation in tissue regeneration could be related to the transflammation process, the capacity of pro-inflammatory signals to modulate epigenetic enzymes such as histone acetyltransferases or histone deacetylases, resulting in epigenetic modifications and increased DNA accessibility that facilitate stem cell reprogramming (Antonio et al., 2015; Lee et al., 2012; Meng et al., 2017; O'Neill, 2012).

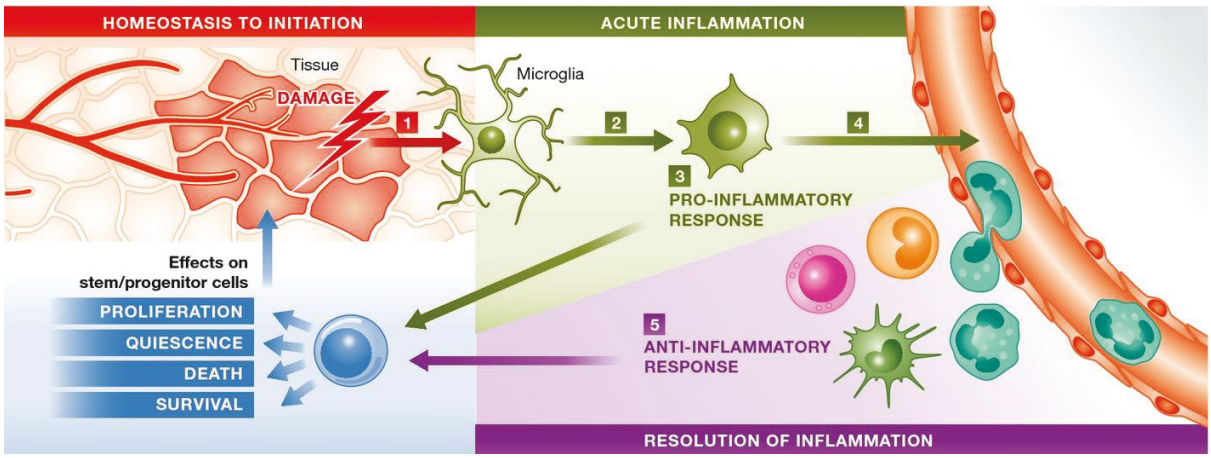



**Figure 26. Generic scheme of initiation and resolution of the inflammatory response.** In case of injury different damage signals are produced (1). These signals are detected by the resident immune cells, in this case microglia, that activate and acquire new morphological and functional characteristics (2), mainly the secretion of several pro-inflammatory cytokines and chemokines (3), initiating acute inflammation and recruiting different peripheral immune cells that subsequently potentiate the inflammation by secreting more pro-inflammatory factors (4). Once the damage is solved, the immune cells move to a reparative phenotype and take and active part in the resolution of the inflammatory response through the secretion of anti-inflammatory factors (5). Depending on the degree and duration of the inflammatory process, the final effect on the tissue stem cells will be either beneficial, or detrimental. From (Kizil et al., 2015).

In the second phase, the proinflammatory response starts to subside with main inflammatory cells, including macrophages, moving to a reparative (M2-like) phenotype characterized by the secretion of anti-inflammatory factors (figure 26) (Eming et al., 2017). This switch to a M2-like cells is required for stem cell differentiation and tissue regeneration in several systems. In zebrafish, M2-like macrophages can promote fin regeneration after amputation (Nguyen-Chi et al., 2017). In mammals, the transition from M1 to M2-like macrophages is required for proper tissue reparation in several organs such as the skeletal muscle (Arnold et al., 2007; Aurora and Olson, 2014; Mounier et al., 2013; Vannella and Wynn, 2017), liver (Dal-Secco et al., 2015), or heart (Horckmans et al., 2017). Thus, macrophages play a crucial role in the biphasic activation of the immune system and are required for the controlled progression of the regenerative process. Beyond macrophages, T regulatory cells (Tregs) also play an important role in this second phase, probably due to their capacity to regulate the activity of other cells in the immune system to suppress the immune response (Lei et al., 2015). For instance, Treg cells are necessary to the regeneration of the heart in zebrafish (Hui et al., 2017). The implication of Tregs on tissue regeneration has also been described in the mammalian skeletal muscle (Burzyn et al., 2013; Castiglioni et al., 2015), bone (Liu et al., 2015), kidney tubules (Gandolfo et al., 2009), pancreatic islet β cells (Zhao et al., 2012), intestine (Andoh et al., 2007) and liver (Lu et al., 2013). Altogether, these results indicate that although the inflammation is required to trigger the regenerative response, resolution of the inflammatory response is also required to allow complete tissue regeneration (Abnave and Ghigo, 2019; King et al., 2012; Vannella and Wynn, 2017). Moreover, the precise regulation of this timely transition from a pro- to an anti-inflammatory response is critical for the regenerative process, as a delayed resolution or an early activation of the anti-inflammatory function compromises tissue regeneration (Abnave and Ghigo, 2019; Arnold et al., 2007; Kotwal and Chien, 2017; Lucas et al., 2010; Perdiguero et al., 2011). For example, chronic inflammation in skeletal muscle in mammals impairs the regenerative process from satellite cells (Perandini et al., 2018). Along the same lines, fine regulation of the macrophagic population, either by inhibition of pro-inflammatory macrophages or by increasing the number of anti-inflammatory macrophages in the damaged adult heart, enhances adult cardiac repair (Lavine et al., 2014). Thus, the two major phases of the inflammatory response must be carefully regulated, with peripheral immune cells and resident tissue macrophages playing a unique and critical role at each step in order to have an efficient regeneration. In the final stage, the damage is solved, and immune cells exit the tissue or are eliminated through apoptosis to completely restore tissue homeostasis (Eming et al., 2017).

*Nota Bene*: this binary classification of the immune phenotypes in M1/M2 is an oversimplification of the immune response to adapt it to the two polars of the inflammatory response. Thus, *in vivo* many overlapping phenotypes may coexist in inflamed tissues during the inflammatory response.

As mentioned in section 2, microglial cells are the resident immune cells of the nervous system specialized in the immune surveillance of the nervous tissue (figure 27). Similar to the role of macrophages in the regeneration of other tissues, in case of damage the activation of microglia and their release of inflammatory factors is required for the regenerative response of the neural tissue. For instance, in both the teleost brain and spinal cord, the activation of microglial cells and the secretion of pro-inflammatory signals are necessary to enhance the proliferation of neural progenitors and subsequently the neurogenesis (figure 28) (Bosak et al., 2018; Jaerve and Müller, 2012; Kizil et al., 2012; Kizil et al., 2015; Kyritsis et al., 2012). The resolution of the inflammation is equally required to achieve neural regeneration. For example, Treg cells have been shown to be necessary to the regeneration of the spinal cord in zebrafish (Hui et al., 2017). Thus, as in other organs, neural tissue regeneration in zebrafish requires the activation and the resolution of the inflammatory response. However, inflammation is still generally considered to hamper regeneration in mammals, as it has been related with decreased proliferation and survival of the precursor cells, ineffective integration of the new neurons into the neural circuitry, or even with the induction of further degeneration (Ekdahl et al., 2003; Ekdahl et al., 2009; Erlandsson et al., 2011; Fitch and Silver, 2008; Iosif et al., 2006; Kyritsis et al., 2014; Monje et al., 2003; Perez-Asensio et al., 2013; Sofroniew and Vinters, 2010; Vannella and Wynn, 2017). The different effect of the inflammation between fish and mammals could be due to an ineffective switch to M2-like microglia and/or a prolonged pro-inflammatory phase. Indeed, an *in vitro* study has shown that TNFα stimulates proliferation of mammalian neural stem cells from the subventricular zone in culture at low doses, but it induces apoptosis at higher doses (Bernardino et al., 2008), supporting the idea that a prolonged pro-inflammatory phase could be related to the restricted neuronal regenerative capacity in mammals. Along this idea, both *in vitro* and *ex vivo* studies have shown that the proliferative and neurogenic capacity of mouse neural stem cells were increased in the presence of M2-polarized microglia (Choi et al., 2017; Matsui and Mori, 2018). In addition, it has been shown that M2-like microglia/macrophages are required to promote remyelination (Lloyd et al., 2019; Miron et al., 2013) and repair after spinal cord injury (Shechter et al., 2009) and stroke (Wattananit et al., 2016) in mice. Altogether, these studies suggest that the inflammatory process could have a positive effect on neural regeneration in mammals, but it requires an accurate coordination of the multi-staged immune response to regulate properly the activation and differentiation of progenitor cells in order to result in an effective repair.

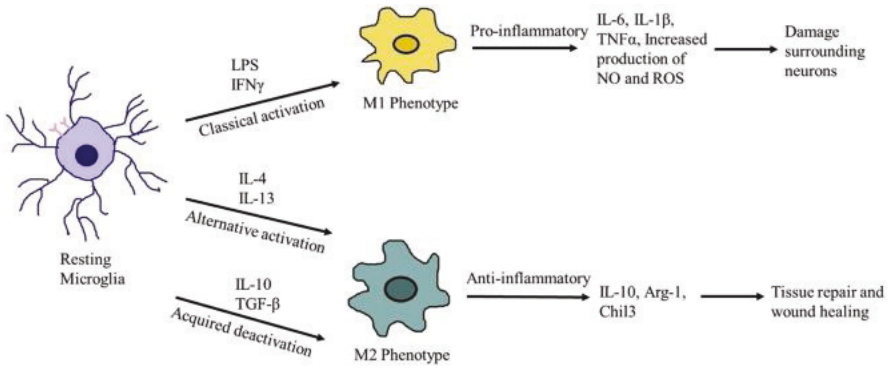



**Figure 27. Binary model of microglia polarized phenotypes.** Depending on the type of stimuli microglia will be activate in a M1-like phenotype (classical activation), characterized by pro-inflammatory functions, or in a M2-like phenotype (alternative activation of acquired deactivation) characterize by anti-inflammatory and tissue repair functions From (Subhramanyam et al., 2019).

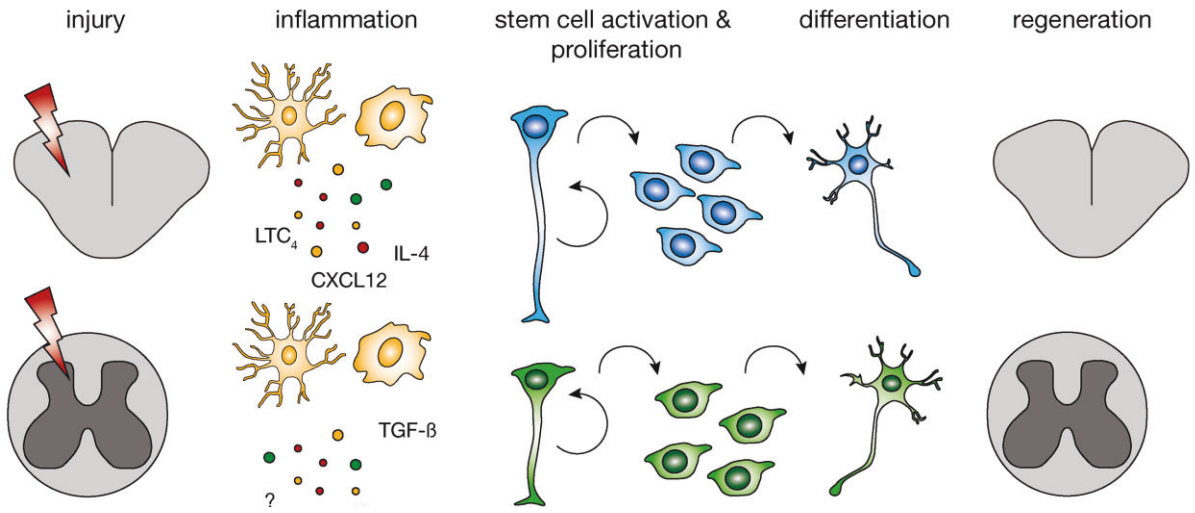



**Figure 28 Inflammatory signals implicated in the regenerative response of the central nervous system in zebrafish.** Tissue damage triggers a rapid and strong inflammatory response characterized by immune cell activation, proliferation and infiltration that will secret several pro-inflammatory factors. These factors stimulate and increase stem cell proliferation. In zebrafish telencephalon and spinal cord, radial glial cells consitute the source of newly generated neurons. IL, interleukin; TNF, tumor necrosis factor; CNTF, ciliary neurotrophic factor; SDF, SDF – stromal cell-derived factor (also known as CXCL12); CCL, CC motif chemokine; LTC4 , leukotriene C4; CysLT, cysteinyl leukotriene (Modified from Bosak et al., 2018)

## INFLAMMATION AND RETINAL REGENERATION

In the retina, as in the brain, microglial cells are the resident macrophages specialized in immunosurveillance. In case of damage, these cells start a rapid and strong inflammatory response characterized by immune cell activation and secretion of several pro-inflammatory factors, proliferation, and infiltration of peripherical immune cells that will enhance the pro-inflammatory environment before transition to a pro-regenerative state and a return to tissue homeostasis. We will see below that this microglia cell activation is required for fish and bird retinal regeneration.

### IN TELEOST FISH

As described in section 4.1 in the teleost fish, TNF-α or IL-6-family cytokines have been shown to promote injury-induced Müller glial cell reprogramming and proliferation (figure 29) (Conner et al., 2014; Elsaeidi et al., 2014; Goldman, 2014; Iribarne et al., 2019; Nelson et al., 2013; Zhao et al., 2014a). Similarly, the growth factor/cytokine Midkine-a has also been shown to be required for Müller cell proliferation upon injury (figure 25) (Nagashima et al., 2019). However, whether or not this effect was mediated through an innate cellular immune response remained unknown. Recently, it has been reported in both larvae or adult zebrafish that in case of rod photoreceptor ablation or damage upon laser-induced injury or puncture wounding, microglial cells are required to promote the regenerative process, as their inhibition by coablation or pharmacological treatments for immune cell suppression impedes Müller cell reactivation, proliferation and retinal regeneration (Conedera et al., 2019; Silva et al., 2020; White et al., 2017). On the other hand, this situation is reversed by microglial repopulation (Conedera et al., 2019). In addition to resident microglia, infiltrating immune cells also appear to participate to the inflammatory response triggered by retinal injury (Hui et al., 2017; Mitchell et al., 2018). Interestingly, if resolution is delayed, the inflammatory response become detrimental, indicating that the time regulation of the inflammatory response is critical for Müller cell-dependent retinal regeneration in zebrafish (White et al., 2017).

The mechanisms underlying Müller cell reactivation and regeneration following microglia-mediated inflammation are just beginning to be explored. Recently, it has been shown that the inflammation is necessary to trigger the mTOR signaling in adult zebrafish Müller cells (Zhang 2020). mTOR signaling has been associated with the upregulation of several reprogramming factors as *Ascl1* or *Lin-28*, cytokines and cell-cycle-related genes, and so with an increase in Müller cell-dependent retinal regeneration (Zhang et al., 2020). Altogether, these results indicate the existence of a positive correlation between acute inflammatory response and Müller cell-dependent retinal regeneration in zebrafish.

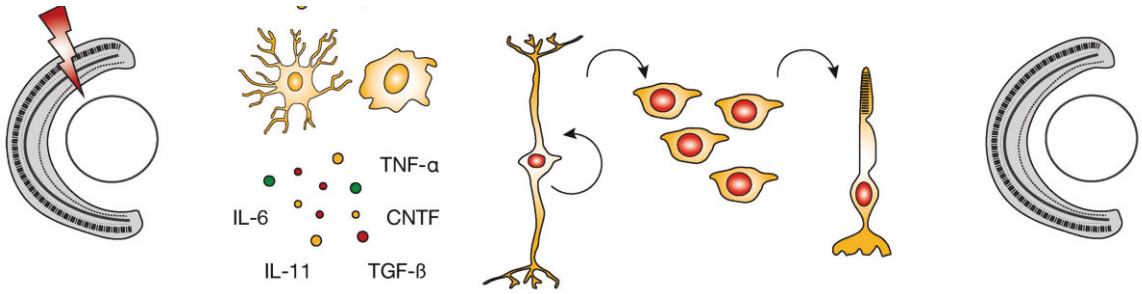



**Figure 29. Inflammatory signals implicated in the regenerative response of the retina in zebrafish.** Tissue damage triggers a rapid and strong inflammatory response characterized by immune cell activation, proliferation and infiltration that will secret several pro-inflammatory factors. These factors stimulate and increase stem cell proliferation. In retina, Müller cell provide the retinal progenitors that proliferate and differentiate into new neurons. IL, interleukin; TNF, tumor necrosis factor; CNTF, ciliary neurotrophic factor; SDF, SDF – stromal cell-derived factor (also known as CXCL12); CCL, CC motif chemokine; LTC4 , leukotriene C4; CysLT, cysteinyl leukotriene. Modified from (Bosak et al., 2018)

### IN CHICKEN

In the chicken retina, the microglia-mediated inflammation is also implicated in the regenerative process. Indeed, clodronate-liposome based ablation of microglia prior retinal injury suppress Müller glia proliferation, suggesting that microglial activation and pro-inflammatory cytokines are necessary for retinal regeneration (Fischer et al., 2014). Accordingly, IL-6 has been shown to be sufficient to stimulate chicken Müller cell proliferation upon injury (Fischer et al., 2014). Interestingly, the avian retina possesses an atypical glial cell named the non-astrocytic inner retinal glial cells (NIRG) that behaves as microglia and thus could provide additional signals to stimulate the proliferation of Müller cells upon injury (Fischer et al., 2010; Hamon et al., 2016a; Zelinka et al., 2012). Regarding the molecular mechanisms underlying this microglia-mediated Müller cell regeneration, it seems that the modulation of the NF-kB signaling by microglia is required to regulate the formation of Müller cell-derived progenitors (Palazzo et al., 2019). Thus, as in zebrafish, microglial cells are required for retinal regeneration in the chick.

### IN MAMMALS

In contrast to the above described positive effect of inflammation in non-mammalian species, the inflammatory process in mammals seems not only to limit the proliferation of Müller cells but even to favor degeneration. Indeed, it has been shown that pro-inflammatory molecules, such as IL-6, CXCL10, and TNF-α, prevent human retinal stem/progenitor proliferation and differentiation *in vitro* (Dick, 2009). Similarly, mouse Müller cell proliferation *in vitro* is decreased in the presence of LPS-activated microglia (Wang et al., 2011). However, this effect of the microglia on Müller cell behavior may be dependent on the *in vitro* culture conditions. Indeed, although Müller cells co-cultured with activated microglia displayed normal neuroprotective and pro-inflammatory effects, they had a reduced gliotic response (Wang et al., 2011). This situation is not found *in vivo*, where in case of damage and subsequent microglia activation, Müller cells undergo reactive gliosis, suggesting that some damage-derived signals influencing the behavior of Müller cells may be missing in the *in vitro* experiments, modifying their response to the microglial-induced inflammation. *In vivo*, different studies have also placed microglial activation as a catalyst of retinal degeneration following damage. For example, suppression of chemoattractive signaling, such as CCL3 or CCL2-CCR2, in a mouse model of retinitis pigmentosa, decreases microglial migration towards the photoreceptor cell layer and slows down the degeneration process (Guo et al., 2012; Kohno et al., 2014). Similarly, genetic (*Cx3cr1*) or pharmacological (minocycline, tamoxifen or progesterone) manipulation of microglial activation as well as microglia depletion using the selective colony stimulating factor 1 receptor (CSF1R) kinase inhibitors PLX3397 or PLX5622, have been shown to delay retinal degeneration (Okunuki et al., 2019; Peng et al., 2014; Roche et al., 2016; Wang et al., 2017a; Wang et al., 2019; Yang et al., 2007; Zabel et

al., 2016; Zhao et al., 2015). Interestingly, this pro-degenerative effect of microglia would not be associated with the intensity or the duration of the inflammatory response, but with the phagocytic activity of microglial cells. Indeed, microglial phagocytosis of apoptotic photoreceptors facilitate their clearance. However, it has been shown that many of the photoreceptors that were engulfed by microglia were negative for multiple markers of apoptosis, suggesting that in case of damage, microglia phagocyte both dead and viable photoreceptors thereby accelerating the degenerative process (Silverman and Wong, 2018a; Zabel et al., 2016; Zhao et al., 2015). Altogether, these studies show that microglia have a detrimental effect on the mammalian retina upon damage. However, it is important to note that none of these *in vivo* studies have examined Müller cell regenerative behavior after microglial modulation following injury. Thus, how the microglia-induced inflammation is implicated in Müller cell-dependent retinal regeneration in mammals remains unknown and is at the center of my PhD project.

## 4.3 CONCLUSION

Increasing our knowledge of the molecular mechanisms underlying Müller cell reactivation upon injury may help to develop new therapeutic strategies based on the stimulation of their regenerative capacities. In the recent years, different factors and signaling pathways have been brought to light as regulators of Müller cell reprogramming, proliferation and neurogenic potential. In addition, it has been shown that the microglial-induced inflammatory response to injury can shape the proliferative and regenerative potential of Müller cells in non-mammalian species. Thus, a better understanding of the mechanisms underlying the bilateral communication between Müller cells and the innate immune system in regenerative and non-regenerative species deserves further investigation.

In this context, my laboratory has focused on the Hippo/ YAP signaling pathway, which has been shown to be related to the regulation of stem cells, the regeneration of several mammalian organs and the inflammatory response. However, there is almost no data regarding its role in the retina and its implication in retinal regeneration.

# 5. YAP/TAZ AS NEW REGENERATIVE FACTORS

## 5.1 THE HIPPO SIGNALING PATHWAY

In 1995 an actor of the Hippo signaling pathway was discovered for the first time when the deletion of the *warts* kinase gene (**WTS**) in *Drosophila* resulted in excess growth of multiple tissues (Justice et al., 1995; Xu et al., 1995). It was not until 2002 that other components were identified: the adaptor proteins Salvador (**SAV**) and Mobs-As-Tumor-Supressor (**MATS**), and in particular the Hippo kinase (**HPO**), which gave its name to the signaling pathway (Piccolo et al., 2014; Yu et al., 2015). In 2005, the downstream effector of the Hippo kinase cascade, the transcriptional co-activator Yorkie (**YKI**), was discovered, and the core of the signaling pathway was stablished (Dong et al., 2007; Huang et al., 2005).

In mammals, this signaling pathway is highly conserved regarding the core component, the signal transduction pathway and the biological functions. The mammalian orthologues of *Hpo, sav, wts*, *mats* and *yki* are MST1/2 (mammalian Ste20-like kinases), SAV1, LATS1/2 (Large tumor suppressor 1 and 2), MOB1 (Mps one binder kinase activator-like 1) et YAP/TAZ (Yes-associated protein/Transcriptional co-activator with PDZ-binding motif, also known as WWTR1, two homologs of *Drosophila* Yorkie) (Dong et al., 2007; Kanai et al., 2000; Ma et al., 2019; Moya and Halder, 2019). As is *Drosophila*, the activation of the Hippo signaling pathway results in the formation of the MST1/2-SAV1 complex, that phosphorylates the LATS1/2 kinases associated with the co-factors MOB1A and MOB1B (Callus et al., 2006; Chan et al., 2005; Praskova et al., 2008; Wu et al., 2003). This kinase cascade ends by phosphorylating and inhibiting both transcriptional co-activators YAP and TAZ via their cytoplasmic retention or their proteasomal degradation (Dong et al., 2007; Lei et al., 2008). When YAP and TAZ are active, they translocate into the nucleus and interact with other transcriptional factors to induce the expression of a wide range of genes (Kim et al., 2018a) (figure 30).

### REGULATION OF THE HIPPO SIGNALING PATHWAY

The activation of the Hippo signaling pathway does not involve a single specific receptor and extracellular ligand. Rather, Hippo signaling is triggered by several microenvironmental stimuli that can be classified in four categories: cell adhesion molecules, membrane-receptors, actin cytoskeleton and membrane-associated proteins/cell junction proteins (Davis and Tapon, 2019; Kodaka and Hata, 2015; Moya and Halder, 2019). Thus, mechanical signals, cellular stress, extracellular stimuli, polarity and adhesion cues are integrated through upstream regulators that then activate the core Hippo pathway components (figure 31 ) (Davis and Tapon, 2019; Hansen et al., 2015; Kodaka and Hata, 2015; Ma et al., 2019; Manning et al., 2020; Meng et al., 2016; Moya and Halder, 2019; Zheng and Pan, 2019)

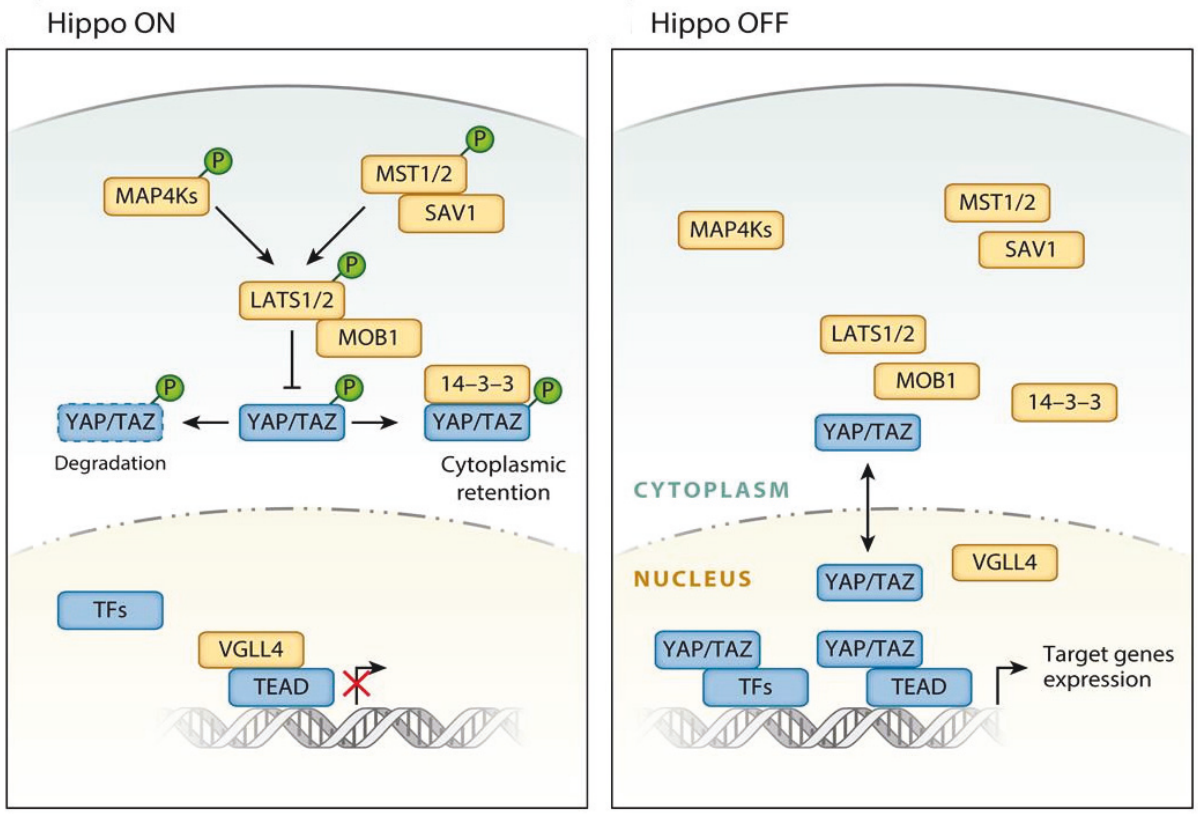



**Figure 30. Schematic representation of the Hippo signaling pathway in mammals.** Several upstream signals trigger the activation of the MST1/MST2, LATS1/LATS2 kinase cascade that ultimately phosphorylates YAP/TAZ proteins (left). Phosphorylation of YAP/TAZ results in the recruitment of 14-3-3 proteins and thus cytoplasmic retention, or in proteolytic degradation. When Hippo signaling pathway is inactive (right), YAP/TAZ are translocated to the nucleus, form a complex with other transcription factors and regulate the activation of their target genes. From (Ma et al., 2019).

Interestingly, it has been recently discovered that the regulatory properties of YAP/TAZ are controlled by liquid-liquid phase separation (Cai et al., 2019b; Lu et al., 2020a).

Moreover, Hippo pathway crosstalk with other signaling pathways to modulate signaling activity at different levels of the Hippo kinase cascade. Of note, YAP/TAZ regulation independently of MST1/2 and LATS1/2 kinases is known as the non-canonical Hippo pathway (Dong et al., 2007; Hansen et al., 2015; Lei et al., 2008; Varelas, 2014). Thus, GPCRs (G protein-coupled receptors) via F-actin, Wnt via β-catenin, BMP and TGFβ via SMADs, and Notch signaling, also regulates YAP/TAZ activation (figure 31) (Hansen et al., 2015; Ma et al., 2019). In addition, different reports have indicated the existence of a positive crosstalk between EGFR signaling and the Hippo pathway. For instance, EGF family ligands like EGF or the receptor tyrosine kinase ERBB4 (EGFR family member v-Erb-b2 avian erythroblastic leukemia viral oncogene homolog 4) promote the translocation of YAP/TAZ to the nucleus and the expression of their target genes in mammalian epithelial (Fan et al., 2013), glial and hepatic (Reddy and Irvine, 2013), renal epithelial (Chen and Harris, 2016) and breast cancer (Haskins et al., 2014) cell cultures. In this context, EGF signaling prevents the activation of LATS by MST via phosphoinositide-dependent kinase-1 and AJUBA proteins, which lead to impairment of SAV1's role in the kinase complex and thus the inhibition of LATS phosphorylation and the activation of YAP/TAZ (Fan et al., 2013; Reddy and Irvine, 2013). In the case of ERBB4 receptor, the interaction with its ligand neuregulin 1 (NRG1) induces the cleavage and the release of its cytoplasmic intracellular domain, which interacts with YAP and facilitates its nuclear translocation and thereby its target gene expression (Haskins et al., 2014; Komuro et al., 2003; Omerovic et al., 2004). Altogether, these studies showed that the activity of YAP/TAZ is regulated by the EGF family of ligands in both LATS-dependent and -independent manners (figure 32). In turn, it seems that YAP/TAZ can also activate the EGF signaling pathway by upregulating the expression of the EGF-like growth factor AREG or HB-EGF in breast epithelial cells or cervical cancer cell respectively (figure 32) (He et al., 2015a; Zhang et al., 2009). Thus, these data, mainly obtained in cell cultures, suggest that there is a two-ways interaction between YAP/TAZ and the EGFR pathway. In various types of cancer, the EGFR-YAP interaction results in chemoresistance or cancer progression (He et al., 2015a; Hsu et al., 2019; Kurppa et al., 2020; Song et al., 2015; Xia et al., 2018). However, the biological role of this EGFR-YAP/TAZ crosstalk is not well understood *in vivo*.

Other examples of protein kinases involved in Hippo/YAP signaling regulation are Src kinase, protein kinase A (PKA); MAP4K and PAR-1/MARK kinase (Chen et al., 2019; Huang et al., 2013; Meng et al., 2015; Wu and Griffin, 2017; Yu and Guan, 2013). Interestingly, it has been shown that dimerization of MST1/2 can lead to their autophosphorylation, and therefore to their activation, without involving upstream kinases (Glantschnig et al., 2002; Praskova et al., 2008). The discovery of all these up and

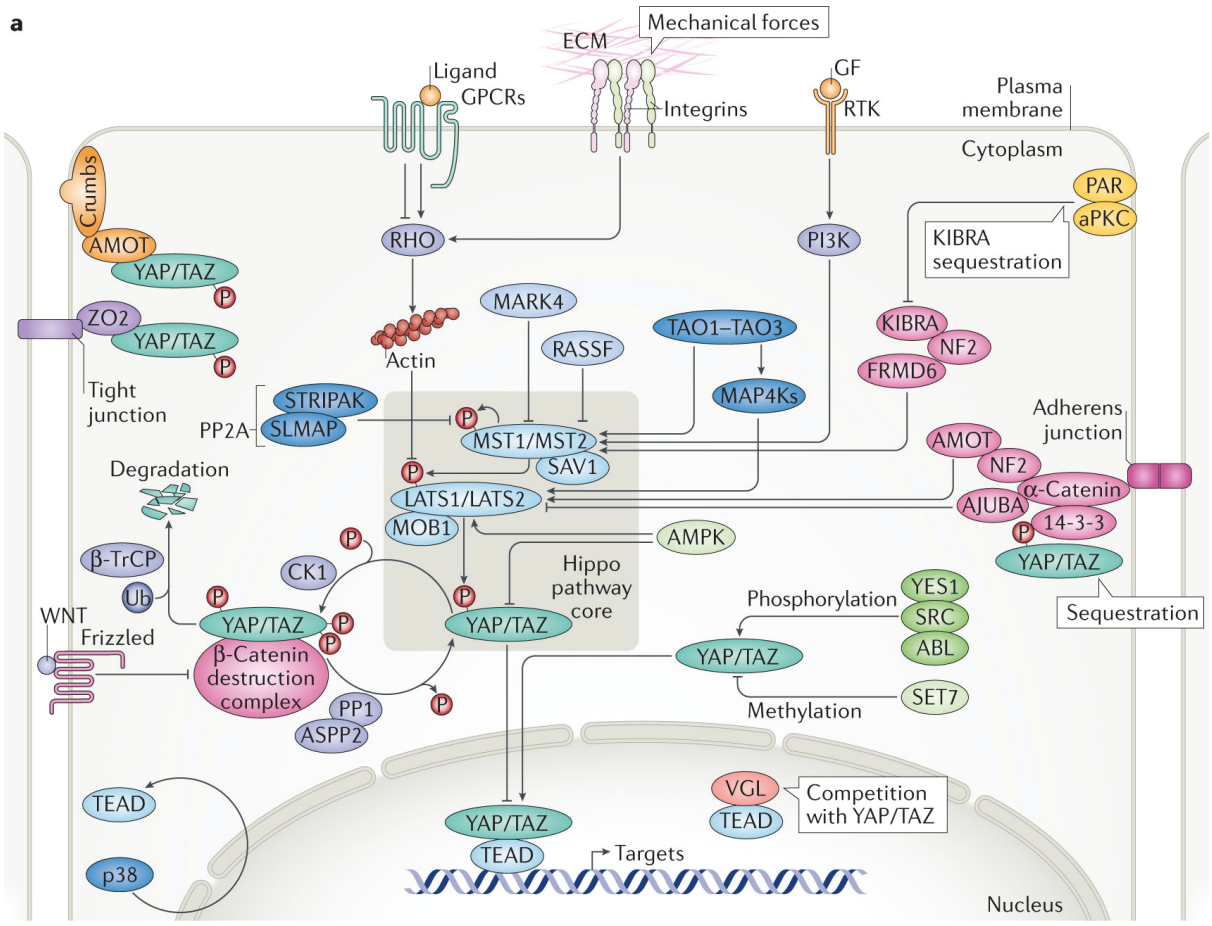



**Figure 31. Vertebrate Hippo signaling pathway components and regulation.** Schematic representation of the different regulators of the Hippo signaling pathway. From (Moya and Halder, 2019)

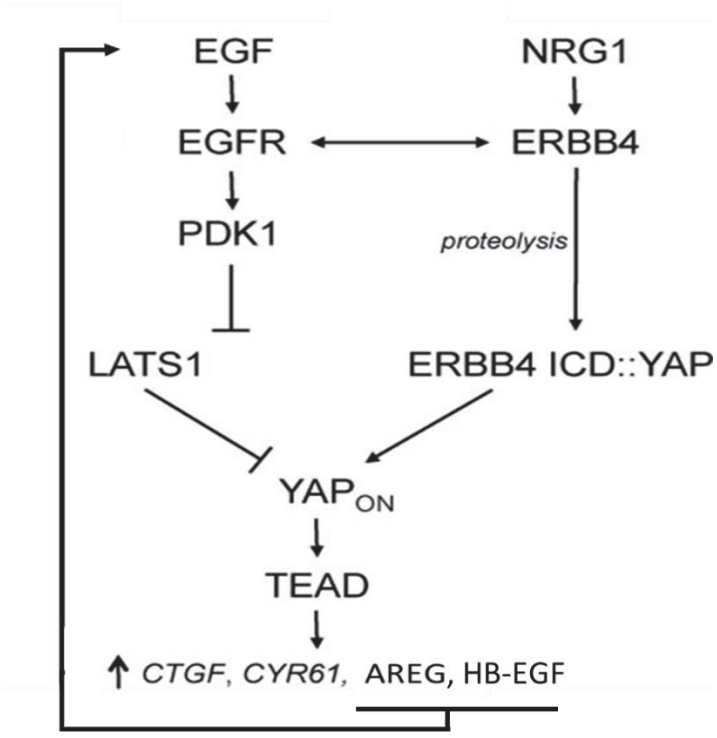



**Figure 32. Crosstalk between EGF and Hippo/YAP signaling pathways in breast cancer cells.** EGF-induced stimulation of EGF receptor (EGFR) leads to an indirect activation of YAP by inhibiting the Hippo kinase LATS1 via PDK1. The proteolytic cleavage of ERBB4 releases a cytoplasmic domain that binds and translocates YAP to the nucleus, allowing the expression of its target genes. In turn, YAP upregulate the expression of the EGF family growth factor AREG and HB-EGF and thereby stimulates the activation of EGFR signaling pathway (Modified from (Haskins et al., 2014))

downstream regulators have expanded the vertebrate Hippo pathway to a complex signaling network with more than 30 components (figure 31).

## POST-TRANSLATIONAL MODIFICATIONS OF YAP/TAZ

As mentioned above, the activity of YAP/TAZ is dependent on their cellular localization, which is mainly controlled by post-translational modifications. These modifications can be made by the canonical cascade of kinases of the Hippo pathway but can also be achieved independently of the MST1/2 and LATS1/2 kinases (Dong et al., 2007; Lei et al., 2008; Varelas, 2014). The negative regulation of YAP/TAZ by LATS1/2 involves the phosphorylation of different serine residues (five and four respectively). The most important phosphorylation sites in the mouse are the serine 127 of YAP (S127) and the serine 89 of TAZ (S89), which has become the principal mode of action for the Hippo pathway (Basu et al., 2003; Huang et al., 2005; Kanai et al., 2000; Lei et al., 2008; Varelas, 2014). When YAP and TAZ are phosphorylated on these residues, they are recognized by 14-3-3 protein and sequestered to the cytoplasm, inhibiting their transcriptional activity (Hao et al., 2008; Varelas, 2014; Zhao et al., 2007). The phosphorylation of residues S381 of YAP and S311 of TAZ by LATS1/2 trigger further phosphorylation by the casein kinase 1δ or 1ε and ubiquitylation by the SCF E3 ubiquitin ligase complex, leading to their degradation by the proteasome (Liu et al., 2010; Zhao et al., 2010). In the same way, phosphorylation of residue S66 of TAZ results in the subsequent phosphorylation of S58 and S62 by the GSK3β kinase and their ubiquitination and proteasome degradation (Huang et al., 2012). Consequently, mutations affecting the serine residues implicated in the negative regulation of YAP/TAZ results in its nuclear translocation and an increase of their activity.

Non-canonical Hippo pathways involve post-translational modifications achieved by different kinases, such as YES, SRC, AKT, C-ABL, JNK, P38, CDK1 or NEK1 (Kodaka and Hata, 2015). These kinases can phosphorylate the same serine residues that the canonical Hippo pathway, leading to the retention and/or degradation of YAP and TAZ or, on the contrary, can phosphorylate other residues such as the tyrosine Y357, leading to YAP stabilization and an increase of its activity (Basu et al., 2003; Levy et al., 2008; Rosenbluh et al., 2012). In addition, other post-translational modifications can also regulate YAP/TAZ activity, such as acetylation, methylation and sumoylation (Hata et al., 2012; Lapi et al., 2008; Oudhoff et al., 2013).

## YAP/TAZ TRANSCRIPTIONAL ACTIVITY

Once YAP/TAZ translocate to the nucleus, and due to the absence of a DNA-binding domain, they have to interact with DNA-binding transcriptional factors to regulate target gene's expression. Among the different partners of YAP/TAZ we can found TEAD, p73, ERBB4, EGR-1, TBX5, RUNXs and SMADS (Kim et al., 2018a; Ma et al., 2019; Pocaterra et al., 2020). The TEAD family is considered the main partner

of YAP/TAZ and is implicated in the control of cell proliferation, growth and survival (Chan et al., 2009; Zhang et al., 2009; Zhao et al., 2008). Similarly, the complex YAP-ERBB4 induces tissue growth (Haskins et al., 2014). P73 and EGR-1 (Early growth response protein 1) interacts with YAP to induce apoptosis (Basu et al., 2003; Kim et al., 2018a; Strano et al., 2005; Zagurovskaya et al., 2009). Regarding TBX5, SMADs and RUNXs, interaction with YAP/TAZ regulates the expression of the target genes of different signaling pathways such as TGF-β, BMPs, β-catenin, or other developmental pathways (Grannas et al., 2015; Heallen et al., 2011; Hong et al., 2005; Jang et al., 2017; Murakami et al., 2005; Rosenbluh et al., 2012; Varelas et al., 2010; Yagi et al., 1999). In addition, my laboratory has recently shown that YAP can also interacts with PKNOX1, a transcriptional factor that regulates multiple aspects of embryonic development and the maintenance of genomic stability (Cabochette et al., 2015).

Target genes regulated by the different YAP/TAZ-DNA-binding transcriptional factor complex are dependent on the tissue and the context. Thus, biological function regulated by YAP/TAZ are diverse, going from the response to mechanical stress and the modification of the extracellular matrix (Calvo et al., 2013; Dupont et al., 2011; Low et al., 2014) and the oxidative stress response (Ashraf and Pervaiz, 2015; Mao et al., 2015), to the induction of apoptosis in response to DNA damage (Hamilton et al., 2009; Mao et al., 2015; Pefani and O'Neill, 2016; Van Der Weyden et al., 2012). In addition, a recent study also suggest a role of YAP as tumor suppresor (Cheung et al., 2020). However, a general study of the YAP/TAZ targets in numerous cell systems have revealed that the majority of the genes regulated by YAP/TAZ are related with cell proliferation. More specifically, YAP/TAZ are involved in all the phases of the proliferative response, from the cell cycle re-entry via regulation of *Ccnd1* (cyclin D1) (Lu et al., 2010; Mizuno et al., 2012) to mitosis, including the control of the transition point G1/S, DNA replication and repair and nucleotide metabolism (Cabochette et al., 2015; Dong et al., 2007; Santinon et al., 2018; Zanconato et al., 2015; Zhao et al., 2008). This involvement in the regulation of the cell cycle underlies YAP/TAZ function in the regulation of stem cell activity. Some biological functions of YAP/TAZ are described in details in section 5.2. Curiously, the widespread accepted transcriptional markers of YAP/TAZ activity, *Ctgf* (Connective Tissue Growth Factor) and *Cyr61* (Cystein-Rich angiogenic inducer 61 or CCN1) are not directly related with cell proliferation, as they are non-structural proteins that reside in the extracellular matrix, but can participate to the proliferative process by the coordination of several signaling pathways (Li et al., 2019a; Mugahid et al., 2020). Along this idea, it has been shown that YAP/TAZ activation also leads to the expression of genes coding for ligands that activate different signaling pathways driving to cell proliferation, as the mentioned EGFR ligands *Areg* (amphiréguline) and *Hbegf* (figure32), but also ligands of Wnt, Hedgehog or Notch signaling pathway (He et al., 2015b; Zhang et al., 2009).

In general, the majority of the studies on the YAP/TAZ functions place YAP/TAZ as transcriptional co-activators of several genes implicated in different cellular process, such as cell growth and proliferation, cell survival, stem cell identity or tissue architecture (Hansen et al., 2015). However, YAP and TAZ are also engaged in transcriptional co-repression. Indeed, around 50% of YAP/TAZ target genes are negatively regulated, indicating that YAP/TAZ perform a widespread effect on gene regulation in both positive and negative ways (Elster et al., 2018; Kim et al., 2015). Thus, it is not surprising that mutations affecting the activity of YAP/TAZ results in the apparition of several diseases, mainly different types of cancer, but also autoimmunity and retinal developmental anomalies. The role of YAP on the retina will be described in detail further in the text.

## 5.2 YAP AND TAZ FUNCTIONS

Although the first recognized role of the Hippo pathway was the regulation of organ size, in the last decade numerous studies have expanded its physiological functions to other processes. In this section, I provide a general vision of the main functions of YAP in development, adult homeostasis and regeneration , and focus on the role of YAP in the nervous system and more specifically in the retina. In addition, giving the importance of the inflammation in my PhD project, I will also describe the recent findings of the role of the Hippo pathway in immune regulation.

### YAP/TAZ IN DEVELOPMENT AND ORGAN GROWTH

YAP and TAZ are essential during development in different species (Varelas, 2014; Zheng and Pan, 2019). For example in mammals, *Yap* knock-out causes embryonic lethality at stage E8.5 due to severe developmental defects, whereas *Taz* knock-out results in a partial lethality at birth while mice that are viable present renal dysfunctions (Hossain et al., 2007; Makita et al., 2008; Morin-kensicki et al., 2006; Tian et al., 2007). The combined knockout potentiates these lethal defects, preventing blastocyst formation and embryonic implantation (Nishioka et al., 2009; Sasaki, 2017).

Numerous studies have related the deleterious defects observed in the YAP/TAZ loss of function phenotypes during development with the regulation of several process in stem cells, including pluripotency and self-renewal, cell type specification and differentiation (Moya and Halder, 2019; Varelas, 2014). For instance, YAP/TAZ are essential for the maintenance of stemness and self-renewal potential of embryonic stem cells (ESCs), for the inhibition of their differentiation (Beyer et al., 2013; Lian et al., 2010; Qin et al., 2012; Tamm et al., 2011; Varelas et al., 2008); and for blastocyst cell fate decision (Marikawa and Alarcón, 2009).

Later on, YAP and TAZ regulate the proliferation of embryonic progenitors and promote organ growth. Thus, the inhibition of MST1/2, LATS1/2 or the expression of a constitutively active form of YAP during mammalian organ development leads to an increase in organ size, mimicking the situation observed in *Drosophila* (Camargo et al., 2007; Dong et al., 2007; Heallen et al., 2011; Lee et al., 2016a; Lu et al., 2010; Moya and Halder, 2019; Song et al., 2010; Von Gise et al., 2012; Xin et al., 2013; Zhou et al., 2009b). For instance, inhibition of the Hippo pathway or *Yap* hyperactivation in mice triggers cardiomyocyte proliferation and heart overgrowth (Heallen et al., 2011). In addition to its proliferative role, YAP/TAZ also have other important functions during organ development, such as in cell type specification and differentiation, or even apoptosis in several species (Davis and Tapon, 2019; Ma et al., 2019; Piccolo et al., 2014; Varelas, 2014; Zheng and Pan, 2019). Altogether, loss of function analysis demonstrate that YAP and TAZ are key factors implicated both at the pre-implantation stages and later on during the embryonic development.

## YAP/TAZ IN ADULT HOMEOSTASIS AND REGENERATION

Multiple studies have shown that, as in embryonic tissues, the overproduction of YAP in adult tissues results in an expansion of the number of stem cells and their progeny and, on the contrary, to an inhibition of cell differentiation (Moya and Halder, 2019; Varelas, 2014; Wang et al., 2017c; Zheng and Pan, 2019). For example, overexpression of YAP or the inhibition of the Hippo pathway results in an expansion of intestinal, epithelial and liver adult stem cells (Cai et al., 2010; Camargo et al., 2007; Imajo et al., 2015; Lee et al., 2008; Lee et al., 2010; Lee et al., 2016b; Schlegelmilch et al., 2011; Zhou et al., 2011). This led to the initial idea that YAP/TAZ were involved in the regulation of adult stem cells and thus in tissue homeostasis. However, the situation is not so simple, as studies using conditional *Yap* loss of function mutants suggest that YAP would be dispensable for stem cell renewal in some mammalian organs such as the mammary glands, pancreas or intestine under physiological conditions (Azzolin et al., 2014; Cai et al., 2010; Chen et al., 2014; Zhang et al., 2010; Zhang et al., 2014). On the contrary, YAP/TAZ activity would be required in pathological conditions for the regeneration of multiple adult tissues.

In regenerative organism such as *Xenopus* tadpoles, the expression of a dominant negative form of YAP prevents limb and tail regeneration after amputation (Hayashi et al., 2014a; Hayashi et al., 2014b). In a flatworm, inhibition of the Hippo pathway promotes regeneration of the whole posterior part after amputation via the recruitment of stem cells, while the inactivation of YAP prevents regeneration (Demircan and Berezikov, 2013). Similarly, in *Drosophila* the inactivation of *Yorkie* prevents the regeneration of the intestine (Staley and Irvine, 2010). In mammals, the level and the activity of

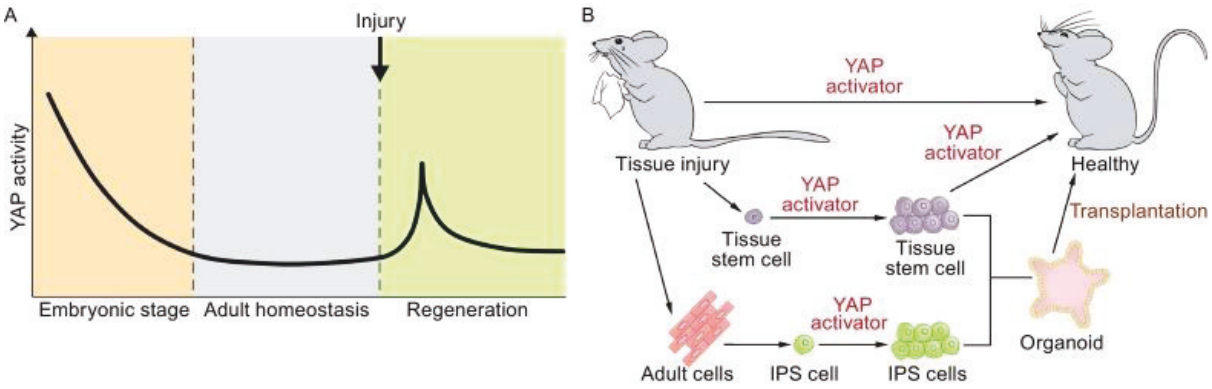



**Figure 33. Hippo signaling in tissue regeneration and regenerative medicine.** (**A**). YAP activity decreases after birth but increases in case of damage. (**B**) Induced YAP activation could trigger tissue regeneration *in vivo* or enhance stem cell expansion *in vitro*. From (Wang et al., 2017c)

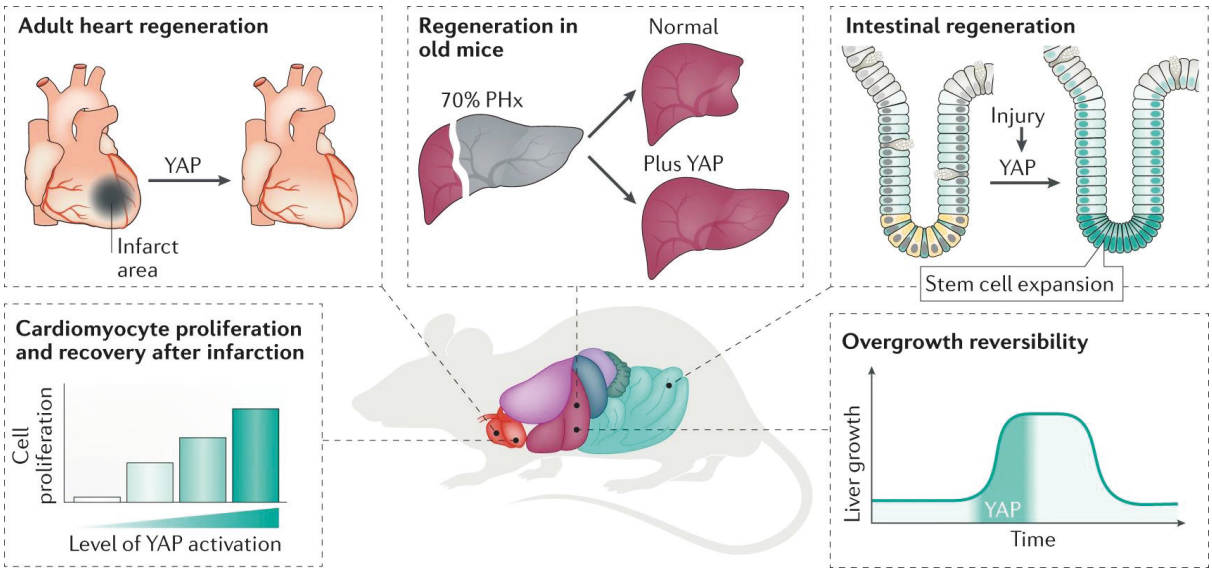



**Figure 34. Effects of YAP/TAZ overactivation in different organs**. Experimental overactivation of YAP/TAZ triggers cell proliferation in several organs, such as liver, heart or intestine, resulting in tissue overgrowth (liver) and/or regeneration. From (Moya and Halder, 2019)

YAP/TAZ dramatically increase in case of damage in the intestine, skin and heart, just before the beginning of the regenerative process (figure 33) (Moya and Halder, 2019). In the absence of *Yap/Taz* the regenerative capacities of some mammalian organs such as the liver, the skin or the lungs are decreased or impeded upon damage (Moya and Halder, 2019; Varelas, 2014; Wang et al., 2017b; Yu et al., 2015). Interestingly, YAP/TAZ are also implicated in the regenerative process even in tissues where YAP/TAZ does not appear to contribute significantly to homeostasis of adult tissues, such as the intestine or the mammary gland (Moya and Halder, 2019). Besides, experimental activation of YAP/TAZ induces cell proliferation in many organs, which lead to overgrowth of tissues, such as the liver, and/or to tissue regeneration (figure 34) (Moya and Halder, 2019). For instance, it has been shown that although the regenerative capacity of the mouse heart is limited, inactivation of the Hippo pathway or overexpression of a constitutively active form of YAP promotes myocardial regeneration after a heart attack (Heallen et al., 2013; Leach et al., 2017; Lin et al., 2014; Xin et al., 2013).

This enhanced regeneration following ectopic YAP/TAZ activation has also been described in diseased organs, such as old and chronically diseased livers, intestine affected with ulcerative colitis and Crohn's disease or aged skin, that cannot regenerate efficiently (Moya and Halder, 2019). However, due to YAP/TAZ function in cell proliferation, a therapeutic approach based on YAP/TAZ overexpression is not risk free, as sustained activation of YAP/TAZ can result in aberrant cell proliferation and tumor formation (Moya and Halder, 2019). In addition, YAP/TAZ can induce changes on tumor environment promoting cancer stem cell behavior, chemo-resistance and metastasis (Misra and Irvine, 2018). Thus, transient overexpression or overactivation approaches should likely be considered. One possibility would be the use of molecule inhibitors of the Hippo signaling pathway, as the XMU-MP1, an inhibitor of MST1/2 which has been shown to facilitate intestinal and hepatic repair upon injury (Fan et al., 2016). Another one is the combination of YAP/TAZ expression using AAV vectors in combination with inducible expression systems such as doxocycline-inducible system or laser-directed activation using optogenetics, which would restrain YAP/TAZ expression to the tissue or cell type of interest, reducing the adverse side effects, and allow a very accurate control of the YAP/TAZ expression (Moya and Halder, 2019).

### YAP FUNCTIONS DURING REGENERATION

How YAP regulates adult stem cell properties during regeneration has not yet been well characterized. First, YAP overexpression was shown to promote their proliferation (Moya and Halder, 2019; Wang et al., 2017c). In addition, it has been suggested that YAP binds directly to promoters of pluripotency genes and induce their reprogramming to a dedifferentiated state (Moya and Halder, 2019; Wang et al., 2017c). Along this line, YAP/TAZ overexpression has been shown to be sufficient to reprogram

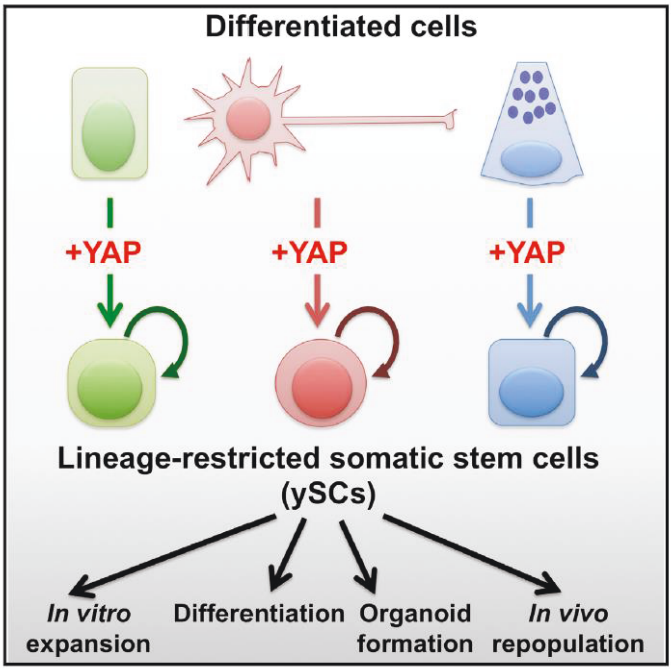



**Figure 35. Reprogramming and lineage conversions of different differentiated cells in response to YAP overexpression.** Transitory expression of exogenous YAP lead to the conversion of differentiated cells into cells presenting stem cells characteristics. From (Panciera et al., 2016)

mature differentiated cells into pluripotent progenitors (figure 35) (Moya and Halder, 2019; Panciera et al., 2016; Panciera et al., 2018; Schlegelmilch et al., 2011; Yimlamai et al., 2014; Yui et al., 2018).

The mechanisms underlying this YAP-dependent cell reprogramming could involve the activation of important regenerative reprogramming factors, such as *Lin28* (Ye et al., 2020). Along this idea, combination of YAP expression or LATS2 or MST1 inhibition with the four factors of Yamanaka (*Klf4, c-myc, Oct4* and *Sox2*) has been shown to increase the efficacy of the reprogramming of mouse and human embryonic fibroblasts into induced pluripotent stem cells (iPCs) (Lian et al., 2010; Qin et al., 2012; Robertson et al., 2017; Takahashi and Yamanaka, 2006). Besides, YAP is implicated in the control of chromatin accessibility, another process implicated in regeneration (Monroe et al., 2019). In addition, YAP and TAZ have also been shown to be implicated in the biogenesis of some miRNA like miR-16, -21, 23a, -29, -107 and -152 via Dicer activation (Chaulk et al., 2014; Tumaneng et al., 2012). Giving the role of miRNA on the regenerative process, this mechanism should also be considered. Altogether, YAP/TAZ function as a reprogramming/proliferative factor during tissue regeneration.

## YAP/TAZ IN THE CENTRAL NERVOUS SYSTEM

The role of YAP in the central nervous system is still far from being deciphered. However, recent studies have placed YAP as an important regulator of neural stem cell self-renewal, the proliferation of neural progenitor cells or the differentiation and activation of glial cells (Bao et al., 2017; Jin et al., 2020; Ouyang et al., 2020; Sahu and Mondal, 2020). Of note, the specific case of the retina will be dealt with below.

Gain and loss of function approaches have determined that YAP is required to regulate the number of neural progenitors during embryogenesis in zebrafish, *Xenopus* and chicken and in the developing mouse hippocampus and ventricular zone by controlling the proliferation and the differentiation of neural progenitors (Cao et al., 2008; Gee et al., 2011; Jiang et al., 2009; Kostic et al., 2019; Lavado et al., 2013; Lavado et al., 2014; Lavado et al., 2018; Milewski et al., 2004; Piccolo et al., 2014; Wang et al., 2017c). However, the role of YAP is not so simple. Indeed, a study in mice has shown that neocortical neurogenesis is not affected in a specific *Yap* mutant of the nervous system (Huang et al., 2016a). Similarly, it seems that YAP is not necessary for the proliferation of adult mouse neural stem cells under physiological conditions (Huang et al., 2016a; Lavado et al., 2013). Strikingly, YAP seems to be implicated in mouse neocortical astrogliogenesis (Huang and Xiong, 2016; Huang et al., 2016a; Huang et al., 2016b). Consequently, the absence of YAP during neocortical development does not affect neurogenesis at birth but leads to neurodegeneration of neocortical neurons probably due to its role in the proliferation of astrocytes (Huang et al., 2016a). Thus, it seems that the developmental functions of YAP/TAZ in mouse neuronal progenitor proliferation and differentiation are dependent on the brain region. Of note, YAP function is required for proper proliferation of progenitors and apical

attachment of ependymal precursor cells, a type of neuroglia that line the ventricular surface in the brain and in the central canal of the spinal cord (Park et al., 2016), suggesting that in the mammalian nervous system, YAP and TAZ are important for both the generation of different glial cells and the generation of neurons. In the peripheral nervous system, YAP is also important via its role in the regulation of Schwan cell proliferation and also in the formation of the peripheral nerves and their myelinization (Poitelon et al., 2016)(Mindos et al., 2017). The role of YAP in repairing central neural tissue has not been studied to date.

## YAP/TAZ IN THE RETINA

As for other organs, precise regulation of YAP and TAZ expression and activity is critical for eye development in different species. Indeed, *Yap* deletion using a morpholino in zebrafish or *Xenopus* results in reduced eye size (Cabochette et al., 2015; Jiang et al., 2009), whereas YAP overexpression in zebrafish or *Xenopus* promotes the proliferation of retinal progenitor cells (Asaoka et al., 2014; Cabochette et al., 2015), resulting in eye growth and increased eye size in *Xenopus* (Cabochette et al., 2015). In mice, mRNA and protein expression analysis of YAP during development have shown that it is highly expressed in retinal progenitor cells and downregulated in newly postmitotic retinal neurons (Kim et al., 2016). Overexpression of YAP in mouse embryonic retinal explants leads to an increase in the number of proliferative retinal progenitor cells, although the effect on the eye size has not been studied (Asaoka et al., 2014; Kim et al., 2016). Besides, studies in zebrafish and mouse showed an implication of YAP and TAZ in the genesis of RPE during development modulating the differentiation process (Asaoka et al., 2014; Jiang et al., 2009; Kim et al., 2016; Liu et al., 2010; Miesfeld et al., 2015). Thus, as in other tissues, YAP and TAZ regulate the balance between proliferation and differentiation of retinal progenitors during eye development.

In the mouse eye, YAP is detected from embryonic stage E10.5 in the distal optic cup and then is ubiquitously expressed in the cornea, lens, RPE, ciliary body, iris and retina (Kim et al., 2016; Williamson et al., 2014; Zhu et al., 2018). Regarding the retina, YAP is expressed in proliferative retinal progenitors during early post-natal stages (Zhang et al., 2012). *Yap* deletion during this period has revealed its involvement in several cellular processes such as cell cycle control, angiogenic activation, progenitor proliferation, junctional integrity maintain, regional fate choice and structural preservation (Choi et al., 2015; Kim et al., 2016; Zhang et al., 2012; Zhu et al., 2018). In the adult retina, a recent study has shown that YAP is required to prevent the dedifferentiation of adult RPE cells (Lu et al., 2020b). In addition, it also suggests that YAP may be implicated in the RPE-mediated visual circuit, as YAP knockout correlates with the inhibition of several important visual cycle genes, such as RPE65,

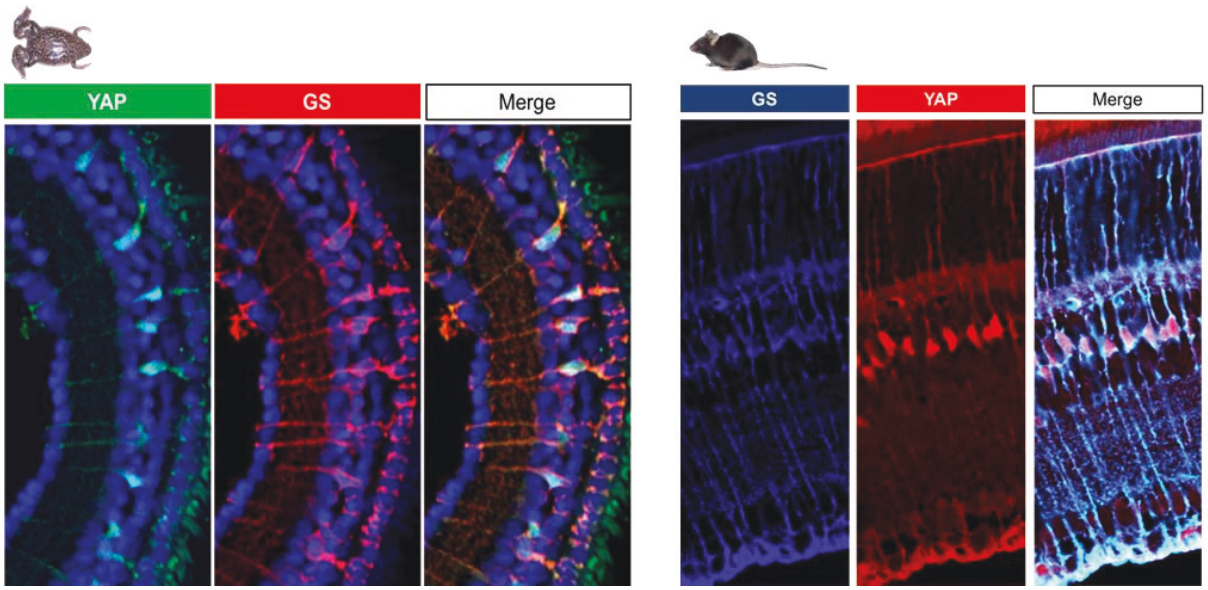



**Figure 36. YAP expression in mouse and *Xenopus* Müller cells.** Retinal sections of *Xenopus* (left) and mouse (right) retinas immunolabeling for YAP. Glutamine synthase (GS) labeling was used as a marker of Müller cells. For *Xenopus*, nuclei are Hoeschst counterstained. Of note, the orientation of the retina is different for each image. The ONL points to the top for the mouse and to the right for the Xenopus images. In both cases, YAP is detected specifically in Müller cells, in both the nucleus and the cytoplasm. From (Hamon et al., 2017; Hamon et al., 2019).

and results in a reduction of RPE pigments, photoreceptor cell death and loss of vision with the age (Lu et al., 2020b). However, the role of YAP and TAZ in the adult neural retina, and more specifically in the regenerative process, is still unknown. Although YAP is known to be expressed in proliferating progenitor cells, my laboratory has shown that it is also expressed specifically in adult retinal Müller cells in both *Xenopus* and mice (figure 36) (Cabochette et al., 2015; Hamon et al., 2017). Further analysis by whole transcriptome sequencing showed that, in case of photoreceptor degeneration in mice, the expression of YAP and its co-factor TEAD1 is increased in reactive Müller cells at both mRNA and protein levels, revealing for the first time a link between the Hippo pathway and the reactivation of Müller cells (Hamon et al., 2017). This data constituted the initial point of my PhD project.

## YAP/TAZ IN THE INFLAMMATORY RESPONSE

In addition to the well-established function of YAP/TAZ in cell proliferation, recent data indicate that multiple core components of the Hippo/YAP pathway are also involved in the regulation of the immune response. Interestingly, some studies also reported that the activation of the immune system can modulate the activation state of Hippo/YAP pathway (Wang et al., 2020a). However, this reciprocal regulation is still not well understood, with few studies implicating both canonical and non-canonical Hippo pathways and reporting conflicting data that remains to be reconciled (Wang et al., 2020a). In this section, I will provide a brief description of the main evidences on the crosstalk between the immune system and the canonical Hippo/YAP pathway, with a special focus on the effect on this interaction on the regenerative response.

### INFLAMMATION AS A REGULATOR OF THE HIPPO/YAP SIGNALING PATHWAY

Insult-related inflammation can upregulate YAP/TAZ signaling through a variety of mechanisms that increase tissue repair and reduce deleterious cytotoxic inflammation. For instance, TLRs, the specialized receptors against conserved microbial signatures, act upstream Hippo pathway and regulates the activity of YAP and/or the regeneration of different tissues. For example, in macrophages activation of TLR4 by LPS exposure results in the upregulation of YAP (Zhou et al., 2019). A similar study has also described an increase of YAP expression via TLR4-AP-1 in liver Kupffer cells *in vitro* and *in vivo* in response to LPS (Song et al., 2019). As another an illustration, in case of myocardial infarction during the mouse neonatal period, the activation of YAP through TLR3 signaling results in enhanced cardiomyocyte proliferation and the functional recovery of the heart (Wang et al., 2018). In addition to TLRs, activation of other inflammatory signaling pathways also regulate YAP regenerative capacity in a direct and/or indirect way. For example, in case of intestinal inflammation, inflammatory cytokines trigger the secretion of the ISLR protein by stromal cells, which are connective tissue cells. ISLR then suppresses Hippo signaling and thus induces YAP activation in epithelial cells, promoting

intestinal regeneration (Xu et al., 2020). In case of experimental colitis induced by dextran sulfate sodium salt (DSS), intestinal inflammation activates the IL-6 co-receptor, gp130, triggers YAP activation via Src and Yes kinases and stimulates epithelial cell proliferation and intestinal regeneration (Taniguchi et al., 2015). In addition, inflammatory signals, especially IL-6, would be required to switch the YAP-apoptotic activity to a cell expansion activity in order to increase tissue regeneration. Indeed, Su and colleagues have shown that YAP-overexpression in hepatocytes results in enhanced apoptosis and impaired proliferation in normal tissue. However, in case of tissue damage and inflammation the overexpression of YAP is associated with an enhanced proliferative response of adult stem cells and liver regeneration (Su et al., 2015).Altogether, these data indicate that inflammation-induced YAP activation may be critical for tissue regeneration.

## YAP AS A REGULATOR OF THE INFLAMMATORY RESPONSE

Insult-related or inflammation-related YAP activation can in turn potentialize and/or resolve the inflammatory process, modulating immune-related phenomenon including regeneration. However, this role of YAP is still quite controversial.

Some studies have reported that in case of damage, the activation of YAP results in an upregulation of the expression of pro-inflammatory cytokines. Accordingly, microarray analysis on mammalian breast cells overexpressing YAP has revealed that many of the YAP targets include several cytokines such as Il6, IL8 or CXCL1 (Kim et al., 2015). However, this goes to the detriment of the regeneration. In liver hepatocytes, YAP overexpression was shown to enhance the expression of pro-inflammatory signals, such as TNFα or IL1-β, activate immune cells and trigger liver fibrosis after injury (Mooring et al., 2020; Song et al., 2019). Consequently, YAP/TAZ deletion leads to opposite effects (Mooring et al., 2020; Song et al., 2019). Similarly, another study has reported that Hippo signaling inhibition, and thus YAP activation in the acinar cells of the pancreas results in a strong inflammation and severe fibrosis, impairing the regenerative process (Liu et al., 2019b). In addition, another study has shown that in the absence of YAP, the inflammatory process in a mouse model of DSS-induced colitis is decreased, suggesting that YAP promotes a pro-inflammatory response (Zhou et al., 2019). In this context, the pro-inflammatory effect of YAP may be linked to its capacity to recruit and regulate M1/M2 polarization of macrophages. Indeed, a study in the liver has shown that YAP activation in hepatocytes increase hepatic inflammation and promotes macrophage infiltration via the production of the chemoattractant protein CCL2 (Kim et al., 2018b). Moreover, it has been recently reported that in the intestine, the increase of YAP expression in macrophages in response to LPS promotes M1-like macrophage polarization and an increase of IL-6 production, while restricting M2 polarization through

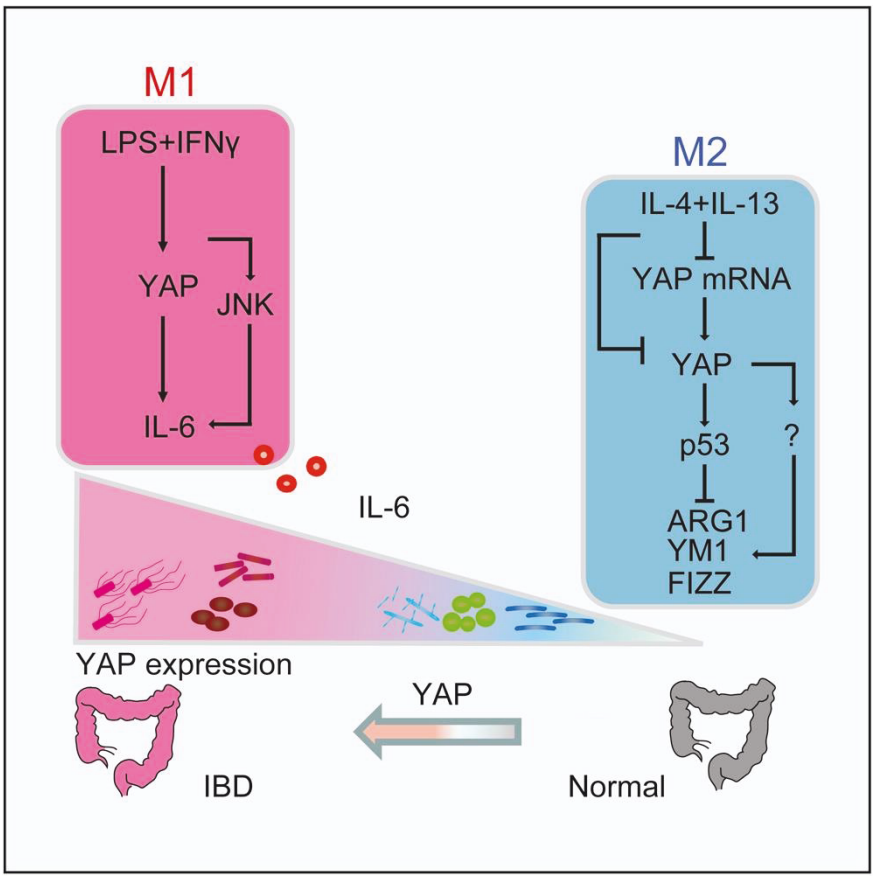



**Figure 37. YAP is differentially regulated in macrophages in response to inflammatory signals.** Proposed model of the differential regulation of YAP in macrophages by LPS/IFNγ and IL-4/13 treatment. LPS-induced M1-phenotype increases YAP expression which in turn promotes Il-6 production, sustains M1-phenotype, and inhibits M2 polarization. IL-4/13 treatment induces M2 polarization and decreases YAP expression. From (Zhou et al., 2019).

p53 transcription (Zhou et al., 2019). A similar study has also described an increase of YAP expression via TLR4-AP-1 in liver Kupffer cells *in vitro* and *in vivo* in response to LPS, that was accompanied by an increase on the expression of pro-inflammatory cytokines (Song et al., 2019). On the contrary, treatment of macrophages with IL-4/13, which has been described as inductors of the anti-inflammatory response, results in a reduction of YAP quantity, suggesting that in response to inflammatory signals the expression of YAP is differentially regulated to drive macrophages to an M1 or M2 fate (figure 37 ) (Zhou et al., 2019). Altogether, these results indicate that in response to damage or inflammation, the expression of YAP is increased to regulate the recruitment and acquisition of M1 macrophage phenotype and trigger the pro-inflammatory response.

On the contrary, other studies have shown that the regenerative effects of YAP are linked to its immunosuppressive effect through the inhibition of the pro-inflammatory response and the recruitment of M2-like macrophages or Treg cells. For exemple, in case of LPS-induced endotoxemia, which is a systematic inflammation induced by the presence of LPS in the bloodstream, YAP is necessary to prevent NF-kB signaling activation and restrain the immune cells recruitment, the endothelial activation and the inflammatory response (Lv et al., 2018). This process is required to avoid lung inflammatory injury and cardiovascular organ failure (Lv et al., 2018). Similarly, in case of pneumococcal infection, the expression and the activity of YAP/TAZ is increased in alveolar epithelial cells to stop NF-kB dependent transcription of inflammatory genes, limiting the excessive and prolonged production of inflammatory mediators and favoring lung alveolar regeneration (figure 38) (LaCanna et al., 2019). In heart, the anti-inflammatory role of YAP has also been described not as related to a direct inhibition of pro-inflammatory signaling pathways, but with the recruitment of Treg cells (Ramjee et al., 2017). Indeed, in case of myocardial infarction, the expression of YAP is required to enhance the expression of the IFN-γ, a known Treg inducer, and trigger the infiltration of Treg cells to localize and limit the inflammation to avoid cardiac fibrosis and improve recovery (Ramjee et al., 2017). Of note, YAP and TAZ functions in Treg cells seem to be opposed. While YAP is highly expressed in immunosuppressive Treg cells and reinforces their functions (Ni et al., 2018), TAZ inhibits Treg differentiation and promotes the development of inflammatory T helper 17 (Th17) cells (Geng et al., 2017). Further studies are thus needed to elucidate this apparently conflicting regulations. Overall, these studies place YAP as a molecular brake to restrict the expression of inflammatory genes and resolve the inflammatory response, avoiding excessive inflammation and improving regeneration and favorizing the return to tissue homeostasis.

So far, both positive and negative influences of YAP on the inflammatory response have been reported. The complexity and the dynamic of the inflammation make it difficult to compare the different information in order to determine if these apparent contradictory functions are dependent

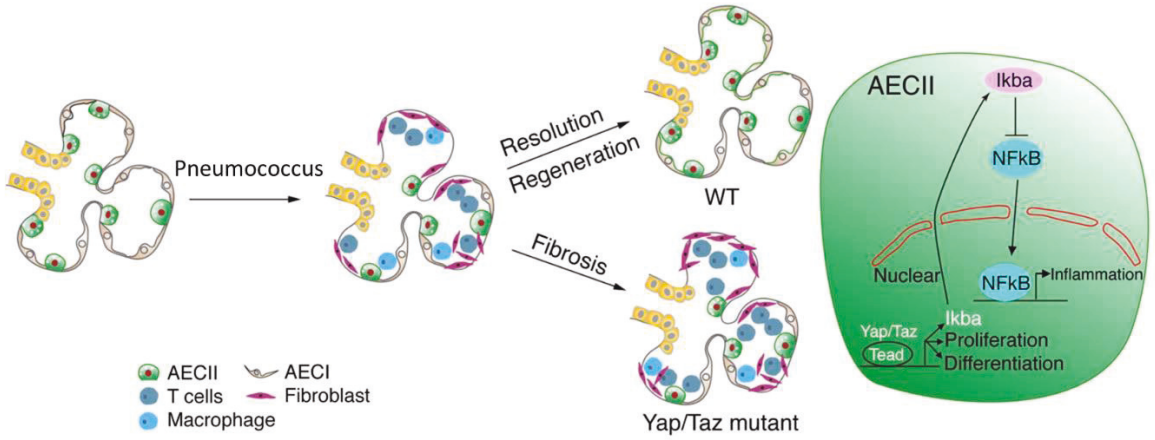



**Figure 38. Schematic model of YAP/TAZ function during alveolar regeneration and resolution of lung inflammation.** In case of bacterial pneumonia, mice that lacked Yap/Taz in alveolar epithelial type II cells (AECIIs) exhibit prolonged inflammatory responses in the lung and a delay in alveolar epithelial regeneration. This impaired alveolar epithelial regeneration is parallel to a failure to upregulate IκBa, the molecule that terminates NF-κB–mediated inflammatory responses. AECI: alveolar epithelial type I cell. Modified from (LaCanna et al., 2019).

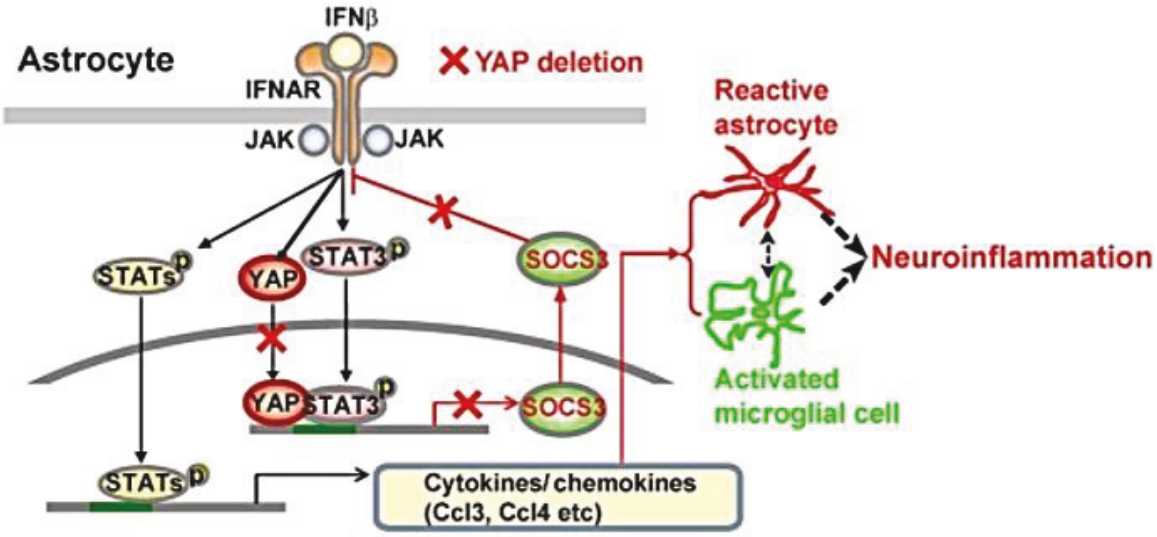



**Figure 39. Schematic model of YAP function in reactive astrocytes and neuroinflammation.** YAP regulates SOCS3 expression in astrocytes in respond to cytokine stimulation as IFNβ to inhibit JAK-STAT signaling pathway and limit the inflammatory response. In the absence of YAP JAK-STAT signaling pathway is hyperactivated and results in microglial recruitment and activation and in an enhanced inflammatory response. From (Huang et al., 2016c).

on the context or the cell type. Further studies are therefore required to explain this apparently contradictory regulation.

### YAP FUNCTION IN INFLAMMATION IN THE CENTRAL NERVOUS SYSTEM

The role of the inflammatory response on YAP-dependent neural progenitor behavior has not been investigated to date. However, in case of neuroinflammation YAP has been shown to have an anti-inflammatory role by preventing reactive astrogliosis. Indeed, upon cytokine stimulation YAP is translocated to the nucleus to interact with p-STAT3 and induce the expression of SOCS3, a protein that is often induced by cytokines that function in a negative-feedback loop to prevent the transduction of cytokines by the JAK-STAT pathway (figure 39) (Huang et al., 2016c). On the contrary, YAP deficiency in astrocytes results in an increase of the JAK-STAT inflammatory pathway activity and the reactive astrogliosis, which drives the recruitment and activation of microglia and neuroinflammation (figure 39) (Huang et al., 2016c). In the lens, RNA profiling analysis have revealed that inflammation genes like TNFα or IL-6 are increased in the absence of YAP, suggesting also an anti-inflammatory role of YAP (He et al., 2019). However, the interplay between YAP and retinal inflammation has hitherto not been investigated.

## 5.3 CONCLUSION

In the quest for new factors involved in Müller cell-dependent regeneration upon injury, YAP/TAZ are candidates of choice. First, YAP/TAZ activation regulates stem cell properties. Second, they induce cell proliferation and accelerate tissue repair in many organs, including those that cannot regenerate effectively in mammals. Third, there are emergent evidences linking the Hippo/YAP pathway and the inflammatory response in the context of the regenerative process. Therefore, studying the role of YAP in retinal Müller cell reactivation in case of damage and its implication in microglia-Müller cell relationships during injury, appears to be necessary in the search for new therapeutic strategies based on the stimulation of retinal endogenous stem cell regenerative capacities.

# OBJECTIVES

# OBJECTIVES

Molecular mechanisms regulating Müller cell behavior have received increasing attention in recent years, with the idea that one day it will be possible to activate the regenerative capacity of these cells in human. That is why, since the discovery of Müller cell neurogenic potential in zebrafish, several signaling pathways have been studied in this context. Strikingly, although YAP function in stem cells and in regeneration point to its potential role in the control Müller cell-dependent retinal regeneration, its function in these retinal cells has never been explored, neither in mice nor in other species. When I arrived in the laboratory, my colleagues were starting to explore the relationship between YAP and Müller cell reactivation. They had found that YAP was specifically expressed in adult retinal Müller cells, and whole transcriptome analysis had revealed the existence of a link between the Hippo pathway and the reactivation of Müller cells. In this context, during the first part of my PhD I contributed to two current projects in the laboratory with the aim to: (I) better understand the role played by YAP in mouse retinas under physiological and pathological conditions, and (II) finding a way to awake the Müller cell regenerative potential in mouse retinas. To this aim, I participated to the characterization of the phenotypes of both YAP heterozygous mice and YAP inducible conditional mice were YAP was specifically deleted in Müller cells. Shortly after, based on the hypothesis that increasing YAP expression and activity could possibly awake Müller cell regenerative potential, I developed an AAV approach to drive YAP activation specifically in Müller cells.

The second part of my PhD project stemmed from the emerging discoveries of inflammation as a regulator of the regenerative process. Although inflammation is considered to hamper retinal regeneration in mammals, there are no studies regarding the influence of inflammation on Müller cell-dependent regenerative process. In addition, recent discoveries on the role of YAP in the regulation of the inflammatory process lead to the hypothesis that it could serve as a hub between the inflammation and the regenerative responses. Both loss and gain of function models of YAP, developed and characterized during the first part of my PhD, constituted useful tools to address this question and contribute to the interconnection of two different fields: stem cell/regeneration and inflammation. Thus, the last objectives of my PhD were (III) to determine the role played by the inflammatory response to injury on mouse Müller cell behavior and (IV) define how YAP fits in this interplay.

In order to facilitate the reading of the document, I have not presented a separated discussion section at the end of the Thesis manuscript, but have instead split the discussion text, so that each result section is either directly followed by the corresponding discussion (Part 1 and Part 2) or combined with the discussion (Part 3 and Part 4).

# RESULTS AND DISCUSSION

# PART 1

## YAP FUNCTION IN THE ADULT RETINA UNDER PHYSIOLOGICAL CONDITIONS

Although the function of YAP during retinal development, where it is implicated in the proliferation and the differentiation of retinal progenitors, has been well described, there is almost no information about the role of YAP in adult retinas. In this context, the laboratory developed a project to understand its role in post-natal and adult retina. Christel Masson was in charge of the study of the role of YAP in both postnatal and adult mammalian retinas under physiological conditions using a loss of function mouse model of YAP. In collaboration with her, I was particularly involved in the statistical analysis of the results. In addition, I performed all the qPCR experiments related to the interaction between YAP and EGFR signaling pathway.

Associated publication:
Christel Masson, **Diana García-García**, Juliette Bitard, Élodie-Kim Grellier, Jérôme E. Roger and Muriel Perron. *Yap* haploinsufficiency leads to Müller cell dysfunction and late-onset cone dystrophy. (2020) **Cell death and disease** 11, Article number: 631

## 1. ANIMAL MODELS

As it has been already mentioned, *Yap* knockout is lethal at embryonic day 8.5 (Morin-kensicki et al., 2006). Thus, in order to investigate YAP function in the adult retina we took advantage of a ***Yap* heterozygous mouse model**. *Yap*$^{+/-}$ mice are viable and fertile, allowing for investigations of the impact of YAP reduced levels at adult stages. Moreover, as YAP is expressed in Müller cells but also in the RPE, we took advantage of a genetic mice line in which *Yap* was conditionally deleted specifically in Müller cells to confirm some of our results. This *Yap* CKO model is described in details in Part 2.

## 2. MAIN RESULTS

- This study in *Yap*$^{+/-}$ mice retina revealed unsuspected dynamics of YAP compensatory mechanisms: YAP decrease is compensated by TAZ at postnatal stages but not anymore in adulthood.
- TAZ-dependent compensatory regulation in *Yap*$^{+/-}$ mice during post-natal stages leads to *Taz* upregulation, subsequently leading to a gain of function-like phenotype, *i.e.* delayed cell cycle exit of retinal progenitor cells.
- Our data led us to propose a model in which the potentiation of EGFR activity by TAZ underlies this delay in cell cycle exit of retinal progenitor cells in *Yap*$^{+/-}$ retina.
- Non-compensated decrease of *Yap* expression in aged retinas leads to *Yap* haploinsufficiency, resulting in altered Müller cell homeostatic function.
- We also discovered late-onset cone degeneration and impaired cone visual function in aged *Yap*$^{+/-}$ mice.

- Conditional *Yap* deletion in Müller glia also leads to Müller cell homeostasis dysfunction and altered pattern of cone opsin expression, suggesting that YAP function in Müller cells is necessary for ensuring the maintenance of Müller cell homeostasis and preserving cone integrity.

# Article nº1

Christel Masson, **Diana García-García**, Juliette Bitard, Élodie-Kim Grellier, Jérôme E. Roger and Muriel Perron

(2020)

# *Yap* haploinsufficiency leads to Müller cell dysfunction and late-onset cone dystrophy

*Cell death and disease*

Masson et al. *Cell Death and Disease* (2020)11:631
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Cell Death & Disease

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# *Yap* haploinsufficiency leads to Müller cell dysfunction and late-onset cone dystrophy

Christel Masson[1], Diana García-García[1], Juliette Bitard[1], Élodie-Kim Grellier[1], Jérôme E. Roger[1] and Muriel Perron[1]

**Abstract**

Hippo signalling regulates eye growth during embryogenesis through its effectors YAP and TAZ. Taking advantage of a *Yap* heterozygous mouse line, we here sought to examine its function in adult neural retina, where YAP expression is restricted to Müller glia. We first discovered an unexpected temporal dynamic of gene compensation. At postnatal stages, *Taz* upregulation occurs, leading to a gain of function-like phenotype characterised by EGFR signalling potentiation and delayed cell-cycle exit of retinal progenitors. In contrast, $Yap^{+/-}$ adult retinas no longer exhibit TAZ-dependent dosage compensation. In this context, *Yap* haploinsufficiency in aged individuals results in Müller glia dysfunction, late-onset cone degeneration, and reduced cone-mediated visual response. Alteration of glial homeostasis and altered patterns of cone opsins were also observed in Müller cell-specific conditional *Yap*-knockout aged mice. Together, this study highlights a novel YAP function in Müller cells for the maintenance of retinal tissue homeostasis and the preservation of cone integrity. It also suggests that YAP haploinsufficiency should be considered and explored as a cause of cone dystrophies in human.

## Introduction

Deregulation of key signalling pathways, such as Wnt or Notch, well known for their involvement in retinal development, are at the origin of retinal diseases in adults[1,2]. Studying developmental signalling pathways can thus allow the identification of possible causes of retinal disorders in adults, pinpointing possible targets for therapeutic approaches. The Hippo signalling tightly regulates eye development, and its deregulation in animal models leads to severe ocular defects (reviewed in[3,4]). This signalling pathway consists of a kinase cascade that ultimately phosphorylates the transcription coactivators YAP (Yes associated protein) and TAZ (WW domain-containing transcription regulator 1), causing their retention in the cytoplasm or their degradation. When the pathway is inactive, YAP/TAZ are translocated to the nucleus, leading to activation of their target genes[5,6]. During retinal development, YAP and TAZ are expressed in all optic vesicle compartments, becoming more prominently expressed in the retinal pigment epithelium (RPE) and the ciliary margin of the optic cup[4,7]. Studies in zebrafish and mice indicate that YAP is necessary for the maintenance of progenitor populations in both the retina and the RPE[4,7–11]. Besides, mutations in Hippo pathway components have been identified in human ocular diseases, such as the Sveinsson's chorioretinal atrophy (SCRA)[12,13], in patients with ocular manifestations of the neurofibromatosis type-2 human disease[14], or in patients exhibiting coloboma[15,16]. Together, these studies have contributed to highlight the important role of the Hippo-YAP pathway in eye development and ocular disorders. Although it has previously been established that YAP remains expressed in the adult retina in both Müller glia and RPE cells[17], its function in the adult eye has received little attention thus far.

To investigate YAP function in the adult, and because *Yap* knockout is lethal at embryonic day 8.5[18], we took advantage of a *Yap* heterozygous mouse model. We found that depletion of one *Yap* allele leads to a transient TAZ

Correspondence: Christel Masson (christel.masson-garcia@universite-paris-saclay.fr) or Jérôme E. Roger (jerome.roger@universite-paris-saclay.fr) or Muriel Perron (muriel.perron@universite-paris-saclay.fr)
[1]Paris-Saclay Institute of Neuroscience, CERTO-Retina France, CNRS, Université Paris-Saclay, Orsay 91405, France
Edited by S. Inoue





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compensatory mechanism at postnatal stages, associated with an increased expression of YAP/TAZ target genes. Consistent with the known critical role of the Hippo pathway in regulating neural progenitor cell proliferation[19], we found that such enhanced TAZ activity in *Yap*$^{+/-}$ postnatal retinas is associated with a gain of function-like phenotype, i.e., delayed cell-cycle exit of retinal progenitors. We identified the regulation of the EGFR (Epidermal Growth Factor Receptor) pathway as a potential underlying mechanism. Conversely, TAZ compensatory regulation declined during aging in *Yap* heterozygous retinas. We found that non-compensated decrease of *Yap* expression in aged retinas leads to altered Müller cell homeostatic function, causing late-onset cone degeneration and thereby impaired cone visual function. This work thus uncovers a novel unexpected role for YAP in cone photoreceptor maintenance and proper vision.

## Methods and material

### Ethics statement

All animal experiments have been carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EEC). All animal care and experimentation were conducted in accordance with institutional guidelines, under the institutional license D 91–272–105. The study protocols were approved by the institutional animal care committee CEEA n°59 and received an authorisation by the "Direction Départementale de la Protection des Populations" under the reference APAFIS#1018–2016072611404304 v1.

### Mice

Mice were kept at 21 °C, under a 12-h light/12-h dark cycle, with food and water supplied *ad libitum*. Heterozygous *Yap*$^{+/-}$ mice were obtained from Sigolene Meilhac (Institut Pasteur, Paris). Briefly, *Yap*$^{flox/+}$ mice[20] were crossed with PKG Cre mice (PGK-Cre transgene is maternally expressed and serves as a tool for early and uniform activation of the Cre site-specific recombinase[21]) to generate the *Yap*$^{+/-}$ mice, that are viable and fertile. *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ mice were obtained as previously described[22] by mating *Yap*$^{flox/flox}$ mice[20] with heterozygous Rax-CreER$^{T2}$ knockin mice[23]. The Cre activity was induced through a single intraperitoneal injection of 4-hydroxytamoxifen (4-OHT; 1 mg/kg) at P10. Genotyping was done by PCR using genomic DNA prepared from mouse tail snips. Primers are provided in Supplementary Table S1.

### Tissue sectioning, immunohistochemistry, and EdU labelling

Eyes of sacrificed animals were rapidly enucleated and dissected in Hanks' Balanced Salt solution (Gibco) to obtain posterior segment eye-cups, which were then fixed in 4% paraformaldehyde, 1X PBS, for 1 h at 4 °C. Eye-cups were then dehydrated, embedded in paraffin and sectioned (7 µm) with a Microm HM 340E microtome (Thermo Scientific). Hematoxylin–eosin (H&E) staining was performed according to the manufacturer's instructions (BBC Biochemical). Stained sections were mounted with Eukitt (Polylabo). Standard immunohistochemistry techniques on paraffin sections were applied with the following specificities: antigen unmasking treatment was done in boiling heat-mediated antigen retrieval buffer (10 mM sodium citrate, pH 6.0) for 20 min. Primary antibody was diluted in ready-to-use diluent (Dako). Primary and secondary antibodies are listed in Supplementary Table S2. Sections were counterstained with 1 µg/ml DAPI (Thermo Fisher Scientific) and mounted with Fluor Save$^{TM}$ reagent (Millipore). For retinal flat mounts, small dorsal incisions were made before fixation to mark the orientation of the retina within the eyecup. For EdU incorporation experiments, mice were given a single 50–100 µl intraperitoneal injection of 10 µM of EdU (Invitrogen) at the indicated stage. EdU incorporation was detected on flat-mounted retinal mouse explants using the Click-iT EdU Imaging Kit (Invitrogen) according to manufacturer's recommendations. For double labelling, EdU labelling was done first, followed by immunostaining.

### Imaging

Fluorescence images were acquired using a LSM710 confocal microscope (Zeiss). Whole retina images were acquired using an AXIOZoom.V16 (Zeiss) using the mosaic mode. Image mosaics of flat-mounted retinas were acquired and combined by the stitching processing method using ZEN Tiles module (Zeiss). Brightfield images of H&E staining were acquired using an AxioImager.M2 microscope. Image processing was performed using Zen 2.1 (Zeiss), Fiji (National Institutes of Health[24]), and Photoshop CS4 software (Adobe) software. The same magnification, laser intensity, gain, and offset settings were used across animals for any given marker.

### Western blotting

Western blot was performed on protein extracts from single retinas, at least on three individuals per condition, unless otherwise specified in the figure legends. Retinas from enucleated eyes were dissected in Hanks' Balanced Salt solution (Gibco) by removing the anterior segment, vitreous body, sclera and RPE and were frozen at −80 °C. Retinas were lysed in P300 buffer (20 mM $Na_2HPO_4$; 250 mM NaCl; 30 mM NaPPi; 0.1% Nonidet P-40; 5 mM EDTA; 5 mM DTT) supplemented with protease inhibitor cocktail (Sigma–Aldrich). For RPE protein extracts, the anterior segment and the retina were removed from enucleated mouse eyes. The RPE was then separated from the choroid by incubating the posterior eyecup (sclera-choroid-RPE) with P300 buffer for 10 min. Protein concentration

was determined using a Lowry protein assay kit (DC Protein Assay; Bio-Rad). Equal amounts of proteins (20 µg/lane) of each sample were loaded, separated by 7.5% SDS-PAGE (Bio-Rad) and transferred onto nitrocellulose membranes. Western blots were then conducted using standard procedures. Primary and secondary antibodies are listed in Supplementary Table S2. An enhanced chemiluminescence kit (Bio-Rad) was used to detect the proteins. Each sample was probed once with anti-α-tubulin antibody for normalisation. Quantification was done using Fiji software (National Institutes of Health[24]).

### Electroretinographic analysis

Electroretinograms (ERGs) were recorded using a Micron IV focal ERG system (Phoenix Research Labs). Mice were dark-adapted overnight and prepared for recording in darkness under dim-red illumination. Mice were anesthetised with intraperitoneal injection of ketamine (90 mg/kg, Merial) and xylazine (8 mg/kg, Bayer), and were topically administered tropicamide (0.5%) and phenylephrine (2.5%) for pupillary dilation. Flash ERG recordings were obtained from one eye. ERG responses were recorded using increasing light intensities ranging from −1.7 to 2.2 log cd.s/$m^2$ under dark-adapted conditions, and from −0.5 to 2.8 log cd.s/$m^2$ under a background light that saturates rod function. The interval between flashes varied from 0.7 s at the lowest stimulus strengths to 15 s at the highest ones. Five to thirty responses were averaged depending on flash intensity. Analysis of a-wave and b-wave amplitudes was performed using LabScribeERG software (https://www.iworx.com/research/software/labscribe). The a-wave amplitude was measured from the baseline to the negative peak and the b-wave was measured from the baseline to the maximum positive peak.

### Fluorescein angiography

Mice were anesthetised as described above. Pupils were dilated using 0.5% tropicamide (Théa) and 5% chlorhydrate phenylephrine (Europhta) eye drops. The mouse was placed on the imaging platform of the Micron IV system (Phoenix Research Labs), and Ocry-gel was applied on both eye to keep the eye moist during the imaging procedure. Mice were injected in tail's vein with 100 µL of 5% fluorescein isothiocyanate dextran (Sigma), and rapid acquisition of fluorescent images ensued for ~5 min.

### RNA extraction and RT-qPCR

RT-qPCR experiment was performed on at least three mice per condition. Total RNA was extracted from a single retina using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA quantity was assessed using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific). Total RNA (500 ng) was reverse transcribed in the presence of oligo-(dT)20 using Superscript II reagents (Thermo Fisher Scientific). For each RT-qPCR, cDNA was used in the presence of EvaGreen (Bio-Rad), and the reactions were performed in triplicates on a CFX96 Real-Time PCR Detection System (Bio-Rad). Differential expression analysis was performed using the ΔΔCt method and normalised using the geometric mean expression of two housekeeping gene, *Rps26* and *Srp72*[25]. For each gene, the relative expression in each sample was calculated using the mean of the controls as the reference (1 a.u.). Primers used are listed in Supplementary Table S1.

### Quantification and statistical analysis

The numbers of labelled cells on retinal sections or retinal flat mounts were manually counted in a defined field (sizes are indicated in the legends). For all such counting, as well as qPCR and western blot analysis, at least three retinas were used per condition. The non-parametric one-tailed Mann–Whitney test was used for all statistical analysis implicating two unpaired groups. For the ERG experiment, where multiple statistical comparisons were needed, a two-way ANOVA followed by Bonferroni's multiple comparison test was used. All results are reported as mean ± SEM. All analyses were performed using GraphPad Prism 8.3 (GraphPad Software, La Jolla California USA) with statistical significance set at $p$ value ≤ 0.05.

## Results

### Compensatory *Taz* regulation in *Yap*$^{+/-}$ postnatal mice

In contrast with the developmental arrest observed in *Yap*$^{-/-}$ mice[18], *Yap* heterozygous mice are viable and fertile. We wondered whether *Yap* gene haploinsufficiency could occur in the adult, and therefore decided to examine, in detail, the molecular and phenotypic consequences of one *Yap* allele deletion. As expected, qPCR and western blot analysis confirmed an approximately two-fold decrease of *Yap* expression in postnatal and adult *Yap*$^{+/-}$ retinas compared to controls (Fig. 1a, b). By immuno-histochemical (IHC) analysis, we found that YAP expression was strongly diminished in both Müller glial and RPE cells, where YAP has previously been shown to be expressed[17] (Fig. 1c). Prior to analysing potential ocular phenotypes in *Yap*$^{+/-}$ mice, we wanted to confirm that the observed *Yap* expression reduction actually leads to the decrease of *Yap* target gene expression. Indeed, this may not be the case since compensatory regulation mechanisms have previously been reported between YAP and TAZ[7,26,27]. In accordance with this, we observed an increase in *Taz* transcript abundance in postnatal *Yap*$^{+/-}$ retinas compared to controls (Fig. 1d). Noticeably, the compensatory upregulation waned in adult stages (after P21). This trend was confirmed at the protein level

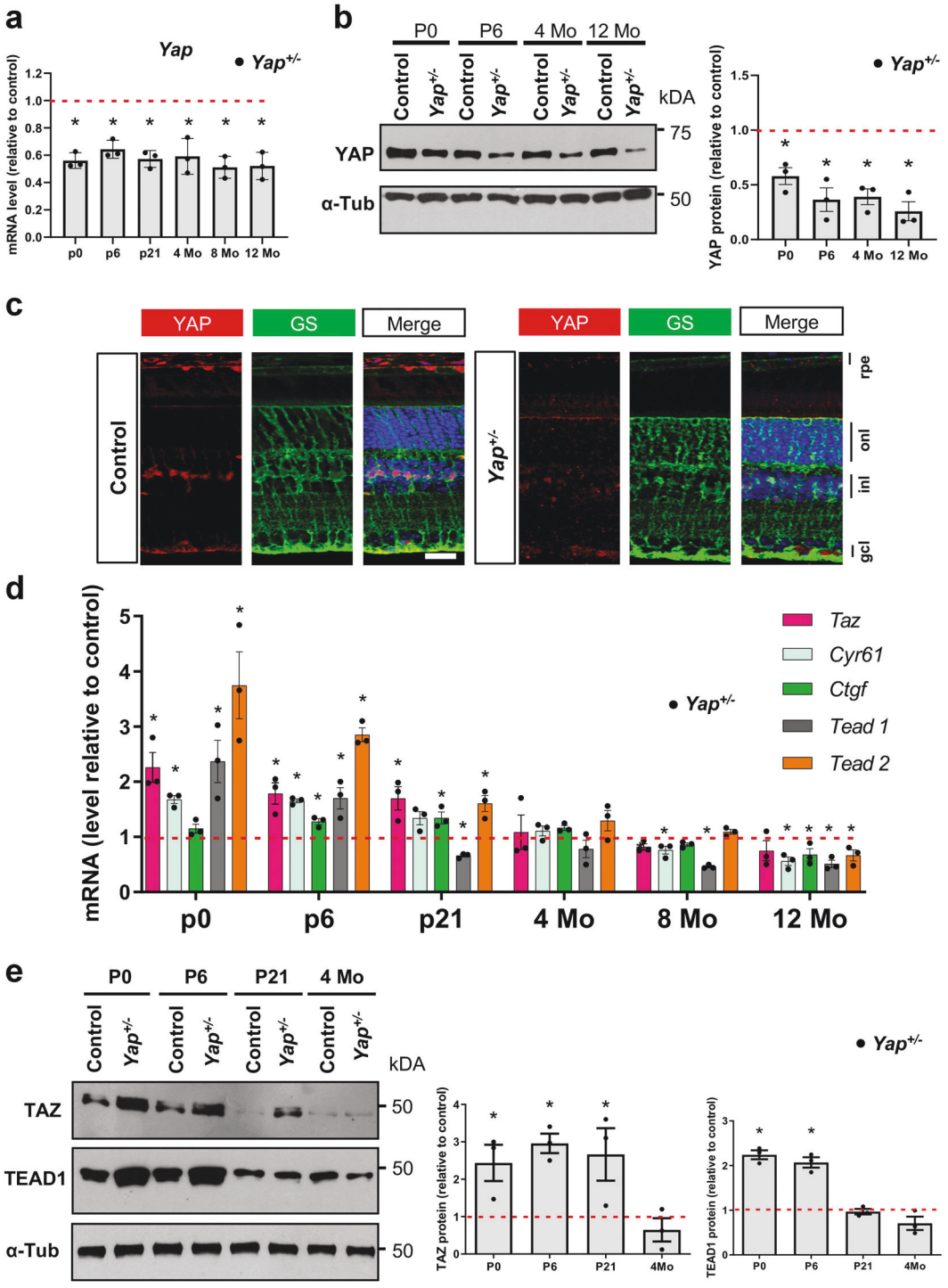


**Fig. 1 Compensatory regulation in *Yap*$^{+/-}$ mice. a** RT-qPCR analysis of *Yap* expression in *Yap*$^{+/-}$ mice retina, relative to wild-type controls (dashed line) ($n = 3$ biological replicates per condition). **b** Analysis of YAP protein expression level by western blot. The quantification is normalised to α-tubulin (α-Tub) signal and relative to wild-type controls at each stage (dashed line) ($n = 3$ biological replicates per condition). **c** 4-month-old mice retinal sections immunostained for YAP (red) and a marker of Müller cells, glutamine synthetase (GS, green). Nuclei are DAPI counterstained (blue). **d** RT-qPCR analysis of *Taz*, *Cyr61*, *Ctgf*, *Tead1*, and *Tead2* expression relative to wild-type controls (dashed line) ($n = 3$ biological replicates per condition). **e** Western blots analysis of TAZ and TEAD1. The quantification is normalised to α-tubulin (α-Tub) signal and relative to controls at each stage (dashed line) ($n = 3$ biological replicates per condition). rpe: retinal pigment epithelium, inl: inner nuclear layer, onl: outer nuclear layer, gcl: ganglion cell layer. All values are expressed as the mean ± SEM. Statistics: Mann–Whitney test, $*p \leq 0.05$. Scale bar: 20 µm.

(Fig. 1e). In order to assess the net outcome of the compensatory mechanism on YAP/TAZ activity, we analysed by RT-qPCR the expression level of *Ctgf* and *Cyr61*, which have been recognised as direct YAP/TAZ target genes[28]. Similar to *Taz* expression profile, we found that both genes were first upregulated at postnatal stages (prior P21), but downregulated from 8-month onwards in $Yap^{+/-}$ mice compared to controls (Fig. 1d). YAP/TAZ interacting transcription factors TEAD1 and TEAD2 followed the same expression profile (Fig. 1d, e). Of note, TEAD3 and TEAD4 are not expressed in the mouse retina[17]. Together, these data reveal the existence of a dynamic regulatory mechanism taking place in $Yap^{+/-}$ mice, that first leads to an increased expression of key YAP related and target genes at postnatal stages due to the compensatory overexpression of TAZ that diminished progressively with aging, with a concomitant decreased expression of these genes in adult mice. This unexpected temporally dynamic compensation prompted us to undertake a thorough phenotypic analysis of $Yap^{+/-}$ mice retina at both postnatal and adult stages, when compensatory mechanisms are occurring or not, respectively.

### Delayed cell-cycle exit of retinal progenitor cells in $Yap^{+/-}$ postnatal mice

*Yap* overexpression in new-born mice was shown to promote retinal cell proliferation, while *Yap* knockdown leads to the opposite phenotype[8]. Therefore, considering the abundance changes of both YAP (decrease) and TAZ (increase) in *Yap* heterozygous postnatal retinas, we wondered how it impacts the proliferative behaviour of retinal progenitor cells. Interestingly, 24 h after EdU intraperitoneal injection at P5 (when most progenitors have exited the cell cycle), four times more EdU-positive cells were detected in the central part of mutant retinas compared to control ones (Fig. 2a, b). This persistence of a population of proliferative cells in $Yap^{+/-}$ P6 retinas was supported by IHC analysis, showing more cells labelled with the cell proliferation markers PCNA and Cyclin D1, compared to controls (Fig. 2c). We found no more EdU-labelled cells in $Yap^{+/-}$ retinas, neither in the central nor in the peripheral region, when EdU injection was performed at P11 (Supplementary Fig. S1), suggesting that $Yap^{+/-}$ late progenitors eventually exited the cell cycle between P6 and P11. We next sought to determine, by EdU birthdating, the fate of these late progenitor cells. Proliferative progenitors in P6 $Yap^{+/-}$ retinas gave rise to both late born neurons (CHX10-positive bipolar cells and Recoverin-positive photoreceptors) and glial cells (SOX9-positive Müller cells) in the central retina (Supplementary Fig. S2A–D). To be able to compare this distribution with a control one, and since no EdU-positive cells were present in the central retina of P6 control individuals, we analysed the fate of EdU cells localised in the periphery of the retina. The proportion of double EdU/CHX10-, EdU/Recoverin- and EdU/SOX9-positive cells among EdU cells was similar in $Yap^{+/-}$ and in control retinas (Supplementary Fig. S2E). Finally, a series of IHC staining on P21 animals did not reveal any significant differences between wild-type and $Yap^{+/-}$ retina regarding markers of rods, cones, bipolar, ganglion, amacrine, horizontal, and Müller cells (data not shown). Together, these data suggest that a subset of $Yap^{+/-}$ retinal progenitor cells have a delayed cell-cycle exit, without exhibiting any apparent bias in their cell fate determination.

### EGFR pathway potentiation in $Yap^{+/-}$ postnatal mice

YAP has been recognised as an integrator of several key signalling pathways. In particular, crosstalk between EGFR and Hippo pathways have been reported[22,29–31]. Knowing that EGFR signalling regulates proliferation of retinal progenitor cells at postnatal stages[32], we hypothesised that the excess of late retinal proliferative progenitor cells in $Yap^{+/-}$ retinas could result from a deregulated EGFR pathway. RT-qPCR analysis at P0 and P6 showed a significant upregulation of *Egfr*, *Erbb2*, *Erbb3*, and *Erbb4* (encoding receptors of the EGFR family: EGFR, Her2, Her3, and Her4, respectively), as well as of *Hbegf* and *Neuregulin 1* (encoding EGFR ligands: HB-EGF and NRG1, respectively), in $Yap^{+/-}$ mice compared to controls (Fig. 3a). From P21, their expression diminished progressively in $Yap^{+/-}$ mice to reach control levels or levels lower than the controls (Fig. 3a). It is established that EGFR signalling acts through the activation of the MAPK, PI3K/AKT, or STAT3 pathways and previous reports have shown that these pathways are activated during proliferation of retinal progenitors cells[33–35]. We thus investigated whether their activity was affected in $Yap^{+/-}$ mice. P-AKT/AKT and P-STAT3/STAT3 ratios were increased in $Yap^{+/-}$ mice compared to controls at P6, while no difference was observed for P-ERK/ERK ratio (Fig. 3b). Together, these results revealed that EGFR pathway activity is potentiated at early postnatal stages in $Yap^{+/-}$ retina, and that this upregulation no longer occurs in adult stages. We therefore propose that the TAZ-dependent compensatory regulation that we observed in $Yap^{+/-}$ mice during postnatal stages may underlie this EGFR pathway activation.

### $Yap^{+/-}$ mice display retinal dysplasia

We next sought to determine whether $Yap^{+/-}$ mice exhibit any ocular defect at adult stages when the TAZ-dependent compensatory regulation is no longer effective. Considering the delay in cell-cycle exit described above at postnatal stages, and because YAP/TAZ are implicated in the control of organ growth[36,37], we examined the size of $Yap^{+/-}$ eyes. No major difference was found when compared to control eyes (Supplementary Fig. S3A). Cataract

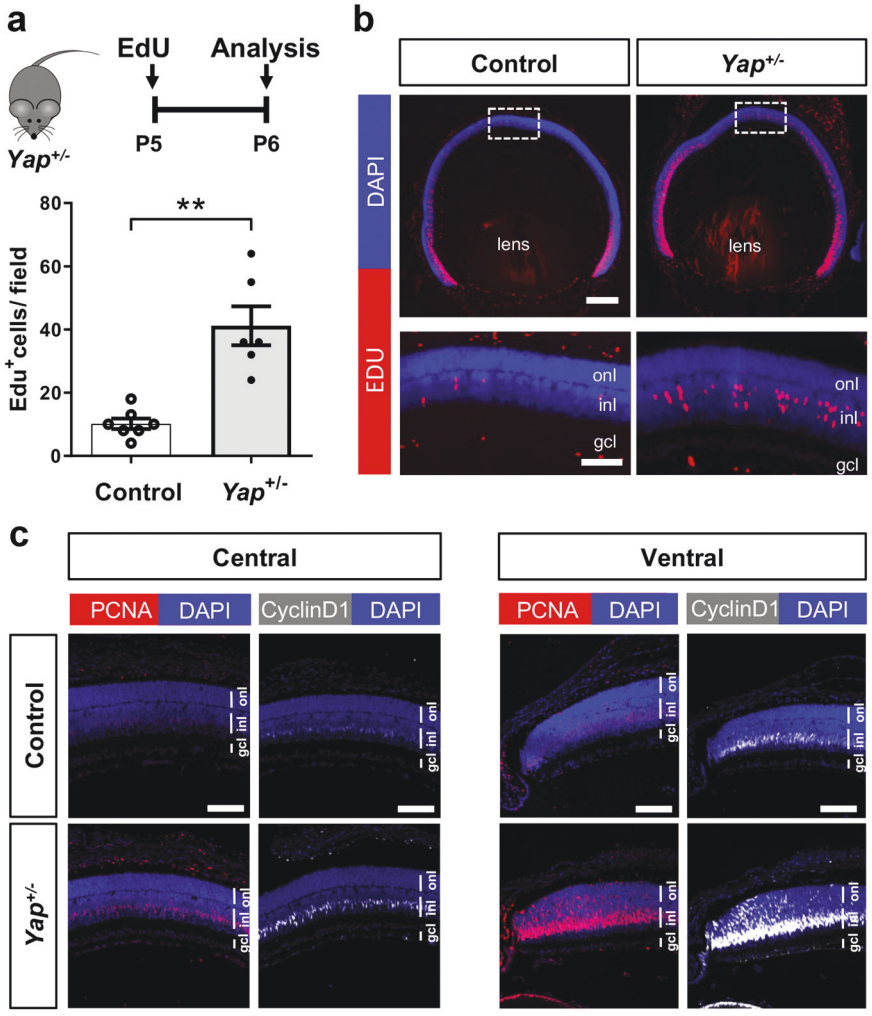


**Fig. 2 Prolonged proliferation of retinal progenitors at postnatal stages in *Yap*$^{+/-}$ mice. a** Timeline diagram of the experimental procedure used in **b**. Wild-type (Control) or *Yap*$^{+/-}$ mice were injected with EdU at P5 and analyzed 24 h later. **b** P6 retinal sections labelled for EdU (red) and stained with DAPI (blue). The delineated areas are enlarged in the bottom panels. Scatter plot with bars represents the number of EdU$^{+}$ cells per field (250 × 250 µm). Means ± SEM from seven control retinas and six *Yap*$^{+/-}$ retinas are shown. **c** P6 retinal sections immunostained for PCNA (red) or Cyclin D1 (grey). Both central and peripheral ventral regions are shown. Nuclei are DAPI counterstained (blue). inl: inner nuclear layer, onl: outer nuclear layer, gcl: ganglion cell layer. Statistics: Mann–Whitney test, $^{**}p \leq 0.01$. Scale bar: 200 µm (**b**) and 50 µm (**c** and enlarged panels in **b**).

was occasionally observed but only in about 8% of *Yap*$^{+/-}$ retinas (not shown). Heterozygous *Yap* loss-of-function in humans can result in coloboma[15,16]. We, however, did not detect any defects in optic fissure closure in *Yap*$^{+/-}$ mouse retina (data not shown). Yet, from P21 onwards, we observed that some mutant retinas displayed one or two dysplastic regions in either the central or dorsal retina (Supplementary Fig. S3B–D). The incidence of this non-fully penetrant phenotype was higher in older mice (Supplementary Fig. S3D). The severity of the dysplasia was highly variable and each cell layer of the retina was susceptible to be affected (data not shown). Dysplasia were, however, never detected in the RPE and analysis of the expression of RPE cell markers suggests that the deletion of a *Yap* allele does not disturb RPE integrity (Supplementary Fig. S4A–C). Outside the dysplasia, no difference in the thickness of either the outer or the inner nuclear layers was observed between *Yap*$^{+/-}$ and control retinas (Supplementary Fig. S4D). This data indicates that the general structure of the adult retina is not affected in *Yap*$^{+/-}$ mice, apart from the dysplastic region.

### *Yap*$^{+/-}$ adult mice display progressive cone photoreceptor degeneration

To assess the impact of one *Yap* allele deletion on adult retinal function, we performed electroretinogram (ERG) recordings in *Yap*$^{+/-}$ mice. Scotopic a- and b-waves were similar between controls and *Yap*$^{+/-}$ mice at all stages

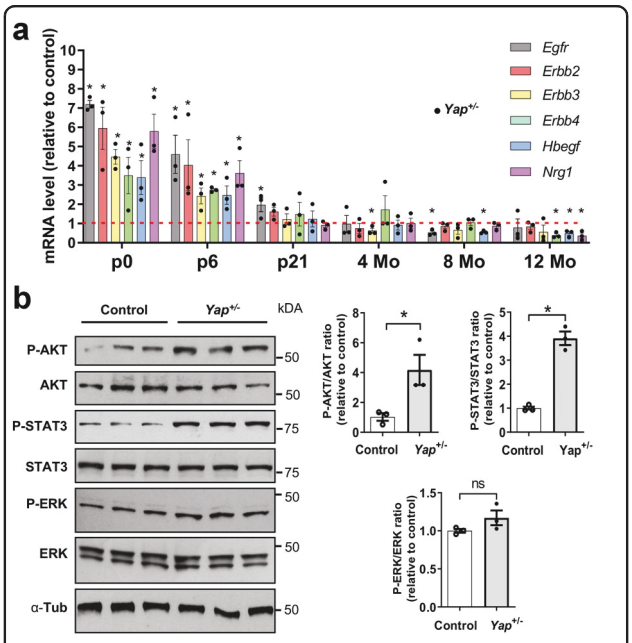


**Fig. 3 EGFR pathway activation in the retina of *Yap*$^{+/-}$ postnatal mice. a** RT-qPCR analysis of various EGFR pathway signaling gene expression (*Egfr, Erbb2, Erbb3, Erbb4, Hbegf*, and *Nrg1*), relative to wild-type controls (dashed lines) ($n = 3$ biological replicates per condition). **b** Analysis of protein expression levels of EGFR signaling pathway components at P6 by western blot. Quantifications of p-AKT/AKT, p-STAT3/STAT3, and p-ERK/ERK ratios are relative to controls ($n = 3$ biological replicates per condition). All values are expressed as the mean ± SEM. Statistics: Mann–Whitney test, $*p \leq 0.05$, ns nonsignificant.

examined (Fig. 4a and Supplementary Fig. S5). This suggests that rod photoreceptors in *Yap*$^{+/-}$ mice function normally (reflected by the a-wave), and that visual signal is properly transmitted through the inner retina (reflected by the b-wave). Strikingly however, photopic b-wave amplitude presented a significant depression in 12-month-old *Yap*$^{+/-}$ mice compared to controls at high intensity stimuli (Fig. 4a). Such specific reduction of the cone-mediated ERG response could signify either defects in the synaptic transmission between cone photoreceptors and bipolar cells and/or the presence of a cone dystrophy. In 12-month-old *Yap*$^{+/-}$ retina, IHC analysis showed a significant decrease in the number of Ribeye-positive puncta, which labels the presynaptic ribbons in photoreceptor terminals (Supplementary Fig. S6). Ribbons with proper horseshoe shape were present close to dendritic process of the rod-bipolar cell postsynaptic terminals, labelled with anti-Protein Kinase C alpha (PKC-α), suggesting a correct synaptic connection between rod photoreceptor and rod-bipolar cells in *Yap*$^{+/-}$ retina. In contrast, some ribbons did not exhibit the typical horseshoe shape suggesting compromised synapse integrity (Supplementary Fig. S6). We next assessed whether these defects were associated with cone photoreceptor defects in *Yap*$^{+/-}$ retina. The number of cones, as inferred by peanut agglutinin (PNA) labelling, was significantly decreased in *Yap*$^{+/-}$ ventral retinas compared to controls, affecting both S-opsin and M-opsin labelled cones (Fig. 4b, c). S- and M-opsin labelling were indeed both reduced in the ventral retina. S-opsin labelling was also severely decreased in the mid-dorsal (the most dorsal part was not analysed considering the occurrence of dysplasia) and the central retina. In addition to this quantitative phenotype, we also found that the remaining staining was abnormal, being more punctuated compared to the fusiform fluorescence in controls. This phenotype is reminiscent of degenerative cones[38]. We confirmed on flat-mounted retinas the punctuated versus the fusiform labelling, as well as the reduced number of PNA, S-opsin, and M-opsin labelled cones in the ventral region of 12-month-old *Yap*$^{+/-}$ mutant mice (Supplementary Fig. S7A). Consistent with a cone degenerative phenotype, we found that Cone Arrestin labelling was also severely decreased in the ventral retina (Supplementary Fig. S7B). The cone phenotype was only detected in old mice, as no difference in PNA, S- or M-opsin expression was observed between control and 1- or 4-month-old *Yap*$^{+/-}$ mice (Supplementary Fig. S8), consistent with the ERG data. Regarding rods, Rhodopsin was localised as expected within rod outer segments and we did not detect any major difference in its distribution between 12-month-old mutant and control retinas (Supplementary Fig. S9A). This result also supports the ERG data showing no rod dysfunction. Altogether, these results demonstrate that heterozygous mutation of *Yap* leads to specific cone dystrophy in aged mice.

## Deregulation of genes important for Müller cells homeostasis in *Yap*$^{+/-}$ mice

Since YAP is expressed in adult Müller cells[17] and Müller cells maintain retinal homeostasis[39], we wondered whether cone degeneration in *Yap*$^{+/-}$ mice could result from altered Müller cell function. First, we showed that Müller cells were properly located within the inner nuclear layer and that their number was not changed in mutant mice (Supplementary Fig. S9B). However, RT-qPCR, western blot and IHC analyses revealed that GFAP (glial fibrillary acidic protein) expression, the most sensitive indicator of retinal stress in Müller cells, was dramatically increased in 12-month-old *Yap*$^{+/-}$ retina compared to controls (Fig. 5a–c). Therefore, we next explored the impact of *Yap* heterozygous mutation on the expression of Müller cell-specific homeostatic regulatory proteins, aquaporin-4 (AQP4) and the potassium channel Kir4.1. Although no significant difference was observed at 8 months, we found that Kir4.1 and AQP4 protein levels were severely reduced in *Yap*$^{+/-}$ mice compared to controls at 12 months of age (Fig. 5d, e). Since Müller cells

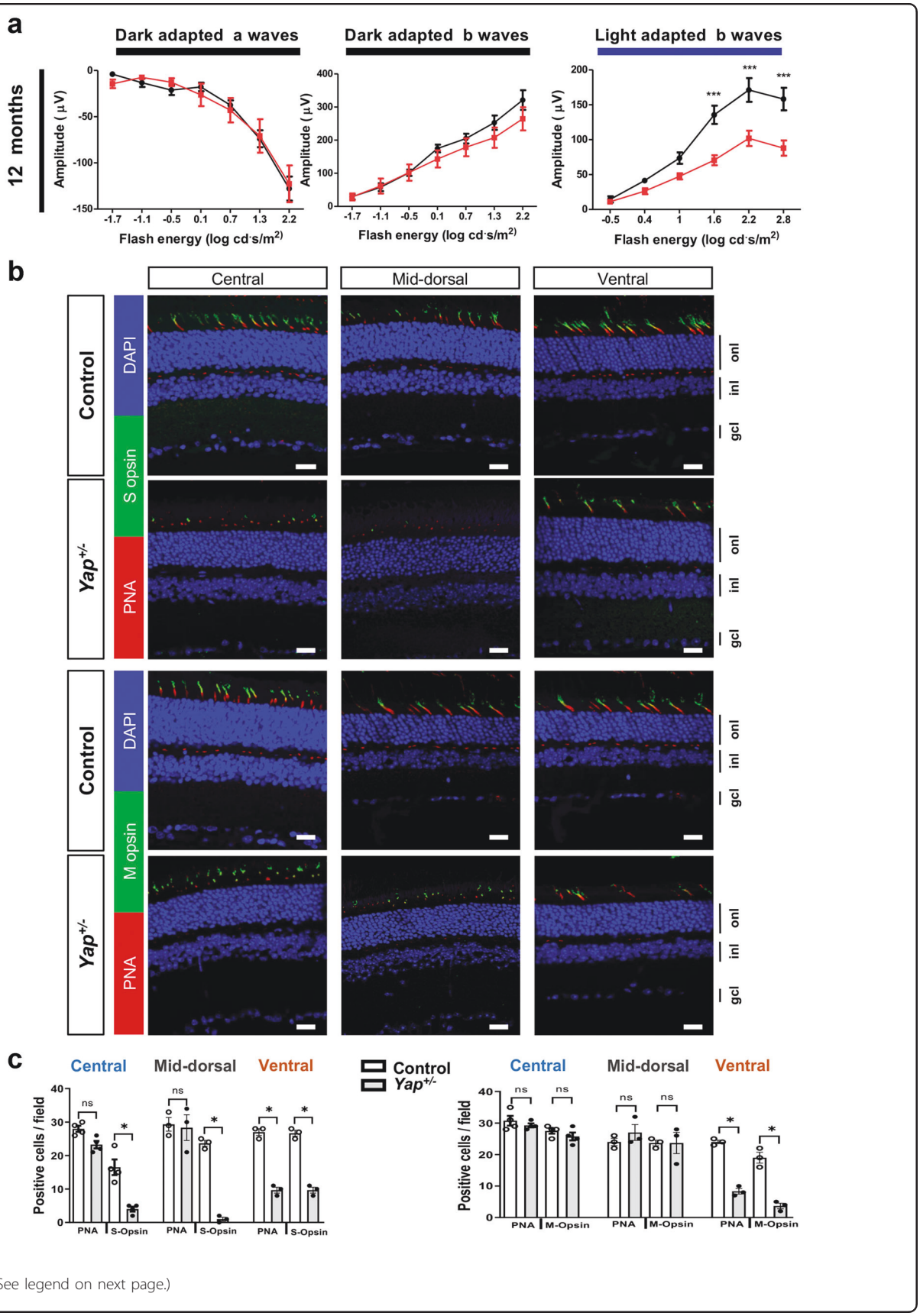


**Fig. 4** (See legend on next page.)

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**Fig. 4 Altered cone-driven vision and decreased expression of cone photoreceptor markers in *Yap*$^{+/-}$ adult mice. a** Quantitative evaluation of the scotopic and photopic a- and b-waves maximum amplitude data from 12-month-old wild-type (black) or *Yap*$^{+/-}$ (red) mice. Mean ± SEM intensity response curves are averaged from nine controls and seven *Yap*$^{+/-}$ mice. Statistics: two-way ANOVA test, ***$p \leq 0.001$. **b** Retinal sections from 12-month-old wild-type (Control) and *Yap*$^{+/-}$ mice, immunostained for cone makers (PNA, S-Opsin, and M-Opsin). Central, mid-dorsal, and ventral regions of retinal sections are shown. Nuclei are DAPI counterstained (blue). **c** Scatter plots with bars represent the number of labelled cells per field (300 × 300 µm). Values are expressed as the mean ± SEM from 3 to 4 retinas per condition. onl: outer nuclear layer, inl: inner nuclear layer, gcl: ganglion cell layer. Statistics: Mann–Whitney test, *$p \leq 0.05$, **$p \leq 0.01$, ns nonsignificant. Scale bars: 20 µm.

have been shown to actively participate at cone opsins recycling[40], we wondered whether the expression of cone-specific visual cycle factors could be altered in *Yap*$^{+/-}$ Müller cells. We thus analysed the expression of cellular retinaldehyde–binding protein (CRALBP), given its key role in cone visual cycle and its expression in Müller cells[41,42]. We observed a reduction of CRALPB labelling in both Müller glia and RPE cells of 12-month-old *Yap*$^{+/-}$ retinas, compared to controls (Fig. 6).

Perturbed ionic channels, such as the potassium channel Kir4.1, in Müller cells is a leading cause of intraretinal blood vessel defects[43]. We thus examined the three retinal vascular plexi in 12-month-old *Yap*$^{+/-}$ mice (Supplementary Fig. S10A). We observed a reduction of the intermediate vascular plexus staining, without apparent blood leakage in 12-month-old *Yap*$^{+/-}$ retinal flat-mounts, compared to controls (Supplementary Fig. S10B and Supplementary Fig. S11). Although recent studies have shown that YAP/TAZ are involved in vascular retinal development[44], no obvious differences could be observed after labelling the three capillary plexi between 8-month-old *Yap*$^{+/-}$ mice and controls (Supplementary Fig. S10B). Such observation ruled out significant developmental defects of vascular networks. Collectively, these data revealed some retinal vasculature defects and altered Müller cell homeostatic function in *Yap*$^{+/-}$ aged mice.

### Conditional *Yap* deletion in Müller glia leads to Müller cell homeostasis dysfunction and altered pattern of cone opsin expression

We next sought to investigate whether Müller glia could be the main cell type in which *Yap* haploinsufficiency mediates the observed cone degenerative phenotype. We took advantage of a transgenic line that we previously generated, *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$, and that allows Cre-mediated conditional gene ablation specifically in Müller cells[22]. It is thereafter named *Yap* CKO while "control" refers to *Yap*$^{flox/flox}$ mice. *Yap* deletion was induced in fully differentiated Müller cells, through 4-OHT intraperitoneal injection at P10 (Fig. 7a). Phenotypic analyses were then conducted on 12-month-old mice. Similar to what we observed in *Yap*$^{+/-}$ aged mice, the expression of Müller cell-specific homeostatic regulatory proteins AQP4 and Kir4.1 were downregulated and GFAP upregulation indicated reactive gliosis (Fig. 7b). Moreover, the expression pattern of S- and M-cone opsins was severely affected compared to control retinas, S-opsin signal being drastically decreased dorsally while M-opsin signal being almost absent ventrally in *Yap* CKO mice (Fig. 7c, d). This phenotype is similar to that observed in *Yap*$^{+/-}$ aged retinas. However, the number of cones, as inferred by PNA labelling, was not significantly changed in *Yap* CKO mice compared to controls (Fig. 7c, d). Together, although the phenotype appears less severe in *Yap* CKO retinas than in *Yap*$^{+/-}$ mice, the sole deletion of *Yap* in Müller cell triggers defects in cone opsin expression. These data thus led us to propose a model in which YAP function in Müller cells would be necessary for ensuring the maintenance of Müller cell homeostasis and preserving cone integrity (Fig. 8).

## Discussion

While YAP function during eye development has been well documented, our data demonstrate the usefulness of *Yap* heterozygous mice to better understand YAP function in the adult retina. Interestingly, our study revealed that the compensatory upregulation of YAP partner, TAZ, could not be sustained throughout life and declined during aging. This dynamic regulatory mechanism results in *Yap* haploinsufficiency only in the aged retina. We propose a model where the maintenance of proliferative progenitor cells in *Yap*$^{+/-}$ postnatal retina would result from the compensatory TAZ activity, leading to a gain of function-like phenotype, mediated by EGFR pathway potentiation (Fig. 8). During aging, when this compensatory mechanism is no longer operational in *Yap*$^{+/-}$ mice, we discovered the occurrence of an age-related cone dystrophy, associated with reduced cone-driven vision. Our data suggest that this phenotype may be the consequence of impaired Müller cell homeostatic function. This study therefore reveals that deregulation of *Yap* gene dosage could cause retinal degenerative diseases.

It has previously been reported that changes in YAP abundance result in compensatory regulation of TAZ to maintain Hippo signalling homeostasis[45]. We interestingly found that one *Yap* allele deletion was indeed accompanied by an increase in *Taz* expression in the retina, as well as an increase in *Tead* gene expression. However,

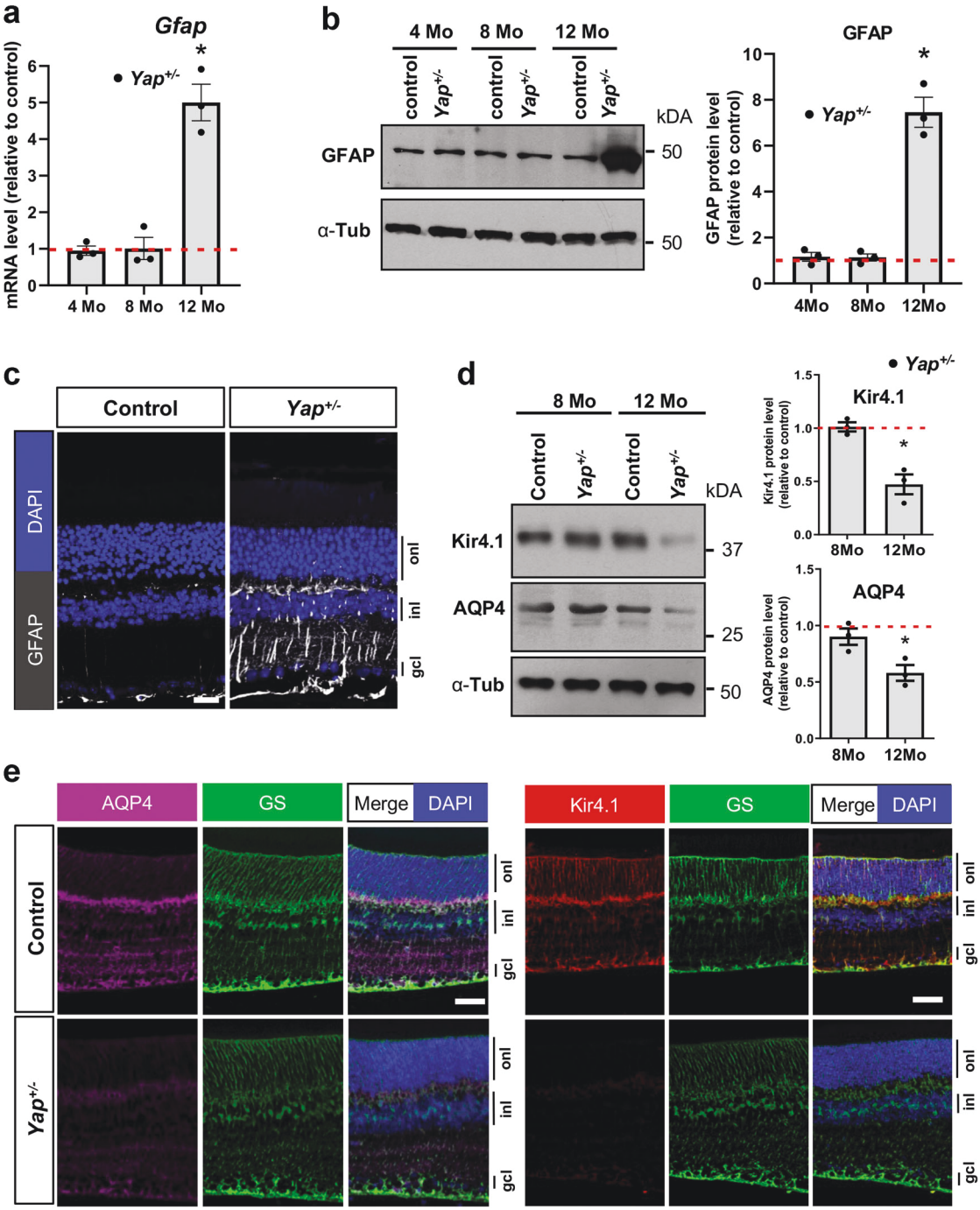


**Fig. 5 Altered Müller glia homeostasis in *Yap*$^{+/-}$ adult mice. a** RT-qPCR analysis of *Gfap* expression, relative to wild-type controls (dashed line). ($n = 3$ biological replicates per condition). **b** Analysis of GFAP protein expression level by western blot. Results are normalised to α-tubulin (α-Tub) signal and expressed relative to controls at each stage (dashed line) ($n = 3$ biological replicates per condition). **c** 12-month-old retinal sections immunostained for GFAP (white). Nuclei are DAPI counterstained. **d** Analysis of Kir4.1 and AQP4 protein expression levels by western blot, in 8 or 12-month-old mice. Results are normalised to α-tubulin (α-Tub) signal and expressed relative to controls at each stage (dashed line) ($n = 3$ biological replicates per condition). **e** 12-month-old retinal sections immunostained for Kir4.1 (red), AQP4 (purple), or the Müller cell marker glutamine synthetase (GS, green). Nuclei are DAPI counterstained (blue). onl: outer nuclear layer, inl: inner nuclear layer, gcl: ganglion cell layer. All values are expressed as the mean ± SEM. Statistics: Mann–Whitney test, $*p \leq 0.05$. Scale bar: 50 µm.

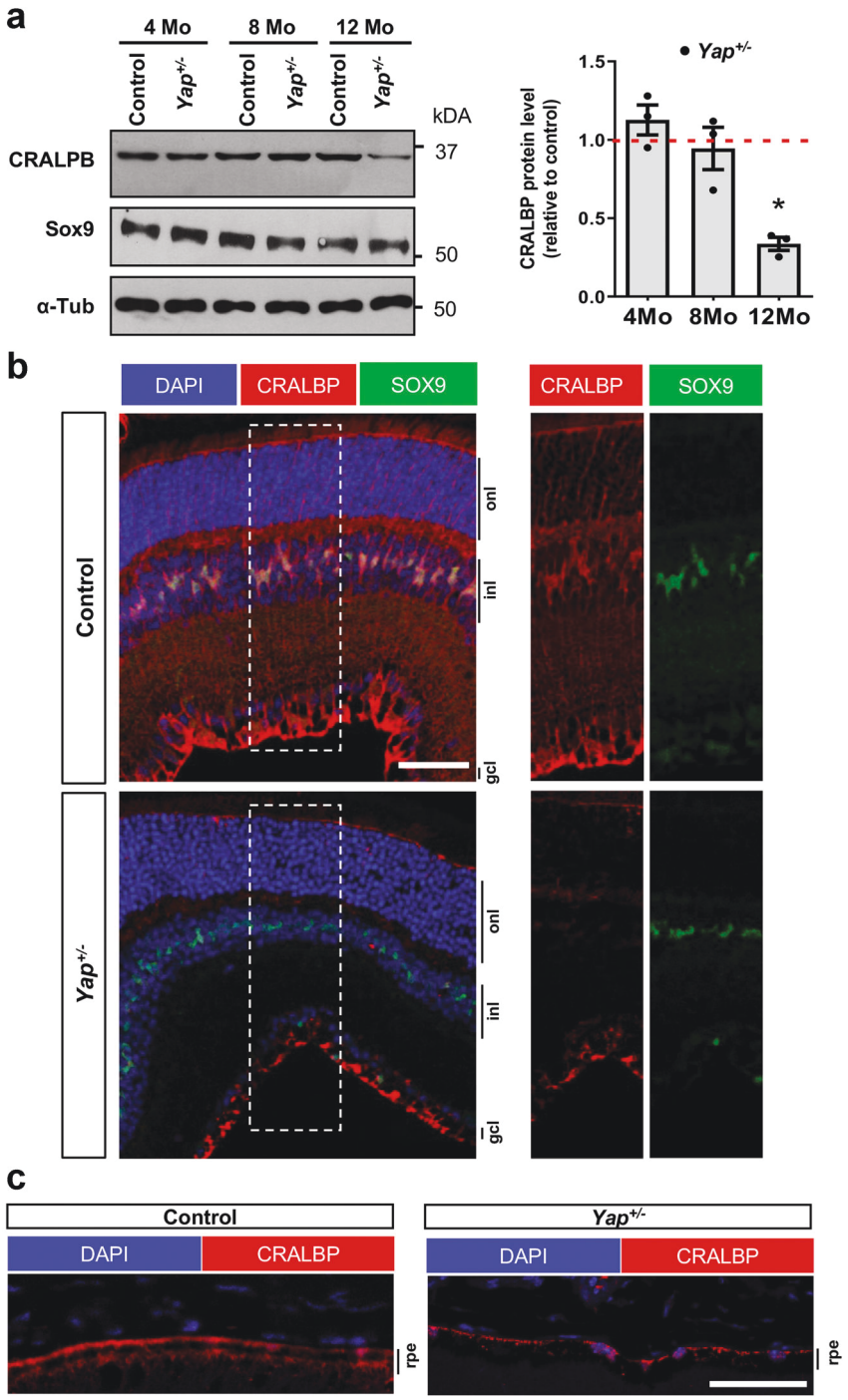


**Fig. 6 Decreased CRALBP expression in *Yap*$^{+/-}$ adult mice retina. a** Analysis of CRALBP protein expression level by western blot. SOX9 serves as a marker of Müller cells. Results are normalised to α-tubulin (α-Tub) signal and expressed relative to controls at each stage (dashed line) ($n = 3$ biological replicates per condition). Values are expressed as the mean ± SEM. **b** 12-month-old retinal sections immunostained for CRALBP (red) and SOX9 (green). Nuclei are DAPI counterstained (blue). The delineated areas (dashed lines) are enlarged in the right panels. **c** 12-month-old RPE sections immunostained for CRALBP (red). Nuclei are DAPI counterstained (blue). inl: inner nuclear layer, onl: outer nuclear layer, gcl: ganglion cell layer, rpe: retinal pigment epithelium. Statistics: Mann–Whitney test, *$p \leq 0.05$. Scale bar: 50 µm (**b**) and 100 µm (**c**).

such rises appeared to go beyond *Yap* compensation as we observed an increase in YAP/TAZ target genes. Interestingly, this dosage compensation system was not maintained in the retina of adult mice after 4 months of age. All these quantitative data on the abundance of YAP/TAZ and their target genes highlight *Yap*$^{+/-}$ mice as an

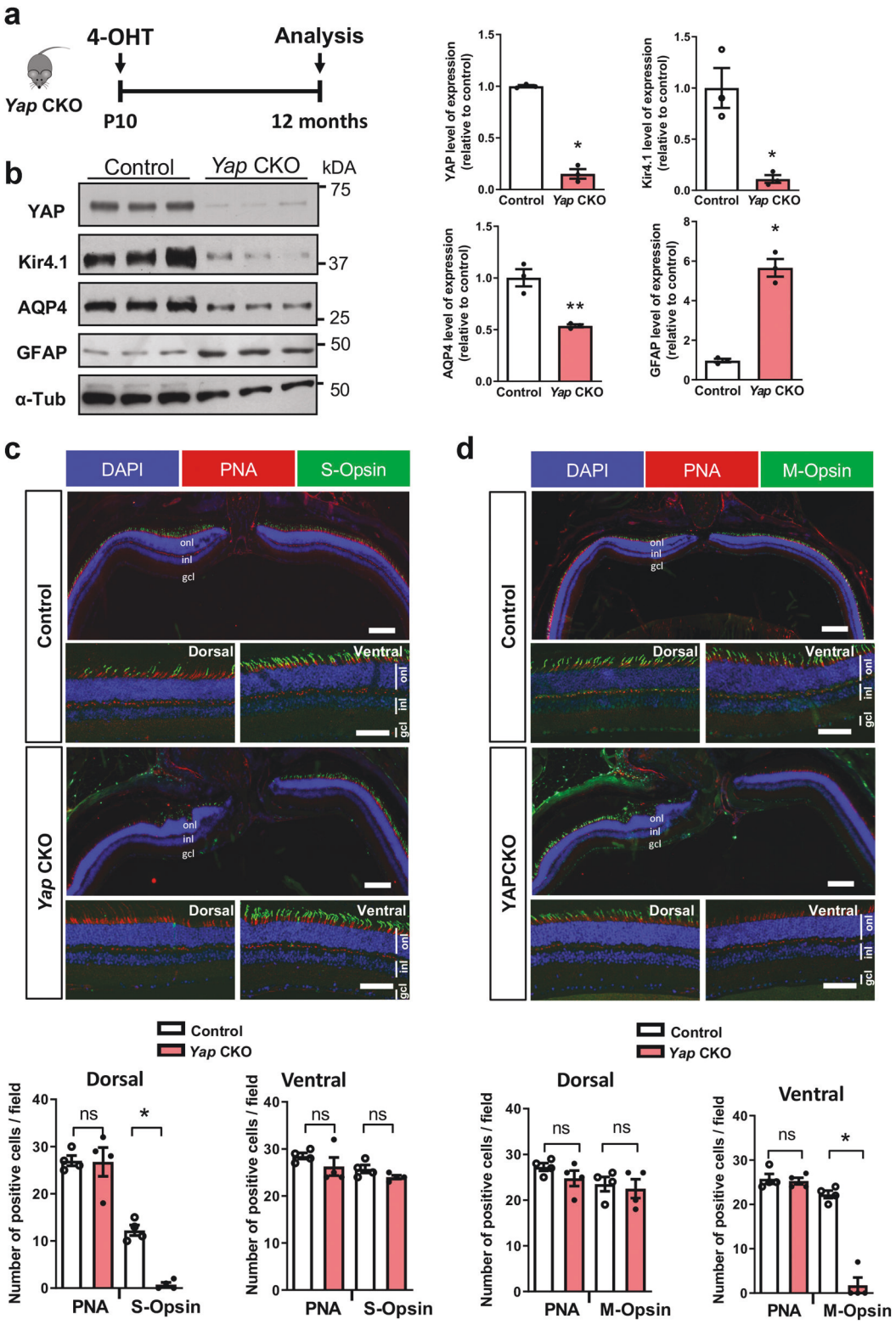


**Fig. 7** (See legend on next page.)

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**Fig. 7 Müller cell dysfunction and decreased expression of S/M-opsin in *Yap* CKO. a** Timeline diagram of the experimental procedure used in **b**. *Yap*$^{flox/flox}$ mice (control) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ mice (*Yap CKO*) received a single dose of 4-OHT at P10 and their retinas were analyzed at 12 months. **b** Analysis of the protein expression level of AQP4, Kir4.1, and GFAP. Results are normalised to α-tubulin (α-Tub) signal and expressed relative to control ($n = 3$ biological replicates per condition). **c, d** Retinal sections from 12-month-old control and *Yap CKO* mice immunostained for S-opsin (green) and PNA (red) (**c**) or M-opsin (green) and PNA (red) (**d**). Dorsal, central and ventral sections of retinal sections are shown. Nuclei are DAPI counterstained (blue). Scatter plots with bars represent the mean number of labelled cells per field (250 × 250 µm) from four retinas per condition. All values are expressed as the mean ± SEM. onl: outer nuclear layer, inl: inner nuclear layer, gcl: ganglion cell layer. Statistics: Mann–Whitney test, *$p \leq 0.05$, **$p \leq 0.01$, ns nonsignificant. Scale bars: 100 µm (central part) (enlarged panels) and 50 µm (dorsal and ventral part).

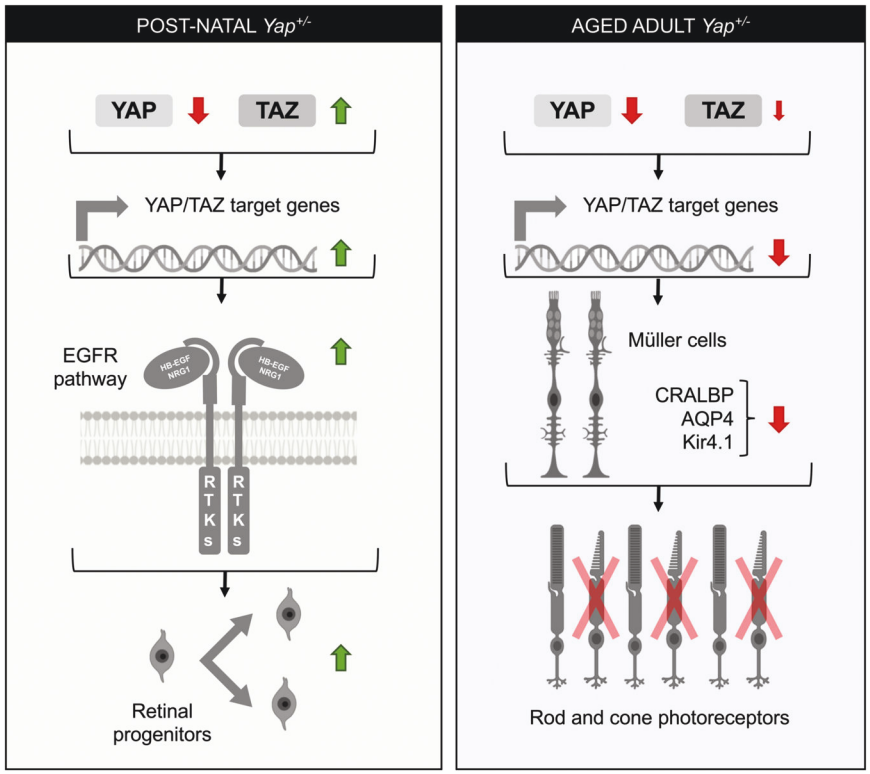


**Fig. 8 Proposed model explaining *Yap*$^{+/-}$ phenotypes at postnatal and adult stages.** Our results revealed the occurrence of a TAZ compensatory mechanism in postnatal *Yap*$^{+/-}$ retina, i.e., the decreased of *Yap* expression (red arrow) is accompanied by an increase of *Taz* expression (green arrow), that results in the upregulation of YAP/TAZ target gene expression. Our data suggest that this leads to prolonged proliferation of postnatal retinal progenitor cells, and that this phenotype may likely result from EGFR pathway potentiation. In the adult *Yap*$^{+/-}$ retina, the compensatory mechanism is no longer effective, and YAP/TAZ target genes expression is diminished. Under such conditions, one *Yap* allele deletion progressively leads to impaired Müller cell homoeostasis. We propose that this may, at least in part, contribute to impaired cone-specific visual cycle and therefore to the cone dystrophy observed in aged *Yap*$^{+/-}$ mice. RTKs Receptor tyrosine kinases. This figure was created with some schemas from ©BioRender—biorender.com.

excellent model to study the role of YAP in the adult retina, when TAZ no longer compensates for *Yap* deletion. Unlike in humans, where heterozygous loss-of-function mutations in *YAP* causes coloboma[15,16], *Yap*$^{+/-}$ mice do not exhibit such congenital malformation of the eye. A plausible hypothesis is that the compensatory mechanism during eye development is not regulated in humans as it is in mice. Interestingly, the phenotype in humans is not fully penetrant, which could indicate potential YAP/TAZ dosage variations between individuals.

YAP overexpression in neonatal mouse retinas was shown to promote cell proliferation and inhibit cell-cycle exit of late retinal progenitors[8]. We also found a delay in cell-cycle exit in *Yap*$^{+/-}$ postnatal retina. We propose that this is the consequence of the compensatory regulation that leads to TAZ level increase. Indeed, despite the genetic *Yap*$^{+/-}$ context, we observed an upregulation of YAP/TAZ target genes at postnatal stages. Since the observed delayed cell-cycle exit does not appear to cause an overall increase in cell number, it may result from

slower cell-cycle kinetics. We have previously shown in *Xenopus* retina that *Yap* knockdown results in perturbed cell-cycle kinetics of retinal stem cells in the ciliary margin[46]. Further analyses are, however, required to measure cell-cycle length in $Yap^{+/-}$ retinal progenitors. Furthermore, our data suggest that this delayed cell-cycle exit of retinal progenitor may be mediated by an increase in EGFR activity. Previous studies in other cell types have demonstrated that YAP and TAZ promote proliferation by potentiating the EGFR pathway[29–31]. This is also consistent with our recent finding suggesting that YAP is a key regulator of the EGFR pathway in adult reactive Müller cells in a degenerative context and that EGFR pathway is required for YAP mitogenic activity[22]. It remains to be investigated whether EGFR pathway components are directly regulated by YAP/TAZ in retinal cells. We raised the hypothesis that the prolonged maintenance of cell proliferation at postnatal stages could be linked to the appearance of dysplastic regions in $Yap^{+/-}$ retina. Indeed, the maintenance of progenitor cells in proliferation may subsequently lead to abnormal folding of retinal layers. Alternatively, considering that YAP regulates key effectors of matrix stiffening[47], we can also hypothesise that the stiffness of the retinal tissue is affected, leading to retinal misfolding. This could underlie the increased incidence of dysplasia observed during aging.

We showed previously that YAP is expressed only in Müller cells in the adult retina[17]. We thus raised the hypothesis that cone degeneration in $Yap^{+/-}$ mice could result from altered Müller cell function. Indeed, Müller cells play many essential roles in retinal maintenance, regulation of synaptic activity, ion, and water homeostasis, and, importantly, in mediating cone visual cycle[39]. Moreover, conditional Müller cell ablation was shown to cause photoreceptor degeneration[48,49]. Our hypothesis is supported by the finding that deleting *Yap* specifically in Müller cells in *Yap* CKO mice also leads to abnormal expression of cone opsins. Both mutants exhibit reactive gliosis and impaired expression of genes involved in Müller cell homeostatic function (AQP4 and Kir4.1) consistent with a model where impaired cone integrity would be caused by Müller cell dysfunction.

We also discovered in $Yap^{+/-}$ Müller glia an altered expression of CRALBP, which is involved in cone-specific visual cycle. Knockdown or mutations in CRALBP gene lead to decreased cone-driven ERG responses in zebrafish, M-cone loss in mice or can lead to severe cone photoreceptor-mediated retinal disease in patients[41,50,51]. Importantly, restoration of CRALBP expression specifically in Müller cells, but not RPE cells, can rescue the sensitivity of CRALBP-deficient cones in the mouse[41]. We can thus raise the hypothesis that the severe decrease of CRALBP levels in Müller cells of aged $Yap^{+/-}$ mice may contribute to the observed cone dysfunction and degeneration. We did not observe any significant decrease in CRALBP levels in *Yap* CKO retinas (data not shown), which may explain, at least in part, the less severe cone phenotype (PNA staining unaffected) compared to the one observed in $Yap^{+/-}$ mice. This could indicate only structural cone defects in *Yap* CKO retinas without bona fide degeneration. Besides, the decreased abundance of YAP in $Yap^{+/-}$ mice in other cell types than Müller cells may also explain the different severity of the phenotype. Although we did not detect any major defects in RPE cells in $Yap^{+/-}$ mice, we observed a decreased expression of CRALBP in RPE cells. We therefore cannot exclude the possibility that the RPE is not functioning properly, contributing to cone degeneration. Similarly, the defect in the intermediate vascular plexus observed in $Yap^{+/-}$ mice could also participate to the degenerative phenotype.

We here did not focus our analysis on extraocular tissues. However, ocular phenotypes in a $Yap^{+/-}$ mouse line were recently examined with an emphasis on the cornea and severe corneal pathology was reported[52]. In this mutant mouse, similar to our findings, retinal defects were detected only in aged mice. However, in contrast to our study, the phenotype was very severe, including retinal detachment, defect in the RPE and important loss of photoreceptors. It remains to be addressed whether genetic background differences could explain these phenotypic variabilities between the two $Yap^{+/-}$ mouse lines.

To conclude, we highlighted in this study a novel role for YAP in the maintenance of cone photoreceptors in adult mice, associated with a regulation of Müller cell homeostasis. Overall, our data showing cone degeneration in $Yap^{+/-}$ mice (*i*) warrant further investigation in patients with *YAP* heterozygous loss-of-function mutations, regarding their cone-driven vision during aging, and (*ii*) identify *Yap* as a novel candidate gene that could account for cone dystrophy in patients with unidentified mutations.

**Acknowledgements**
We are thankful to S. Meilhac, Jeff Wrana, and Seth Blackshaw for mouse lines. We are grateful to S. Lourdel for her help with the maintenance of mouse colonies. This research was supported by grants to M.P. from the FRM (Fondation pour la Recherche Médicale), Association Retina France, Fondation Valentin Haüy, DIM Région Île de France and UNADEV. DGG is a FRM fellow.

**Conflict of interest**
The authors declare that they have no conflict of interest.



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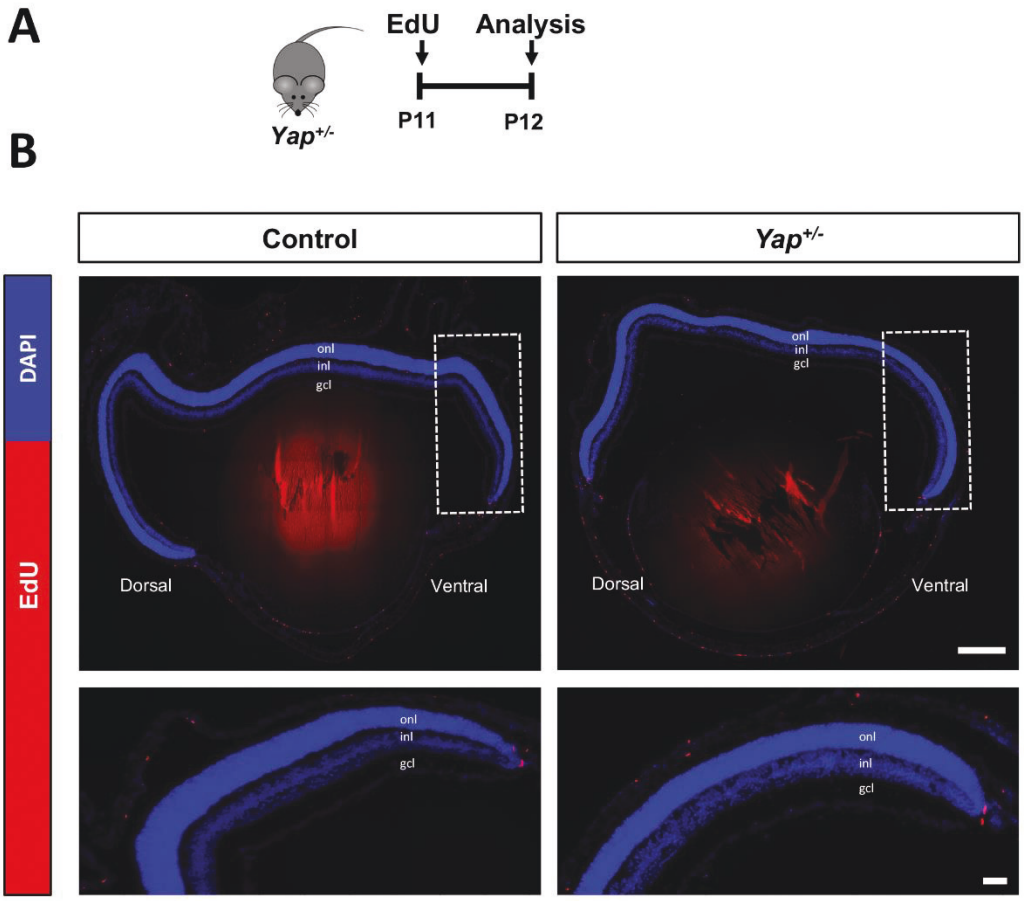
## Supplemental Information

# *Yap* haploinsufficiency leads to Müller cell disfunction and late-onset cone dystrophy

**Christel Masson, Diana García-García, Juliette Bitard, Élodie-Kim Grellier, Jérôme E. Roger and Muriel Perron**

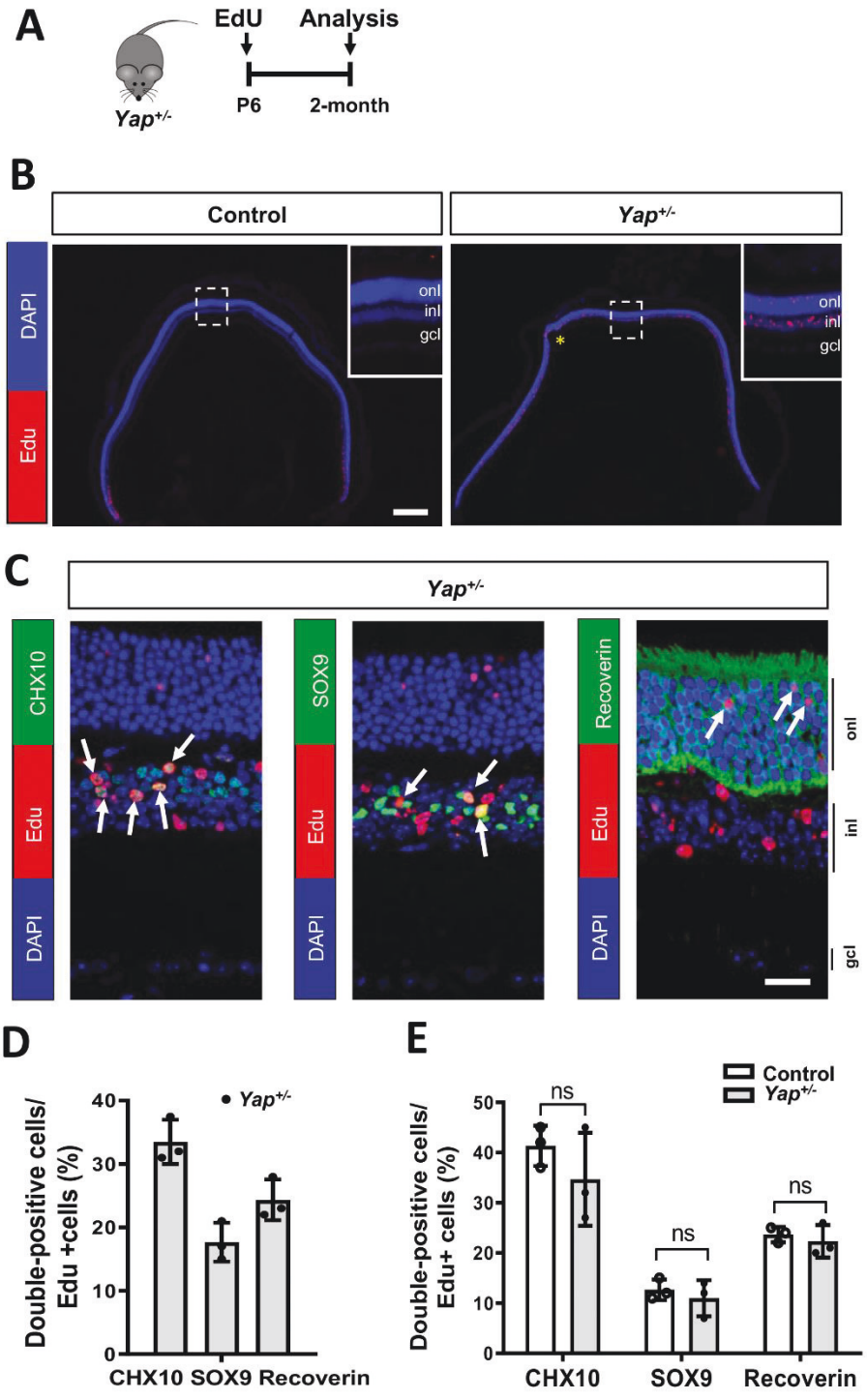
**Supplementary Figure S1. Absence of retinal progenitor proliferation from P11 *Yap*$^{+/-}$ mice.** (**A**) Timeline diagram of the experimental procedure used in B. Wild-type (Control) or *Yap*$^{+/-}$ mice were injected with EdU at P11 and the presence of EdU-positive cells was assessed 24 hours later (P12). (**B**) Retinal sections labelled for EdU (red) and DAPI counterstained (blue). Ventral areas are enlarged in the bottom panels. INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer. Scale bar: 200 µm and 50 µm (enlarged panels).

# Supplementary Figure S1



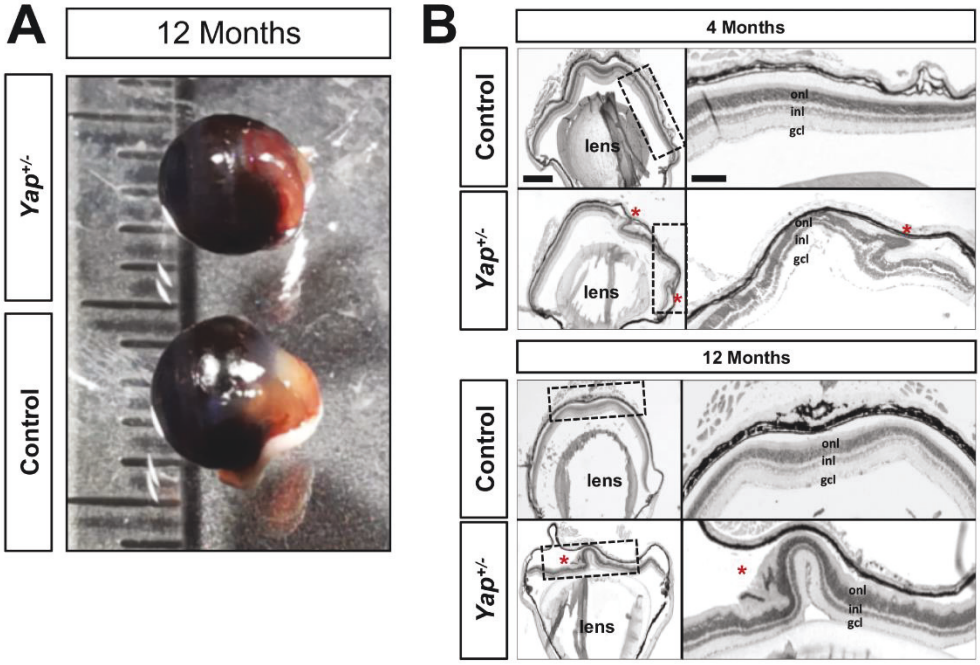
**Supplementary Figure S2. Correct cell fate of retinal progenitors with delayed cell cycle exit in *Yap*$^{+/-}$ mice** (**A**) Timeline diagram of the birthdating experimental procedure used in B. Wild-type (Control) or *Yap*$^{+/-}$ mice were injected with EdU at P6 and the fate of EdU-positive cells was analysed in 2-month-old mice. (**B**) Retinal sections labelled for EdU (red) and DAPI counterstained (blue). The delineated areas are enlarged in the insets. The asterisk marks the location of a dysplastic region. (**C**) Retinal sections labelled for EdU (red) and stained for the indicated markers (SOX9, CHX10, Recoverin, green) and DAPI counterstained (blue). Arrows point to double labelled cells. (**D, E**) Quantification of double labelled cells among EdU-positive cells per field (400 µm x 400 µm) in the central (D) or peripheral (E) retinal region. Mean values ± SEM from 3 retinas per condition are shown. INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer. Statistics: Mann-Whitney test, ns: non-significant. Scale bar: 200 µm (B), 50 µm (C).

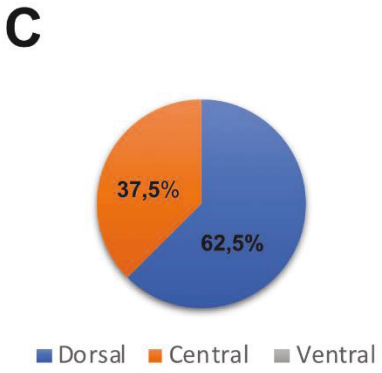
Supplementary Figure S2

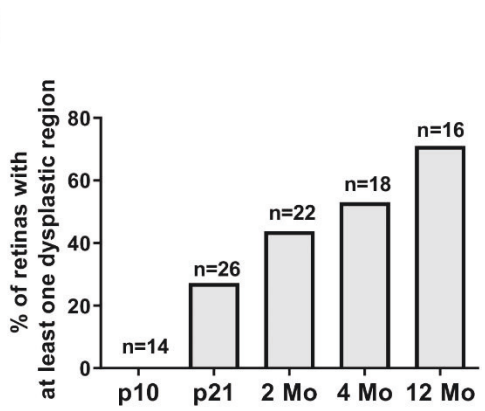


**Supplementary Figure S3. One allele deletion of *Yap* does not alter eye size but generate retinal dysplasia**. (**A**) Enucleated eyes from wild type (Control) and $Yap^{+/-}$ 12-month-old mice. (**B**) H&E staining of retinal sections in 4 and 12-month-old wild type (Control) and $Yap^{+/-}$ mice. Red asterisks indicate the position of dysplastic regions. Area delineated with dashed black lines are enlarged in panels on the right showing finger-like protrusions of $Yap^{+/-}$ retinal layers toward the RPE. (**C**) Number of retinas with a dorsal, central or ventral dysplasia in $Yap^{+/-}$ retinas expressed in percentage of the total number of retinas with a dysplasia (n=96 retinas from stage p21 to 12 month-old). (**D**) Incidence of retinal dysplasia in control and $Yap^{+/-}$ mice at different stages. INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer. Scale bar: 200 µm and 50 µm (enlarged panels).

# Supplementary Figure S3

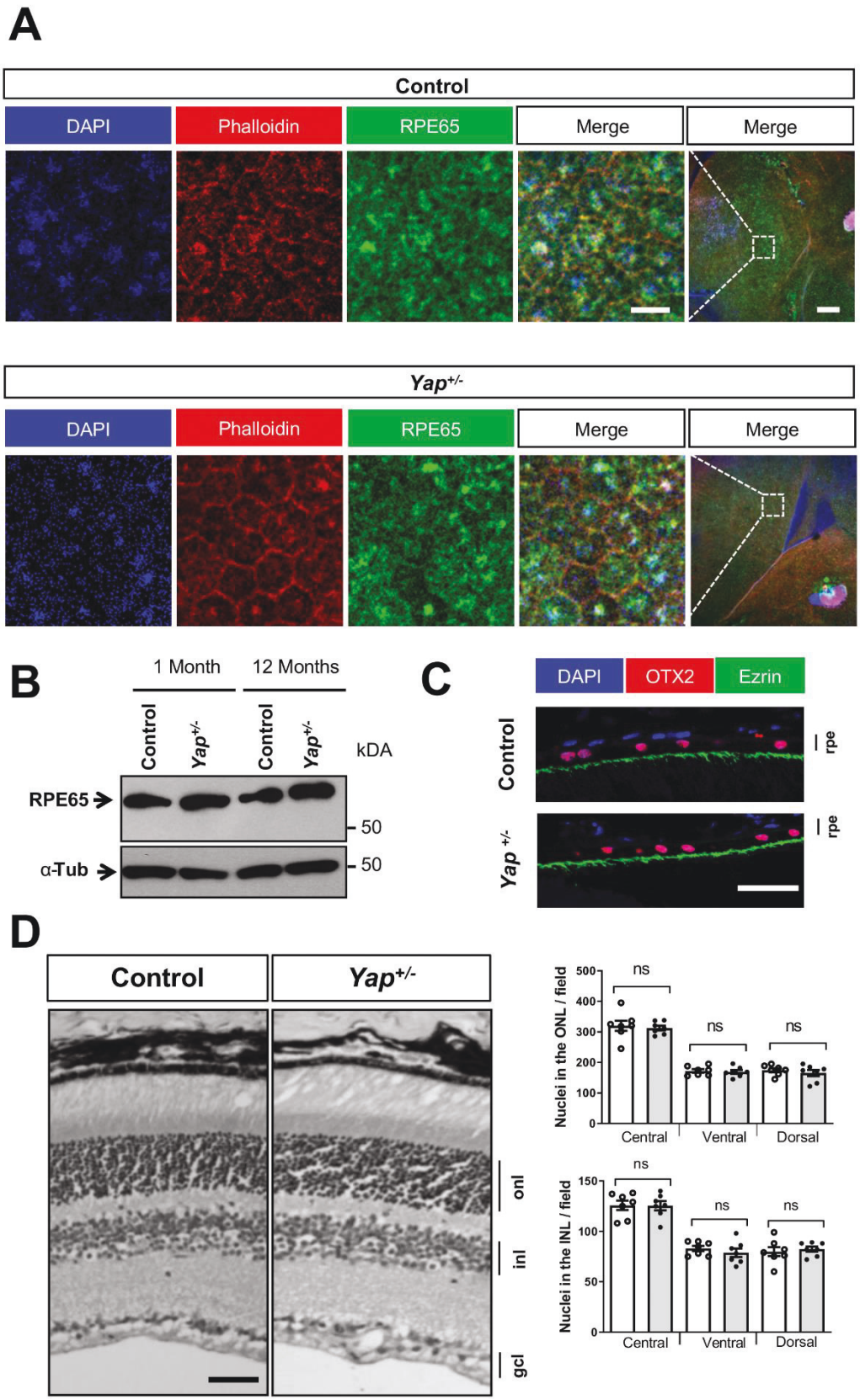


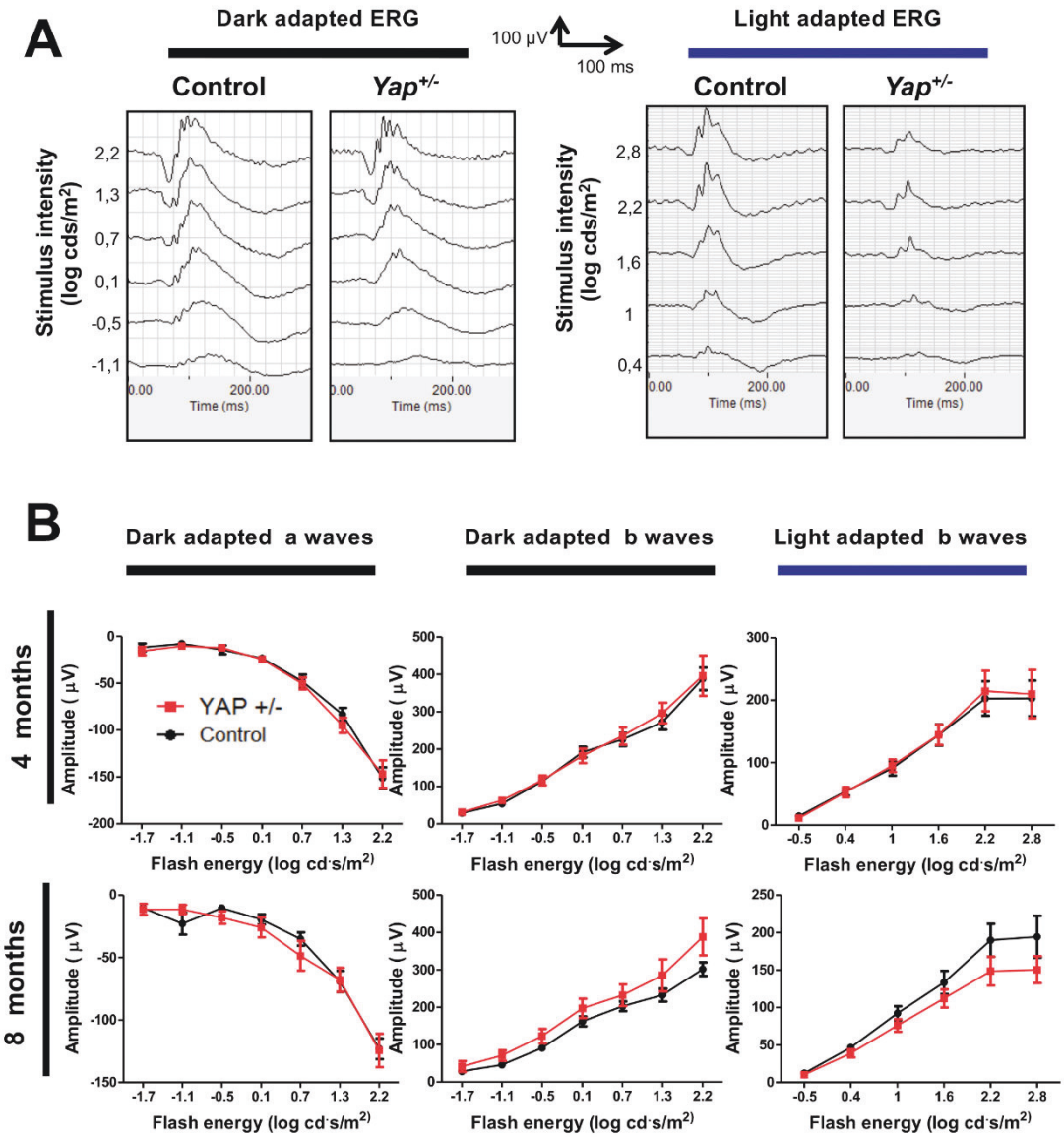
**Supplementary Figure S4. Normal appearance of the RPE and normal retinal thickness in *Yap*$^{+/-}$ mice**. (**A**) RPE flat mounts from 12-month-old wild type (control) and *Yap*$^{+/-}$ mice immunostained for Phalloidin (red) and RPE65 (green). Nuclei are DAPI counterstained (blue). (**B**) Analysis of RPE65 and α-tubulin (α-Tub) level of expression in 1 or 12-month-old mice. (**C**) RPE sections from 12-month-old mice immunostained for OTX2 (red) and Ezrin (green). Nuclei are DAPI counterstained (blue). RPE: retinal pigment epithelium. **(D)** H&E staining of retinal sections from 12-month-old wild type (Control) and *Yap*$^{+/-}$ mice. A region away from a dysplastic region is shown. Histogram represents the measurement of the outer and inner nuclear layer thickness. Mean values ± SEM from three independent retinas are shown. INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer. Mean ± SEM from 7 retinas per condition are shown. Statistics: Mann-Whitney test, ns: non-significant. Scale bar: 20 µm and 100 µm (enlarged panels) (A), 10 µm (C), 50 µm (D).

# Supplementary Figure S4



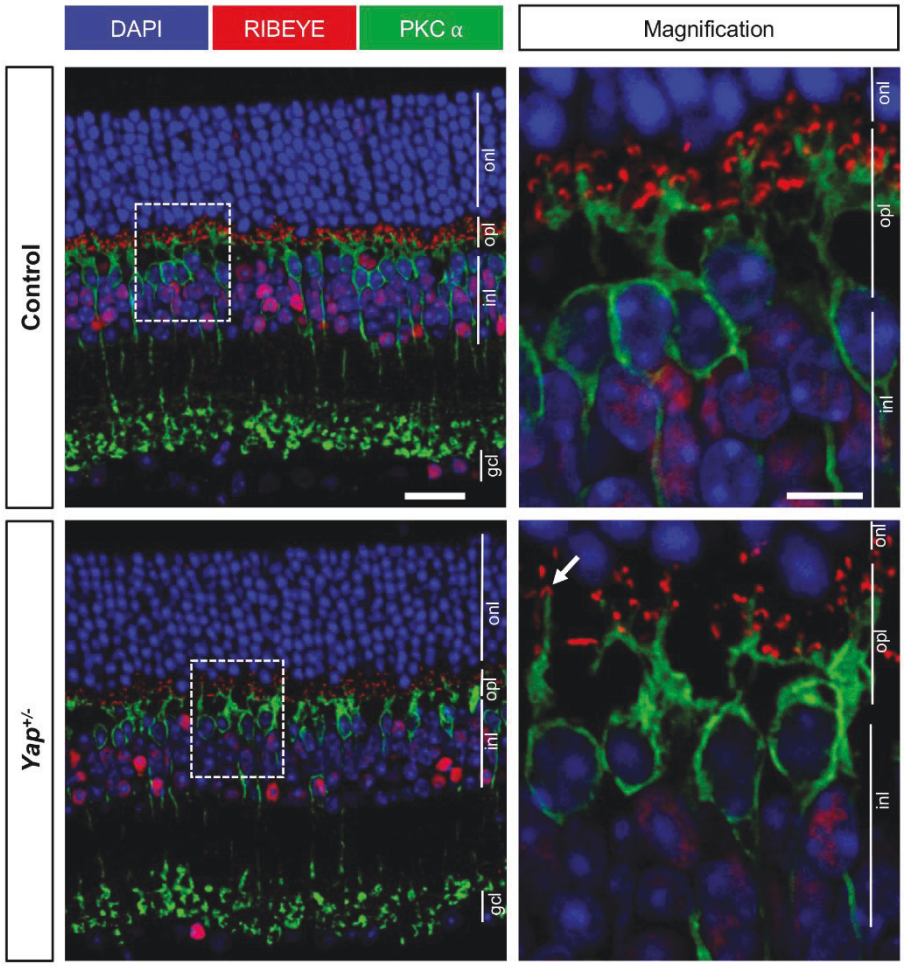
**Supplementary Figure S5. Altered cone-driven vision in *Yap*$^{+/-}$ adult mice.** (**A**) Representative ERG intensity series for scotopic (dark-adapted) and photopic (light-adapted) responses in 12-month-old wild-type (Control) and *Yap*$^{+/-}$ mice. (**B**) Quantitative evaluation of the scotopic and photopic a- and b-waves maximum amplitude data from 4- and 8-month-old wild-type (black) or *Yap*$^{+/-}$ (red) mice. Mean ± SEM intensity response curves are averaged from: 8 controls and 7 *Yap*$^{+/-}$ biological replicates of 4- month-old mice; 9 controls and 8 *Yap*$^{+/-}$ biological replicates of 8-month-old mice.

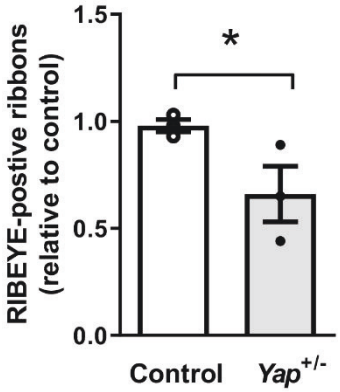
# Supplementary Figure S5



**Supplementary Figure S6**. **Altered architecture of the photoreceptor ribbon synapse in 12-month-old *Yap*$^{+/-}$ adult mice**. 12-month-old retinal sections immunostained for PKC-α (green) and RIBEYE (red). Delineated areas (dashed lines) are enlarged in the right panels. Nuclei are DAPI counterstained (blue). An arrow indicates a ribbon with a normal horseshoe shape, facing PKC-α labelled rod-bipolar cell post-synaptic terminals in *Yap*$^{+/-}$ retinas. The scatter plot with bars indicates the number of labelled RIBEYE positive ribbons per field (150 µm x 150 µm). Values are expressed as the mean ± SEM from 3 retinas per condition. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Statistics: Mann-Whitney test, ns: non-significant. Scale bar: 20 µm and 50 µm (enlarged panels).

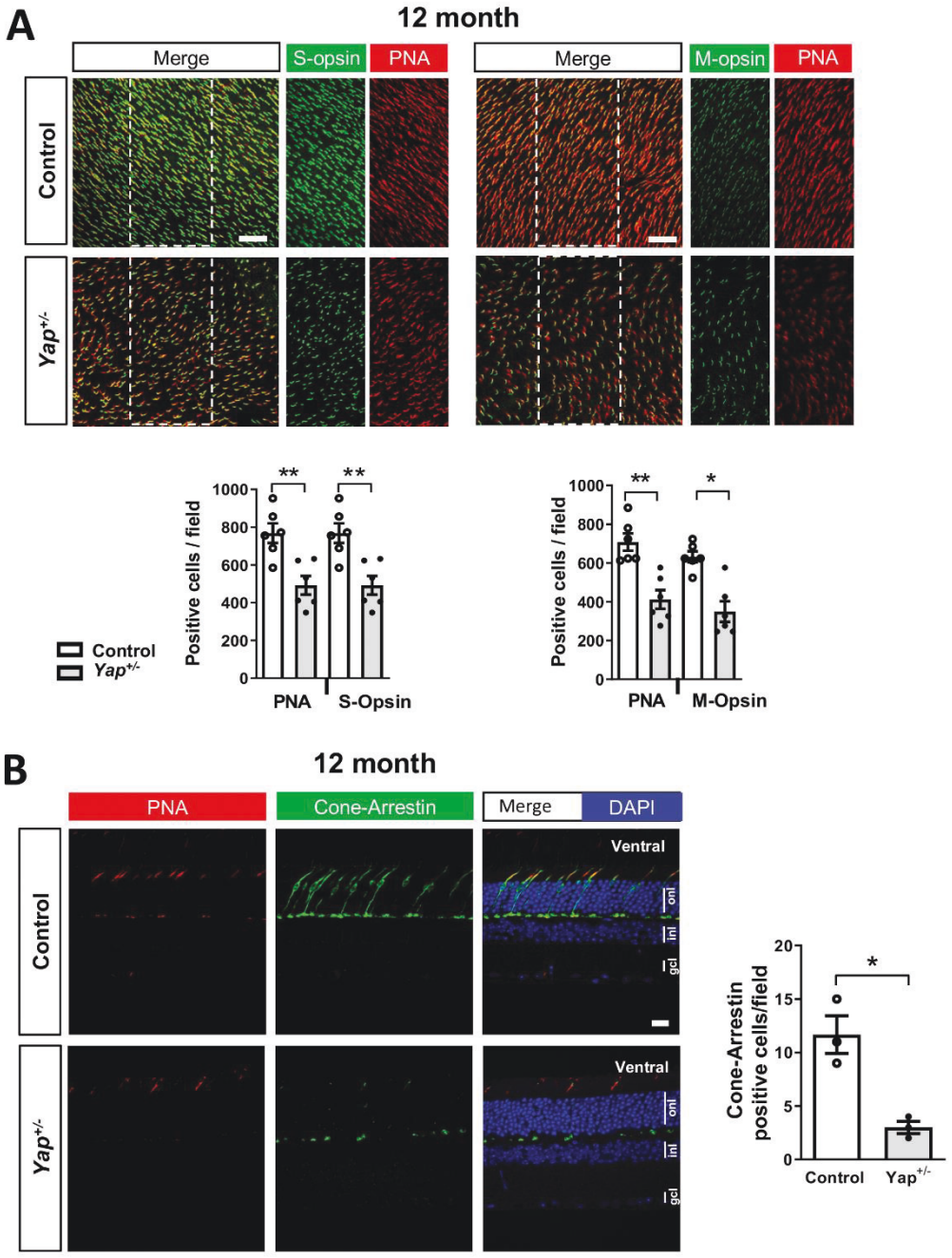
# Supplementary Figure S6



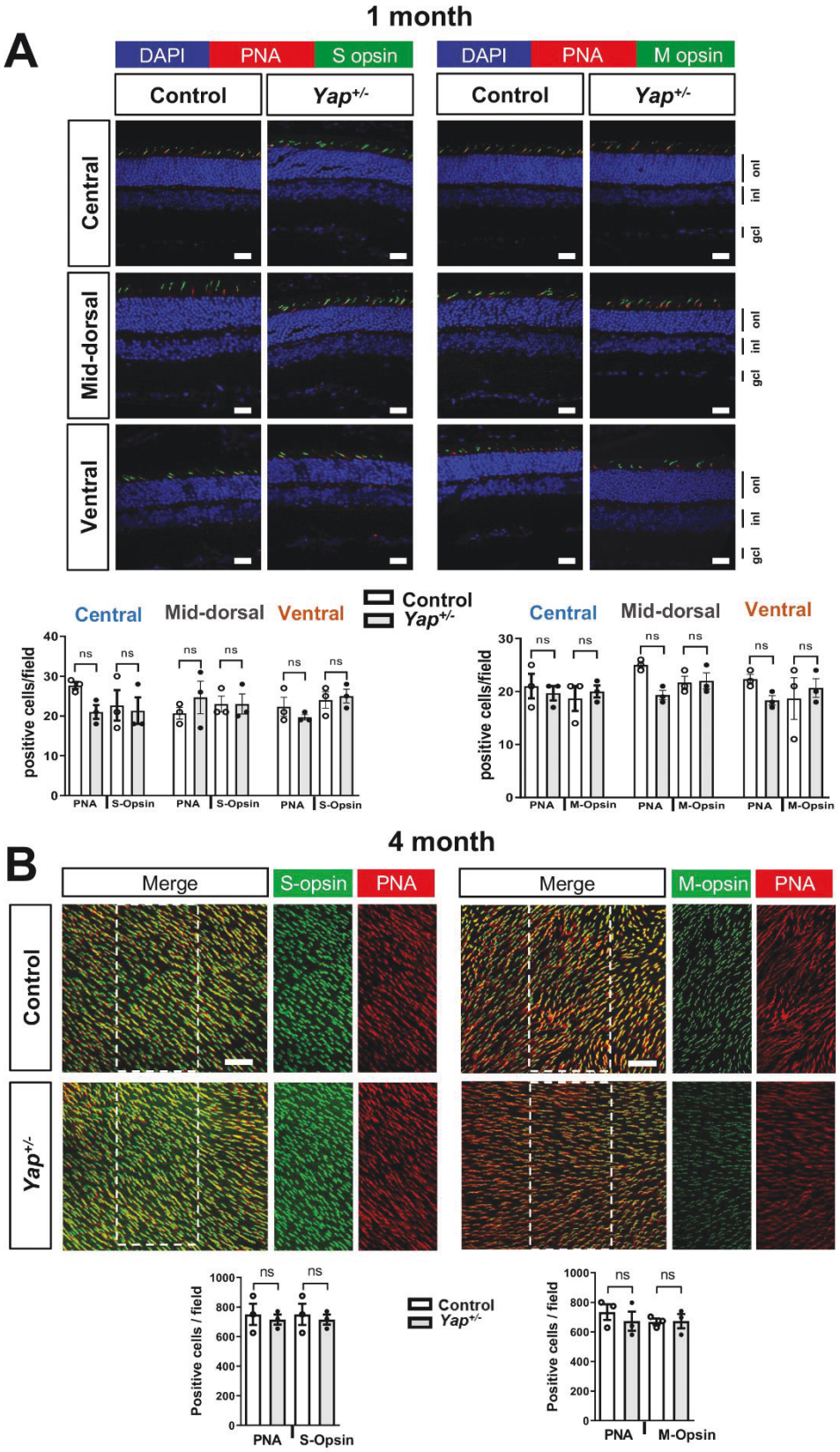
**Supplementary Figure S7. Decreased expression of cone markers in 12-month-old *Yap*$^{+/-}$ mice retinas. (A)** Retinal flat-mounts from 12-month-old wild type (control) and *Yap*$^{+/-}$ mice, immunostained for cone makers (PNA, S-Opsin and M-Opsin) in the ventral part of the retina. Histograms indicate the number of labelled cells per field (400 µm x 400 µm). Values are expressed as the mean ± SEM from 6 retinas per condition. **(B)** 12-month-old mouse retinal sections immunostained for PNA (red) and Cone Arrestin (green). Nuclei are DAPI counterstained (blue). Scatter plots with bars represent the number of Cone Arrestin labelled cells per field (150 µm x 150 µm). Values are expressed as the mean ± SEM from 3 retinas per condition. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar: 50 µm (A), 20 µm (B). Statistics: Mann-Whitney test, *$p \leq 0.05$, **$p \leq 0.01$.

Supplementary Figure S7



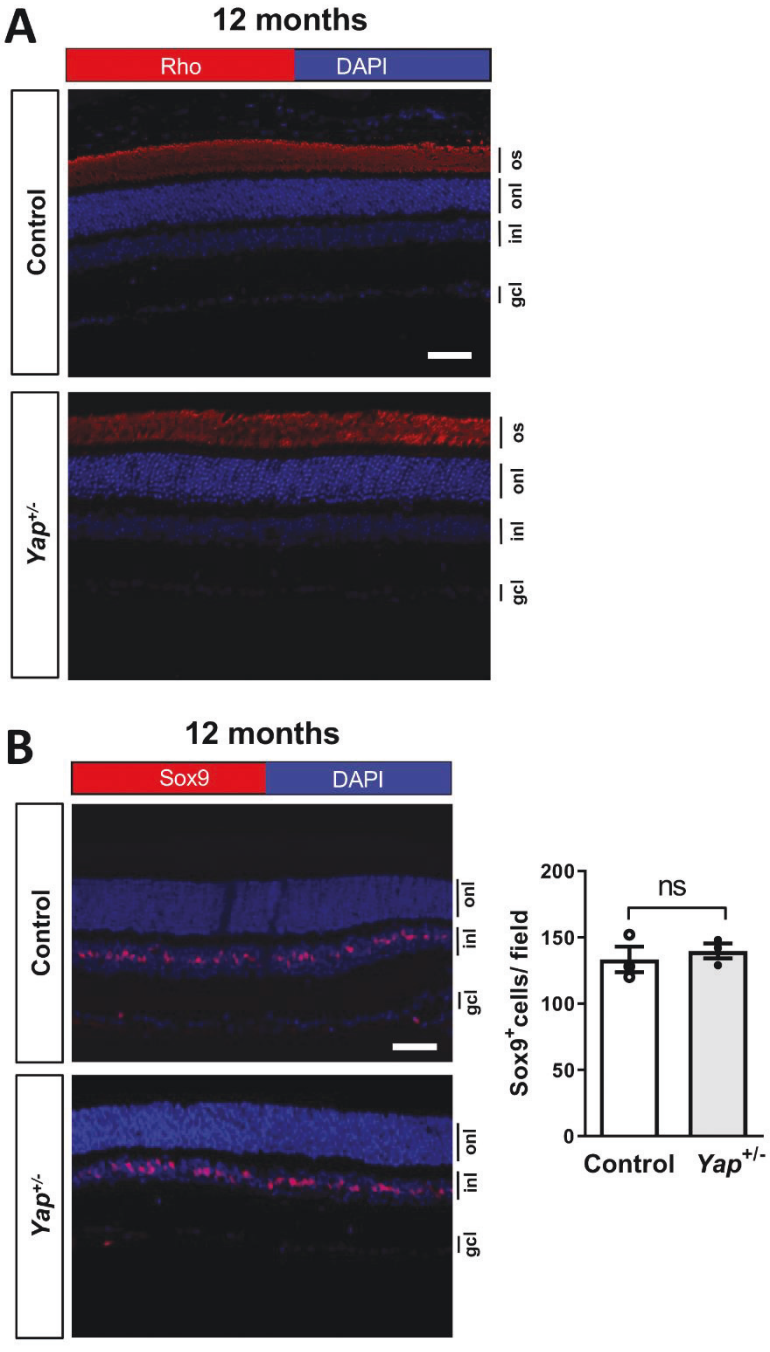
**Supplementary Figure S8. Normal expression of cone markers in 1- and 4-month-old *Yap*$^{+/-}$ mice**. **(A)** 1-month-old retinal sections immunostained for cone markers (PNA, S-opsin, M-opsin). Scatter plots with bars represent the quantification of labelled cells per field (300 µm x 300 µm). Central, mid-dorsal and ventral regions of retinal sections are shown. Mean values ± SEM from 3 retinas per condition are shown. INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer. Statistics: Mann-Whitney test, ns: non-significant. **(B)** 4-month-old retinal flat-mounts immunostained for cone markers (PNA, S-opsin, M-opsin). Scatter plots with bars represent the quantification of labelled cells per field (400 µm x 400 µm). Mean values ± SEM from 3 retinas per condition are shown. Statistics: Mann-Whitney test, ns: non-significant. Scale bare : 20 µm (A), 50 µm (B).

# Supplementary Figure S8



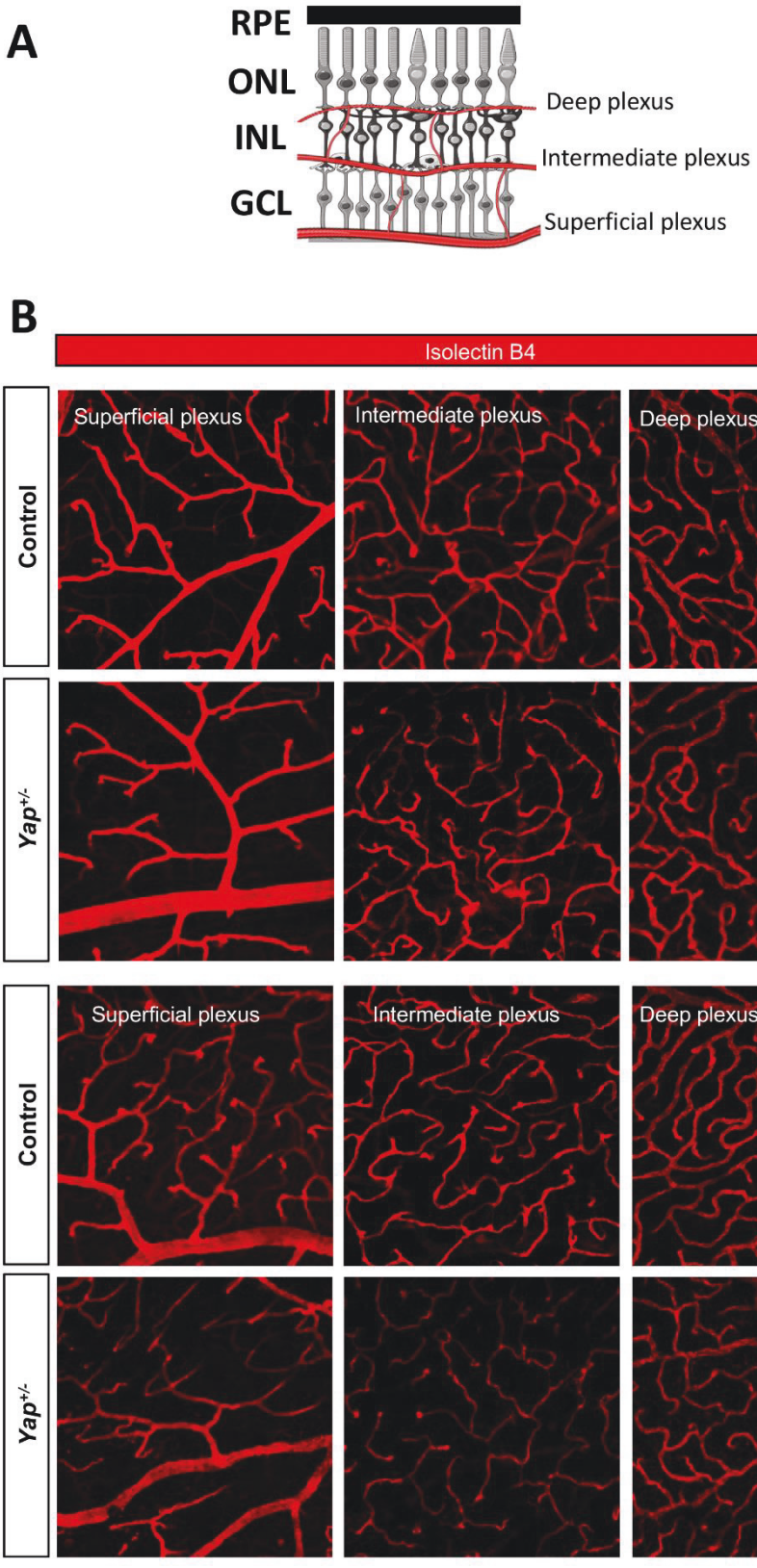
**Supplementary Figure S9. Normal expression of Rhodopsin and normal number of Müller cells in *Yap*$^{+/-}$ adult mice**. 12-month-old retinal sections immunostaining for Rhodopsin (Rho, red) **(A)** or for SOX9 (green) **(B)**. Nuclei are DAPI counterstained (blue). Scatter plots with bars represents the quantification of labelled cells per field (1200 µm x 1200 µm). Mean values ± SEM from 3 retinas per condition are shown. GCL: ganglion cell layer, INL: inner nuclear layer; ONL: outer nuclear layer; OS: outer segment. Statistics: Mann-Whitney test, ns: non-significant. Scale bar: 50 µm.

Supplementary Figure S9



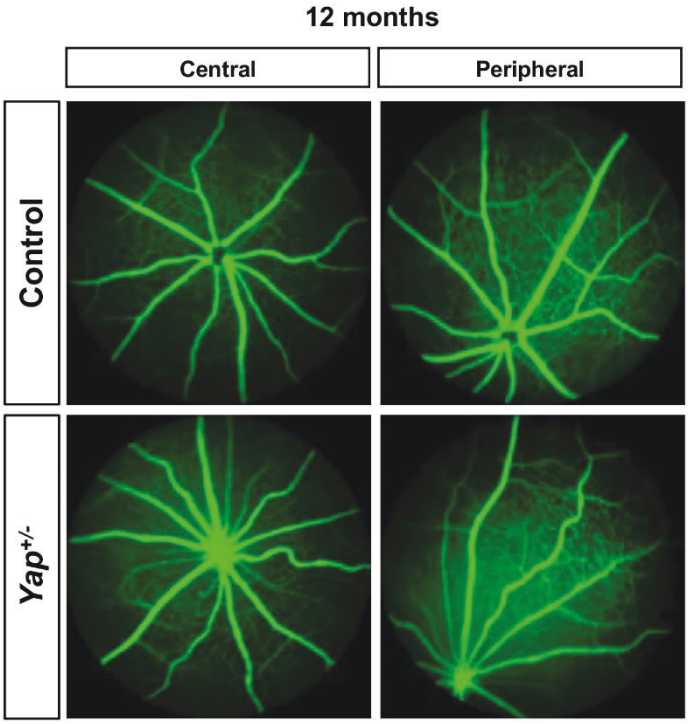
**Supplementary Figure S10. Alteration of intermediate retinal vascular plexus in *Yap*$^{+/-}$ adult mice.** (**A**) Schematic representation of the three-retinal plexi: deep plexus, intermediate plexus and superficial plexus (red) in the mouse retina. This figure was created with some schemas adapted from Servier Medical Art by Servier (licensed under a Creative Commons Attribution 3.0 Unported License). (**B**) 8 and 12-old-month retinal flat-mounts stained for endothelial cells with isolectin $B_4$ (red) in the superficial, intermediate, and deep plexi (n=3 mice per condition). INL: inner nuclear layer; ONL: outer nuclear layer; GCL: ganglion cell layer; RPE: retinal pigment epithelium. Scale bar: 100 µm.

Supplementary Figure S10



**Supplementary Figure S11. No blood leakage in *Yap*$^{+/-}$ adult mice.** Fluorescein angiography in 12-month-old mouse retina showing no signs of vascular leakage approximately 5 min after intra-peritoneal injection of sodium fluorescein. Scale bar: 200 µm.

Supplementary Figure S11



## Table S1: List of primers

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| ***Ctgf (Connective tissue growth factor)*** | GCCCTAGCTGCCTACCGACT | AGAACAGGCGCTCCACTCTG |
| ***Cyr61 (Cysteine-rich angiogenic inducer 61)*** | TGAAGAGGCTTCCTGTCTTTGG | CGGCACTCTGGGTTGTCATT |
| ***Egfr (Epidermal growth factor receptor)*** | TGGCATCATGGGAGAGAACA | GTACAGTTGGCGTGGCATAG |
| ***Erbb2 (Erb-B2 tyrosine kinase receptor 2)*** | AACTGCAGTCAGTTCCTCCG | GTGCTTGCCCCTCACATACT |
| ***Erbb3 (Erb-B2 tyrosine kinase receptor 3)*** | AGTCCGGGAGATTACAGGCT | CGATGGTCGTCAGGTTGGAA |
| ***Erbb4 (Erb-B2 tyrosine kinase receptor 4)*** | CCTTGCCATCCAAACTGCAC | GGTAAAGTGGAATGGCCCGT |
| ***Gfap (Glial fibrillary acidic protein)*** | CCAGTTACCAGGAGGCACTTG | CGATGTCCAGGGCTAGCTTAA |
| ***Hbegf (Heparin binding epidermal growth factor)*** | GCATCCAAAGTGATCGCTGC | GACAACACTGCGGCCAGAAA |
| ***Nrg1 (Neuregulin 1)*** | GAGTGCAGACCCATCTCTCG | CCAGGGCTTCTCCCATCTTC |
| ***Rax-CreERT2 (genotyping)*** | TTCCCGCAGAACCTGAAGAT | CCCCAGAAATGCCAGATTAC |
| ***Rps26 (Mitochondrial ribosomal S26 protein)*** | CCCAAGGATAAGGCCATCAAG | AAGCACGTAGGCGTCGAAGA |
| ***Srp72 (Signal Recognition Particle 72)*** | CACCCAGCAGACAGACAAACTG | GCACTCATGGTAGCGTTCCA |
| ***Taz/wwtr1 (WW domain containing transcription regulator 1)*** | AGACCCCAGGAAGGTGATGAA | TGTTGGTGATTCATTGCGAGAT |
| ***Tead1 (TEA domain family member 1)*** | TCTGGGCGGACTTAAACTGC | GAACCTCGCATACTCCGTCTCT |
| ***Tead2 (TEA domain family member 2)*** | CGGAAGCCTGGTTATTGAGC | TTCCAGAAGAGCAAGGAGGG |
| ***Yap (Yes-associated-protein 1)*** | TCCTGATGGATGGGAGCAAG | CTCTGGTTCATGGCAAAACGA |

**Table S2 : List of antibodies.** IHC: immunohistochemistry, WB: western blot.

| Antigene | Host | Supplier | Reference | Dilution (IHC) | Dilution (WB) |
|---|---|---|---|---|---|
| **Primary antibodies** | | | | | |
| **AKT** | mouse | SIGMA | P2482 | | 1:1,000 |
| **Alpha-tubulin** | mouse | SIGMA | T5168 | | 1:20,000 |
| **AQP4** | rabbit | Alomone labs | AQP4-004 | 1:300 | 1:3000 |
| **CHX10** | mouse | Santa cruz | SC365519 | 1:250 | |
| **CRALPB** | mouse | abcam | ab15051 | 1:50 | 1:500 |
| **CtBP2/ribeye** | mouse | BD Bioscience | BD612044 | 1:50 | |
| **Cycline D1** | rabbit | abcam | ab134175 | 1:100 | |
| **ERK1/2 (p44/42 MAPK)** | rabbit | Cell Signaling | 9102 | | 1:1,000 |
| **EZRIN** | mouse | Neomarkers | MS-661PABX | 1 :1,000 | |
| **GFAP** | rabbit | Dako | Z0334 | 1:500 | |
| **GFAP** | goat | Abcam | ab53554 | | 1:1,500 |
| **Glutamine synthetase** | mouse | Abcam | ab64613 | 1:1,500 | 1:10,000 |
| **Isolectin gs-ib4 alexa 594** | G.simplicifolia | Thermo Fisher Scientific | 121413 | 1:1,500 | |
| **Kir4.1** | rabbit | Alomone labs | APC-035 | 1:300 | 1:500 |
| **Lectin PNA alexa 568** | peanut | Thermo Fisher Scientific | L32458 | 1:500 | |
| **OTX2** | rabbit | abcam | ab114138 | 1:200 | |
| **P-AKT (Ser473)** | rabbit | Cell Signaling | 9271 | | 1:1,000 |
| **PCNA** | mouse | Dako | Mo879 | 1:500 | |
| **P-ERK1/2 (Thr202/Tyr204)** | rabbit | Cell Signaling | 4370 | | 1:1,000 |
| **Phalloidin alexa 568** | A.phalloides | Thermo Fisher Scientific | A12380 | 1:50 | |
| **PKC alpha** | rabbit | Sigma | P4334 | 1:400 | |
| **P-Stat3 (Ty705)** | mouse | Cell Signalling | 9138S | 1:1000 | |
| **Recoverin** | rabbit | millipore | AB5585 | 1:100 | |
| **Rhodopsin** | mouse | EMD Millipore | MAB5316 | 1:2,000 | |
| **M-opsin** | rabbit | EMD Millipore | AB5407 | 1:1000 | |
| **Stat3** | mouse | Cell Signalling | 9139S | 1:1000 | |
| **S-opsin** | rabbit | EMD Millipore | AB5407 | 1:500 | |
| **Sox9** | rabbit | EMD Millipore | AB5535 | 1:300 | |
| **RPE65** | mouse | Thermo fisher Scientific | SC53483 | 1:500 | 1:1,000 |
| **TAZ** | Rabbit | Abcam | Ab110239 | | 1:1,000 |
| **TEAD-1** | Rabbit | Abcam | Ab133533 | | 1:500 |
| **YAP** | mouse | Abcam | ab56701 | 1:50 | 1:1,000 |
| **Secondary antibodies** | | | | | |
| **Alexa 488 anti-mouse IgG1** | goat | Thermo Fisher Scientific | A21121 | 1:200 | |
| **Alexa 555 anti-mouse IgG1** | goat | Thermo Fisher Scientific | A21127 | 1:200 | |
| **Alexa 488 anti-mouse IgG2a** | goat | Thermo Fisher Scientific | A21141 | 1:200 | |
| **Alexa 647 anti-rabbit** | goat | Thermo Fisher Scientific | A21244 | 1:200 | |
| **HRP anti-mouse IgG** | goat | Sigma-Aldrich | A4416 | | 1:5,000 |
| **HRP anti-rabbit IgG** | donkey | GE Health | NA934V | | 1:5,000 |
| **HRP anti-goat IgG** | rabbit | Sigma-Aldrich | A5520 | | 1:5,000 |

# DISCUSSION AND PERSPECTIVES

## IS YAP REALLY DISPENSABLE IN ADULT RETINA?

In several mammalian organs such as the intestine, the pancreas, the liver or the mammary gland, the absence of major defects in the adult tissue in conditional mutants of loss of function of *Yap* suggested unexpectedly that this factor may be dispensable to maintain normal homeostasis (Azzolin et al., 2014; Cai et al., 2010; Chen et al., 2014; Zhang et al., 2010; Zhang et al., 2014). To address whether *Yap* could play a role in the adult retina, we took advantage of a *Yap* heterozygous mice (*Yap*$^{+/-}$) since *Yap* knockout is lethal on embryonic day 8.5 (Morin-kensicki et al., 2006). Although heterozygous mice are often phenotypically normal, detailed phenotypic analysis can reveal specific defects. In line with this, we found no obvious phenotype in adult retina (2 to 8 months of age). However, it does not mean that *Yap* is dispensable but that its function may be compensated by TAZ, which we demonstrated up to 4 months of age. Despite that double deficiency of YAP and TAZ has been reported as having little consequences on intestinal homeostasis (Azzolin et al., 2014), a double YAP/TAZ knockout would be necessary to fully assess their role in adult retina.

## YAP IS A KEY FACTOR FOR CONE SURVIVAL AND PROPER VISION

Very interestingly, the TAZ compensation disappears in aged *Yap*$^{+/-}$ mice and thus allows to assess YAP function in aged retinas. Surprisingly, we found a severe effect on cone survival and proper cone-driven vision. A recent manuscript also reported the analysis of *Yap*$^{+/-}$ phenotype and, consistent with our work, revealed an aged-dependent phenotype (Kim et al., 2019). However, they found much stronger defects (retinal detachment accompanied by loss of photoreceptors) but they did not analyze whether only cone or also rods were affected. We hypothesized that the different phenotype severity was due to different genetic background. In any case, these data have an important impact for human retinal diseases since they reveal that a single copy deletion of *Yap* could lead to progressive photoreceptor degeneration. It would thus be interesting to systematically look for *Yap* mutations in patients with cone dystrophies for which the mutated gene has not yet been identified. A collaboration with geneticists in ophthalmology in Montpellier has been established in this context.

## MÜLLER CELL-DEPENDENT YAP FUNCTION PREVENTS CONE DEGENERATION UPON AGING

As YAP is expressed in Müller cells but also in the RPE, we thus took advantage of *Yap* cKO mice, in which *Yap* is specifically deleted in Müller cells from P10 onwards, to assess whether the cone degenerative phenotype that we observed in $Yap^{+/-}$ is only linked to its role in Müller cells. Indeed, the RPE is critical for maintaining visual system function of both rod and cone photoreceptors, whereas the Müller cells are involved only in the visual cycle of cônes (Travis et al., 2007). We also found cone defects in aged *Yap* cKO mice suggesting that indeed YAP expression decrease in Müller cells in $Yap^{+/-}$ eventually leads to cone degeneration. However, the phenotype was less severe in *Yap* cKO. One hypothesis is that *Yap* deletion in the RPE in $Yap^{+/-}$ may also contribute to the phenotype. Crossing *Yap* flox line with a line expressing the Cre only in the RPE could help to address this issue. And assessing whether we can rescue the phenotype by restoring *Yap* expression only in Müller cells or only in RPE in $Yap^{+/-}$ could also be a strategy.

## YAP MAINTAINS PROPER MÜLLER CELL HOMEOSTASIS

We next wondered what was going wrong in Müller cells in both *Yap* cKO or $Yap^{+/-}$. In both lines, we observed a decrease in Kir4.1 or AQP4, proteins that are crucial for the maintenance of retinal homeostasis. Indeed, these proteins are involved in ionic and water homeostasis (Reichenbach and Bringmann, 2013). Interestingly, they are also associated directly or indirectly with the glutamate metabolism (Li et al., 2014; Nishikawa et al., 2016; Reichenbach and Bringmann, 2013). For example, a decrease in Kir4.1 lessen the transport of potassium through Müller cells and causes cell depolarization, impairing glutamate uptake (Reichenbach and Bringmann, 2013). Therefore, we can speculate that alterations in the expression of these proteins could result in alteration of potassium conductance, osmotic gradient and/or glutamate recycling by Müller cells, leading to retinal stress and neurotoxicity by glutamate accumulation in the extracellular medium and thus, eventually, to cone degeneration. In accordance with this idea, the expression of GFAP, the most sensitive indicator of retinal stress in Müller cells, was dramatically increased in aged $Yap^{+/-}$ mice compared to controls, but not before. In addition to Kir4.1 and AQP4 diminution, we also found in aged $Yap^{+/-}$ mice that the reduction of YAP quantity correlated with a decrease in the expression of CRALBP, one of the proteins required for Müller cell visual cycle, which may also contribute to the observed cone dysfunction and degeneration. Of note, we did not observe this down-regulation in the *Yap* CKO mice, which may explain, at least in part, the less severe cone phenotype (PNA staining unaffected) compared to the one observed in $Yap^{+/-}$ mice.

Alternatively, the decrease in the expression of Kir4.1, AQP4 or CRALBP could also be a consequence, and not a cause, of cone defects observed in *Yap* cKO or *Yap*$^{+/-}$ as it has been reported that these Müller cell markers are often affected in retinal diseases (Bringmann et al., 2006; Bringmann et al., 2009; Lewis et al., 1994; Lieth et al., 1998; Tenckhoff et al., 2005). However, unpublished data from a researcher in the laboratory, Juliette Bitard, revealed that Kir4.1 or AQP4 expression is decrease long before cone defects in *Yap* cKO mice retina. Together, these data strongly suggest that YAP is required for the maintenance of Müller cell physiological functions. RNAseq data would be helpful to provide a more global view of Müller cell transcriptomic defects in *Yap* cKO or *Yap*$^{+/-}$ mice during retina aging. Such analysis is underway in the laboratory.

Very interestingly, a link between YAP and mesenchymal stem cell aging has recently been reported (Fu et al., 2019). Indeed, it was shown that YAP represses human mesenchymal stem cell (hMSC) senescence: *Yap*$^{-/-}$ hMSCs exhibited accelerated senescence while lentiviral gene transfer of YAP could rejuvenate aged hMSCs and ameliorate osteoarthritis symptoms in mouse models. In *Drosophila*, YAP/Yorkie was shown to protect germline stem cells from age-associated loss (Francis et al., 2019). A recent paper has highlighted deregulated genes in old Müller cells compared to young one (Lin et al., 2019). It would be very interesting to assess whether Shh10 AAV-mediated YAP overexpression could prevent such aged marks and therefore delay retinal senescence.

# PART 2

## YAP FUNCTION IN THE ADULT RETINA UNDER DEGENERATIVE CONDITIONS

Different studies have reported a crucial role of YAP in tissue regeneration and stem cells. Thus, in parallel with the study of YAP in Müller cells in physiological conditions and in line with the previous data of the laboratory indicating that YAP might be implicated in the reactivation of Müller cells during degenerating conditions (Hamon et al., 2017), a PhD student in the laboratory, Annaïg Hamon, developed a project to understand the role of YAP in Müller cells in adult retinas in case of photoreceptor cell death using a loss of function approach. In addition, a post-doctoral fellow in the laboratory, Divya Ail, started to study the role of YAP in Müller cells during retinal regeneration in *Xenopus laevis*. Based on the hypothesis that YAP expression and activity might potentially awaken the regenerative potential of Müller cells, I developed a gain of function model of YAP using an AAV as a vector to express a constitutively active form of YAP specifically in Müller cells. Using this model, I studied the impact of YAP overexpression in Müller cell reactivation both *ex vivo* and *in vivo*. In addition, I investigate whether the YAP-EGFR interaction was required for the mitogenic effect of YAP on Müller cells.

Associated publication:
Annaïg Hamon*, **Diana García-García***, Divya Ail*, Juliette Bitard, Albert Chesneau, Deniz Dalkara, Morgane Locker, Jérôme E. Roger and Muriel Perron. Linking YAP to Müller glia quiescence exit in the degenerative retina. (2019) **Cell reports** 27: 1712-1725

*These authors contributed equally

## 1. ANIMAL MODELS

In order to determine the function of YAP precisely in Müller cells, my laboratory generated a genetic mouse line in which *Yap* was conditionally deleted specifically in Müller cells thanks to the Cre-Loxp system. Briefly, *Yap*$^{flox/flox}$ mice were obtained from Jeff Wrana's lab (Reginensi et al., 2013) and crossed with heterozygous Rax-CreER$^{T2}$ knock-in mice or double heterozygous Rax-CreER$^{T2}$,R26-CAG-lox-stop-lox-TdTom (Ai9) mice from Seth Blackshaw's lab (Pak et al., 2014) to generate *Yap*$^{flox/flox}$*;Rax-CreER*$^{T2}$ (*Yap* CKO) and *Yap*$^{flox/flox}$;Rax-CreER$^{T2}$;Ai9 (*Yap* CKO; Ai9) mice. Cre activity was induced through a single intraperitoneal injection of 4-hydroxyta-moxifen (4-OHT) at P10. Ai9 is a reporter allele of the Cre recombinase.

To induce degenerative conditions, we used different models. First, we used a **pharmacological model** to induce photoreceptor degeneration. Chemically induced retinal degeneration was performed through a single intraperitoneal injection of N-Methyl-N-nitrosourea (MNU), an alkylant reagent that lead to DNA damage and apoptosis. Second, we used a **genetic model** of retinitis pigmentosa, the *rd10* mice. These mice have an autosomal recessive mutation in the 13$^{th}$ exon of the *Pde6b* gene (cGMP phosphodiesterase 6B) (Chang et al., 2002). This mutation leads to rod photoreceptor cell death from

16 days after birth (P16). In the last stages of the disease the death of rods is followed by cone cell death (Barhoum et al., 2008; Gargini et al., 2007; Pennesi et al., 2012). This genetic model was crossed with *Yap* CKO mice to generate a genetic model of retinal degeneration in which YAP could be specifically deleted in Müller cells. To generate the *Yap$^{flox/flox}$;Rax-CreERT2;rd10* line (*Yap* CKO;rd10), *Yap$^{flox/flox}$;Rax-CreER$^{T2}$* mice were crossed with homozygous *rd10* mice. Third, we used a **spontaneous model** of *ex vivo* retinal degeneration, the retinal explant culture (Alarautalahti et al., 2019; Johnson et al., 2016; Müller et al., 2017).

The gain of function model of YAP was developed by the overexpression of a constitutively active form of YAP (YAP5SA) specifically in Müller cells. This YAP has a mutation in the main phosphorylation sites, which impedes its inactivation by LATS1/2. To deliver this YAP5SA, we established a collaboration with Deniz Dalkara (Institut de la Vision, Paris) to generate an adeno-associated virus (AAV) variant, ShH10, which selectively targets Müller cells (Klimczak et al., 2009), coding for YAP5SA.

For the *Xenopus* experiments, heat-shock-inducible dominant-negative YAP transgenic line Tg (*hsp70:dnYap-GFP, cryga:tdTomato*) (*Tg(dnYAP)*) (Hayashi et al., 2014a) was obtained from the UK *Xenopus* resource center (EXRC). Heat-shock-mediated dnYAP expression was induced by shifting the animals from 18-20ºC to 34ºC for 30 min as previously described (Hayashi et al., 2014a). The transgenic *Xenopus* line *Tg(Rho:GFP-NTR)* used for conditional rod ablation was generated in the lab and previously described (Langhe et al., 2017). Photoreceptor degeneration was induced by bathing the froglets in a 10 mM MTZ solution for 1 week (Langhe et al., 2017). As another degenerative model, retinal mechanical injury was performed by poking the retina once under a stereomicroscope with a needle without damaging the cornea or the lens.

## 2. MAIN RESULTS

- YAP is required for *Xenopus* Müller glia cell proliferation in response to injury.
- In the mouse, YAP is required for cell cycle gene upregulation that accompanies Müller cell reactivation following retinal injury and as such is required for mouse Müller glia exit from quiescence upon degeneration.
- YAP overexpression reprograms mouse Müller glia into highly proliferative cells in an *ex vivo* model of retinal regeneration.
- YAP overexpression triggers Müller glia cell-cycle re-entry *in vivo* even in the absence of retinal damage.
- YAP positively regulates the expression of EGFR pathway components in mouse reactive Müller cells.
- YAP mitogenic effects on Müller cells requires EGFR pathway activity.

# Article nº2

Annaïg Hamon*, **Diana García-García***, Divya Ail*, Juliette Bitard, Albert Chesneau, Deniz Dalkara, Morgane Locker, Jérôme E. Roger and Muriel Perron

(2019)

# Linking YAP to Müller glia quiescence exit in the degenerative retina

*Cell reports*

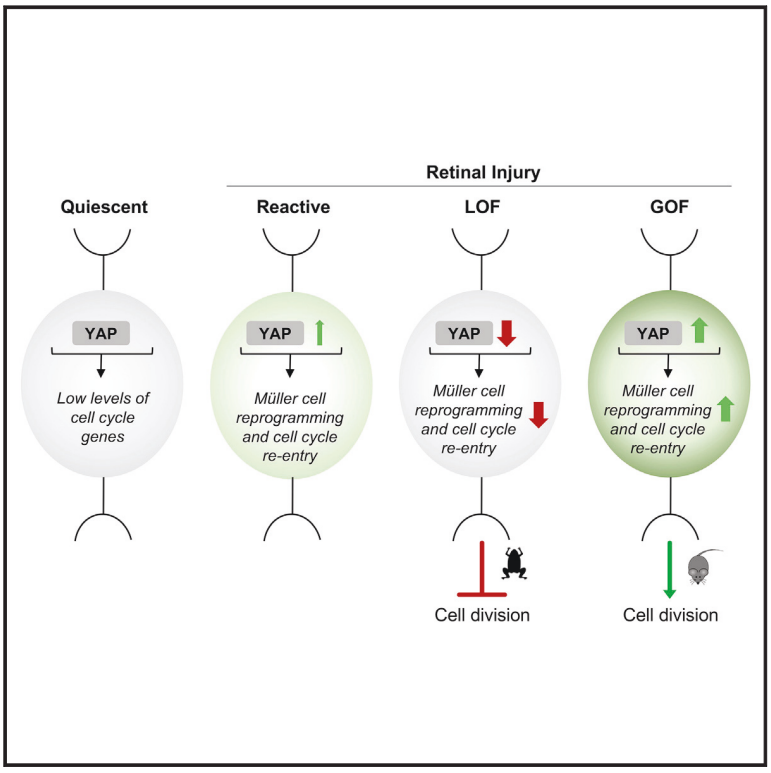
*These authors contributed equally

Article

Cell Reports

# Linking YAP to Müller Glia Quiescence Exit in the Degenerative Retina

## Graphical Abstract




## Authors

Annaïg Hamon, Diana García-García, Divya Ail, ..., Morgane Locker, Jérôme E. Roger, Muriel Perron

## Correspondence

jerome.roger@u-psud.fr (J.E.R.), muriel.perron@u-psud.fr (M.P.)

## In Brief

While fish and amphibian Müller cells behave as retinal stem cells upon injury, their regenerative potential is limited in mammals. Hamon et al. show that YAP is required for their cell-cycle re-entry in *Xenopus* and is sufficient in mouse to awake them from quiescence and trigger their proliferative response.

## Highlights

- YAP is required for *Xenopus* Müller glia proliferation in response to injury
- YAP is required for mouse Müller glia exit from quiescence upon degeneration
- YAP5SA reprograms mouse Müller glia into highly proliferative cells
- YAP functionally interacts with EGFR signaling in Müller cells

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May 7, 2019 
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# Linking YAP to Müller Glia Quiescence Exit in the Degenerative Retina

Annaïg Hamon,[1,3] Diana García-García,[1,3] Divya Ail,[1,3] Juliette Bitard,[1] Albert Chesneau,[1] Deniz Dalkara,[2] Morgane Locker,[1] Jérôme E. Roger,[1,*] and Muriel Perron[1,4,*]

[1]Paris-Saclay Institute of Neuroscience, CERTO-Retina France, CNRS, Univ Paris Sud, Université Paris-Saclay, Orsay 91405, France
[2]Sorbonne Université, UPMC Univ Paris 06, INSERM, CNRS, Institut de la Vision, Paris, France
[3]These authors contributed equally
[4]Lead Contact
*Correspondence: jerome.roger@u-psud.fr (J.E.R.), muriel.perron@u-psud.fr (M.P.)
https://doi.org/10.1016/j.celrep.2019.04.045

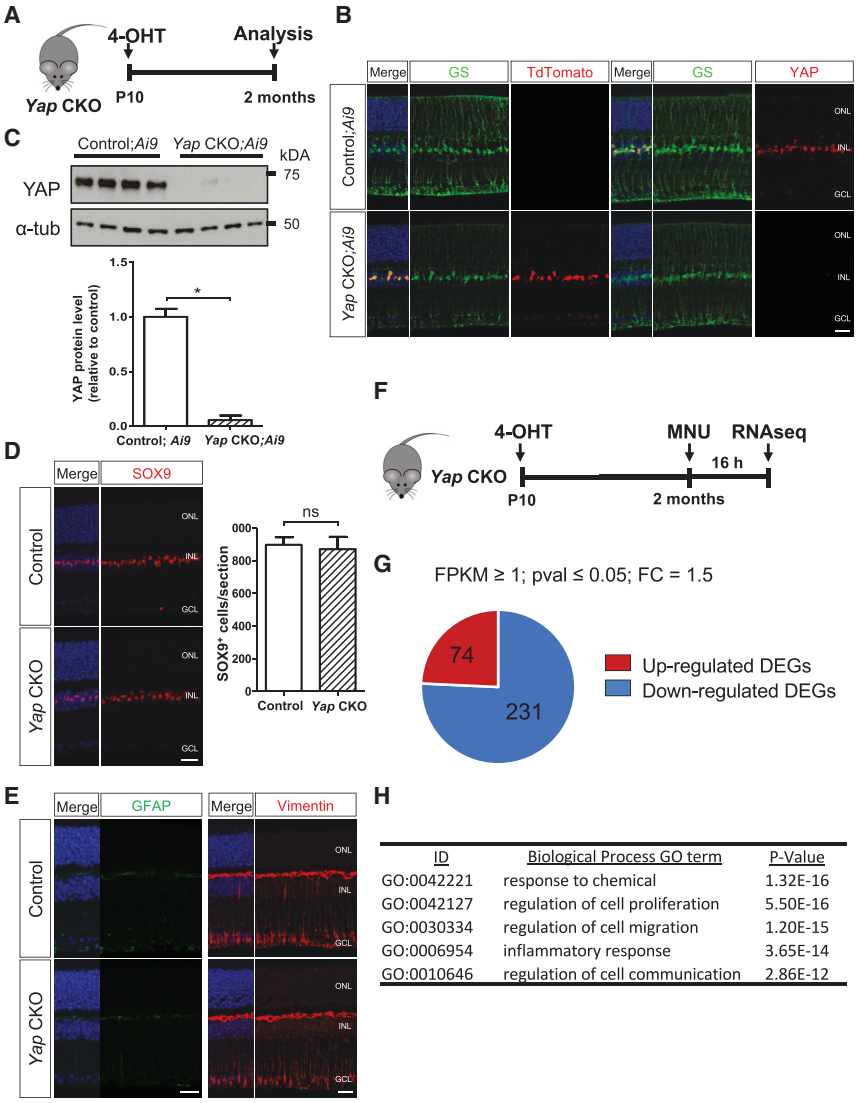
## SUMMARY

**Contrasting with fish or amphibian, retinal regeneration from Müller glia is largely limited in mammals. In our quest toward the identification of molecular cues that may boost their stemness potential, we investigated the involvement of the Hippo pathway effector YAP (Yes-associated protein), which is upregulated in Müller cells following retinal injury. Conditional *Yap* deletion in mouse Müller cells prevents cell-cycle gene upregulation that normally accompanies reactive gliosis upon photoreceptor cell death. We further show that, in *Xenopus*, a species endowed with efficient regenerative capacity, YAP is required for their injury-dependent proliferative response. In the mouse retina, where Müller cells do not spontaneously proliferate, YAP overactivation is sufficient to induce their reprogramming into highly proliferative cells. Overall, we unravel a pivotal role for YAP in tuning Müller cell proliferative response to injury and highlight a YAP-EGFR (epidermal growth factor receptor) axis by which Müller cells exit their quiescence state, a critical step toward regeneration.**

## INTRODUCTION

Neurodegenerative retinal diseases, such as retinitis pigmentosa or age-related macular degeneration, ultimately lead to vision loss, as a consequence of photoreceptor cell death. Driving retinal self-repair from endogenous neural stem cells in patients represents an attractive therapeutic strategy. Among cellular sources of interest are Müller cells, the major glial cell type in the retina (Bringmann et al., 2006). In certain species, such as zebrafish or *Xenopus*, they behave as genuine stem cells, endowed with the ability to reprogram into a progenitor-like state upon retinal damage, proliferate, and regenerate lost photoreceptors (Hamon et al., 2016; Langhe et al., 2017; Wan and Goldman, 2016). In mammals, however, their proliferative response to injury is extremely limited. Following acute retinal damage, mouse Müller glial cells rapidly re-enter the G1 phase of the cell cycle, as inferred by increased *cyclin* gene expression, but they rarely divide (Dyer and Cepko, 2000). Suggesting that they nonetheless retain remnants of repair capacities, their proliferation and neurogenic potential can be stimulated, by supplying exogenous growth factors such as heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), by overexpressing the proneural gene *Ascl1a*, or via cell fusion with Wnt-activated transplanted stem cells (Hamon et al., 2016; Jorstad et al., 2017; Sanges et al., 2016; Ueki et al., 2015; Wilken and Reh, 2016). Our understanding of the genetic and signaling network sustaining Müller cell stemness potential is, however, far from being complete. Identifying novel molecular cues is thus of utmost importance to foresee putative candidates that could be targeted for regenerative medicine. We here investigated whether the Hippo pathway effector YAP might influence Müller cell reactivation and how it would intersect with other critical signaling pathways.

The Hippo pathway is a kinase cascade that converges toward two terminal effectors, YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif). Both are transcriptional coactivators of TEAD family transcription factors. The Hippo pathway emerged as a key signaling in a wide range of biological processes (Fu et al., 2017), including stem cell biology (Barry and Camargo, 2013; Mo et al., 2014). Of note, it proved to be dispensable under physiological conditions in some adult stem cells, such as mammary gland, pancreatic, intestinal, and importantly, neural stem cells (Azzolin et al., 2014; Chen et al., 2014a; Huang et al., 2016; Zhang et al., 2014). It can nonetheless become essential under pathological conditions, as described, for example, in the context of intestinal regeneration following injury (Barry et al., 2013; Gregorieff et al., 2015). YAP status in adult neural tissue repair has hitherto never been investigated. We recently discovered that YAP and TEAD1 are specifically expressed in murine Müller cells, and that their expression and activity are enhanced upon retinal damage (Hamon et al., 2017). We thus sought to determine whether YAP could be required for injury-induced Müller glia reactivation. We found in mouse that YAP triggers cell-cycle gene upregulation in Müller glial cells following photoreceptor cell death. In line with the idea of a conserved role in Müller cell-cycle re-entry, blocking YAP function in *Xenopus* results in a dramatically reduced proliferative response following acute retinal damage or photoreceptor cell ablation. Finally, we report that the limited proliferative response of murine Müller glia can be circumvented




| ID | Biological Process GO term | P-Value |
|---|---|---|
| GO:0042221 | response to chemical | 1.32E-16 |
| GO:0042127 | regulation of cell proliferation | 5.50E-16 |
| GO:0030334 | regulation of cell migration | 1.20E-15 |
| GO:0006954 | inflammatory response | 3.65E-14 |
| GO:0010646 | regulation of cell communication | 2.86E-12 |

**Figure 1. *Yap* CKO Retinas Exhibit Altered Transcriptional Response to Injury**

(A) Timeline diagram of the experimental procedure used in (B–E). *Yap*$^{flox/flox}$ mice with or without the *Ai9* reporter allele (control or control;*Ai9*) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ mice with or without the *Ai9* reporter allele (*Yap* CKO or *Yap* CKO;*Ai9*) received a single dose of 4-OHT at P10 and were analyzed at P60.

(B) Retinal sections immunostained for TdTomato, glutamine synthetase (GS; a Müller cell marker), or YAP.

(C) Western blot analysis of YAP expression on retinal extracts. α-Tubulin labeling was used to normalize the signal. n = 4 mice for each condition.

(D) Retinal sections immunostained for SOX9. n = 3 mice for each condition.

(E) Retinal sections immunostained for GFAP or Vimentin.

(F) Timeline diagram of the experimental procedure used in (G) and (H). *Yap*$^{flox/flox}$ (control) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ (*Yap* CKO) mice received a single dose of 4-OHT at P10 and a single dose of MNU at 2 months. Retinas were then subjected to RNA-sequencing 16 h later.

(G) Pie chart representing the number of DEGs found to be upregulated or downregulated in MNU-injected *Yap* CKO retinas compared with MNU-injected control ones.

(H) Results of Gene Ontology (GO) enrichment analysis exemplifying six over-represented GO biological processes related to retinal response to injury.

In (B), (D), and (E), nuclei were counterstained with DAPI (blue). Mann-Whitney test, *p ≤ 0.05. All results are reported as mean ± SEM. Scale bars, 20 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ns, non-significant; ONL, outer nuclear layer. See also Figures S1 and S2 and Table S1.

and significantly enhanced by YAP overexpression. We further show such YAP mitogenic function relies on its interplay with epidermal growth factor receptor (EGFR) signaling. As a whole, this study highlights the critical role of YAP in driving Müller cells to exit quiescence and thus reveals a potential target for regenerative medicine.

## RESULTS

### *Yap* Conditional Knockout in Mouse Müller Cells Does Not Compromise Their Maintenance under Physiological Conditions

To investigate the role of YAP in murine Müller glia, we generated a *Yap*$^{flox/flox}$; *Rax-CreER*$^{T2}$ mouse line allowing for Cre-mediated conditional gene ablation specifically in Müller cells (Pak et al., 2014; Reginensi et al., 2013). It is thereafter named *Yap* CKO (conditional knockout), whereas "control" refers to *Yap*$^{flox/flox}$ mice. *Yap* deletion was induced in fully differentiated Müller cells, through 4-hydroxytamoxifen (4-OHT) intraperitoneal injection at postnatal day (P) 10 (Figure 1A). Phenotypic analyses were then conducted on 2-month-old mice. We first confirmed Müller cell-specific *Cre* expression and *Yap* deletion efficiency in *Yap* CKO mice carrying the Rosa26-CAG-lox-stop-lox-TdTomato reporter (*Ai9*) transgene (Figures 1B and 1C). We next wondered whether expression of TAZ (the second effector of the Hippo pathway) could be increased in our model and thus potentially compensate for *Yap* deletion, as previously reported in mammalian cell lines (Finch-Edmondson et al., 2015). In these physiological conditions, TAZ protein level was actually similar in *Yap* CKO and control mice (Figure S1A), suggesting an absence of compensatory mechanisms. Finally, we assessed global retinal organization and function in *Yap* CKO mice. Immunostaining for various retinal neuron markers and electroretinogram (ERG) recordings under scotopic and photopic conditions revealed neither structural nor functional difference compared with control retinas (Figures S1B–S1D). Müller cells, whose identity was assessed with Sox9 labeling, were also normally distributed (Figure 1D). They did not display any sign of stress

reactivity, as inferred by the expression of intermediate filament proteins, glial fibrillary acidic protein (GFAP), and Vimentin (Figure 1E). Taken together, these results demonstrate that lack of YAP expression in Müller cells from P10 does not impact the overall retinal structure, neuron and glia maintenance, nor the visual function in 2-month-old mice.

### *Yap* Deletion Impairs Mouse Müller Cell Reactivation upon Photoreceptor Degeneration

We next investigated YAP function in a degenerative context. Retinal degeneration was triggered in *Yap* CKO mice through *in vivo* methylnitrosourea (MNU) injections, a well-established paradigm for inducible photoreceptor cell death (Chen et al., 2014b). In order to evaluate the impact of *Yap* deletion on Müller glia early response to injury, all of the analyses were performed 16 h after MNU injection, at the onset of photoreceptor cell death (Figures 1F and S2A). As previously described (Hamon et al., 2017), YAP protein expression level was upregulated in wild-type (WT) retinas upon MNU injection, and as expected, we found it effectively decreased in our MNU-injected *Yap* CKO model (Figure S2B). In contrast with the physiological situation, this was accompanied by a compensatory increase of TAZ levels (Figure S2C). Yet, this enhanced expression is likely insufficient to entirely compensate for the loss of YAP activity, as inferred by the downregulation of *Cyr61*, a well-known YAP/TAZ target gene (Lai et al., 2011; Figure S2D). We next assessed GFAP expression as a marker of reactive gliosis upon MNU injection. Interestingly, we found it increased at both the mRNA and protein levels in *Yap* CKO mice compared with control ones, reflecting a potential higher degree of retinal stress (Figures S2E and S2F). This led us to further investigate the molecular impact of *Yap* deletion in reactive Müller cells, through a large-scale transcriptomic analysis comparing non-injected WT mice, MNU-injected control mice, and MNU-injected *Yap* CKO mice. This allowed identifying 305 differentially expressed genes (DEGs), 75% of them being downregulated in MNU-injected *Yap* CKO retinas compared with MNU-injected control ones (Figure 1G; Table S1). The top-enriched biological processes they belong to include "response to chemical," "regulation of cell proliferation," and "inflammatory response." This strongly suggests that lack of YAP expression profoundly alters Müller cell transcriptional response to retinal injury (Figure 1H).

### *Yap* Knockout Prevents Cell-Cycle Gene Upregulation in Mouse Reactive Müller Cells

As we were seeking for a potential function of YAP in Müller cell reactivation, we focused our interest on identified genes related to the GO group "regulation of cell proliferation." *Z* score-based hierarchical clustering for the 70 corresponding DEGs revealed three distinct clusters (Figure S3A). One particularly caught our attention because the 52 DEGs it contains appear less responsive to injury in the absence of YAP. These are indeed: (1) expressed at very low levels in wild-type mice, (2) strongly upregulated in MNU-injected control mice, (3) while being only moderately enriched in MNU-injected *Yap* CKO mice. This is the case, for instance, of four cell-cycle regulator coding genes, *Ccnd1*, *Ccnd2*, *Ccnd3*, and *Cdk6* (Figure 2A). Further validation was conducted by qPCR, immunostaining, and western blot for Cyclin D1 and Cyclin D3, which are specifically expressed in Müller cells. This confirmed their downregulation upon MNU injection in *Yap* CKO mice compared with controls (Figures 2B–2D). Noticeably, we found that the pluripotent leukemia inhibitory factor (LIF) and the reprogramming factor Klf4 follow the same profile, suggesting that the reprogramming process that initiates along with Müller cell-reactive gliosis is also impaired by *Yap* loss of function (Figure S3B).

To strengthen our results in a model closer to human retinal disease, we generated *Yap* CKO;*rd10* mice by breeding the *Yap* CKO line into the *rd10* background (*Pde6b*$^{rd10}$ line, a model of retinitis pigmentosa) (Chang et al., 2007; McLaughlin et al., 1995). We next assessed Cyclin D1 and Cyclin D3 expression at P20 (Figures S4A and S4B), which corresponds to the period of intense rod cell death in *rd10* mice (Chang et al., 2007). As observed with the MNU model, protein levels for both cyclins were increased in Müller cells upon photoreceptor degeneration, and this upregulation was impaired in *Yap* CKO retinas. Taken together, these results demonstrate YAP involvement in the injury-induced transcriptional activation of cell-cycle genes, which likely reflects its role in pushing Müller cells out of their quiescent state. Of note, a YAP-dependent control of Cyclin D1 and D3 expression was also observed in physiological conditions (Figures S4C and S4D), suggesting that YAP regulates the basal level of cell-cycle genes in quiescent Müller cells as well.

### Inhibition of YAP Prevents Müller Glia Proliferation upon Acute Retinal Damage or Selective Photoreceptor Cell Ablation in *Xenopus laevis*

All of the above results converge to the idea that YAP triggers cell-cycle re-entry of quiescent Müller glia upon injury. Because this process is not complete in murine Müller cells (they reactivate G1 phase genes but rarely divide), we turned to the frog to strengthen our hypothesis. *Xenopus* is an animal model endowed with regenerative capacity, in which Müller cells efficiently respond to injury by intense proliferation (Langhe et al., 2017). We first confirmed that, within the *Xenopus* central neural retina, YAP expression is restricted to Müller cells (Figure 3A) (Cabochette et al., 2015), as observed in mouse. We next sought to assess the impact of YAP inhibition by taking advantage of a *Xenopus laevis* transgenic line, hereafter named *Tg(dnYAP)*, in which a heat shock promoter (Hsp70) drives the expression of a dominant-negative YAP variant (Hayashi et al., 2014a; Nishioka et al., 2009; Figures 3B, S5A, and S5B). We first verified that heat-shocked *Tg(dnYAP)* embryos displayed a small eye phenotype, as previously demonstrated following *Yap*-Morpholino injection (Cabochette et al., 2015). Moreover, we found that this defective eye growth could be rescued by overexpressing an inducible and constitutively active form of YAP, YAPS98A-GR (Figures S5C–S5E). At latter developmental stages, two YAP target genes, *Ctgf* (connective tissue growth factor) and *Cyr61*, were downregulated in *Tg(dnYAP)* tadpole retinas upon heat-shock induction (Figure S5F). Altogether, these results validated the efficacy and specificity of dnYAP in inhibiting endogenous YAP function. We next assayed Müller cell proliferative response in a model of stab injury (Figure 3C). Importantly, we previously demonstrated that a majority of proliferating cells found at the injury site are indeed Müller cells

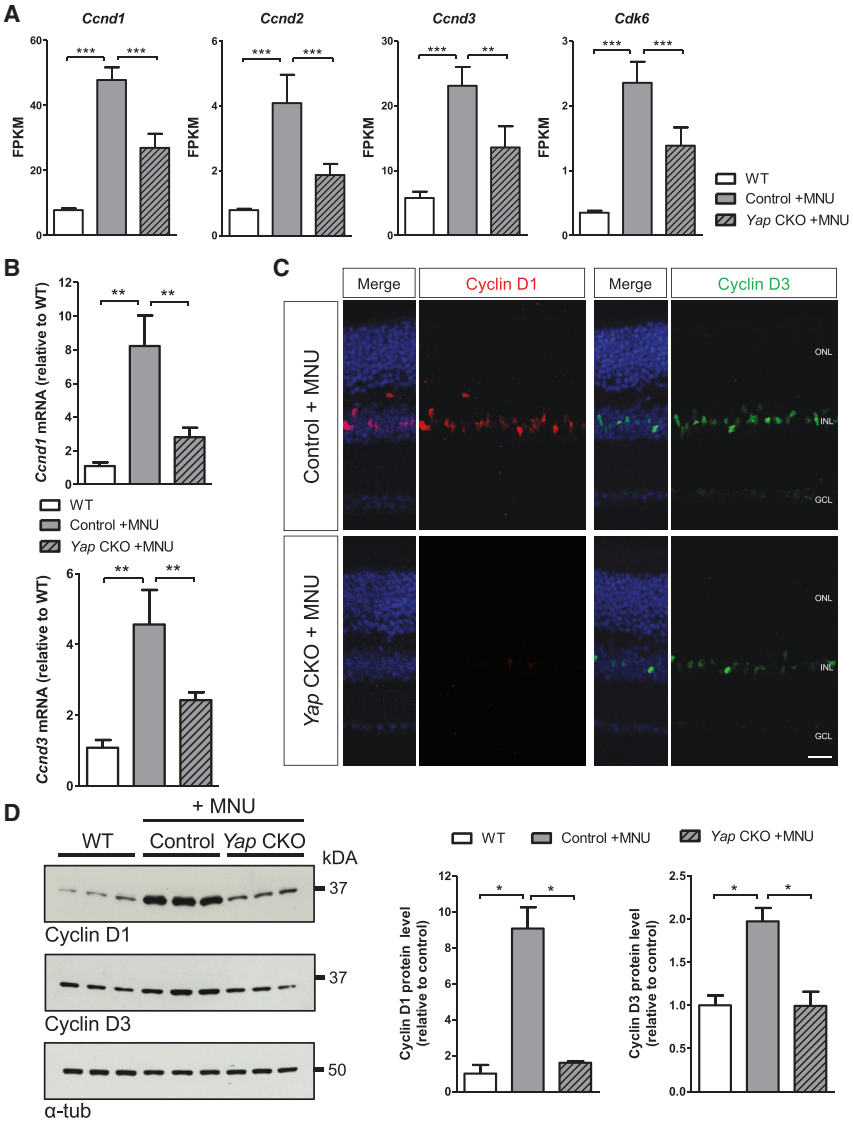



**Figure 2. Cell-Cycle Gene Upregulation in Response to MNU Injection Is Compromised in *Yap* CKO Retinas**

(A) Relative RNA expression (in fragments per kilobase of exon per million fragments mapped [FPKM]; data retrieved from the RNA-seq experiment) of *Ccnd1*, *Ccnd2*, *Ccnd3*, and *Cdk6* in retinas from non-injected WT mice or control and *Yap* CKO mice injected with 4-OHT and MNU as shown in Figure 1F.

(B) qRT-PCR analysis of *Ccnd1* and *Ccnd3* expression in the same experimental conditions (at least five biological replicates per condition were used).

(C) Retinal sections from control and *Yap* CKO mice, immunostained for Cyclin D1 or D3. Nuclei are counterstained with DAPI (blue).

(D) Western blot analysis of Cyclin D1 and D3 expression on retinal extracts from WT, control, and *Yap* CKO mice. α-Tubulin labeling was used to normalize the signal. n = 3 mice for each condition. Mann-Whitney test (except in A where p values were obtained using EdgeR), *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001. Scale bar: 20 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. See also Figures S3 and S4.

(Langhe et al., 2017). Comparison of 5-bromo-2′-deoxyuridine (BrdU) incorporation in heat-shocked and non-heat-shocked wild-type retinas confirmed that heat shock does not affect proliferation by itself (Figure 3D). In contrast, the number of BrdU-labeled cells at the injury site was reduced by about 60% in heat-shocked *Tg(dnYAP)* tadpole retinas compared with controls (non-heat-shocked transgenic animals or heat-shocked non-transgenic siblings; Figure 3D). Of note, loss of YAP activity did not affect Müller cell density (Figure S5G), ruling out the possibility that defective BrdU incorporation might be due to an impaired cell survival. Finally, YAP requirement for Müller glia proliferative response to stab injury could be confirmed at post-metamorphic stage in froglets, with *Tg(dnYAP)* retinas exhibiting a reduction of 80% of BrdU-positive cells compared with controls (Figures 3E and 3F).

We next sought to reinforce these data in a model closer to the mouse MNU or *rd10* paradigms. In this purpose, we turned to a *Xenopus laevis* transgenic line that we previously established, allowing for conditional selective rod cell ablation (Langhe et al., 2017) [*Tg(Rho:GFP-NTR)*, hereafter named *Tg(NTR)*] (Figure 4A). This transgenic line expresses the nitroreductase (*NTR*) gene under the control of the *Rhodopsin* promoter, and photoreceptor degeneration can be induced by adding the enzyme ligand metronidazole (MTZ) to the tadpole rearing medium. Here again, we previously showed that about 80% of cells that proliferate upon rod cell ablation are indeed Müller cells (Langhe et al., 2017). The *Tg(dnYAP)* and *Tg(NTR)* lines were crossed to generate double-transgenic animals, in which inhibition of YAP can be triggered by heat shock, and photoreceptor degeneration by MTZ treatment (Figure 4B). As observed above with the stab injury, Müller cell proliferative response to rod cell death was dramatically reduced in double *Tg(dnYAP;NTR)* froglet retinas compared with *Tg(NTR)* control ones (Figure 4C). Altogether, although we cannot completely rule out the possibility of dnYAP off-target effects on Müller cell behavior, these data strongly support the idea that in different lesional contexts, YAP is required for *Xenopus* Müller glia cell-cycle re-entry and proliferation.

## Forced YAP Expression in Mouse Müller Glial Cells Stimulates Their Proliferation Both *Ex Vivo* and *In Vivo*

Based on the above data on *Xenopus*, we next wondered whether mouse Müller cell inability to proliferate upon injury (despite cell-cycle gene reactivation) might be linked to insufficient levels

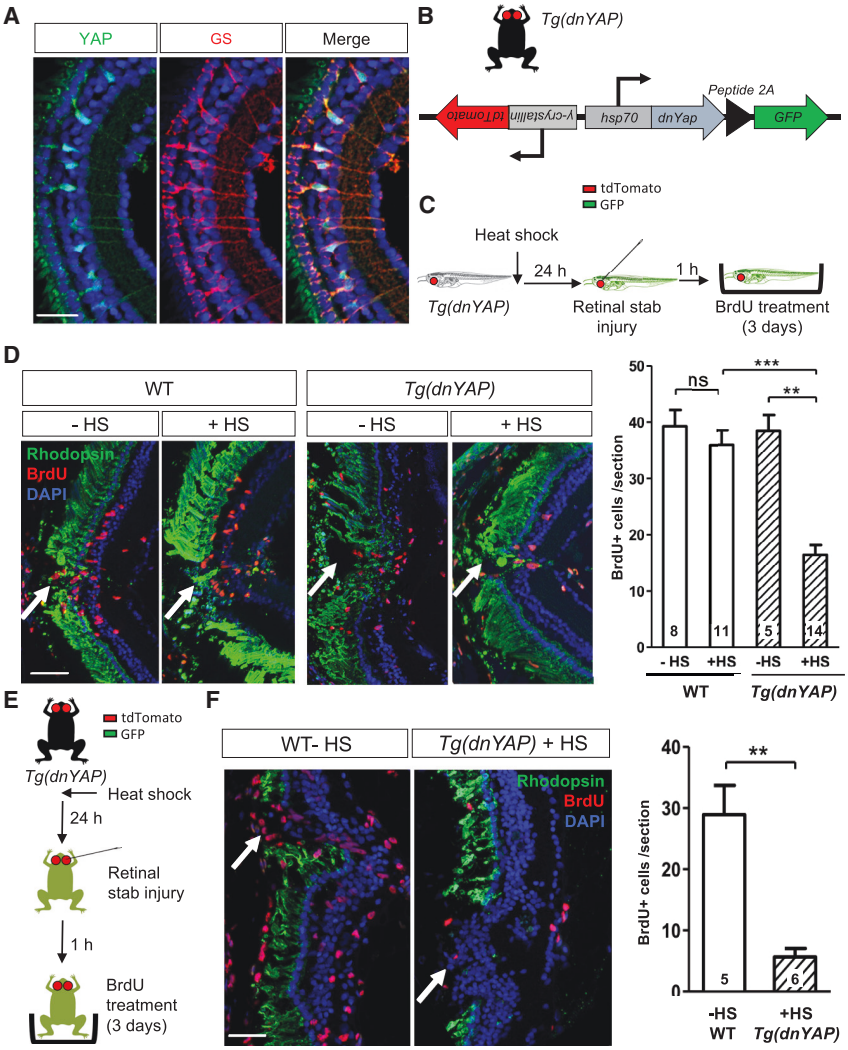



**Figure 3. Inhibiting YAP Activity in *Xenopus* Reduces the Proliferative Retinal Response to a Stab Injury**

(A) Retinal section from a stage 45 WT *Xenopus laevis* tadpole, immunostained for YAP and glutamine synthetase (GS; a marker of Müller cells). Nuclei are counterstained with Hoechst (blue).

(B) Schematic representation of the heat-shock-inducible dominant-negative *Yap* transgene (*dnYap*). *Tg(dnYAP)* transgenic animals can be selected based on tdTomato expression in the lens (driven by the γ-crystallin promoter). Heat-shocked efficiency can be assessed through GFP expression.

(C and E) Timeline diagrams of the experimental procedures used in (D) and (F), respectively. WT or *Tg(dnYAP)* pre-metamorphic tadpoles (stage 54–58; D) or froglets (stage 61–66; F) were heat-shocked, injured in the retina 24 h later, and transferred 1 h post-lesion in a BrdU solution for 3 more days.

(D and F) Retinal sections from animals that were heat-shocked (+HS) or not (−HS), immunostained for rhodopsin and BrdU. Nuclei are counterstained with DAPI (blue). Arrows point to the injury site. The number of retinas tested for each condition is indicated on the corresponding bar. Mann-Whitney test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. All results are reported as mean ± SEM. Scale bars, 25 μm (A), 50 μm (D and F). ns, non-significant. See also Figure S5.

of YAP activity. To investigate this hypothesis, we decided to overexpress in mouse Müller cells a FLAG-tagged mutated YAP protein, YAP5SA, which is insensitive to Hippo pathway-mediated cytoplasmic retention (Zhao et al., 2007). To deliver this constitutively active form of YAP, we took advantage of an adeno-associated virus (AAV) variant, ShH10, which selectively targets Müller cells (Klimczak et al., 2009; Figures 5A and S6A–S6C). We then started by infecting retinal explants, a spontaneous model of retinal degeneration (Müller et al., 2017). We first found that levels of Cyclin D1 were significantly increased upon AAV-YAP5SA transduction compared with that of AAV-GFP-infected controls (Figures 5B and 5C). We next analyzed Müller cell proliferative activity through an EdU incorporation assay. In explants overexpressing YAP5SA, EdU labeling was strongly enhanced, with numerous patches containing a high density of EdU-positive cells (Figure 5D) found in regions with the highest infected cell density (as assessed by FLAG immunostaining; data not shown). Further quantitative analyses within the explant inner nuclear layer revealed that after a 7-day culture: (1) about 50% of YAP5SA-expressing cells were EdU labeled (Figures S6D and S6E), (2) the great majority (more than 88%) of EdU-positive cells were Müller cells (as inferred by their Sox9 labeling and their position; Figures S6F and S6G), and (3) up to ~25% of Müller cells were proliferating in AAV-YAP5SA-infected explants (Figure 5E). Of note, after such a 7-day culture, EdU-positive cells were mainly found in the explant periphery, where neurons are presumably more prone to degenerate (more exposed than those in the center). Importantly, however, after longer culture time period (12 days instead of 7), proliferation spread into the whole infected explant, and the percentage of EdU-labeled Müller cells then reached more than 75% (Figure 5F).

We next assessed the mitogenic potential of YAP5SA *in vivo*, following intravitreal AAV injection in adult mice (Figures 5G and 5H). Only rare proliferative cells were observed in control retinas. In contrast, many EdU-positive cells were found in retinas transduced with AAV-YAP5SA. Co-labeling with glutamine synthetase or SOX9 on retinal sections confirmed that a majority of these had a Müller cell identity (Figure S7A and data not shown). YAP5SA can thus trigger Müller glia cell-cycle re-entry *in vivo*. Altogether, these data reveal that YAP overactivation is sufficient to override the dormancy of murine Müller glial cells and boost their proliferative potential. Additionally, as shown in Figures S7B and S7C, increased expression of *Ascl1* was observed

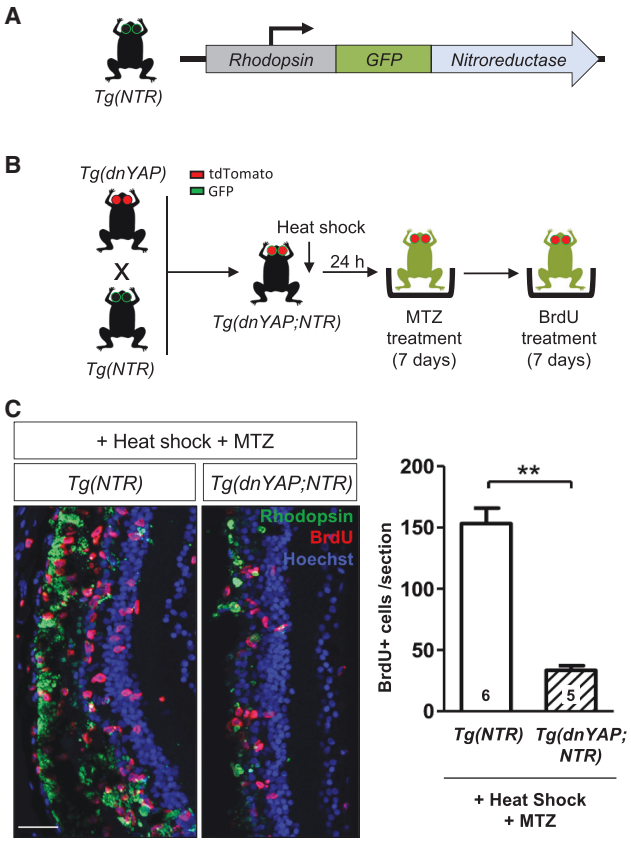



**Figure 4. Inhibiting YAP Activity in *Xenopus* Reduces the Proliferative Retinal Response to Photoreceptor Degeneration**

(A) Schematic representation of the transgene carried by the *Tg(NTR)* line. Transgenic animals can be selected based on GFP expression in rod cells (driven by the *Rhodopsin* promoter).

(B) Timeline diagram of the experimental procedures used in (C). *Tg(dnYAP)* were crossed with *Tg(NTR)* to generate double-transgenic *Tg(dnYAP;NTR)* animals. *Tg(dnYAP;NTR)* froglets (stage 61–66) were heat-shocked, transferred 24 h later for 7 days in a MTZ solution, and finally exposed for 7 more days to BrdU.

(C) Retinal sections from control [*Tg(NTR)*] and *Tg(dnYAP;NTR)* animals, immunostained for rhodopsin and BrdU. Nuclei are counterstained with Hoechst (blue). Note the scattered green staining indicative of photoreceptor degeneration. The number of retinas tested for each condition is indicated on the corresponding bar. Mann-Whitney test, $^{**}p \leq 0.01$. All results are reported as mean ± SEM. Scale bar: 50 μm.

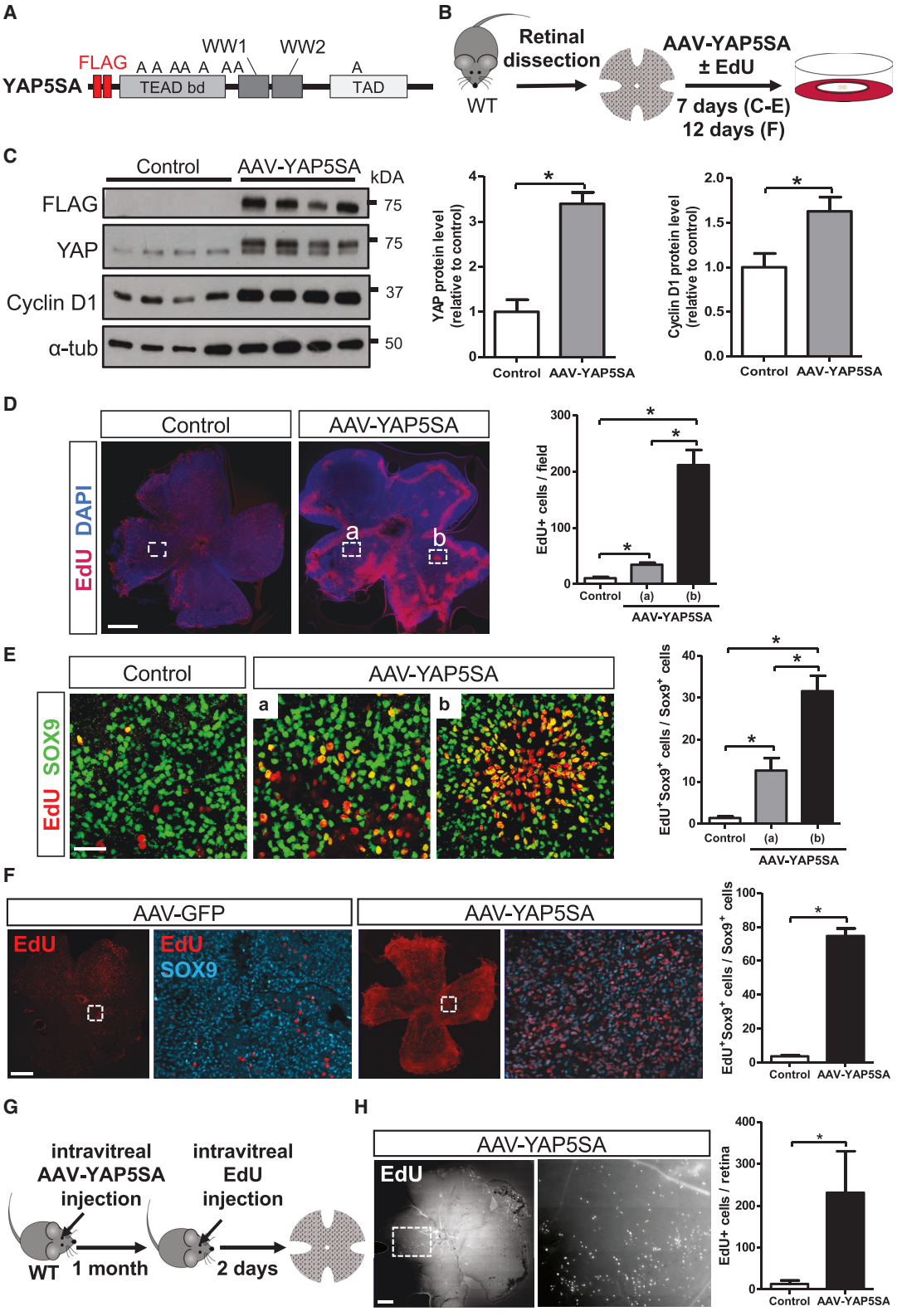
in AAV-YAP5SA-infected retinas. This is reminiscent of the zebrafish situation, where such upregulation occurs in dedifferentiating Müller cells in response to retinal injury (Ramachandran et al., 2010).

## Interfering with *Yap* Expression Affects EGFR Signaling in Mouse Reactive Müller Cells

We then sought to identify the molecular mechanisms underlying YAP effect on Müller glia cell-cycle re-entry. Besides cell-cycle genes, our RNA-sequencing (RNA-seq) dataset and pathway analysis also revealed a deregulation of several members of the EGFR pathway in the *Yap* CKO degenerative background (Figures S3A and S3C). Importantly, EGFR signaling is well known for its mitogenic effects on Müller cells during retinal degeneration. Two EGFR ligands, namely EGF or HB-EGF, have in particular been shown to stimulate Müller glia proliferation in zebrafish, chick, or rodents (Close et al., 2006; Karl et al., 2008; Todd et al., 2015; Wan et al., 2012, 2014). As observed with cell-cycle genes, both *Egfr* and two ligand-coding genes (*Hbegf* and *Neuregulin 1*) failed to be properly up-regulated upon MNU injection in *Yap* CKO retinas compared with control ones (Figure 6A). Expression of another EGFR-coding gene, *Erbb4* (named also *Her4*), did not appear sensitive to MNU injection in control retinas but was still found significantly decreased in MNU-injected *Yap* CKO mice (Figure 6A). In order to decipher whether these deregulations might be associated with defective EGFR signaling activity, we next assessed the status of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/AKT (also known as protein kinase B) pathways, which are known to be required for Müller cell proliferative response to growth factor treatment upon injury (Beach et al., 2017; Ueki and Reh, 2013; Wang et al., 2016). Western blot analysis revealed activation of the extracellular signal-related kinase (ERK) and AKT signaling pathways following MNU injection in control retinas. In the *Yap* CKO context, this increase was significantly attenuated, reflecting lower signaling activation (Figure 6B). Importantly, phosphorylated-ERK (P-ERK) immunostaining confirmed that this decrease was indeed happening in Müller cells: P-ERK labeling (which is barely detectable in non-injured retinas; data not shown) was localized in Müller cell nuclei and processes upon MNU injection and exhibited differential enhancement in control mice (strong signal) compared with *Yap* CKO ones (weaker signal; Figure 6C). Together, these results suggest that YAP is required for proper EGFR signaling through its transcriptional control on both EGFR ligands and receptors. In line with this idea, we found that YAP5SA was sufficient to upregulate *Egfr* expression upon AAV intraocular injections (Figures 6D and 6E). *Hbegf* levels showed a slight trend upward in retinas infected with AAV-YAP5SA, but this did not reach the level of statistical significance. This further supports the idea that YAP regulates the EGFR pathway in Müller glia.

## YAP Mitogenic Effects on Müller Cells Requires EGFR Pathway Activity

In order to investigate whether this YAP-EGFR interaction might converge on cell-cycle gene regulation, we attempted to rescue the *Yap* CKO phenotype through EGFR signaling activation. To this aim, we first decided to treat *Yap* CKO explants with HB-EGF, because this EGFR ligand failed to be properly upregulated upon MNU treatment in the absence of YAP and, in contrast, was found increased upon infection with AAV-YAP5SA (Figures 6A and 6E). As expected from our previous observations in MNU and *rd10* mice (Hamon et al., 2017), we found an increase in YAP level accompanying the degenerative process in explants (Figures 7A and 7B). Moreover, as described above with both paradigms (Figures 2D and S4B), this correlated with Cyclin D1 upregulation (Figures 7C and 7D, compare lanes 1 and 2), and this response was impaired in *Yap* CKO explants (Figure 7D,

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compare lanes 2 and 3). Following HB-EGF addition, Cyclin D1 levels were indistinguishable between *Yap* CKO and control explants (Figure 7D, compare lanes 4 and 5). We next attempted an *in vivo* rescue following intravitreal injection of HB-EGF in *Yap* CKO*;rd10* mice. We found that expression of both Cyclin D1 and Cyclin D3 could be restored to the untreated control level (Figures 7E and 7F, compare lanes 2 and 5). Finally, to strengthen the idea of YAP acting upstream the EGFR pathway in Müller glia cell-cycle gene regulation, we assessed whether blocking the EGFR pathway could impair YAP5SA mitogenic effects on Müller glia. We found indeed that pharmacological inhibition of Erk phosphorylation using explant treatment with U0126 (Figures 7G and 7H) dramatically decreased YAP5SA-dependent EdU incorporation in Müller cells (Figure 7I). By demonstrating the EGFR signaling requirement downstream of YAP activity, this result strongly supports a model whereby *in vivo* functional interaction between the two pathways promotes Müller cell proliferative response.

## DISCUSSION

Through back-and-forth investigations in both mouse and *Xenopus* retinas, we discovered a pivotal role for YAP in the regulation of Müller cell response to injury. We in particular reveal that YAP triggers their exit from quiescence in a degenerative context. In *Xenopus*, this is accompanied with intense proliferation, but not in mouse. We, however, demonstrate that enhancing YAP activity is sufficient to boost the naturally limited proliferative potential of mammalian Müller glia. In addition, our findings unravel a YAP-EGFR axis in Müller glia cell-cycle reentry that sheds a new light on the genetic network underlying their recruitment following retinal injury.

YAP knockout in several mammalian organs, such as liver, pancreas, intestine, and mammary gland, unexpectedly suggested that this factor is dispensable to maintain normal adult tissue homeostasis (Piccolo et al., 2014). In line with this, we did not observe any major abnormalities in *Yap* CKO retinal morphology and function. We, however, revealed YAP requirement in reactive Müller glia. Reactive gliosis occurs upon retinal stress or injury (Bringmann et al., 2009) and includes a series of characteristic morphological and molecular changes. Importantly, a feature of reactive Müller cells is their exit from a quiescent G0 state. Although their cell cycle rarely reaches S phase, G0 to G1 progression is in particular materialized by the upregulation of genes encoding components of Cyclin D-Cyclin-dependent kinase (CDK) complexes, known to drive early to mid-G1 phase progression (Suga et al., 2014). We found that many cell-cycle genes, including *Ccnd* and *Cdk* genes, are downregulated in *Yap* CKO reactive Müller cells, suggesting that this process is impaired in the absence of YAP. Many different transcription factors have been identified that directly regulate *Ccnd1* promoter (Wang et al., 2004). Remarkably, YAP has been described as one of them in cancer cells (Mizuno et al., 2012). In addition, *Ccnd1* was shown to be activated by YAP overexpression in the chick neural tube (Cao et al., 2008). However, in that study, the authors reported that YAP is not required for its basal transcription. In contrast, we found in Müller cells that YAP is necessary both to maintain basal levels of Cyclin D1 in physiological conditions and for enhancing its expression upon injury. This reinforces the hypothesis that *Ccnd1* may be a direct YAP target gene in Müller glia. This could also be the case for *Cdk6*, as previously reported in a human fibroblastic cell line (Xie et al., 2013). Although our data suggest that YAP functions upstream of the EGFR pathway in the regulation of cell-cycle genes, we thus do not exclude EGFR-independent mechanisms as well.

Although dispensable in several homeostatic contexts, YAP is now well recognized as a central player in the regeneration of diverse tissues in different organisms (Barry and Camargo, 2013). As far as *Xenopus* is concerned, its importance in tissue repair had previously been demonstrated in the context of epimorphic limb and tail regeneration (Hayashi et al., 2014a, 2014b). We here bring new insights to the field by highlighting its requirement for *Xenopus* Müller cell proliferation in a lesioned or degenerative context. In mammals, YAP overexpression or Hippo pathway inhibition was already reported to stimulate regeneration of several injured organs, such as the heart, liver,

**Figure 5. YAP Overexpression in Mouse Müller Cells Triggers Their Proliferative Response**

(A) Schematic representation of the constitutively active FLAG-tagged YAP5SA construct. The "A" letters indicate the positions of the serine-to-alanine substitutions. TAD, transcription activation domain; TEAD bd, TEAD binding domain; WW1 and WW2, WW domains.

(B) Timeline diagram of the experimental procedure used in (C–F). Retinas from WT mice were flattened, infected with AAV-GFP (control) or AAV-YAP5SA, and cultured for 7 (C–E) or 12 days (F). In (D–F), EdU was added to the culture medium.

(C) Western blot analysis of YAP (using an anti-FLAG or an anti-YAP antibody) and Cyclin D1 expression. α-Tubulin labeling was used to normalize the signal. n = 4 explants for each condition.

(D) EdU labeling on whole flat-mounted retinas, 7 days after AAV infection. Nuclei are counterstained with DAPI (blue). The "b" box corresponds to a region exhibiting a high concentration of EdU-positive cells (~20-fold increase compared with the control), whereas the "a" box shows an area outside such patches (~3-fold increase compared with the control). n = 3 explants for each condition.

(E) Enlargement of the explant regions delineated with white boxes in (D), showing $EdU^+/SOX9^+$ doubled-labeled cells (yellow) in the inner nuclear layer. n = 3 explants for each condition. In AAV-YAP5SA-infected explants, $EdU^+$ (D) or $EdU^+/SOX9^+$ (E) cells were quantified outside (a) or inside (b) EdU patches.

(F) EdU and Sox9 co-labeling on whole flat-mounted retinas, 12 days after AAV infection. Enlargements show the inner nuclear layer of explant regions delineated with white boxes. n = 3 explants for each condition.

(G) Timeline diagram of the experimental procedure used in (H). WT mice were intravitreally injected with AAV-GFP (control) or AAV-YAP5SA. EdU was administered into the eye 1 month later. Retinas were subsequently flattened and subjected to EdU labeling.

(H) EdU labeling of a flat-mounted retina following *in vivo* infection with AAV-YAP5SA. The enlargement shows the retinal region delineated with the white box. n = 7 (control) or 12 (AAV-YAP5SA) retinas.

Mann-Whitney test, *$p \leq 0.05$. All results are reported as mean ± SEM. Scale bars, 500 μm (D, F, and H); 20 μm (E).

See also Figures S6 and S7.

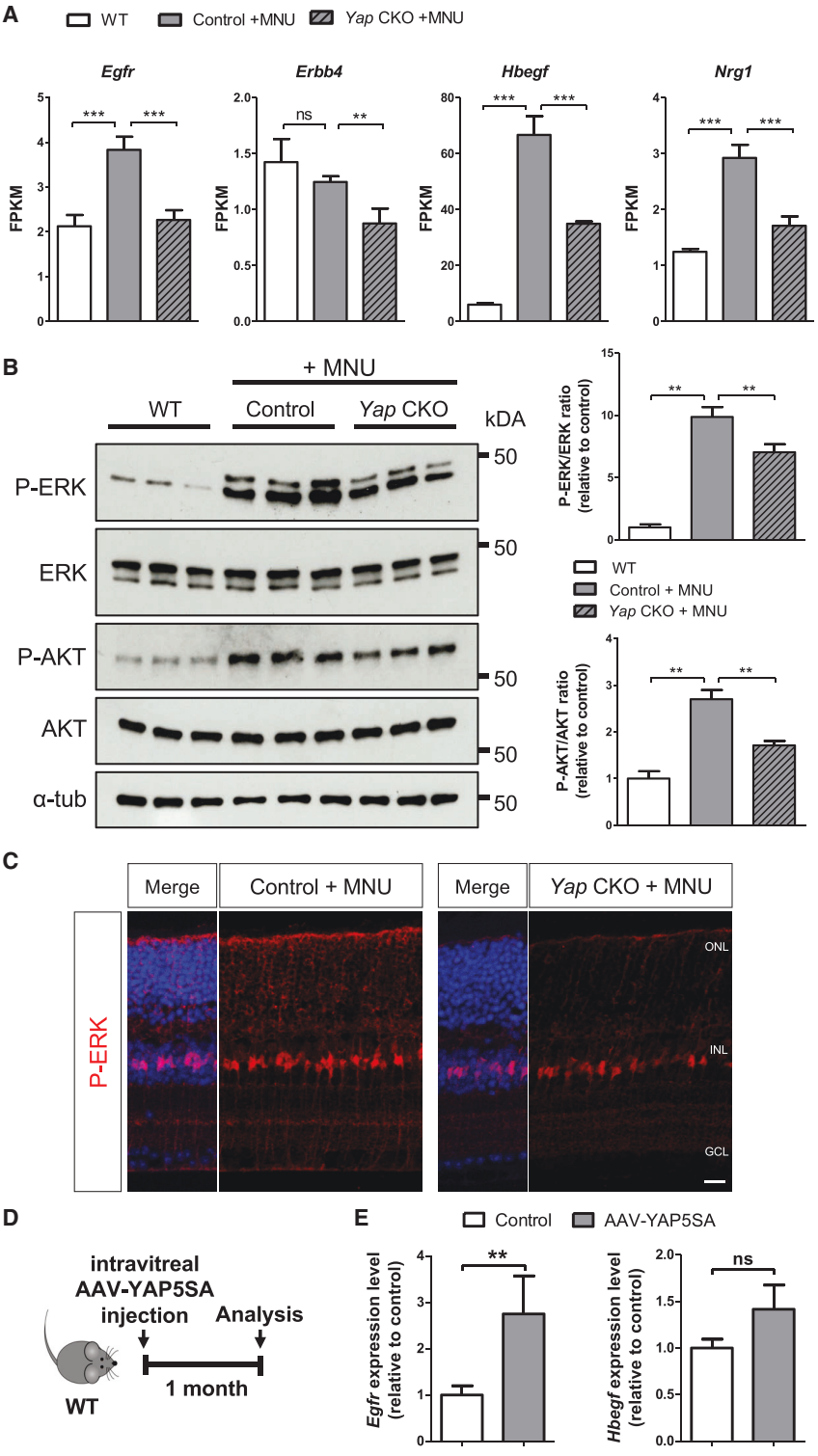


**Figure 6. YAP Regulates the Expression of EGFR Signaling Components**

(A) Relative RNA expression (in fragments per kilobase of exon per million fragments mapped [FPKM]; data retrieved from the RNA-seq experiment) of *Egfr*, *Erbb4*, *Hbegf*, and *Neuregulin1* (*Nrg1*), in retinas from non-injected WT mice or control and *Yap* CKO mice injected with 4-OHT and MNU as shown in Figure 1F.

(B) Western blot analysis of P-ERK/ERK and P-AKT/AKT ratios on the same experimental conditions. α-Tubulin labeling was used to normalize the signal. n = 6 mice for each condition.

(C) Retinal sections from control and *Yap* CKO mice, immunostained for P-ERK. Nuclei are counterstained with DAPI (blue).

(D) Timeline diagram of the experimental procedure used in (E). WT mice were intravitreally injected with AAV-GFP (control) or AAV-YAP5SA. Retinas were then harvested 1 month later for qPCR analysis.

(E) qRT-PCR analysis of *Hbegf* and *Egfr* expression (10 biological replicates per condition). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Mann-Whitney test (except in A where p values were obtained using EdgeR), $*p \leq 0.05$, $**p \leq 0.01$; $***p \leq 0.001$. All results are reported as mean ± SEM. ns, non-significant. Scale bar: 20 μm.

See also Figure S3.

or intestine (Johnson and Halder, 2014; Loforese et al., 2017; Wang et al., 2018). Furthermore, it was recently discovered that YAP/TAZ can act as reprogramming factors, able to turn differentiated cells into their corresponding somatic stem cells (Monroe et al., 2019; Panciera et al., 2016). We here report that enhancing YAP activity awakes quiescent Müller cells and powerfully boosts their proliferative properties both *ex vivo* and *in vivo*. This might be accompanied by their dedifferentiation as inferred from the up-regulation of *Ascl1* expression. Investigating this issue will require assessing whether other retinal progenitor markers are re-expressed in these proliferating Müller cells in order to further characterize their identity. Whether the increased proliferation of YAP-overexpressing Müller cells leads to the production of new neuronal cells will also be an important point to address. Interestingly, following conditional overexpression of YAP5SA, Rueda et al. (2019 [this issue of *Cell Reports*]) confirmed Müller glia cell-cycle re-entry and further showed that a very small subset of reactivated cells may indeed differentiate into neurons. Given YAP efficient mitogenic activity, getting higher rates of differentiation will presumably require (1) controlling its expression in a defined time period using genetic tools allowing for its transient activation (for instance, a doxycycline-inducible construct as previously described) (Panciera et al., 2016); and (2) overexpressing afterward transcription factors known to promote

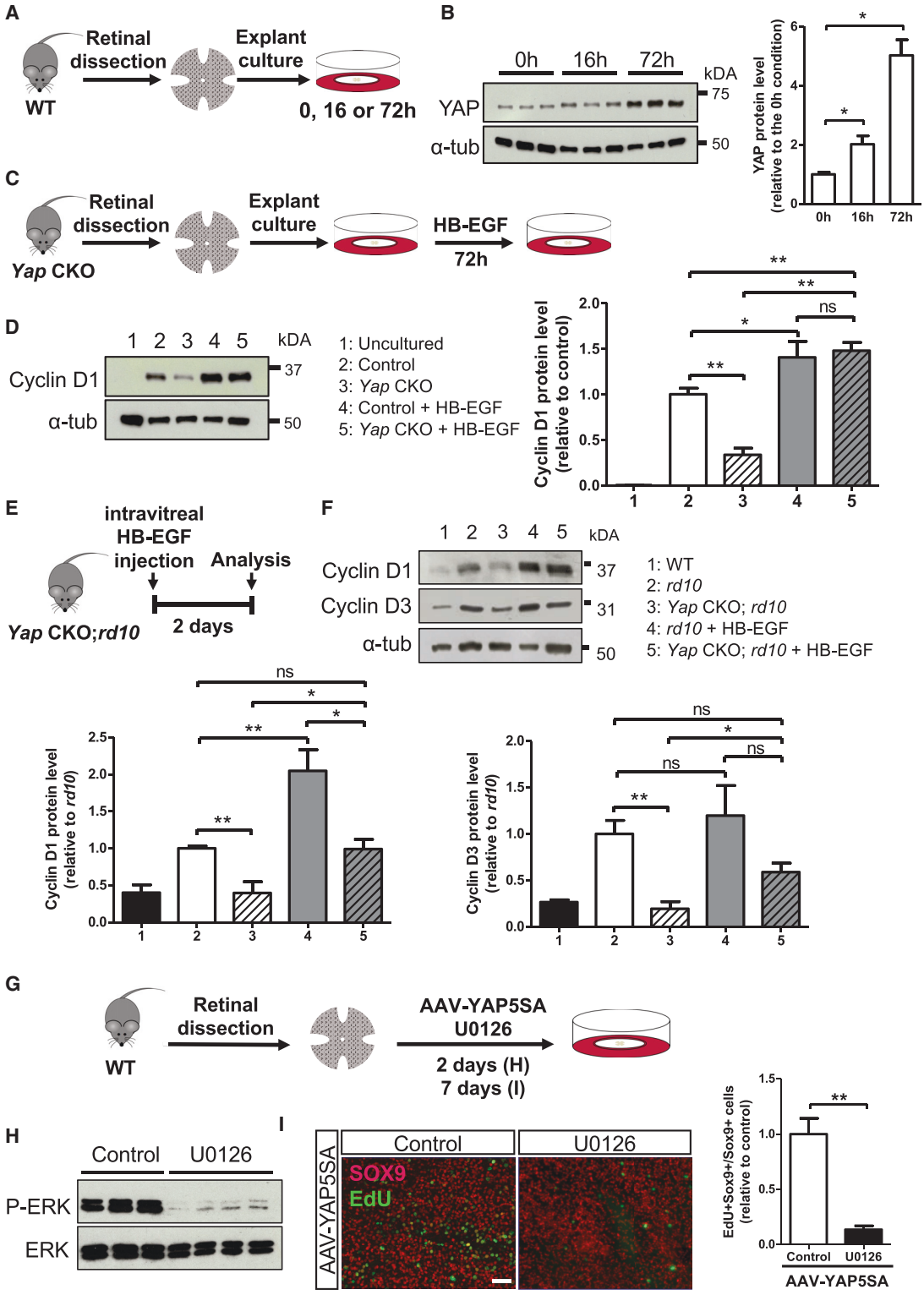


**Figure 7. EGFR Signaling Is Required for YAP-Induced Proliferation of Müller Cells**

(A) Timeline diagram of the experimental procedure used in (B). Retinas from WT mice were flattened and cultured for 0, 16, or 72 h.

(B) Western blot analysis of YAP expression on retinal explant extracts. α-Tubulin labeling was used to normalize the signal. n = 3 mice for each condition.

*(legend continued on next page)*

neuronal specification, as reported in a recent study describing Wnt-dependent retinal regeneration (Yao et al., 2018).

YAP is now well recognized as a molecular hub connecting several key signaling pathways (Barry and Camargo, 2013). In animal models harboring retinal regeneration properties such as zebrafish or chick (Hamon et al., 2016; Kaur et al., 2018; Wan and Goldman, 2016), several factors, like Notch, Wnt, or Shh, were shown to regulate Müller cell proliferative response. Given the known interplay between YAP and these pathways in various cellular contexts (Lin et al., 2012; Yu et al., 2015), it would be interesting to seek for their potential functional interactions in *Xenopus* Müller cell-dependent retinal regeneration. In mammals, Hippo/Wnt cross-talks are in particular well documented (Hansen et al., 2015), and Wnt is known to efficiently stimulate Müller cell proliferation following injury (Yao et al., 2018). However, our RNA-seq analysis did not highlight Wnt among the pathways that are deregulated upon *Yap* deletion. In contrast, we here revealed that YAP is required for proper expression and activity of EGFR pathway components in Müller cells following retinal degeneration. Such functional interaction was previously reported in other contexts. The EGFR ligand amphiregulin (AREG) was in particular shown to be regulated by YAP in human mammary epithelial cells or in cervical cancer cells (He et al., 2015a; Zhang et al., 2009). We did not identify *Areg* as deregulated in *Yap* CKO retinas, but we retrieved in our RNA-seq dataset four genes encoding either ligands (HB-EGF and Neuregulin1) or receptors (EGFR and ERBB4) of the pathway. Interestingly, all were reported as direct YAP target genes in human ovarian cells (He et al., 2015b), suggesting that it could be the case in Müller cells as well. Besides, our results demonstrate that EGFR signaling activity is required for YAP mitogenic effects on Müller cells. Considering that EGFR signaling is a key pathway inducing Müller glia cell-cycle re-entry (Close et al., 2005, 2006; Karl et al., 2008; Löffler et al., 2015; Todd et al., 2015; Ueki and Reh, 2013; Wan et al., 2012, 2014), such functional interaction brings YAP at the core of Müller cell reactivation mechanisms. Altogether, we propose the YAP-EGFR axis as a central player in Müller glia response to retinal damage. Interestingly, this is reminiscent of the intestinal regeneration situation, where YAP-dependent EGFR signaling has previously been reported to drive tissue repair upon injury (Gregorieff et al., 2015). Noteworthy, it was reported that EGF-induced proliferation of Müller cells is greatly altered with age, with a proportion of proliferating cells decreasing from 86% at P8 to 9% at P12 following 6 days of explant culture (Löffler et al., 2015). YAP5SA appears much more potent as we estimated the proportion of proliferative Müller cells at about 25% at P30 following 7 days of explant culture. In addition, although EGF can stimulate Müller glia proliferation in a degenerative context, it does not have any mitogenic effect in undamaged chick or mouse retinas (Todd et al., 2015). The same holds true for the proneural transcription factor ASCL1, which promotes Müller cell reprogramming and proliferation following retinal damage, but not in the intact retina (Ueki et al., 2015). Contrasting with these data, we observed that forced YAP5SA expression *in vivo* is sufficient to promote Müller glia cell-cycle re-entry in a non-degenerative context. Altogether, this strongly suggests that YAP not only regulates the EGFR pathway but probably other ones, which results in robust mitogenic stimulation of Müller glia and this even in the uninjured retina.

As a whole, by identifying YAP as a powerful inducer of Müller glia proliferation, our findings open new avenues for research aimed at developing therapeutic strategies based on endogenous repair of the retina.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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(C) Timeline diagram of the experimental procedure used in (D). Retinas from control and *Yap* CKO mice were cultured for 72 h with or without HB-EGF.
(D) Western blot analysis of Cyclin D1 expression on retinal explant extracts. α-Tubulin labeling was used to normalize the signal. n = 3 explants for the uncultured condition; n = 6 explants for all other conditions.
(E) Timeline diagram of the experimental procedure used in (F). WT, *rd10*, or *Yap* CKO;*rd10* mice were intravitreally injected with PBS (control vehicle) or HB-EGF. Retinas were then harvested 2 days later.
(F) Western blot analysis of Cyclin D1 and Cyclin D3 expression on retinal extracts. α-Tubulin labeling was used to normalize the signal. n = 3–5 retinas for each condition.
(G) Timeline diagram of the experimental procedure used in (H) and (I). Retinas from WT mice were flattened, infected with AAV-YAP5SA, and cultured for 2 or 7 days in the presence of U0126 or vehicle (control).
(H) Western blot analysis of pERK and ERK expression. Shown are results from three different explants for each condition.
(I) EdU and Sox9 co-labeling in the inner nuclear layer of retinal explants. n = 6 explants for the control and 5 for the U0126-treated condition.
Mann-Whitney test, *p ≤ 0.05, **p ≤ 0.01. All results are reported as mean ± SEM. ns, non-significant. Scale bar: 50 μm.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.045.

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## AUTHOR CONTRIBUTIONS

A.H., D.G.-G., D.A., J.B., A.C., and J.E.R. designed and performed the experiments and analyzed the data; D.D. supervised AAV production; M.L. revised the manuscript; M.P. designed the study, analyzed the data, wrote the manuscript with the help of A.H. and D.A., and supervised the study.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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

## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse monoclonal anti-Akt | Sigma-Aldrich | Cat#P2482; RRID: AB_260913 |
| Mouse monoclonal anti-alpha-tubulin | Sigma-Aldrich | Cat#T5168; RRID: AB_477579 |
| Mouse monoclonal anti-alpha-tubulin | Sigma-Aldrich | Cat#T9026; RRID: AB_477593 |
| Rabbit polyclonal anti-arrestin | EMD Millipore | Cat#AB15282; RRID: AB_1163387 |
| Mouse monoclonal anti-Brn3a | Santa Cruz | Cat#Sc-8429; RRID: AB_626765 |
| Rabbit polyclonal anti-calbindin D-28k | Swant | Cat#300; RRID: AB_10000347 |
| Mouse monoclonal anti-calretinin | EMD Millipore | Cat#MAB1568; RRID: AB_94259 |
| Rat monoclonal anti-CD68 | Bio-Rad UK | Cat#MCA1957; RRID: AB_322219 |
| Rabbit polyclonal anti-cyclin D1 | Abcam | Cat#ab134175; RRID: AB_2750906 |
| Mouse monoclonal anti-cyclin D3 | Cell Signaling | Cat#2936; RRID: AB_2070801 |
| Rabbit polyclonal anti-p44/42 MAPK (ERK1/2) | Cell Signaling | Cat#9102; RRID: AB_330744 |
| Rabbit polyclonal anti-FLAG | Cell Signaling | Cat#F7425; RRID: AB_439687 |
| Rabbit polyclonal anti-GFAP | Dako | Cat#Z0334; RRID: AB_10013382 |
| Goat polyclonal anti-GFP | Abcam | Cat#ab6673; RRID: AB_305643 |
| Mouse monoclonal anti-glutamine synthetase | Abcam | Cat#ab64613; RRID: AB_1140869 |
| Peanut anti-Lectin PNA alexa 568 conjugate | Thermo Fisher Scientific | Cat#L32458 |
| Rabbit polyclonal anti-phospho-Akt (Ser473) | Cell Signaling | Cat#9271; RRID: AB_329825 |
| Rabbit polyclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | Cell Signaling | Cat#4370; RRID: AB_2315112 |
| Rabbit polyclonal anti-Red Fluorescence Protein | Rockland | Cat#600-401-379; RRID: AB_2209751 |
| Mouse monoclonal anti-Rhodopsin | EMD Millipore | Cat#MAB5316; RRID: AB_2156055 |
| Mouse monoclonal anti-Rhodopsin | EMD Millipore | Cat#MABN15; RRID: AB_10807045 |
| Rabbit polyclonal anti-opsin blue | EMD Millipore | Cat#AB5407; RRID: AB_177457 |
| Rabbit polyclonal anti-SOX9 | EMD Millipore | Cat#AB5535; RRID: AB_2239761 |
| Rabbit polyclonal anti-TAZ | Abcam | Cat#ab110239 |
| Rabbit polyclonal anti-Vimentin | Santa Cruz | Cat#sc-7557; RRID: AB_793998 |
| Mouse monoclonal anti-YAP | Abcam | Cat#ab56701; RRID: AB_2219140 |
| Rabbit monoclonal anti-YAP | Abcam | Cat#ab52771; RRID: AB_2219141 |
| Goat anti-mouse IgG1, Alexa 488 | Thermo Fisher Scientific | Cat#A21121; RRID: AB_141514 |
| Donkey anti-rabbit, Alexa 488 | Thermo Fisher Scientific | Cat#A21206; RRID: AB_141708 |
| Goat anti-mouse IgG1, Alexa 555 | Thermo Fisher Scientific | Cat#A21127; RRID: AB_141596 |
| Goat anti-mouse IgG2b, Alexa 555 | Thermo Fisher Scientific | Cat#A21147; RRID: AB_1500897 |
| Goat anti-mouse IgG2a, Alexa 555 | Thermo Fisher Scientific | Cat#A21127; RRID: AB_141596 |
| Donkey anti-rabbit, Alexa 555 | Thermo Fisher Scientific | Cat#A31572; RRID: AB_162543 |
| Goat anti-mouse, Alexa 488 | Thermo Fisher Scientific | Cat#A11001; RRID: AB_2534069 |
| Goat anti-mouse, Alexa 594 | Thermo Fisher Scientific | Cat#A11005; RRID: AB_141372 |
| Goat anti-rat, Alexa 594 | Thermo Fisher Scientific | Cat#A11007; RRID: AB_141374 |
| Goat anti-rabbit, Alexa 488 | Thermo Fisher Scientific | Cat#A11008; RRID: AB_143165 |
| Goat anti-rabbit, Alexa 594 | Thermo Fisher Scientific | Cat#A11012; RRID: AB_141359 |
| Goat anti-mouse IgG HRP conjugate | Sigma-Aldrich | Cat#A4416; RRID: AB_258167 |
| Donkey anti-rabbit IgG HRP conjugate | GE Health | Cat#NA934; RRID: AB_772206 |

(*Continued on next page*)

***Continued***

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and Virus Strains | | |
| AAV-GFP | Klimczak et al., 2009 | N/A |
| AAV-YAP5SA | This paper | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| 4-hydroxytamoxifen | Sigma-Aldrich | Cat#H6278 |
| Human recombinant HB-EGF | R&D systems | Cat#259-HE |
| U0126 | Abcam | Cat# ab120241 |
| 1-Methyl-1-nitrosourea (MNU) | Trinova Biochem | N/A |
| Metronidazole (MTZ) | Sigma-Aldrich | Cat# M-183 |
| BrdU | Roche | Cat#10280879001 |
| EdU | Thermo Fisher Scientific | Cat# D1756 |
| DAPI | Thermo Fisher Scientific | Cat#62248 |
| Hoechst | Sigma-Aldrich | Cat#H6024 |
| MS-222 | Sigma-Aldrich | Cat#A5040-25G |
| Rompun 2% (Xylasine) | Bayer | N/A |
| Imalgene 500 (Kétamine) | MERIAL SAS | N/A |
| Dexamethasone | Sigma-Aldrich | Cat#D4902 |
| Critical Commercial Assays | | |
| RNA 6000 Nano Kit | Agilent Technologies | Cat#5067-1511 |
| TruSeq Stranded mRNA Library Preparation Kit | Illumina | Cat#20020594 |
| RNeasy mini kit | QIAGEN | Cat#74104 |
| NucleoSpin RNA Plus kit | Macherey Nagel | Cat#740984.50 |
| mMessage mMachine kit | Life Technologies | Cat#AM1340 |
| DC Protein Assay | Bio-Rad | Cat# 500-0116 |
| SuperScript II Reverse Transcriptase kit | Thermo Fisher Scientific | Cat#18064-071 |
| SsoFast EvaGreen supermix | Bio-Rad | Cat#172-5204 |
| SYBR select master mix | Thermo Fisher Scientific | Cat#4472908 |
| DeadEnd Fluorometric TUNEL System | Promega | Cat#G3250 |
| Click-iT Plus EdU Alexa Fluor 555 Imaging Kit | Thermo Fisher Scientific | Cat#C10338 |
| Click-iT Plus EdU Alexa Fluor 647 Imaging Kit | Thermo Fisher Scientific | Cat#C10640 |
| Click-iT Plus EdU Alexa Fluor 488 Imaging Kit | Thermo Fisher Scientific | Cat#C10337 |
| Deposited Data | | |
| RNaseq dataset | This paper | GEO: GSE121858 |
| RNaseq dataset | Hamon et al., 2017 | GEO: GSE94534 |
| Experimental Models: Organisms/Strains | | |
| Mouse: C57Bl6/J | Jackson Laboratory | N/A |
| Mouse: B6.CXB1-*Pde6b*$^{rd10}$/J | Bo Chang; Chang et al., 2007 | N/A |
| Mouse: *Yap*$^{flox/flox}$ | Jeff Wrana; Reginensi et al., 2013 | N/A |
| Mouse: *Rax-CreER*$^{T2}$ | Seth Blackshaw; Pak et al., 2014 | N/A |
| Mouse: *Rax-CreER*$^{T2}$, *R26-CAG-lox-stop-lox-TdTom (Ai9)* | Seth Blackshaw; Pak et al., 2014 | N/A |
| *Xenopus laevis*: WT | Centre de Ressources Biologiques Xénopes (CRB) | N/A |
| *Xenopus laevis*: *Tg(hsp70:dnYap-GFP, cryga:tdTomato)* | UK *Xenopus* resource center ; Hayashi et al., 2014b | N/A |
| *Xenopus laevis: Tg(Rho:GFP-NTR)* | Langhe et al., 2017 | N/A |
| Oligonucleotides | | |
| Primers for genotyping, see Table S2 | | N/A |
| Primers for RT-qPCR, see Table S2 | | N/A |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Recombinant DNA | | |
| Plasmid: pCMV-flag-YAP2-5SA | Kun-Liang Guan | Addgene plasmid#27371; RRID: Addgene_27371 |
| Plasmid: pCS2-YapS98A | Cabochette et al., 2015 | N/A |
| Plasmid: pCS2-flag-YapS98A-GR | This paper | N/A |
| Software and Algorithms | | |
| LabScribe software | iWorx | https://www.iworx.com/research/software/labscribe/ |
| Fiji | National Institutes of Health | https://fiji.sc/ |
| HISAT2 2.1.0 algorithm | Kim et al., 2015; Pertea et al., 2016 | N/A |
| FeatureCounts algorithm | Liao et al., 2014 | http://bioinf.wehi.edu.au/featureCounts/ |
| EdgeR algorithm | Robinson et al., 2010 | N/A |
| PANTHER | | http://pantherdb.org/ |
| Kyoto Encyclopedia of Genes and Genome | Kanehisa Laboratories | https://www.genome.jp/kegg/ |
| GOplot | Walter et al., 2015 | http://wencke.github.io |
| Zen software | Zeiss | N/A |
| Photoshop CS4 software | Adobe | N/A |
| GraphPad Prism 5.01 | GraphPad Software | https://www.graphpad.com/ |

## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Muriel Perron (muriel.perron@u-psud.fr).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal experiments have been carried out in accordance with the European Community Council Directive of 22 September 2010 (2010/63/EEC). All animal cares and experimentations were conducted in accordance with institutional guidelines, under the institutional license D 91-272-105 for mice and the institutional license C 91-471-102 for *Xenopus*. The study protocols were approved by the institutional animal care committee CEEA n°59 and received an authorization by the ''Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche'' under the reference APAFIS#1018-2016072611404304v1 for mice experiments and APAFIS#998-2015062510022908v2 for *Xenopus* experiments.

### Mouse lines and degenerative models

Mice were kept at 21°C, under a 12-hour light/12-hour dark cycle, with food and water supplied *ad libitum*. *Yap*$^{flox/flox}$ mice were obtained from Jeff Wrana's lab (Reginensi et al., 2013) and crossed with heterozygous *Rax-CreER*$^{T2}$ knock-in mice or double heterozygous *Rax-CreER*$^{T2}$, *R26-CAG-lox-stop-lox-TdTom (Ai9)* mice from Seth Blackshaw's lab (Pak et al., 2014) to generate *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ (*Yap CKO*) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$;*Ai9* (*Yap CKO; Ai9*) mice. Primer sequences used for genotyping tail snip genomic DNA are provided in Table S2. Cre activity was induced through a single intraperitoneal injection of 4-hydroxytamoxifen (4-OHT; 1 mg) at P10, as previously described (Pak et al., 2014). To generate the *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$;*rd10* line (*Yap CKO; rd10*), *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ mice were crossed with homozygous *rd10* mice (*Pde6b*$^{rd10}$; a model of retinitis pigmentosa, with a mutation in *phosphodiesterase-6b* (*Pde6b*) gene (Chang et al., 2007); The Jackson Laboratory, Bar Harbor, ME, USA). Chemically-induced retinal degeneration was performed through a single intraperitoneal injection of 1-Methyl-1-nitrosourea (MNU, Trinova Biochem) at 60 mg/kg body weight, as previously described (Hamon et al., 2017). All experiments involving adult mice were performed with male or female mice that were 3 to 8 weeks. No difference between sexes was observed in any retinal phenotype.

### *Xenopus* lines and regeneration models

*Xenopus laevis* tadpoles were obtained by conventional procedures of *in vitro* or natural fertilization, staged according to Nieuwkoop and Faber method (Nieuwkoop and Faber, 1994) and raised at 18-20°C. Heat-shock-inducible dominant-negative YAP transgenic line *Tg(hsp70:dnYap-GFP, cryga:tdTomato)* (Hayashi et al., 2014b) (*Tg(dnYAP)*) was obtained from the UK *Xenopus* resource center (EXRC). Heat-shock-mediated dnYAP expression was induced by shifting the animals from 18-20°C to 34°C for 30 min as previously described (Hayashi et al., 2014b). The transgenic *Xenopus* line *Tg(Rho:GFP-NTR)* (*Tg(NTR)*) used for conditional rod ablation was

generated in the lab and previously described (Langhe et al., 2017). Photoreceptor degeneration was induced by bathing the froglets in a 10 mM MTZ (Sigma-Aldrich) solution for 1 week (Langhe et al., 2017). Retinal mechanical injury was performed as previously described (Langhe et al., 2017), by poking the retina once under a stereomicroscope with a needle (Austerlitz Insect Pins, 0.2 mm), without damaging the cornea or the lens. For cell proliferation assays, *Xenopus* tadpoles (i.e., pre-metamorphic individuals) or froglets (i.e., post-metamorphic individuals) were immersed in a solution containing 1 mM BrdU (5′-bromo-2′-deoxyuridine, Roche) for 3 days or 1 week, as indicated. The solution was renewed every other day.

## METHOD DETAILS

### Microinjection in *Xenopus* embryos

The *Xenopus* construct *YapS98A,* encoding a constitutively active YAP protein (Ser-98 residue substituted with an alanine), was provided by S Gee and S Moody (Gee et al., 2011) and subcloned into pCS2$^{+}$ (pCS2-*YapS98A*) (Cabochette et al., 2015). A glucocorticoid-inducible FLAG-tagged version of YAPS98A (YAPS98A-GR) was then generated by subcloning in frame the *YapS98A* coding sequence from pCS2-*YapS98A* plasmid into the EcoRI and XhoI sites of pCS2-*flag-GR* (Talikka et al., 2002) (pCS2-*flag-YapS98A-GR*). Following *in vitro* transcription (mMessage mMachine kit, Life Technologies), 200 pg of mRNA were injected at the one-cell stage. *LacZ* mRNAs were injected as controls. Activity of the chimeric YAPS98A-GR protein was induced by addition of 4 mg/ml dexamethasone (dex, Sigma-Aldrich) into the embryo rearing medium.

### Mouse retinal explants

Retinas from enucleated P30 eyes were dissected in Hanks' Balanced Salt solution (GIBCO) by removing the anterior segment, vitreous body, sclera and RPE. They were then flat-mounted onto a microporous membrane (13 mm in diameter; Merk Millipore) in a twelve-well culture plate, with the ganglion cell layer facing upward. Each well contained 700 μL of culture medium, consisting in DMEM-Glutamax (GIBCO) supplemented with 1% FBS, 0.6% D-Glucose, 0.2% $NaHCO_3$, 5mM HEPES, 1% B27, 1% N2, 1X Penicillin-Streptomycin. Culture medium containing 100 ng/mL human recombinant HB-EGF (R&D systems), 10 μM of U0126 (Abcam) or vehicle was added from the beginning of the explant culture. Explants were maintained at 37°C in a humidified incubator with 5% $CO_2$. Half of the culture medium was changed daily. For proliferation assays, 20 mM EdU was applied 7 or 12 days before fixation.

### AAV production and retinal transduction

Human YAP5SA cDNA was amplified by PCR from pCMV-flag-YAP2-5SA plasmid (a gift from Kunliang Guan, Addgene plasmid#27371; http://addgene.org/27371; RRID:Addgene_27371) and subcloned into an AAV transfer plasmid, where the expression is driven by the minimal cytomegalovirus (CMV) promoter (Klimczak et al., 2009). AAV vectors were produced as already described using the co-transfection method and purified by iodixanol gradient ultracentrifugation (Choi et al., 2007). AAV vector stocks were tittered by qPCR using SYBR Green (Thermo Fisher Scientific) (Aurnhammer et al., 2012). The previously engineered AAV-GFP (Klimczak et al., 2009) was used as a control. $10^{11}$ vg of AAV-GFP or AAV-YAP5SA were applied on mouse retinal explants for viral transduction. Infected explants were cultured for 7 or 12 days as indicated before further western blot analysis or EdU labeling.

### Intravitreal injection

Mice were first anesthetized through intraperitoneal injection of ketamine (90 mg/ kg, Merial) and xylazine (8 mg/kg, Bayer). They were then topically administered tropicamide (0.5%) and phenylephrine (2.5%) for pupillary dilation. While applying gentle pressure around the eye socket to extrude the eye, a 30-gauge needle was passed through the sclera just behind the limbus, into the vitreous cavity. Injection of 2 μl of AAV ($10^{13}$ vg/ml) or 1 μl of 100 μg/mL HB-EGF (R&D systems) or 1 μl of 1 μg/μl EdU (Thermo Fisher Scientific) was made with direct observation of the needle in the center of the vitreous cavity.

### Electroretinography

Electroretinograms (ERGs) were recorded using a Micron IV focal ERG system (Phoenix Research Labs). Mice were dark-adapted overnight, prepared for recording in darkness under dim-red illumination and finally anesthetized as described above. Flash ERG recordings were obtained from one eye. ERG responses were recorded using increasing light intensities ranging from −1.7 to 2.2 log cd·s/m$^2$ under dark-adapted conditions, and from −0.5 to 2.8 log cd·s/m$^2$ under a background light that saturates rod function. The interval between flashes varied from 0.7 s at the lowest stimulus strength to 15 s at the highest one. Five to thirty responses were averaged depending on flash intensity. Analysis of a-wave and b-wave amplitudes was performed using LabScribeERG software. The a-wave amplitude was measured from the baseline to the negative peak and the b-wave was measured from the baseline to the maximum positive peak.

### Western-blotting

Each protein extract was obtained from a single retina, quickly isolated and frozen at −80°C. Retinas were then lysed in P300 buffer (20 mM $Na_2HPO_4$; 250 mM NaCl; 30 mM NaPPi; 0.1% Nonidet P-40; 5 mM EDTA; 5mM DTT) supplemented with protease inhibitor

cocktail (Sigma-Aldrich). Lysate concentration was determined using the Lowry protein assay kit (DC Protein Assay; Bio-Rad) and 20 μg/lane of each sample were loaded for western-blot analysis. Between 3 and 6 individuals were tested per condition. Primary/secondary antibodies and their corresponding working dilutions are listed in Table S3. Protein detection was performed using an enhanced chemiluminescence kit (Bio-Rad). Each sample was probed once with anti-α-tubulin antibody for normalization. Quantification was done using Fiji software (National Institutes of Health) (Schindelin et al., 2012).

### Whole transcriptome sequencing and data analysis

Whole transcriptome analysis was performed on three independent biological replicates from MNU-injected control and MNU-injected *Yap* CKO retinas at P60 and compared to WT dataset previously published (Hamon et al., 2017) (GEO accession number GSE94534). After harvesting, both retinas for each animal were immediately frozen. RNA was extracted using Nucleospin RNA Plus kit (Macherey-Nagel). RNA quality and quantity were evaluated using a BioAnalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies). Stranded RNA-Seq libraries were constructed from 100 ng high-quality total RNA (RIN > 8) using the TruSeq Stranded mRNA Library Preparation Kit (Illumina). Paired-end sequencing of 40 bases length was performed on a NextSeq 500 system (Illumina). Pass-filtered reads were mapped using HISAT2 2.1.0 and aligned to mouse reference genome GRCm38 (Kim et al., 2015; Pertea et al., 2016). Count table of the gene features was obtained using FeatureCounts (Liao et al., 2014). Normalization, differential expression analysis and FPKM (fragments per kilobase of exon per million fragments mapped) values were computed using EdgeR (Robinson et al., 2010). An FPKM filtering cutoff of 1 in at least one of the 6 samples was applied. A *p value* of less than or equal to 0.05 was considered significant and a fold change cutoff of 1.5 was applied to identify differentially expressed genes. GO annotation was obtained using PANTHER classification system and pathways analysis was done using the Kyoto Encyclopedia of Genes and Genome (KEGG). Data visualization was done using GOplot R package (Walter et al., 2015).

### RNA extraction and RT-qPCR

Total RNA was extracted from mouse neural retina or whole *Xenopus* tadpoles using RNeasy mini kit (QIAGEN) or NucleoSpin RNA Plus kit (Macherey Nagel), respectively. RNA concentration was assessed using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific). Total RNA was reverse transcribed in the presence of oligo-(dT)20 using Superscript II reagents (Thermo Fisher Scientific). For each RT-qPCR reaction, 1.5 ng of cDNA was used in triplicates in the presence of EvaGreen (Bio-Rad) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Differential expression analysis was performed using the ΔΔCt method using the geometric mean of *Rps26, Srp72* and *Tbp* as endogenous controls (Vandesompele et al., 2002) for mouse genes, and of *Rpl8* and *Odc1* as endogenous controls for *Xenopus* genes. Relative expression of each gene in each sample was calculated using the mean of the controls as the reference (1 a.u.). Primers are listed in Table S2. RT-qPCR experiments were performed on at least 5 mice or 3 tadpoles per condition, allowing for statistical analysis.

### Histology & Immunofluorescence

Pre- and post- metamorphic *Xenopus* individuals were anesthetized in 0.4% MS222 (Sigma-Aldrich) and then fixed in 1X PBS, 4% paraformaldehyde, overnight at 4°C. For mice, the eyes of sacrificed animals were rapidly enucleated and dissected in Hanks' Balanced Salt solution (GIBCO) to obtain posterior segment eye-cups, which were then fixed in 1X PBS, 4% paraformaldehyde for 1 hr at 4°C. Fixed samples were dehydrated, embedded in paraffin and sectioned (7μm) with a Microm HM 340E microtome (Thermo Scientific). Fixed retinal explants were sectioned (7μm) with a Microm HM550 cryostat (Thermo Scientific), following embedment in 1X PBS, 7,5% gelatin, 10% sucrose. Immunostaining on retinal sections or whole explants was performed using standard procedures with the following specificities: (i) An antigen unmasking treatment was done in boiling heat-mediated antigen retrieval buffer (10 mM sodium citrate, pH 6.0) for 20 min; (ii) For *Xenopus* sections, this was followed by a 45 min treatment in 2N HCl at room temperature; (iii) Incubation timing was increased at all steps for immunolabelling on retinal explants. Primary/secondary antibodies and their corresponding working dilutions are listed in Table S3. Nuclei were counterstained with 1μg/ml DAPI (Thermo Fisher Scientific) or Hoechst (Sigma-Aldrich). H&E staining was performed using standard procedure.

### TUNEL assay and EdU labeling

Detection of apoptotic cells was conducted on retinal sections using DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. EdU incorporation was detected using the Click-iT EdU Imaging Kit (Thermo Fisher Scientific) following the manufacturer's recommendations. Coverslips were mounted using Fluorsave Reagent (Millipore, USA).

### Imaging

Fluorescence and brightfield images were acquired using an ApoTome-equipped AxioImager.M2 microscope or a Zeiss LSM710 confocal microscope. Whole retinal explants were imaged using an AxioZoom.v16 (Zeiss). Image mosaics of whole explants or flat mounted retinas were acquired and combined by the stitching processing method using ZEN Tiles module (Zeiss). Images were processed using Zen (Zeiss), Fiji (National Institutes of Health) and Photoshop CS4 (Adobe) softwares. The same magnification, laser intensity, gain and offset settings were used across animals for any given marker.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Quantifications in *Xenopus*: all labeled cells were counted manually. 3 to 10 sections were analyzed for each retina, and an average number was calculated from at least 5 retinas. BrdU-positive cells were counted within the *Xenopus* neural retina (after exclusion of the ciliary marginal zone). The size of dissected *Xenopus* eyes was estimated by measuring the surface of the corresponding pictures using Photoshop CS4 (Adobe) software. Quantifications in mouse: mean number of labeled cells in mouse retinal explants were calculated from 3 different fields of $10^4$ $\mu m^2$ per retina and using 3 explants per condition (for quantifications on whole explants) or from one field of $10^4$ $\mu m^2$ per section and using minimum 3 different retinas per condition (for quantifications on explant sections). SOX9-positive mouse Müller cells in Figure 1 were quantified by considering one entire retinal section from 3 different mice for each condition. Statistical analysis was performed with GraphPad Prism 5.01 (GraphPad Software, La Jolla California USA) using the non-parametric Mann-Whitney test in all experiments, except ERG, for which we applied a two-way ANOVA test. p value $\leq 0.05$ was considered significant. All results are reported as mean ± SEM.

## DATA AND SOFTWARE AVAILABILITY

RNaseq dataset from MNU-injected control and MNU-injected *Yap* CKO retinas has been deposited at the Gene Expression Omnibus under the ID code GEO: GSE121858.

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## Supplemental Information

# Linking YAP to Müller Glia Quiescence Exit in the Degenerative Retina

**Annaïg Hamon, Diana García-García, Divya Ail, Juliette Bitard, Albert Chesneau, Deniz Dalkara, Morgane Locker, Jérôme E. Roger, and Muriel Perron**

# Figure S1

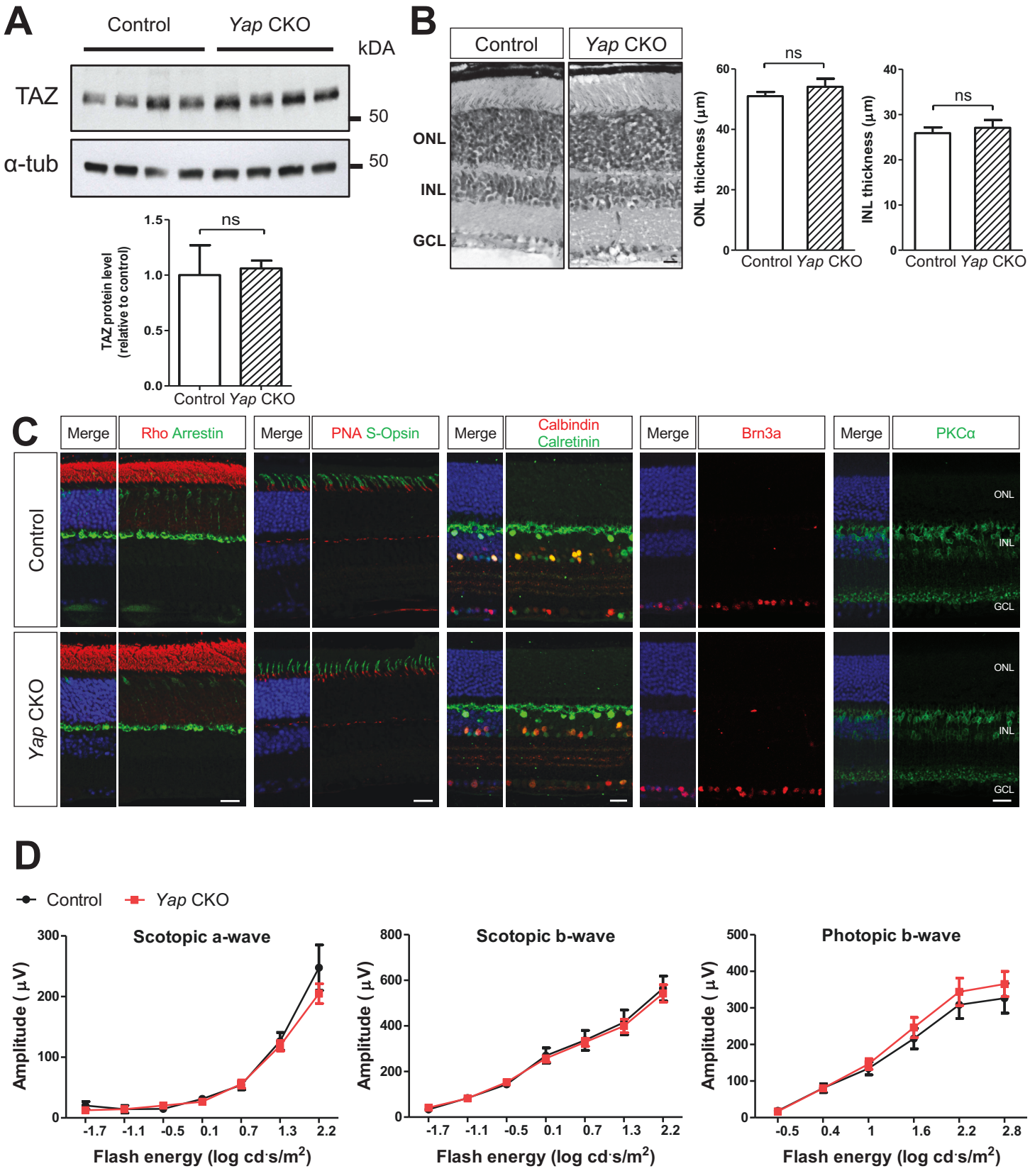

A
Control
Yap CKO
kDA
TAZ
α-tub
50
50
TAZ protein level (relative to control)
ns
Control Yap CKO
B
Control
Yap CKO
ONL
INL
GCL
ONL thickness (μm)
ns
Control Yap CKO
INL thickness (μm)
ns
Control Yap CKO
C
Merge
Rho Arrestin
Merge
PNA S-Opsin
Merge
Calbindin
Calretinin
Merge
Brn3a
Merge
PKCα
Control
Yap CKO
ONL
INL
GCL
D
Control
Yap CKO
Scotopic a-wave
Amplitude (μV)
Flash energy (log cd·s/m²)
Scotopic b-wave
Amplitude (μV)
Flash energy (log cd·s/m²)
Photopic b-wave
Amplitude (μV)
Flash energy (log cd·s/m²)

**Figure S1. *Yap* CKO mice do not show any major retinal defects. Related to Figure 1. (A)** Western-blot analysis of TAZ expression on retinal extracts from Control and *Yap* CKO mice injected with 4-OHT as shown in Figure 1A. α-tubulin labelling was used to normalize the signal. Quantification: n=4 mice for each condition. **(B)** H&E staining on retinal sections from Control and *Yap* CKO mice injected with 4-OHT as shown in Figure 1A. Quantification of INL and ONL thickness in the central retina: n=4 mice for each condition. **(C)** Retinal sections from Control and *Yap* CKO mice injected with 4-OHT as shown in Figure 1A, immunostained as indicated for a rod marker (Rhodopsin, Rho), cone photoreceptor markers (Arrestin, lectin PNA and S-Opsin), a horizontal cell marker (Calbindin), an amacrine cell marker (Calretinin), a ganglion cell marker (Brn3a) and a bipolar cell marker (PKCα). Nuclei are counterstained with DAPI (blue). **(D)** ERG-derived intensity response curves of the average scotopic a-wave, scotopic b-wave and photopic b-wave, in Control (n=6) and *Yap* CKO (n=9) mice injected with 4-OHT as shown in Figure 1A. Two-way ANOVA tests revealed no significant differences. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Statistics: Mann-Whitney test (A, B), ns: non-significant. Scale bars: 50 μm (B), 20 μm (C).

# Figure S2

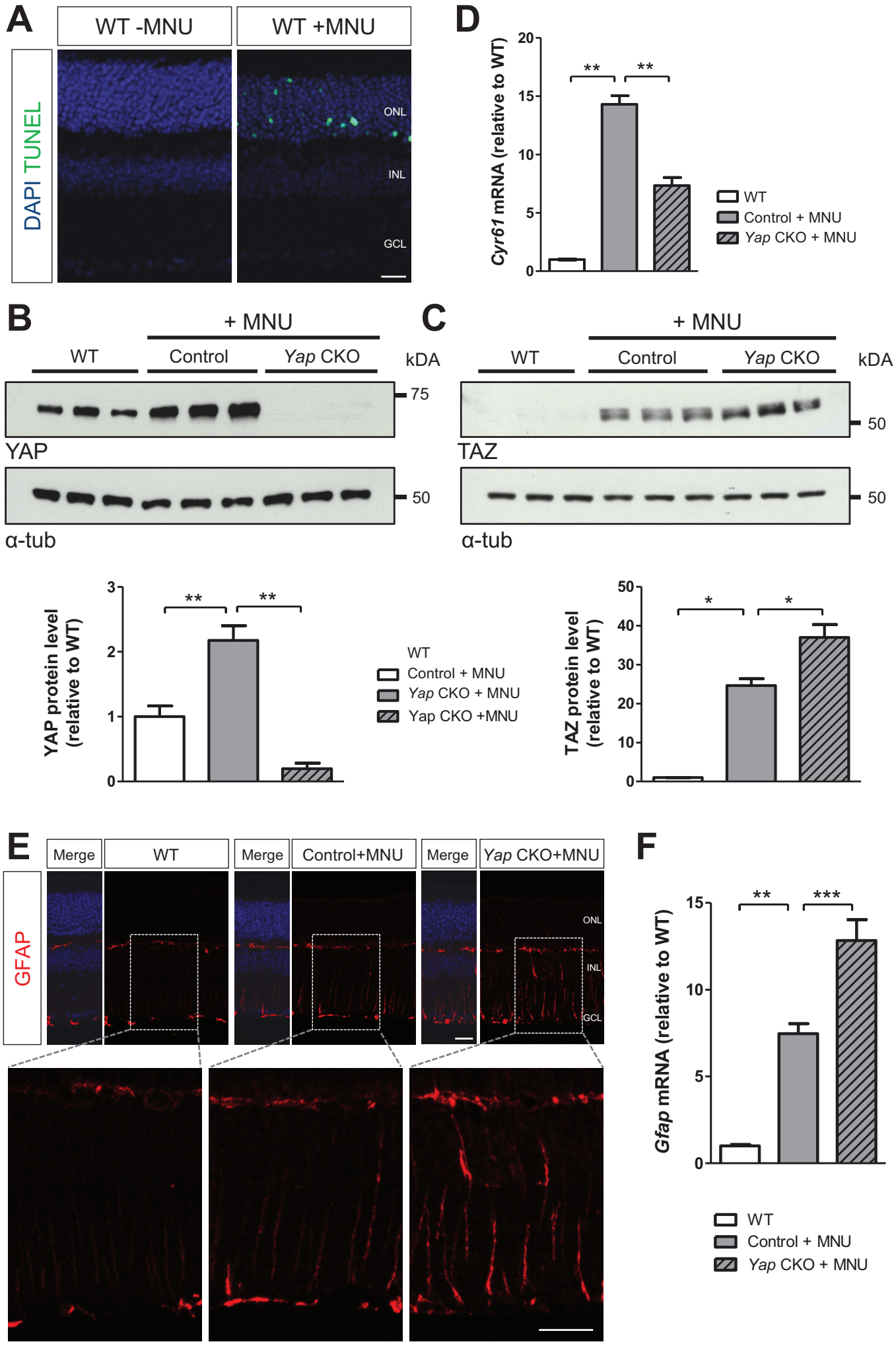
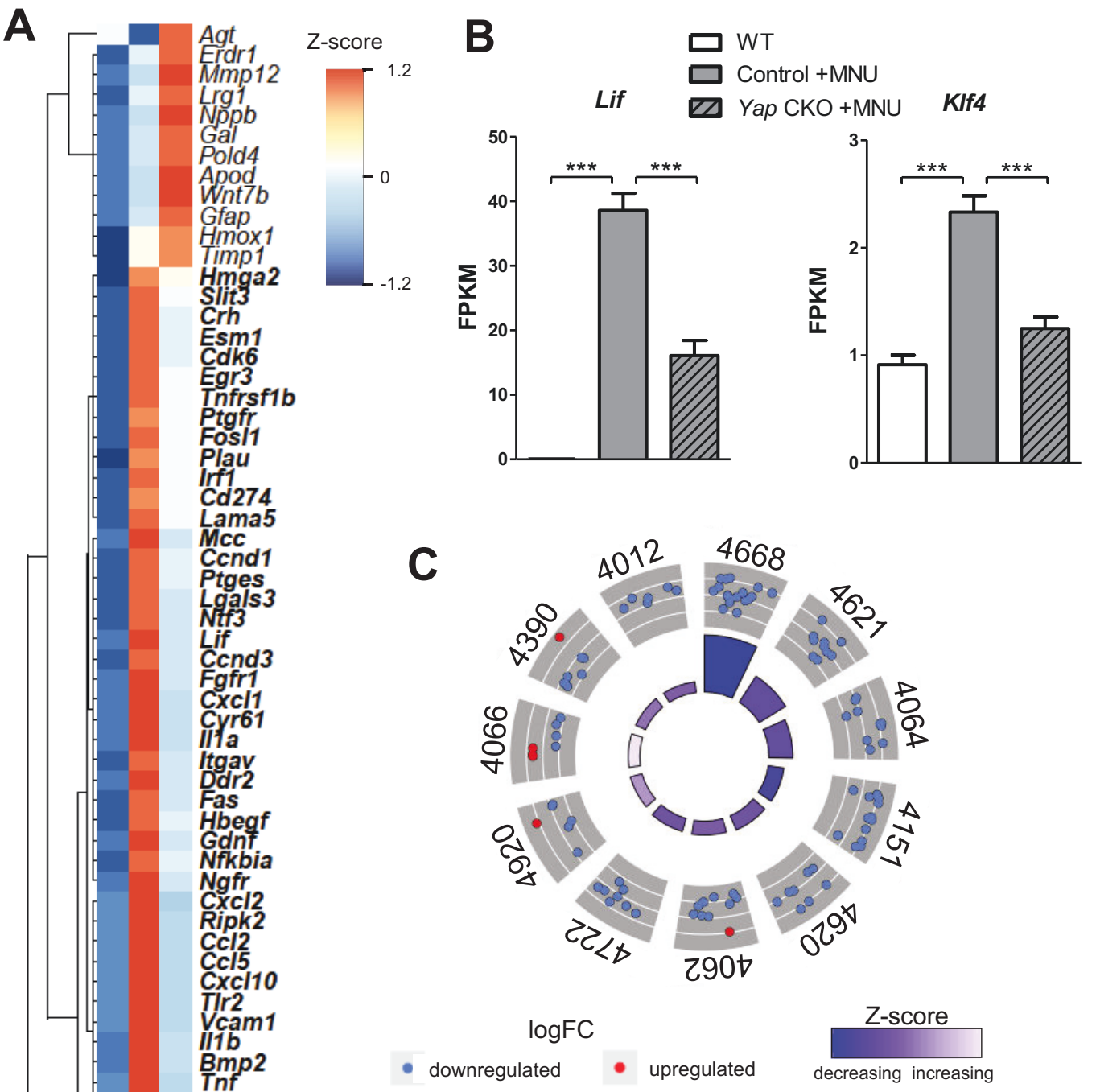

A
WT -MNU
WT +MNU
DAPI TUNEL
ONL
INL
GCL
D
Cyr61 mRNA (relative to WT)
20
15
10
5
0
**
**
WT
Control + MNU
Yap CKO + MNU
B
+ MNU
WT
Control
Yap CKO
kDA
75
YAP
50
α-tub
C
+ MNU
WT
Control
Yap CKO
kDA
50
TAZ
50
α-tub
YAP protein level (relative to WT)
3
2
1
0
**
**
WT
Control + MNU
Yap CKO + MNU
Yap CKO +MNU
TAZ protein level (relative to WT)
50
40
30
20
10
0
*
*
E
Merge
WT
Merge
Control+MNU
Merge
Yap CKO+MNU
GFAP
ONL
INL
GCL
F
Gfap mRNA (relative to WT)
15
10
5
0
**
***
WT
Control + MNU
Yap CKO + MNU

**Figure S2. YAP/TAZ expression in *Yap* CKO retinas following MNU-induced degeneration. Related to Figure 1. (A)** TUNEL assay on retinal sections from non-injected and MNU-injected WT mice (16 hours post-injection). Nuclei are counterstained with DAPI (blue). **(B, C)** Western-blot analysis of YAP and TAZ expression on retinal extracts from non-injected WT mice or from Control and *Yap* CKO mice injected with 4-OHT and MNU as shown in Figure 1F. α-tubulin labelling was used to normalize the signal. Quantification: n=3 mice for each condition. **(D)** RT-qPCR analysis of *Cyr61* expression on the same experimental conditions (at least 5 biological replicates per condition were used). **(E)** Retinal sections immunostained for GFAP. Nuclei are counterstained with DAPI (blue). The regions delineated with dashed lines are enlarged in the lower panels. **(F)** RT-qPCR analysis of *Gfap* expression (at least 5 biological replicates per condition were used). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Statistics: Mann-Whitney test, *p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001. Scale bar: 20 µm.

# Figure S3




| ID | Pathway | Gene count | FDR |
|---|---|---|---|
| 4668 | TNF signaling pathway | 18 | 4.85E-13 |
| 4621 | NOD-like receptor signaling pathway | 10 | 2.09E-07 |
| 4064 | NF-kappa B signaling pathway | 10 | 6.75E-06 |
| 4151 | PI3K-Akt signaling pathway | 15 | 0.000389 |
| 4620 | Toll-like receptor signaling pathway | 8 | 0.000464 |
| 4062 | Chemokine signaling pathway | 10 | 0.000945 |
| 4722 | Neurotrophin signaling pathway | 8 | 0.00126 |
| 4920 | Adipocytokine signaling pathway | 6 | 0.00251 |
| 4066 | HIF-1 signaling pathway | 7 | 0.00337 |
| 4390 | Hippo signaling pathway | 8 | 0.00458 |
| 4012 | ErbB signaling pathway | 6 | 0.00492 |

**Figure S3. Deregulated genes and pathways in *Yap* CKO mice. Related to Figures 2 and 6. (A)** Hierarchical clustering analysis of the 70 DEGs that belong to the GO Biological Process “regulation of cell proliferation” (GO:0042127). The resulting heatmap represents z-score expression values for each condition. Genes in bold are those that are (*i*) expressed at very low levels in non-injected WT mice, (*ii*) strongly upregulated in MNU-injected Control mice, (*iii*) only moderately enriched in MNU-injected *Yap* CKO mice. **(B)** Relative RNA expression (in FPKM; data retrieved from the RNA-seq experiment) of *Klf4 and Lif*, in retinas from non-injected WT mice or Control and *Yap* CKO mice injected with 4-OHT and MNU as shown in Figure 1F. **(C)** Pathway analysis of the RNA-seq dataset comparing non-injected WT mice or MNU-injected Control and *Yap* CKO mice. The eleven most deregulated signaling pathways identified by Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment analysis are shown. Downregulated (blue dots) and upregulated (red dots) genes within each signaling pathway are plotted based on logFC. Z-score bars indicate if an entire signaling pathway is more likely to be increased or decreased based on the behavior of genes it contains. The table shows pathway identity number (ID) depicted on the circle, associated pathway name, gene count and False Discovery Rate (FDR). Note that, in addition to the *Egfr* ligand- and receptor-coding genes mentioned in the result section, other deregulated genes belonging to the PI3K-AKT and ErbB signaling pathways were also retrieved (15 and 6 associated DEGs, respectively). Statistics: P-values were obtained using EdgeR, *** $p \leq 0.001$.

# Figure S4

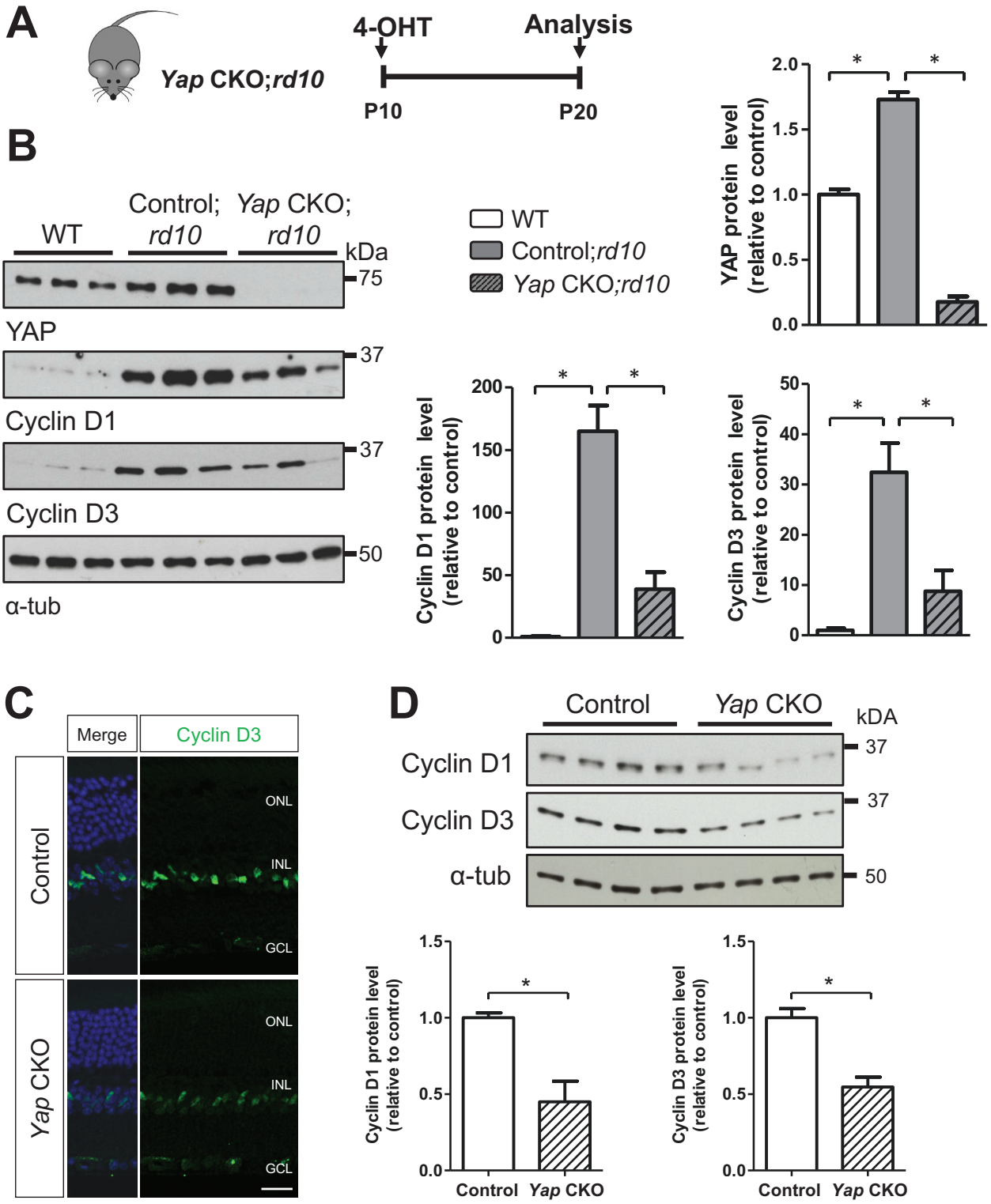

A
Yap CKO;rd10
4-OHT
Analysis
P10
P20
B
WT
Control; rd10
Yap CKO; rd10
kDa
75
YAP
37
Cyclin D1
37
Cyclin D3
50
α-tub
WT
Control;rd10
Yap CKO;rd10
YAP protein level (relative to control)
Cyclin D1 protein level (relative to control)
Cyclin D3 protein level (relative to control)
C
Merge
Cyclin D3
Control
Yap CKO
ONL
INL
GCL
D
Control
Yap CKO
kDA
Cyclin D1
37
Cyclin D3
37
α-tub
50
Cyclin D1 protein level (relative to control)
Cyclin D3 protein level (relative to control)
Control
Yap CKO

**Figure S4. YAP loss of function results in decreased Cyclin D1/D3 expression in *rd10* degenerating retinas or in a physiological context. Related to Figure 2.** (**A**) Timeline diagram of the experimental procedure used in B. *Yap*$^{flox/flox}$;*rd10* (Control;*rd10*) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$*;rd10* (*Yap* CKO*;rd10*) mice received a single dose of 4-OHT at P10 and were analyzed at P20. (**B**) Western-blot analysis of YAP, Cyclin D1 and Cyclin D3 expression. α-tubulin labelling was used to normalize the signal. Quantification: n=3 mice for each condition. **(C)** Retinal sections from 2-month-old Control and *Yap* CKO mice in physiological conditions, immunostained for Cyclin D3. Nuclei are counterstained with DAPI (blue). (**D**) Western-blot analysis of Cyclin D1 and Cyclin D3 expression on retinal extracts from 2-month-old Control and *Yap* CKO mice in physiological conditions. α-tubulin labelling was used to normalize the signal. Quantification: n=4 mice for each condition. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Statistics: Mann-Whitney test, *p≤ 0.05. Scale bar: 20 µm.

# Figure S5

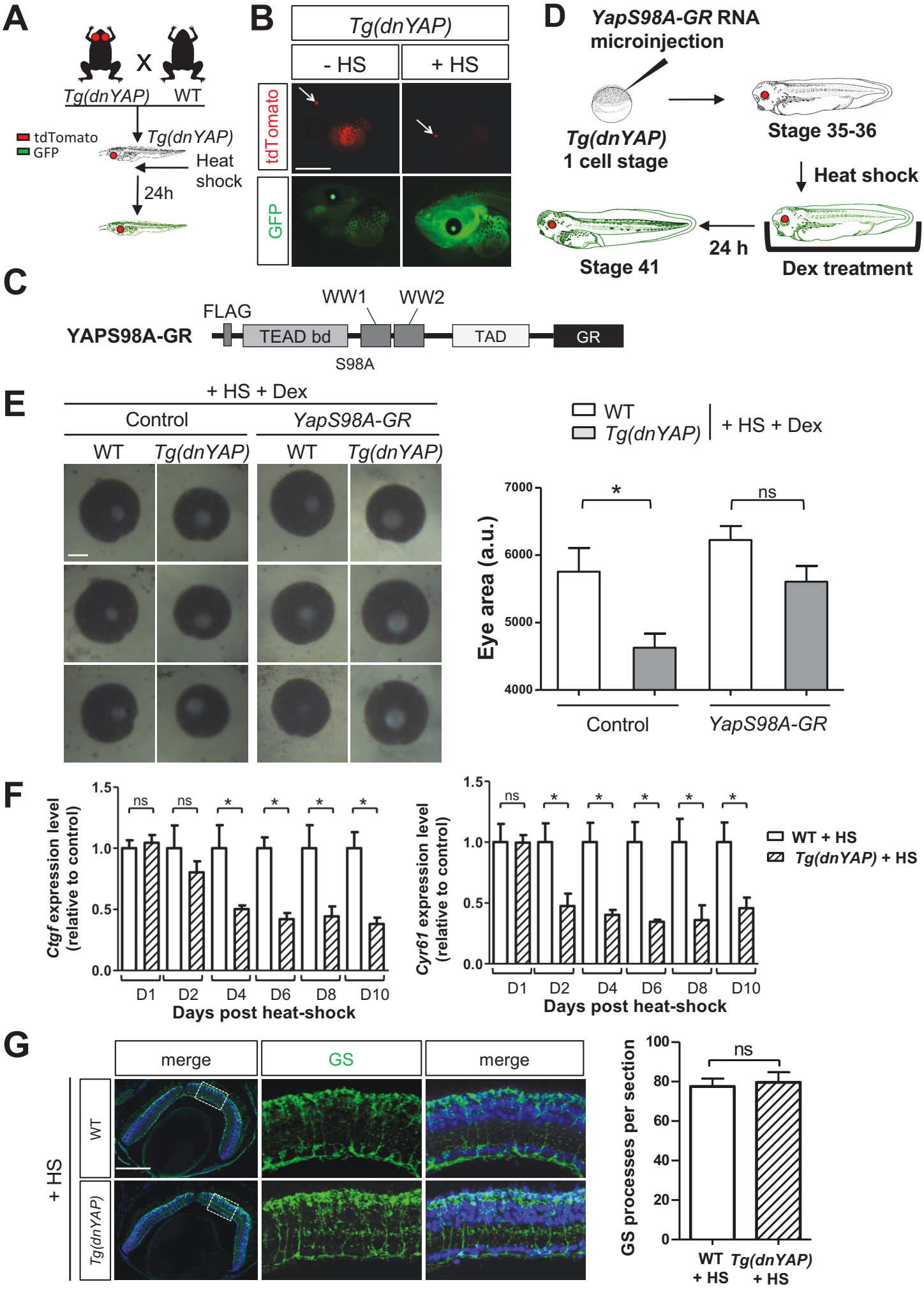

A
X
Tg(dnYAP)
WT
tdTomato
GFP
Tg(dnYAP)
Heat shock
24h
B
Tg(dnYAP)
- HS
+ HS
tdTomato
GFP
D
YapS98A-GR RNA microinjection
Tg(dnYAP) 1 cell stage
Stage 35-36
Heat shock
Dex treatment
24 h
Stage 41
C
YAPS98A-GR
FLAG
WW1
WW2
TEAD bd
TAD
GR
S98A
E
+ HS + Dex
Control
YapS98A-GR
WT
Tg(dnYAP)
WT
Tg(dnYAP)
WT
Tg(dnYAP)
+ HS + Dex
Eye area (a.u.)
7000
6000
5000
4000
*
ns
Control
YapS98A-GR
F
Ctgf expression level (relative to control)
Cyr61 expression level (relative to control)
1.5
1.0
0.5
0.0
ns
*
D1
D2
D4
D6
D8
D10
Days post heat-shock
WT + HS
Tg(dnYAP) + HS
G
merge
GS
merge
+ HS
WT
Tg(dnYAP)
GS processes per section
100
80
60
40
20
0
ns
WT + HS
Tg(dnYAP) + HS

**Figure S5. Validation of the *Xenopus Tg(dnYAP)* line. Related to Figure 3.** (**A**) Timeline diagram of the experimental procedures used in B, F and G. WT *Xenopus* were crossed with *Tg(dnYAP)* individuals. *Tg(dnYAP)* tadpoles were selected within the progeny, heat-shocked and harvested 24 hours later. (**B**) Images of tadpole anterior region. Presence of the transgene is assessed by tdTomato lens expression (red, arrows). Of note, tdTomato is very bright and its fluorescence can also be seen in the GFP channel. Heat shock effectiveness is assessed by GFP expression in the entire tadpole. (**C**) Schematic representation of the *Xenopus* YAPS98A-GR construct. The Ser-98 residue, which is the target of Lats phosphorylation (Ser-127 in mammalian YAP), has been substituted with alanine (S98A). YAPS98A is therefore insensitive to Hippo pathway-mediated inhibition (Zhao et al., 2007) and thus constitutively active. This YAPS98A construct is fused to the ligand-binding domain of the glucocorticoid receptor (GR), which allows its conditional activation upon addition of dexamethasone (Dex) to the tadpole rearing medium. TAD: transcription activation domain; TEAD bd: TEAD binding domain, WW1&2: WW domains. **(D)** Timeline diagram of the experimental procedure used in E. *LacZ* mRNA (control) or *YapS98A-GR* mRNA were injected in *Tg(dnYAP)* fertilized eggs. Embryos were then heat-shocked at stage 35-36 and transferred 1 hour later for 24 hours in a Dex solution. (**E**) Images of the dissected tadpole eyes. Areas were quantified on pictures from 8 tadpoles for each condition. (**F**) RT-qPCR analysis of two YAP target genes, *Ctgf* and *Cyr61*, in WT or *Tg(dnYAP)* tadpoles harvested 1 to 10 days after the heat shock (3 biological replicates per condition). For each time point, the control transcript level is set to 1. (**G**) Retinal sections from heat-shocked WT or *Tg(dnYAP)* tadpoles, immunostained for glutamine synthetase (GS, green). Nuclei are counterstained with Hoechst (blue). The regions delineated with dotted lines are enlarged in the right panels. For quantification, the number of GS-positive processes were counted. Quantification: n=5 tadpoles for WT and n= 6 tadpoles for *Tg(dnYAP)* condition. Statistics: Mann-Whitney test, *p≤ 0.05; ns: non-significant. Scale bars: 1 mm (B), 100 µm (E) or 50 µm (G).

# Figure S6

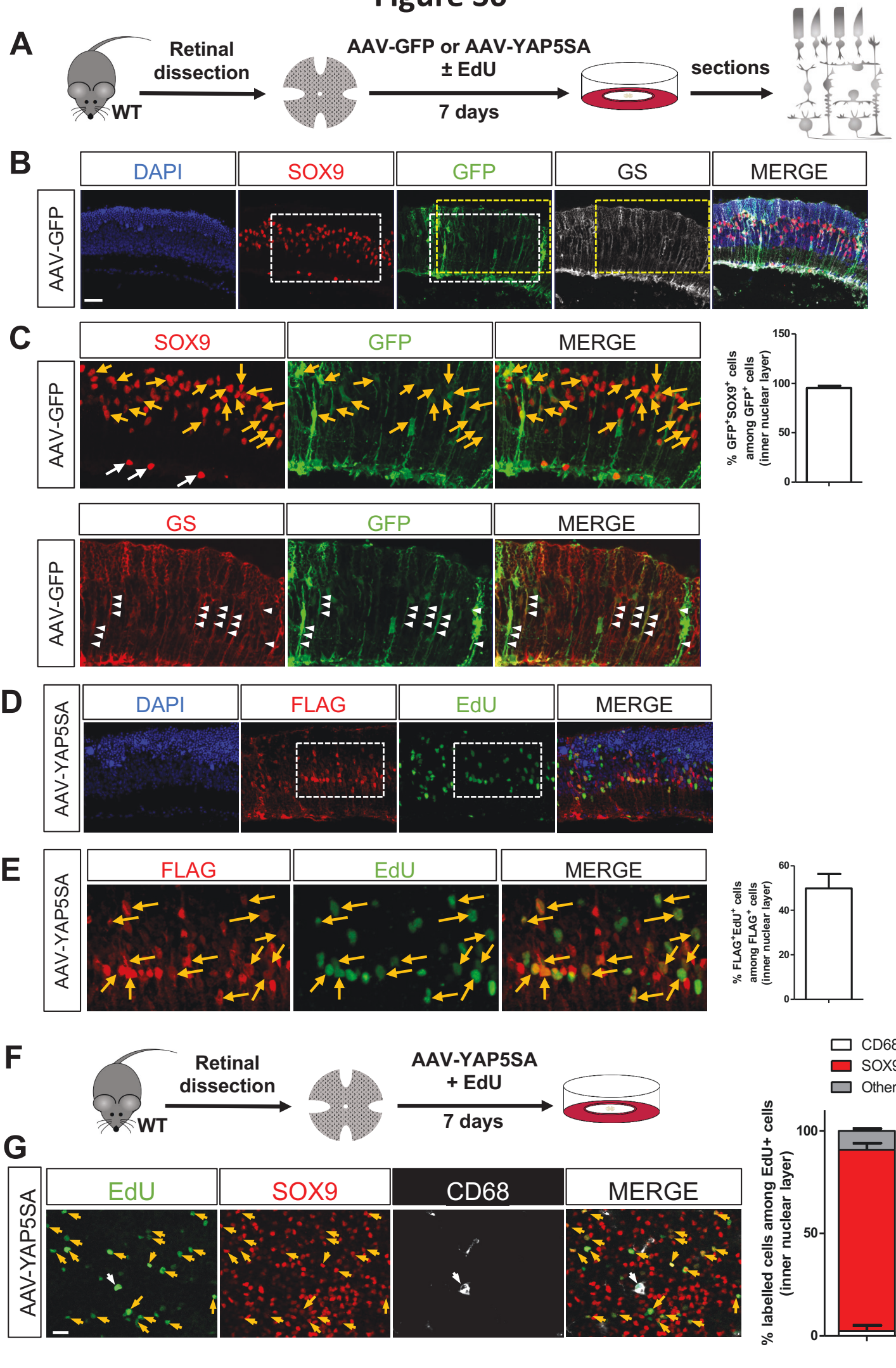
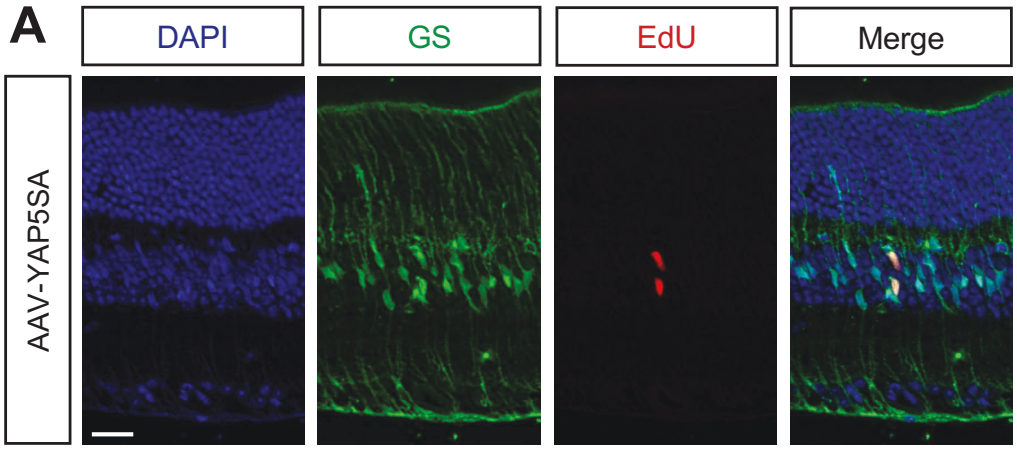
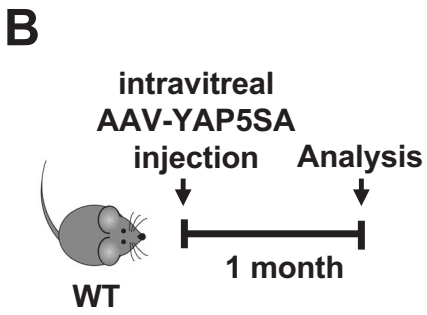

A
Retinal dissection
WT
AAV-GFP or AAV-YAP5SA ± EdU
7 days
sections
B
DAPI
SOX9
GFP
GS
MERGE
AAV-GFP
C
SOX9
GFP
MERGE
AAV-GFP
% GFP+SOX9+ cells among GFP+ cells (inner nuclear layer)
150
100
50
0
GS
GFP
MERGE
AAV-GFP
D
DAPI
FLAG
EdU
MERGE
AAV-YAP5SA
E
FLAG
EdU
MERGE
AAV-YAP5SA
% FLAG+EdU+ cells among FLAG+ cells (inner nuclear layer)
60
40
20
0
F
Retinal dissection
WT
AAV-YAP5SA + EdU
7 days
G
EdU
SOX9
CD68
MERGE
AAV-YAP5SA
CD68
SOX9
Others
% labelled cells among EdU+ cells (inner nuclear layer)
100
50
0

**Figure S6. YAP overexpression in mouse Müller cells triggers their proliferative response. Related to Figure 5. (A)** Timeline diagram of the experimental procedure used in B-E. Retinas from WT mice were flattened, infected with AAV-GFP or AAV-YAP5SA and sectioned. In D, E, EdU was added to the culture medium. **(B)** GFP, Sox9 and GS co-labelling on AAV-GFP-transduced retinal explant sections. Nuclei are counterstained with DAPI. **(C)** Enlargements of retinal regions delineated with white (upper panel) and yellow (lower panel) boxes in B. Infected Müller cells are pointed by yellow arrows ($Sox9^+/GFP^+$ nuclei) or white arrowheads ($GS^+/GFP^+$ fibers). As depicted on the graph, almost 100% of GFP-labelled cells in the inner nuclear layer are Sox9-positive Müller cells. Of note, although ShH10AAV was shown to preferentially infect Müller cells, it also targets retinal astrocytes (Klimczak et al., 2009), which are Sox9-positive as well. However, these cells are localized in the inner surface of the retina (white arrows), and can thus be easily distinguished from Müller cells based on their position. Quantification: n=3 explants. **(D)** FLAG staining of YAP5SA-expressing cells (see schematic of the construct in Figure 5A) coupled to EdU labelling on retinal explant sections. **(E)** Enlargement of the region delineated with a white box in D. Yellow arrows point to $FLAG^+/EdU^+$ cells in the inner nuclear layer. Note that a week after infection, about 50% of infected cells are proliferating. Quantification: n=3 explants. **(F)** Timeline diagram of the experimental procedure used in G. Retinas from WT mice were flattened, infected with AAV-YAP5SA and cultured for 7 days in the presence of EdU. **(G)** EdU, Sox9 and CD68 co-labelling in the inner nuclear layer of flat mounted retinas. Note that $Sox9^+$ Müller cells represent the majority of proliferating cells. Only rare $CD68^+$ reactive microglial cells were identified among the EdU-labelled population. Quantification: n=3 explants for each condition. Scale bars: 50 µm (B and D) or 20 µm (G).

# Figure S7


A
DAPI
GS
EdU
Merge
AAV-YAP5SA


B
intravitreal
AAV-YAP5SA
injection
Analysis
1 month
WT

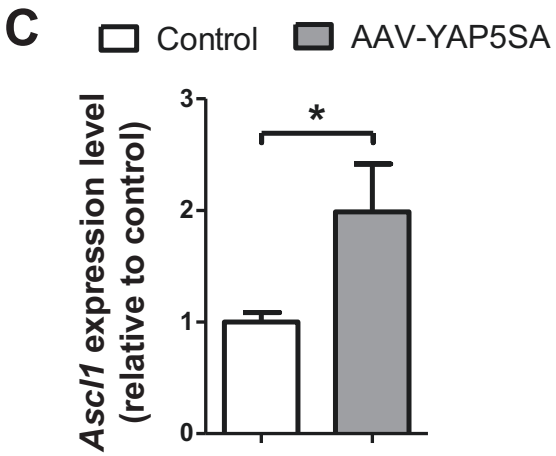

C
Control
AAV-YAP5SA
Ascl1 expression level
(relative to control)
3
2
1
0
*

**Figure S7. *YAP5SA* triggers Müller cell proliferation and *Ascl1* up-regulation *in vivo*. Related to Figure 5. (A)** EdU and glutamine synthetase (GS) co-labelling on a retinal section following *in vivo* infection with AAV-YAP5SA and EdU injection as depicted in Figure 5G. Nuclei are counterstained with DAPI (blue). **(B)** Timeline diagram of the experimental procedure used in C. WT mice were intravitreally injected with AAV-GFP (Control) or AAV-YAP5SA. Retinas were then harvested one month later for qPCR analysis. **(C)** RT-qPCR analysis of *Ascl1* (10 biological replicates per condition). Statistics: Mann-Whitney test, *p≤ 0.05. Scale bars: 20 µm.

| Gene | LogFC | pvalue | Gene | LogFC | pvalue | Gene | LogFC | pvalue | Gene | LogFC | pvalue | Gene | LogFC | pvalue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2700038G22Rik | 0,78 | 0,002339109 | Serpine3 | 3,74 | 1,41E-07 | Col9a2 | -0,69 | 0,021354054 | Hbegf | -0,93 | 1,22E-07 | Pgm5 | -0,66 | 0,01184783 |
| 8430408G22Rik | 1,16 | 0,001037516 | Slc47a2 | 0,91 | 0,00010045 | Col9a3 | -0,74 | 0,019713884 | Heph | -1,12 | 3,87E-06 | Phldb1 | -0,80 | 0,000264039 |
| A930036K24Rik | 0,90 | 0,002548412 | Snord104 | 0,93 | 0,002804991 | Colec12 | -1,73 | 2,28E-08 | Herc6 | -0,82 | 0,00120464 | Piezo1 | -0,65 | 0,000579816 |
| Acot1 | 1,03 | 1,52E-05 | Sprr1a | 1,96 | 0,000121067 | Crh | -0,74 | 0,007951762 | Hmga2 | -0,64 | 0,031411996 | Pik3r5 | -0,79 | 0,001338209 |
| Adam8 | 1,16 | 0,000100127 | Steap4 | 0,77 | 0,001647729 | Crhbp | -0,93 | 0,000110666 | Hsd17b2 | -0,82 | 0,000150664 | Plau | -0,70 | 0,000408957 |
| Agt | 0,75 | 0,007174048 | Timp1 | 0,63 | 0,005852127 | Crispld2 | -0,64 | 0,002987806 | Icam1 | -1,42 | 1,61E-14 | Plaur | -0,80 | 0,000725776 |
| Akr1b8 | 0,84 | 0,000571571 | Tmem159 | 0,83 | 0,000114452 | Crybg3 | -0,81 | 7,04E-05 | Ifit1 | -0,78 | 0,014708827 | Plxnd1 | -0,90 | 5,99E-05 |
| Aldh1a3 | 1,17 | 2,88E-06 | Tmem252 | 0,95 | 0,000188769 | Cxcl1 | -1,47 | 3,41E-06 | Ifit3 | -0,84 | 0,013260031 | Pnrc2 | -0,68 | 2,54E-05 |
| Alpk2 | 1,52 | 0,017723232 | Tmem256 | 0,62 | 0,007019929 | Cxcl10 | -1,76 | 5,82E-06 | Ifit3b | -0,77 | 0,015011305 | Podn | -2,12 | 9,45E-09 |
| Apod | 1,10 | 0,006703564 | Umodl1 | 0,59 | 0,038915374 | Cxcl2 | -2,16 | 6,39E-05 | Il1a | -1,31 | 1,33E-05 | Prss23 | -1,16 | 1,14E-07 |
| AW047730 | 0,69 | 0,019575443 | Wnt7b | 1,50 | 0,000157792 | Cyp39a1 | -0,89 | 0,000313006 | Il1b | -1,53 | 1,46E-05 | Ptgds | -0,65 | 0,004665045 |
| BC064078 | 1,08 | 0,000539679 | Xist | 12,09 | 1,14E-07 | Cyr61 | -1,26 | 2,90E-09 | Iqgap2 | -0,71 | 0,001515865 | Ptges | -0,89 | 0,001255908 |
| Bfsp1 | 3,83 | 6,62E-05 | Zmynd15 | 0,68 | 0,016019621 | Ddr2 | -0,79 | 0,005446161 | Irak2 | -0,91 | 3,30E-09 | Ptgfr | -0,76 | 6,87E-05 |
| Bfsp2 | 3,55 | 0,000152613 | 1700016P03Rik | -1,04 | 0,000360121 | Dio2 | -1,03 | 0,000183826 | Irf1 | -0,76 | 4,27E-05 | Qrfpr | -1,08 | 8,11E-06 |
| Calca | 1,01 | 0,000126586 | 1810011O10Rik | -0,76 | 0,008802628 | Dio3 | -0,71 | 0,043534676 | Ism2 | -0,80 | 0,029814411 | Rab11fip1 | -0,63 | 0,004541084 |
| Casd1 | 1,12 | 0,002921064 | 2900052N01Rik | -1,04 | 3,60E-06 | Egfr | -0,80 | 2,43E-05 | Itga7 | -0,65 | 0,013390582 | Rab20 | -0,63 | 0,008907992 |
| Ccdc24 | 0,66 | 0,046889841 | 9130015L21Rik | -0,94 | 0,002537487 | Egr2 | -0,62 | 0,008133733 | Itgav | -0,69 | 0,000249966 | Rab38 | -1,13 | 0,00028141 |
| Ccl6 | 0,68 | 0,048893461 | Abi3bp | -0,75 | 0,00075485 | Egr3 | -0,80 | 0,018945204 | Itgb8 | -0,89 | 2,65E-05 | Rarres2 | -1,75 | 1,16E-10 |
| Cdk5rap1 | 0,78 | 0,000487897 | Adamts1 | -0,91 | 2,02E-05 | Eif4a-ps4 | -0,71 | 0,020482351 | Itpripl2 | -1,05 | 1,22E-08 | Rbm46 | -0,89 | 0,000631068 |
| Chl1 | 0,62 | 0,019779106 | Adamts5 | -0,59 | 0,001477544 | Erbb4 | -0,62 | 0,004339885 | Kank1 | -0,81 | 0,000154733 | Rel | -0,76 | 0,000365105 |
| Cited1 | 0,61 | 0,015581668 | Adamtsl1 | -0,80 | 6,55E-06 | Esm1 | -0,88 | 0,020977607 | Kcnj13 | -1,41 | 0,002792169 | Rin1 | -0,98 | 0,019579529 |
| Clrn1 | 0,91 | 0,000268916 | Adgrg6 | -0,89 | 2,05E-05 | F5 | -2,50 | 2,58E-12 | Klf10 | -0,60 | 0,000309421 | Ripk2 | -1,32 | 1,01E-07 |
| Crym | 0,61 | 0,025697537 | Ahnak | -0,86 | 9,75E-06 | Fam107a | -1,38 | 3,86E-05 | Klf4 | -0,85 | 9,23E-05 | Rnd1 | -1,18 | 1,93E-10 |
| Cyp26b1 | 0,81 | 0,035457967 | Aldh1a1 | -0,62 | 0,002152026 | Fam129a | -1,51 | 1,05E-07 | Klhdc7a | -2,16 | 1,79E-09 | Rsad2 | -1,63 | 0,00115145 |
| Erdr1 | 0,77 | 0,025764298 | Aldh1l1 | -0,73 | 0,000911001 | Fam135b | -0,64 | 0,000920242 | Lama5 | -0,70 | 0,003365956 | S100a6 | -0,73 | 0,000169777 |
| Fabp5 | 0,74 | 4,26E-05 | Anxa1 | -1,07 | 2,17E-08 | Fas | -1,11 | 4,82E-06 | Lgals3 | -1,09 | 5,26E-08 | Samd9l | -0,70 | 0,008427654 |
| Fam181b | 1,07 | 0,001867425 | Arc | -0,73 | 0,007025691 | Fat4 | -0,63 | 0,000546096 | Lif | -1,27 | 1,20E-09 | Scarf2 | -0,77 | 0,000395311 |
| Fcrls | 1,19 | 0,000257085 | Arhgap22 | -1,20 | 1,99E-08 | Fblim1 | -0,59 | 0,002373593 | Lrp2 | -1,11 | 0,000471439 | Sele | -0,77 | 0,029915836 |
| Gal | 0,84 | 0,001193966 | Arhgef37 | -0,62 | 0,005348799 | Fbln1 | -2,37 | 3,12E-16 | Lrrfip1 | -0,60 | 0,00013679 | Sema3b | -0,61 | 0,006049126 |
| Gfap | 1,07 | 6,82E-05 | Arrdc4 | -1,19 | 9,94E-12 | Fbln7 | -0,93 | 0,000790538 | Ltbp1 | -0,64 | 0,015004817 | Slc13a4 | -2,07 | 7,71E-12 |
| Glb1l3 | 1,70 | 0,00255268 | Atp13a3 | -0,64 | 0,000494045 | Fbn1 | -1,72 | 1,66E-11 | Ltbp2 | -2,07 | 3,26E-10 | Slc16a7 | -0,62 | 0,002639187 |
| Gm17135 | 0,67 | 0,029556677 | Atp1a2 | -1,24 | 3,10E-06 | Fgfr1 | -0,70 | 4,42E-05 | Madcam1 | -1,26 | 0,001002273 | Slc17a8 | -0,70 | 9,54E-05 |
| Gm29595 | 0,73 | 0,006218653 | Axl | -1,03 | 2,29E-06 | Fmod | -3,21 | 8,45E-19 | Maff | -0,68 | 0,000795482 | Slc22a8 | -0,97 | 0,001856352 |
| Gm6063 | 0,61 | 0,022643434 | Best2 | -3,30 | 8,83E-11 | Folh1 | -1,70 | 4,58E-09 | Matn2 | -0,97 | 9,33E-05 | Slc24a4 | -0,72 | 1,80E-05 |
| Hebp1 | 0,71 | 0,00540347 | Birc3 | -1,45 | 7,61E-08 | Folr1 | -0,67 | 0,002412074 | Mcc | -0,66 | 0,000735645 | Slc26a4 | -5,07 | 5,79E-21 |
| Hmox1 | 0,60 | 0,018000143 | Bmp2 | -0,98 | 0,000168454 | Fosl1 | -0,85 | 1,21E-07 | Mfap4 | -2,16 | 7,48E-08 | Slc6a12 | -0,64 | 0,001221169 |
| Klf2 | 0,75 | 0,001337118 | Bmp7 | -0,80 | 0,000161331 | Foxi3 | -1,03 | 0,004296497 | Mmp3 | -1,20 | 0,007704463 | Slc6a13 | -2,04 | 1,22E-11 |
| Krt24 | 0,67 | 0,012226146 | Bst2 | -0,60 | 0,035821637 | Fstl3 | -1,12 | 7,06E-05 | Mrc2 | -1,03 | 0,001403912 | Slc7a2 | -1,14 | 2,57E-06 |
| Lcn2 | 0,97 | 0,001272808 | Btc | -0,64 | 0,029242102 | Fut4 | -0,84 | 0,002447017 | Msrb3 | -0,67 | 0,001562477 | Slc8b1 | -0,97 | 5,28E-06 |
| Lfng | 0,72 | 0,018681201 | Ccl2 | -2,10 | 9,58E-22 | Fzd10 | -0,75 | 0,008830919 | Mtmr10 | -0,64 | 9,17E-05 | Slit3 | -0,76 | 0,00178327 |
| Lgsn | 3,14 | 1,28E-05 | Ccl5 | -1,46 | 0,000215263 | Gbp2 | -1,59 | 6,45E-09 | mt-Nd6 | -0,70 | 0,028191079 | Sned1 | -2,25 | 1,14E-13 |
| Lhx1os | 0,63 | 0,008805038 | Ccl7 | -1,81 | 8,56E-13 | Gbp3 | -1,09 | 0,001395044 | Myl9 | -0,62 | 0,013021151 | Sod3 | -1,05 | 0,000245296 |
| Lman1 | 0,65 | 0,000941447 | Ccnd1 | -0,84 | 8,63E-05 | Gbp5 | -2,01 | 3,53E-10 | Mylk2 | -2,84 | 2,26E-13 | Sostdc1 | -0,99 | 0,000285233 |
| Lrg1 | 0,96 | 0,002006736 | Ccnd2 | -1,12 | 1,23E-05 | Gbp7 | -0,77 | 0,002412135 | Myof | -0,60 | 0,000507279 | Stx11 | -1,22 | 7,74E-08 |
| Mc4r | 0,83 | 0,003200516 | Ccnd3 | -0,77 | 0,002783318 | Gbp9 | -0,71 | 0,01180802 | NA | -1,09 | 0,045898626 | Tagln | -1,74 | 1,00E-10 |
| Mip | 5,72 | 3,91E-05 | Cd274 | -0,66 | 0,02238986 | Gdf15 | -1,18 | 0,037136065 | Neurl3 | -1,31 | 1,56E-06 | Tap2 | -0,69 | 0,003378966 |
| Mmp12 | 1,60 | 1,03E-08 | Cdk6 | -0,78 | 0,001178631 | Gdnf | -1,15 | 0,00122088 | Nfkb1 | -0,64 | 0,000108345 | Tfrc | -0,76 | 0,022474384 |
| Neurog2 | 0,79 | 0,002302779 | Cdsn | -1,21 | 0,02824172 | Gja1 | -0,78 | 0,002973756 | Nfkb2 | -0,67 | 0,000980692 | Timp3 | -1,07 | 1,58E-08 |
| Nppa | 0,61 | 0,03380138 | Cflar | -0,63 | 0,00029771 | Gja10 | -0,67 | 0,008490054 | Nfkbia | -0,72 | 6,21E-05 | Tlr2 | -1,81 | 2,97E-11 |
| Nppb | 0,86 | 0,019631125 | Ch25h | -0,89 | 0,000830986 | Glipr2 | -0,65 | 0,002194762 | Nfkbie | -1,19 | 1,53E-07 | Tmc7 | -0,70 | 0,002033536 |
| Npy | 0,95 | 1,15E-06 | Chrnb1 | -0,91 | 4,06E-05 | Glra1 | -0,70 | 0,000504522 | Ngfr | -0,80 | 0,003263194 | Tnf | -1,59 | 0,003932158 |
| Pgam2 | 1,43 | 0,000454884 | Chrnb4 | -0,85 | 0,013033656 | Glycam1 | -0,77 | 0,041785345 | Nid2 | -0,96 | 2,28E-05 | Tnfaip2 | -2,00 | 3,81E-09 |
| Pold4 | 1,08 | 7,94E-05 | Cldn1 | -0,76 | 0,02372547 | Gm16346 | -1,12 | 0,002764131 | Nrg1 | -0,79 | 1,76E-05 | Tnfrsf1b | -0,71 | 0,002262262 |
| Ppp1r14a | 0,65 | 0,03607081 | Cldn19 | -1,48 | 0,000108616 | Gm25047 | -0,78 | 0,028091673 | Ntf3 | -0,84 | 0,006765431 | Tnfsf10 | -1,15 | 4,95E-05 |
| Rab26os | 0,72 | 0,003063572 | Cldn2 | -3,58 | 2,78E-16 | Gm7694 | -0,66 | 0,019302583 | Ogn | -3,28 | 3,07E-15 | Trim30a | -0,89 | 0,015062511 |
| Raxos1 | 3,17 | 4,75E-20 | Clic4 | -0,64 | 0,00719126 | Gpx3 | -2,42 | 2,95E-14 | Olfml2a | -2,52 | 5,54E-13 | Trim47 | -0,71 | 0,000419793 |
| Rnf207 | 0,62 | 0,048324244 | Cmpk2 | -0,70 | 0,009097483 | Gsn | -0,62 | 0,005799794 | Optc | -1,58 | 1,59E-06 | Vcam1 | -1,31 | 7,37E-12 |
| Rny1 | 1,39 | 0,0050627 | Cnn2 | -1,52 | 2,71E-11 | Gstm2 | -2,29 | 4,39E-07 | Osgin1 | -0,78 | 0,000806615 | Vcan | -0,78 | 0,013561307 |
| Rps27 | 0,60 | 0,004101469 | Col18a1 | -1,16 | 2,63E-05 | Gulp1 | -0,70 | 0,002203686 | P2ry2 | -0,69 | 0,000254153 | Yap1 | -1,60 | 1,14E-16 |
| Rps27rt | 1,13 | 0,011549715 | Col1a2 | -1,91 | 2,22E-09 | H2-Q4 | -0,65 | 0,003117113 | Parp14 | -0,94 | 0,000855147 | Zfp185 | -1,47 | 2,78E-07 |
| Scgb3a1 | 0,66 | 0,029622518 | Col9a1 | -0,88 | 0,003048003 | H2-Q6 | -1,03 | 0,000606513 | Penk | -1,93 | 4,51E-15 | Zic1 | -0,61 | 0,002782867 |

**Table S1. List of the 305 deregulated genes (up in red, down in blue) in MNU-injected *Yap* CKO mice compared to MNU-injected Control mice. Related to Figure 1.**

| GENE NAME | SPECIES | FORWARD | REVERSE |
|---|---|---|---|
| *Ai9 (genotyping)* | *mus musculus* | CTGTTCCTGTACGGCATGG | GGCATTAAAGCAGCGTATCC |
| *Ascl1* | *mus musculus* | TCTCCTGGGAATGGACTTTG | GGTTGGCTGTCGGTTTGTT |
| *Ccnd1* | *mus musculus* | CCCAACAACTTCCTCTCCTG | TCCAGAAGGGCTTCAATCTG |
| *Ccnd3* | *mus musculus* | CGAGCCTCCTACTTCCAGTG | GGACAGGTAGCGATCCAGGT |
| *Cyr61* | *mus musculus* | TGAAGAGGCTTCCTGTCTTTGG | CGGCACTCTGGGTTGTCATT |
| *Egfr* | *mus musculus* | TGGCATCATGGGAGAGAACA | GTACAGTTGGCGTGGCATAG |
| *Gfap* | *mus musculus* | CCAGTTACCAGGAGGCACTTG | CGATGTCCAGGGCTAGCTTAA |
| *Hbegf* | *mus musculus* | GCATCCAAAGTGATCGCTGC | GACAACACTGCGGCCAGAAA |
| *Rax-CreERT$^2$ (genotyping)* | *mus musculus* | TTCCCGCAGAACCTGAAGAT | CCCCAGAAATGCCAGATTAC |
| *Rps26* | *mus musculus* | CCCAAGGATAAGGCCATCAAG | AAGCACGTAGGCGTCGAAGA |
| *Srp72* | *mus musculus* | CACCCAGCAGACAGACAAACTG | GCACTCATGGTAGCGTTCCA |
| *Tbp* | *mus musculus* | CACAGGAGCCAAGAGTGAAGAAC | ATCACAGCTCCCCACCATGT |
| *Ctgf* | *xenopus leavis* | CCTTCCCACGAAGAGTCAAA | GCAAAGCAGGTCCGACTAAG |
| *Cyr61* | *xenopus leavis* | GACTCTTCAGCACGGGCTAC | GGGAGTCTGTGTGTCCCTGTGT |
| *Odc1* | *xenopus leavis* | GCTTCTGGAGCGGGCAAAGGA | CCAAGCTCAGCCCCCATGTCA |
| *Rpl8* | *xenopus leavis* | CCACGTGTCCGTGGTGTGGCT | GCGCAGACGACCAGTACGACG |

**Table S2. List of primers. Related to the STAR Methods section.**

| | | | | | Experiments on mouse | | Experiments on *Xenopus* |
|---|---|---|---|---|---|---|---|
| **Antigene** | Host | Supplier | Reference | Dilution (WB) | Dilution (IF) | Dilution (IF-FM) | Dilution (IF) |
| **Primary antibodies** | | | | | | | |
| **AKT** | mouse | SIGMA | P2482 | 1:1,000 | | | |
| **Alpha-tubulin** | mouse | SIGMA | T5168 | 1:10,000 | | | |
| **Alpha-tubulin** | mouse | SIGMA | T9026 | 1: 5,000 | | | |
| **Arrestin** | rabbit | EMD Millipore | AB15282 | | 1:1,000 | | |
| **Brn3a** | mouse | Santa Cruz | sc-8429 | | 1:100 | | |
| **BrdU** | | | | | | | 1:200 |
| **Calbindin D-28k** | rabbit | Swant | 300 | | 1:100 | | |
| **Calretinin** | mouse | EMD Millipore | MAB1568 | | 1:1,000 | | |
| **CD68** | rat | Bio-Rad UK | MCA1957GA | 1:1,000 | | 1:1,000 | |
| **Cyclin D1** | rabbit | Abcam | ab134175 | 1:1,000 | 1:100 | | |
| **Cyclin D3** | mouse | Cell Signaling | 2936 | 1:1,000 | 1:100 | | |
| **ERK1/2 (p44/42 MAPK)** | rabbit | Cell Signaling | 9102 | 1:1,000 | | | |
| **FLAG** | rabbit | Sigma | F7425 | 1:1000 | 1:300 | | |
| **GFAP** | rabbit | Dako | Z0334 | | 1:500 | | |
| **GFP** | goat | Abcam | ab6673 | | 1:1,000 | | |
| **Glutamine synthetase** | mouse | Abcam | Ab64613 | | | | 1:200 |
| **Lectin PNA alexa 568 conjugate** | peanut | Thermo Fisher Scientific | L32458 | | 1:500 | | |
| **P-AKT (Ser473)** | rabbit | Cell Signaling | 9271 | 1:1,000 | | | |
| **P-ERK1/2 (Thr202/Tyr204)** | rabbit | Cell Signaling | 4370 | 1:1,000 | 1:100 | | |
| **Red Fluorescence Protein** | rabbit | Rockland | 600-401-379 | | 1:100 | | |
| **Rhodopsin** | mouse | EMD Millipore | MAB5316 | | 1:2,000 | | |
| **Rhodopsin** | mouse | EMD Millipore | MABN15 | | | | 1:500 |
| **S-opsin** | rabbit | EMD Millipore | AB5407 | | 1:500 | | |
| **SOX9** | rabbit | EMD Millipore | AB5535 | | 1:300 | 1:300 | |
| **TAZ** | rabbit | Abcam | ab110239 | 1:1,000 | | | |
| **Vimentin** | rabbit | Santa Cruz | sc-7557 | | 1:200 | | |
| **YAP** | mouse | Abcam | ab56701 | 1:1,000 | 1:50 | | |
| **YAP** | rabbit | Abcam | ab52771 | | | | 1:100 |
| **Secondary antibodies** | | | | | | | |
| **Alexa 488 anti-mouse IgG1** | goat | Thermo Fisher Scientific | A21121 | | 1:500 | | |
| **Alexa 488 anti-rabbit** | donkey | Thermo Fisher Scientific | A21206 | | 1:500 | | |
| **Alexa 555 anti-mouse IgG1** | goat | Thermo Fisher Scientific | A21127 | | 1:500 | | |
| **Alexa 555 anti-mouse IgG2b** | goat | Thermo Fisher Scientific | A21147 | | 1:500 | | |
| **Alexa 555 anti-mouse IgG2a** | goat | Thermo Fisher Scientific | A21127 | | 1:500 | | |
| **Alexa 555 anti-rabbit** | donkey | Thermo Fisher Scientific | A31572 | | 1:500 | | |
| **Alexa 488 anti-mouse** | goat | Thermo Fisher Scientific | A11001 | | | | 1:1,000 |
| **Alexa 594 anti-mouse** | goat | Thermo Fisher Scientific | A11005 | | | | 1:1,000 |
| **Alexa 594 anti-rat** | goat | Thermo Fisher Scientific | A11007 | | | | 1:1,000 |
| **Alexa 488 anti-rabbit** | goat | Thermo Fisher Scientific | A11008 | | | | 1:1,000 |
| **Alexa 594 anti-rabbit** | goat | Thermo Fisher Scientific | A11012 | | | | 1:1,000 |
| **HRP anti-mouse IgG** | goat | Sigma-Aldrich | A4416 | 1:5,000 | | | |
| **HRP anti-rabbit IgG** | donkey | GE Health | NA934V | 1:5,000 | | | |

**Table S3. List of antibodies. Related to the STAR Methods section.** WB: western blot, IF: immunofluorescence, IF-FM: immunofluorescence on flat mounted retinas.

# DISCUSSION AND PERSPECTIVES

## YAP AS A STIMULATOR OF MÜLLER CELLS' REGENERATIVE POTENTIAL

Recent discoveries pointing to a function of YAP in the regulation of adult stem cells and in the regeneration of several tissues make it a good candidate for regenerative medicine (Moya and Halder, 2019). In the retina, we have shown that YAP is involved in the reactivation of Müller cells by triggering their exit from quiescence in a degenerative context. In addition, YAP overexpression powerfully boosted their proliferative properties both *ex vivo* and *in vivo*. Noteworthy, the increase in the Müller cells' mitogenic potential induced by YAP has been revealed to be more efficient than the stimulation of Müller cell proliferation with growth factors, notably EGF. First, although Müller cell proliferation has been reported after EGF stimulation, its efficacy depends on the age of the animals. Thus, while 86% of Müller cells proliferate after EGF-treatment at P8, only 9% of Müller cells proliferate when the EGF treatment is performed in retinal explants from mice at P12 (Löffler et al., 2015). Compared with YAP5SA, about 25% of Müller cells proliferate in retinal explants from mice at P30 following 7 days of culture and increase up to 75% after 12 days of culture. This age-dependent effect of Müller cell proliferation has been also reported after forced transgenic expression of ASCL1 *in vivo*, which is no more efficient past 2 weeks of age (Ueki et al., 2015), reinforcing the idea of YAP as a powerful stimulator of the mitogenic potential of Müller cells, as it is capable to induce their proliferation in adult retinas. Second, although EGF can stimulate Müller cell proliferation in the retinal explants' degenerative context, it does not have any mitogenic effect in undamaged chick or mouse retinas (Todd et al., 2015). The same applies to the proneural transcription factor ASCL1, which stimulates the reprogramming and proliferation of Müller cells following retinal damage but not in intact retina (Ueki et al., 2015). On the contrary, overexpression of YAP5SA is sufficient to trigger Müller cell proliferation in vivo in a non-degenerative context. Only Wnt signaling has been proved to have a similar efficiency than YAP5SA, as forced expression of wild-type β-catenin in adult mouse retina without retinal injury results in a robust proliferative response of Müller cells comparable to retinal injury combined with treatment of the mitogenic growth factor (Yao et al., 2016).

## DIVERS MECHANISMS TO OVERACTIVATE YAP AND INDUCE MÜLLER CELL PROLIFERATION

In addition to the AAV-mediated overexpression of a constitutively active form of YAP, genetic loss or bypass of Hippo signaling within adult MGs also results in spontaneous MG proliferation, suggesting that activation of the Hippo signaling act as a regulatory mechanism blocking mammalian Müller cell proliferation (Rueda et al., 2019). Interestingly, although in both cases manipulation of the Hippo

signaling pathway leads to the proliferation of Müller cells, the genetic overexpression of YAP5SA using the *Yap5SA* transgenic mouse (Rueda et al., 2019) is slightly less efficient compared to the AAV-YAP5SA overexpression. Indeed, just 17% of Müller cells proliferate in case of NMDA-induced damage in the *Yap*5SA transgenic mouse versus 25% in AAV-YAP5SA infected retinal explants after 7 days of culture.

This differences in the efficacy may be related to the different overexpression strategy. For instance, in the transgenic mouse the YAP5SA transgene is randomly inserted into the genome and is driven by the ubiquitous CAG promoter upon GLAST-CreERT2-mediated recombination (Rueda et al., 2019). Strikingly, the expression of YAP5SA in this model requires a 5-day tamoxifen treatment (Rueda et al., 2019). To generate our *Yap* CKO mice, just 2 tamoxifen injections (performed at P10 and P14) were necessary to highly reduce the protein level of YAP. On the contrary, their *Yap* and *Taz* knock-out model still possess around 60% of *Yap* and *Taz* mRNA expression after three injections of tamoxifen per week for 3 weeks, indicating some inefficiencies in the Cre-mediated recombination of the floxed loci in their model (Rueda et al., 2019). In addition, they used a GLAST ubiquitous promoter which led to a mosaic expression of the Cre-recombinase in the adult Müller cells (Rueda et al., 2019). Compared to our Rax-Cre system, all the Müller cells were specifically targeted, suggesting that the inefficiency observed in their Cre-recombinant models may also be related to the Cre-mosaic expression. Of note, although it has been reported that the AAV-ShH10 have some tropism for a subset of NeuN (Rbfox3)+ ganglion and amacrine cells, it remains highly selective for Müller cells as almost 100% of Müller cells are infected compared to 2% of infected interneurons and 4% of retinal ganglion cells (Byrne et al., 2013; Klimczak et al., 2009; Yao et al., 2016). Thus, although genetic modification of the Hippo signaling pathway is sufficient to induce the proliferation of Müller cells, other alternatives should be considered in order to increase the efficacy of the process.

## YAP/TAZ AS DIRECT REGULATORS OF CYCLIN D1 EXPRESSION

Regarding the mechanisms involved in the YAP-dependent proliferative function, we have related the proliferative effect of YAP on Müller cells to its capacity to trigger cell cycle re-entry, as many cell-cycle genes, including CCND and CDK genes, are downregulated in YAP CKO reactive Müller cells. More specifically, we found in Müller cells that YAP is necessary both to maintain basal levels of Cyclin D1 and D3 in physiological conditions and for enhancing their expression upon injury. Interestingly, a similar effect on Cyclin D1 and D3 expression was found in a double YAP/TAZ cKO, suggesting that TAZ it is not involved in the regulation of the cell cycle re-entry (Rueda et al., 2019).

Many different transcription factors have been identified that directly regulate CCND1 promoter (Wang et al., 2004). In cancer cells, YAP has been described as one of them (Mizuno et al., 2012). In

Müller cells, a recent ChIP-qPCR analyse have showed the presence of a TEAD motif upstream the Cyclin D1 start site that was highly enriched for YAP (Rueda et al., 2019). Together with our results, these data suggest that YAP/TEAD are likely a direct transcriptional regulator of Cyclin D1 in Müller cells.

## YAP WITHIN A COMPLEX SIGNALING NETWORK

Connections between YAP and other signaling pathways involved in the reactivation of Müller cells have already been described. For example, YAP interacts with MAPK, TGFβ, Wnt ou Notch pathways in different cell types during development or in tumor formation (figure 40) (Mauviel et al., 2012; Varelas and Wrana, 2012; Zhang et al., 2015), but also during organ regeneration. For instance, in the intestine the YAP-dependent stem cell reprogramming and regeneration is reliant on Wnt and EGFR signaling pathways (Gregorieff et al., 2015). In addition, in the postnatal retina of *Yap*$^{+/-}$ mice we discovered a correlation between the compensatory mechanism by TAZ and the increase in EGFR activity, similarly to what had been previously described in other tissues (Chen and Harris, 2016; He et al., 2015; Yang et al., 2016), that suggested a function of both YAP/TAZ in the activation of the EGFR pathway. This hypothesis was proven in degenerative conditions in our *Yap* cKO model, where we found that the upregulation of YAP in case of damage was required to the genetic upregulation of several components of the EGFR pathways, as well as of its activity (figure 41). Moreover, we showed that EGFR signaling activity is required for YAP mitogenic effects on Müller cells. Altogether, this points to the existence of a YAP-EGFR axis that trigger Müller cell-cycle re-entry in response to retinal damage. Interestingly, it has been shown in other tissues that the EGFR pathway also regulates YAP activity (Fan et al., 2013; Reddy and Irvine, 2013; Chen and Harris, 2016; Haskins et al., 2014). Although we have not investigated such interaction in Müller cells in response to damage, we do not exclude the possibility of the existence of a positive feedback loop in which the EGFR pathway would modulate the Hippo signaling (figure 41).

However, it is probably not the only crosstalk that YAP establishes in the retina in case of damage. Indeed, as discussed above YAP overexpression is more potent than EGF treatment. One hypothesis to explain this increased proliferative effect is that, as mentioned above, it likely directly regulates some cell cycle genes. Another possibility is that, in addition to EGFR pathway, YAP regulates other signaling pathways involved in retinal regeneration to trigger a robust mitogenic stimulation of Müller cells. Thus, it would be interesting to study the relationship of YAP with other regenerative signaling pathways such as Notch or Wnt in Müller cells under degenerative conditions. Both signaling pathways are indeed good candidates because they are involved in the Müller cell-dependent retinal regeneration and they are known to interact with YAP in other cell types (figure 40)(Zhang et al., 2015).

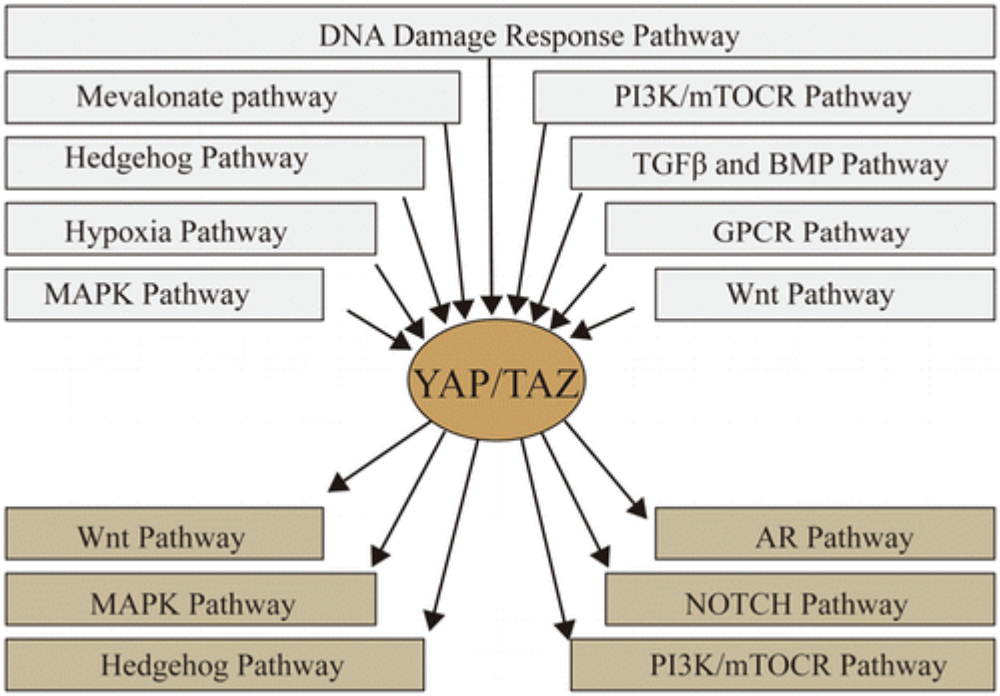



**Figure 40. YAP/TAZ crosstalk with other signaling pathways in mammals.** MAPK, Wnt, TGFβ/BMP, GPCR, PI3K-mTOR or Hedgehog signaling pathways regulates the activation of YAP/TAZ. In turn, YAP/TAZ regulates MAPK, Wnt, TGFβ/BMP, PI3K-mTOR, Hedghehog or Notch. From (Zhang et al., 2015).

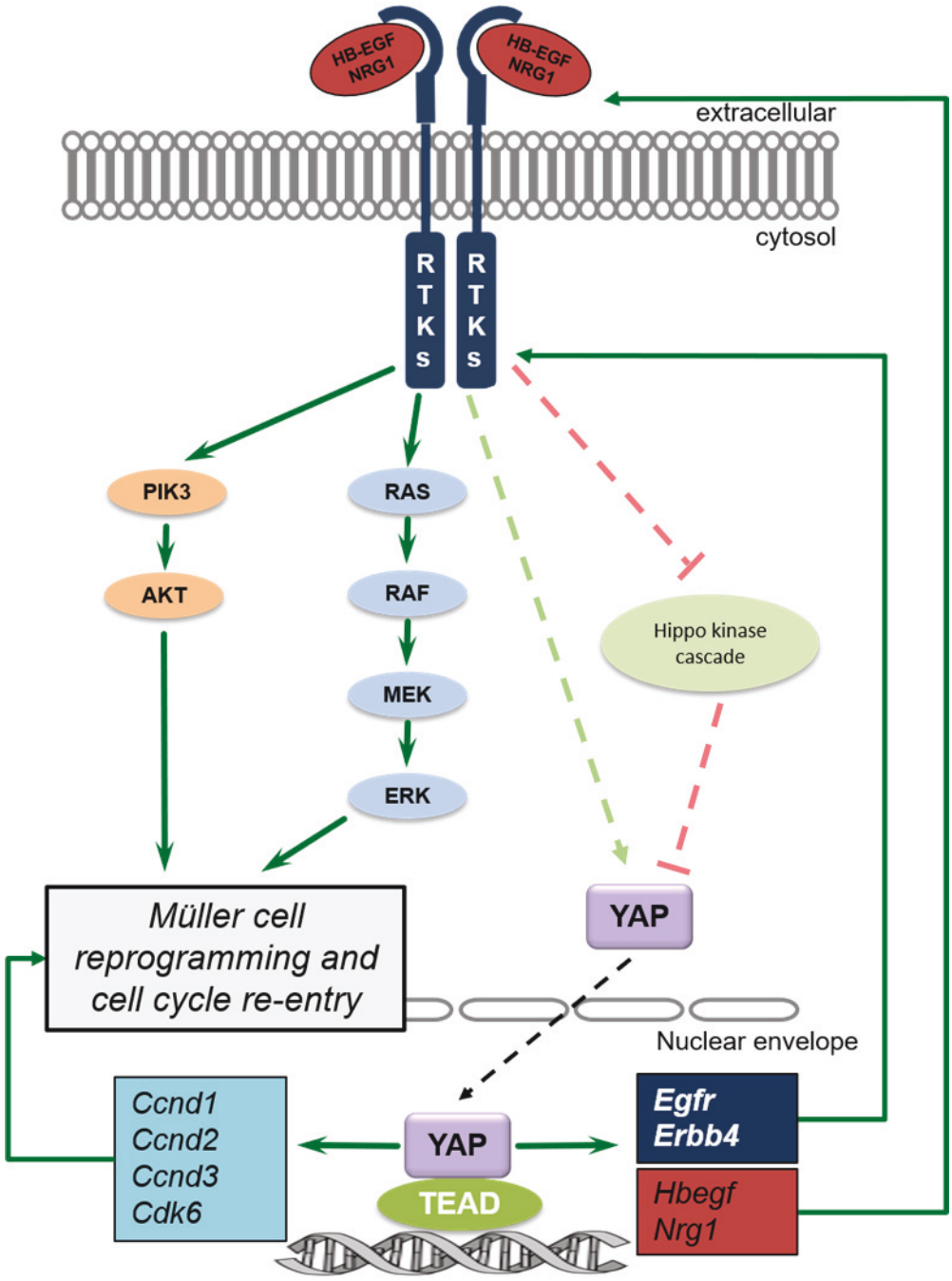



**Figure 41. Proposed model of YAP-EGFR pathway interaction in Müller glia cell cycle re-entry upon injury.** Our results support the idea that YAP and EGFR signaling functionally interact in a retinal degenerative context to induce cell cycle genes in Müller glia. YAP likely acts upstream the EGFR pathway by regulating the expression of EGFR receptors (EGFR and ERBB4) and ligands (HBEGF and NRG1). The EGFR pathway would in turn activate Müller cell reprograming and cell cycle re-entry, as previously reported(Close et al., 2006; Karl et al., 2008; Todd et al., 2015; Wan et al., 2012; Wan et al., 2014). Of note, YAP may also independently impact these processes through direct regulation of cell cycle and reprogramming genes, such as those identified in our RNAseq dataset. Green and red dashed arrows indicate the possible feedback loop between EGFR and Hippo pathways.

In addition, interactions between the EGFR/MAPK and Notch or Wnt have been described in zebrafish retinas or in mice during intestinal regeneration respectively (Gregorieff et al., 2015; Wan et al., 2012). Giving our reported interaction between YAP and EGFR pathway, we do not exclude a possible existence of a functional interaction between all the different signaling pathways with YAP at the core of Müller cell reactivation mechanisms. However, neither Notch nor Wnt signaling pathways come out as particularly deregulated in our RNAseq analyzes, suggesting that the interaction among them could be done at a protein level or through a common gene regulatory network required for Müller cell proliferation, and not by direct modulation of the genetic expression of the components of the signaling pathway. Of note, in the absence of YAP we observed a deregulation on the BMP pathway. It would be thus interesting to perform further analysis regarding the interaction between BMP and YAP signaling pathways in Müller cells.

## YAP-DEPENDENT MÜLLER CELL PROLIFERATION GENERATES NEW NEURONS?

These functions of YAP make it worth considering to use it to induce the reprogramming of Müller cells and stimulate their regenerative potential in a similar way as for the heart, liver or intestine (Johnson and Halder, 2013; Wang et al., 2017). However, whether the increased proliferation of YAP-overexpressing Müller cells leads to the production of new neuronal cells is an important point to address. Indeed, most of the studies focused on mammalian Müller cell-dependent regeneration reported that only a very small subset of reactivated cells may differentiate into neurons (Das et al., 2006; Karl et al., 2008; Ooto et al., 2004; Osakada and Takahashi, 2007; Wan et al., 2008; Yao et al., 2018). This is also likely the case for YAP, as another study, published in the same journal issue than our own article, showed by single cell RNAseq that only very few YAP-overexpressing Müller cells became retinal neurons (Rueda et al., 2019). One possibility is that the forced induction of the Müller cell proliferation by YAP overexpression generates a stress during the replication of DNA that would trigger cell death mechanisms as apoptosis. In fact, the overexpressed YAP might also be responsible for this switch to apoptosis, as it has been reported that YAP interacts with p73 and promote apoptosis in response to DNA damage *in vitro* (Lapi et al., 2008; Strano et al., 2005). In the same line, it could be possible that homeostasis mechanisms would lead to Müller cell death in an attempt to control the excessive proliferation of those cells. In our retinal explants, the density of Müller cells remains unchanged between YAP5SA and GFP conditions (data not shown). Although we have not studied if our proliferative Müller cells generate new neurons, this result suggested that the proliferative Müller cells may indeed been dying. To determine if YAP overexpression in Müller cells was associated with an increase in cell apoptosis as it has been described in intestine (Su et al., 2015), I performed TUNEL analysis in retinal explants infected with AAV-YAP5SA. AAV-GFP infected retinal explants were used as

controls. Although I detected a slightly increase in the number of TUNEL positive cells between AAV-YAP5SA and AAV-GFP infected retinal explants, further analysis has to be done in order to assess these results (data not shown). Finally, the reduced transformation of Müller cells into retinal neurons may also be related with the inhibitory effect of YAP on stem cell differentiation. In fact, several studies have reported that YAP is necessary to stem cell proliferation but impedes their differentiation during development (Beyer et al., 2013; Lian et al., 2010; Tamm et al., 2011).

In order to favor neuron regeneration following YAP overexpression, it would be important to be able to perform a temporary overexpression, just to trigger quiescence exit Müller cells and boost their proliferation. Then, in a second phase, it could be pertinent to overexpress photoreceptor specification factors to favor neuronal differentiation of Müller cell-derived retinal progenitors. Such a two-step reprogramming approach has recently been developed by Chen's laboratory (ref). In this work, the authors obtained Müller glia proliferation by stimulating Wnt signaling and then Crx, Otx2 and Nrl were overexpressed to force these proliferative Müller cells to differentiate into rod photoreceptors. Noticeably, this could restore some visual responses in a mouse model of congenital blindness. Of note, a complete different interpretation of the data has been raised (Wang et al., 2020). It could be possible indeed that the existing photoreceptors in the blind mice were repaired either because they took up the virus carrying the corrective gene (none-specific cell infection could indeed occur), or because of gene transfer from Müller glia (this has recently been observed from transplanted photoreceptors to resident photoreceptors). In either case, the visual restauration would not result from Müller-derived photoreceptors, but from existing photoreceptors with restored function. It is thus absolutely crucial to perform lineage labeling to fully prove that functioning photoreceptors really derive from reactive Müller glia. Of note, this two-step method could not be necessary, as a recent study has shown that the knockdown of Ptbp1 (polypyrimidine tract-binding protein 1) using an *in vivo* viral delivery of a new RNA-targeting CRISPR system CasRx is sufficient to trigger Müller cell conversion into retinal ganglion cells and partially restore the visual function in NMDA-injured retinas (Zhou et al., 2020). Again, lineage labeling should be done to fully prove that these ganglion cells really derive from reactive Müller cells. Strikingly, this downregulation of PTBP1 is also sufficient to convert mouse and human astrocytes to functional neurons (Qian et al., 2020). So, even if complementary analysis has to be done to assess that the "new created" photoreceptors and ganglion cells are indeed derived from Müller cells, these studies constitute a big hope for regenerative ophthalmic therapy.

In order to regulate the timing of YAP overexpression, with the idea of a two-step strategy (first increase proliferation then differentiation), we could use doxycycline (Doxy)-inducible *Yap* lentiviral vectors that have proved sufficient to reprogram differentiated cells into stem cells (Panciera et al.,

2016). Another option would be to replace the overexpression of YAP by a temporary activation of YAP using a pharmacological approach. For example, XMU-MP-1, a selective inhibitor of MST1/2 kinases, favors the translocation of YAP to the nucleus and the expression of its target genes, acting like a stimulant of cell proliferation, tissue repair and regeneration in different models of hepatic and intestine damage (Fan et al., 2016). During my PhD, I had the opportunity to work with this pharmacological agent. The repeated intraperitoneal injection of this drug in the MNU model of retinal degeneration revealed an increased expression of genes involved in cell proliferation (such as CCND1, data not shown). However, my experiments did not show a conclusive action of YAP in those process, as no inhibition of the Hippo signaling pathway was found (data not shown). Although more studies should be done to clearly assess the efficacy of this drug in triggering YAP-dependent Müller cell proliferation, the use of this pharmacological approach as a new therapeutic strategy might be challenging. However, several pharmacological studies are currently in course to develop activators and inhibitors of the Hippo signaling pathway, as the recent discovered pan-TEAD small molecule that acts as a surrogate for TEAD palmitoylation making him act as a negative dominant (Holden et al., 2020); or the quinolinol, that stabilize YAP/TAZ protein levels and increase their activity (Pobbati et al., 2019). Along with that, it would be interesting to test this last component in our retinal model. So, we hope that new components triggering YAP activity could be discovered in the near future and tested in a retinal regenerative context.

# PART 3

## MICROGLIA-MÜLLER CELL CROSSTALK IN THE MAMMALIAN RETINAL REGENERATION

Although it has been described that the inflammatory response can have a beneficial effect in Müller cell-dependent retinal regeneration in regenerative species, there are very few studies regarding the effect of inflammation in retinal regeneration in mammals. Giving the role of the inflammatory response in the regeneration of other mammalian organs, I develop a project to explore the potential impact of inflammatory signaling on Müller cell proliferative behavior. This study is still under progress, but the main data are presented here.

## 1. METHODOLOGY

In this study, I took advantage of retinal explant culture as a retinal degeneration model. The inflammation was induced or inhibited using **pharmacological tools**. To induce the inflammation, I used LPS, a bacterial lipopolysaccharide that is recognized by the toll-like receptor TLR4. To ablate microglia, I used PLX3397 (pexidartinib), a kinase inhibitor that blocks the colony stimulating factor receptor 1 (CSF-1R), a receptor necessary for microglia activity and viability.

## 2. MAIN RESULTS

- LPS-induced reactive microglia provide signals able to stimulate the proliferation of mouse Müller cells.
- Surprisingly however, these LPS-induced proliferating Müller cells do not reprogram into progenitor-like cells.
- Ablation of microglia inhibits Müller cell proliferation, revealing the role of microglia in shaping the injury response of Müller cells to injury.

# Part 3

**Diana García-García**, Lorena Vidal-Gil, Catherine Hottin, Juliette Bitard and Muriel Perron

# Hyper-activated microglia awake Müller cell proliferative potential but inhibit their reprogramming capacities

*Ongoing work*

**ABSTRACT**

Mammals have a very limited retinal regenerative capacity compared to fish or amphibians. Recently, microglia-dependent inflammation has been pointed as a new positive modulator of Müller cell regenerative response in fish and chick. On the other hand, the role of neuroinflammation in mammalian nervous system regeneration remains elusive. Here we sought to determine the role of inflammatory signals in mammalian Müller cell behavior in a degenerative retina. Using *ex vivo* retinal explants, we discovered that chronic immune challenge is able to elicit Müller glia cell cycle re-entry and that this proliferative response requires hyper-activated microglia. Our data point to IL-6 as one of the inflammatory cytokines involved in this inflammatory-induced Müller cell proliferation. Strikingly however, this proliferative response is accompanied by the inhibition of Müller cell reprogramming into neurogenic progenitors. Altogether, our results support a model in which inflammatory signals released from over-stimulated microglia have a positive impact on Müller cell proliferation but do not confer any neurogenic potential. As a conclusion, this study brings novel insights into the mechanisms that enable mammalian Müller cell to exit their quiescence state and about differences between mammalian and non-mammalian species regarding their regenerative capacities.

# INTRODUCTION

Retinal damage or neurodegenerative retinal diseases lead to vision loss in mammals. In contrast, some vertebrate species can regenerate their retina following injury thanks to the reprograming of Müller glia into retinal stem cells. Müller cells are the main type of glial cells in the vertebrate retina where they perform a range of functions that contribute to the maintenance of retinal homeostasis and visual function (Devoldere et al., 2019). In case of neuronal damage, they undergo gliosis and exhibit a tendency to re-enter into the cell cycle. In zebrafish or *Xenopus*, this process is efficient and accompanied by a neurogenic competence which results in the regeneration of retinal neurons (Goldman, 2014; Hamon et al., 2016; García-García et al., 2020). In mammals, some restrictions appear already in the proliferative capacity, resulting in a negligible level of regeneration in case of retinal degeneration (Langhe and Pearson, 2019; Wilken and Reh, 2016; García-García et al., 2020). In the last decade, several signalling pathways, such as Wnt, Hedgehog, Hippo, have been reported as involved in the control of the proliferation and neurogenic potential of Müller cells (Langhe and Pearson, 2019; García-García et al., 2020). Signaling related to innate immunity is now also emerging as a key regulator of Müller cell-mediated retinal regeneration (see review García-García et al., 2020). Microglia are the resident immune cells of the retina. Although recent evidences indicate that microglia participate in neurodegeneration processes under pathological conditions in mammals (Silverman and Wong, 2018b), these cells appear necessary for Müller cell proliferation following injury in regenerative species, such as zebrafish and chick (Conedera et al., 2019; Fischer et al., 2014; Palazzo et al., 2019; Silva et al., 2020; White et al., 2017). In addition to resident microglia, infiltrating immune cells also appear to participate to the regenerative response following retinal damage (Hui et al., 2017; Mitchell et al., 2018). Yet, how immune cells and Müller cells interact and cooperate is not well understood. TNF-α and IL-6-family cytokines have been shown to promote Müller cell reprogramming and proliferation in the chick and/or the fish retina upon injury (Conner et al., 2014; Elsaeidi et al., 2014; Fischer et al., 2014; Goldman, 2014; Iribarne et al., 2019; Nelson et al., 2013; Zhao et al., 2014). Similarly, the cytokine midkine in zebrafish is required to trigger Müller cell cycle progression and proliferation, as its suppression lead to a blockage of Müller cells in the G1 phase and an inhibition in retinal regeneration upon photoreceptor degeneration (Nagashima et al., 2020). Regarding the mechanisms regulating the effect of inflammatory cytokines on Müller cell-dependent regeneration, it seems that they trigger key signalling pathways in Müller cells such as mTOR in the fish (Zhang et al., 2020) or NF-κB in the chick (Palazzo et al., 2020). Altogether, these data support a positive influence of inflammatory signals on Müller cell-dependent retinal regeneration in regenerative species. In mammals, microglial cells have been shown to be involved in the proliferation of retinal precursor cells during postnatal development in mice (Kuse et al.,

2018). However, how inflammation influences Müller cell behavior in mammals remains unknown. We thus took advantage of the mouse retinal explant *ex vivo* model to determine the impact of an immune challenge on Müller cell proliferation. We discovered that hyper-activated microglia can trigger the re-entry into the cell cycle of a subpopulation of Müller cells. Our data also suggest that this mitogenic effect may be due, at least in part, to the pro-inflammatory cytokine IL-6. Finally, we revealed that inflammatory-induced proliferative Müller cells do not reprogram into neurogenic retinal progenitors. As a whole, this study brings new insights into the mechanisms underlying mammalian Müller cell behaviour under pathological condition, highlighting that inflammatory signals released from over-stimulated microglia have a positive impact on Müller cell proliferation but inhibit their neurogenic potential.

# MATERIAL AND METHODS

**Ethics statement and mice**

All animal experiments have been carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EEC). All animal care and experimentation were conducted in accordance with institutional guidelines, under the institutional license D 91-272-105. The study protocols were approved by the institutional animal care committee CEEA n°59 and received an authorization by the "Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche" under the reference APAFIS#1018-2016072611404304v1. Wild-type C57Bl/6JRj mice were kept at 21°C, under a 12-hour light/12-hour dark cycle, with food and water supplied *ad libitum*. All experiments involving adult mice were performed with male or female mice that were 3 to 8 weeks of age. No difference between sexes was observed in any retinal phenotype.

**Mouse retinal explants**

Retinas from enucleated eyes were dissected in Hanks' Balanced Salt solution (GIBCO) by removing the anterior segment, vitreous body, sclera and RPE. The retina was then cut radially into four equal-sized pieces and flat-mounted onto a microporous membrane (13 mm in diameter; Merk Millipore) in a twelve-well culture plate, with the ganglion cell layer facing upward. Each well contained 1 mL of culture medium, consisting in DMEM-Glutamax (GIBCO) supplemented with 1% FBS, 0.6% D-Glucose, 0.2% NaHCO3, 5mM HEPES, 1% B27, 1% N2, 1X Penicillin-Streptomycin. Culture medium containing 5 ng/mL LPS from Escherichia coli O111:B4 (Lypopolysacharide, Sigma), 20 ng/ml of recombinant human IL-6 (Interleukin-6, R&D Systems), 2 or 10 µM of PLX3397 (Pexidartinib, Clinisciences) or vehicle was added from the beginning of the explant culture. Explants were maintained at 37°C in a humidified incubator with 5% CO2. Half of the culture medium was changed

every two days. One drop of medium was added daily over the retinal explants to avoid drying. For proliferation assays, 10 mM EdU was applied at the indicated days of culture before fixation.

**Western-blotting**

Each protein extract was obtained from a single retina, quickly isolated and frozen at −80°C. Retinas were then lysed in P300 buffer (20 mM Na2HPO4; 250 mM NaCl; 30 mM NaPPi; 0.1% Nonidet P-40; 5 mM EDTA; 5mM DTT) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysate concentration was determined using the Lowry protein assay kit (DC Protein Assay; Bio-Rad) and 15 µg/lane of each sample were loaded for western-blot analysis. At least 7 individuals were tested per condition. Primary/secondary antibodies and their corresponding working dilutions are listed in Table S2. Protein detection was performed using an enhanced chemiluminescence kit (Bio-Rad). Each sample was probed once with anti-α-tubulin antibody for normalization. Quantification was done using Fiji software (National Institutes of Health) (Schindelin et al., 2012).

**RNA extraction and RT-qPCR**

Total RNA was extracted from mouse neural retina using RNeasy mini kit (QIAGEN). RNA concentration was assessed using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific). Total RNA was reverse transcribed in the presence of oligo-(dT)20 using Superscript II reagents (Thermo Fisher Scientific). For each RT-qPCR reaction, 1.5 ng of cDNA was used in triplicates in the presence of EvaGreen (Bio-Rad) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Differential expression analysis was performed using the ΔΔCt method using the geometric mean of *Rps2*6, *Srp7*2 and *Tbp* as endogenous controls (Vandesompele et al., 2002). Relative expression of each gene in each sample was calculated using the mean of the controls as the reference (1 a.u.). Primers are listed in Table S1. RT-qPCR experiments were performed on at least 7 mice per condition, allowing for statistical analysis.

**Histology and Immunofluorescence**

Retinal explants were fixed in 1X PBS, 4% paraformaldehyde for 20 minutes at room temperature. Explants were washed in PBS and then incubated for 30 minutes at room temperature in blocking solution, which consisted of Dako diluent (Dako (Agilent)) and 0.3% Triton X-100 (Sigma-Aldrich UK). Explants were then incubated in mouse monoclonal primary antibodies for 48 to 72 hours at 4°C, washed thoroughly, and incubated in fluorescent secondary antibody for 24-48 hours at 4°C. Explants were finally washed and whole-mounted on slides or blocked during 1h at room temperature in PBS 1X containing 5% normal goat serum (Invitrogen Inc.) and 0.3% Triton X-100

(Sigma-Aldrich UK) before treated for EdU labeling detection as described below. Primary/secondary antibodies and their corresponding working dilutions are listed in Table S2.

**TUNEL assay and EdU labelling**

Detection of apoptotic cells was conducted on retinal sections using DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. For double labelling, TUNEL labelling was done first, followed by immunostaining. EdU incorporation was detected using the Click-iT EdU Imaging Kit (Thermo Fisher Scientific) according to manufacturer's recommendations. For double labelling, immunostaining was done first, followed by EdU labelling. Coverslips were mounted using Fluorsave Reagent (Millipore, USA).

**Imaging**

Fluorescence images were acquired using an ApoTome-equipped AxioImager.M2 microscope or a Zeiss LSM710 confocal microscope. Images were processed using Zen (Zeiss) software. The same magnification, laser intensity, gain and offset settings were used across animals for any given marker.

**Quantification and Statistical Analysis**

Mean number of labeled cells in mouse retinal explants were calculated from 8 different fields of $10^4$ $\mu m^2$ per retina (2 in each quarter) and using at least 7 explants per condition. Statistical analysis was performed with GraphPad Prism 8.03 (GraphPad Software, La Jolla California USA) using the non-parametric Mann-Whitney test in all experiments. p value $\leq 0.05$ was considered significant. All results are reported as mean ± SEM.

# RESULTS AND DISCUSSION

**Microglia become hyper-activated in response to LPS immune challenge in degenerating retinal explants**

To examine the effect of inflammation on Müller cell proliferation, we used adult mouse retinal explants cultured in the presence of lipopolysaccharide (LPS), a bacterial endotoxin commonly used to stimulate the pro-inflammatory response of microglia both *in vitro* and *in vivo* (Hoogland et al., 2015; Lively and Schlichter, 2018; Lund et al., 2006). We used retinal explants as this technique (*i*) preserves the complex 3D organisation of the retina, but at the same time (*ii*) offers the simplicity of *ex vivo* experimentation, (*iii*) represents a common model of retinal degeneration, and last but not least (iv) allows studying microglia-dependent inflammatory response, as the recruitment of peripheral macrophages from the blood circulation cannot occur. First, we sought to characterize the dynamics of the inflammatory response to LPS treatment in retinal explants, by analysing the time course of microglial activation after 1 to 7 days *ex vivo* (DEV) culture (Figure 1A). To this aim, we studied the immunoreactive profile of CD68, a lysosomal protein enriched in phagocytic cells, and as such, the most widely used marker of microglial activation (Korzhevskii and Kirik, 2016). In control explants, CD68 labelling was observed from DEV3 and increased up to DEV7 (Figure 1B, C). Other signs of activation were also evident as microglia acquired an amoeboid shape with short thick processes, compared to highly ramified resting microglia, and exhibited increased rate of proliferation (data not shown). This result is consistent with the expected activation of resident microglial cells upon retinal explant degeneration. We found that LPS treatment did not trigger, at any culture time points, further increase in the number of CD68-labelled cells (Figure 1B, C), nor any changes in microglial cell morphology or in their distribution in the different layers of the explants (data not shown). On the contrary, the number of CD68-positive microglia was even reduced at DEV3 compared to control (Figure 1C). Since LPS was previously described as able to make CD68-activated microglia become hyper-reactive (producing an amplified and prolonged proinflammatory response) (Witcher et al., 2015), we analyzed the expression of typical pro-inflammatory factors, Il6, Il1β and TNFα, as well as the expression of the chemotactic molecule CCL2, a key mediator of microglia activation. The mRNA levels of all these factors are increased from DEV1 onwards, in both LPS-treated and control retinas (Figure 1D). However, chronic exposure to LPS dramatically increased the expression of these genes at all-time points compared to controls (Figure 1D). Altogether, these data suggest that activated microglia in degenerating retinal explants can become hyper-activated by a second insult, and confirm the efficacy of LPS immune challenge.

**Microglia, and to a lesser extend Müller cells, mediate LPS-induced immune response in retinal explants**

Since Toll-like receptor (TLR) 4, the LPS receptor, is not only expressed in microglial cells but also in Müller cells (Lin et al., 2013), we wanted to evaluate whether the increase in inflammatory signals following LPS treatment could be attributed not only to its effect on microglia cells but also through Müller cell direct activation, as previously reported in a stable murine Müller cell line (Lin et al., 2013). Along this line, it is widely accepted that Müller cells contribute to the inflammatory response by secreting several inflammatory factors (Eastlake et al., 2015; Kumar et al., 2013). To assess Müller cell contribution to the LPS-induced inflammatory response in retinal explants, we decided to perform LPS treatment on explants depleted of microglial cells by using the CSF1R inhibitor PLX3397 (Figure 2A). The dose-dependent decrease in the number of CD68-labelled cells attested the effectiveness of PLX3397-dependent microglial depletion after 7 days of culture (Figure 2B, C). The effect was maintained even in the presence of LPS (Figure 2B, C). We then analyzed the production of the four pro-inflammatory factors, CCL2, IL-6, IL1-β and TNFα, 7 days after LPS treatment in the presence of 10 µM PLX3397 (Figure 2D). The increase in *Il1-ß* mRNA levels observed following LPS treatment, was completely abolished by PLX3397 exposure, suggesting that *Il1-ß* upregulation was only due to microglial cells. *Ccl2*, *Il-6*, and *Tnfα* upregulation following LPS challenge was also strongly impaired but the reduction did not reach control levels. Although this could be attributed to remaining microglial cells (there is still around 30% of microglia in 10 µM PLX3397 retinal explants compared to controls), Müller cells likely also play a key role in LPS-driven regulation of a set of proinflammatory signals. In the future, in order to unambiguously distinguish the cellular sources of pro-inflammatory factor secretion following LPS treatment, we aim at analyzing their expression following magnetic cell sorting of microglia and Müller cells (in collaboration with Florian Sennlaub team, Institut de la Vision).

**LPS immune challenge does not trigger enhanced retinal damage nor Müller cell stress response in explants**

Historically, prolonged microglial activation in the central nervous system in mammals has been associated with detrimental effects in the neural tissue (Ekdahl et al., 2003; Ekdahl et al., 2009; Erlandsson et al., 2011; Fitch and Silver, 2008; Kyritsis et al., 2014; Monje et al., 2003; Perez-Asensio et al., 2013; Vannella and Wynn, 2017). For instance, sustained and increased release of cytokines from microglial cells following 100ng/mL LPS treatment has been associated with a significant increase in retinal explant neurotoxicity (Bauer et al., 2016). Although we used a much lower dose of LPS (5 ng/mL), we logically wondered whether in our culture conditions, LPS challenge could also

enhance retinal damage and/or stress. To examine the apoptotic response in retinal explants following LPS exposure, we determined the ratio between two pro- and anti-apoptotic members (*Bax* and *Bcl2* respectively), a well-recognized predictor of apoptosis extent. We observed an upregulation of the *Bax/Bcl2* mRNA ratio from DEV1 onwards in control explants, in accordance with the known cell death occurring *ex vivo* (Figure 3A). However, no significant differences in *Bax* and *Bcl2* mRNA levels between control and LPS-treated retinal explants were observed, indicating that the apoptotic response was similar in both conditions (Figure 3A). This result was further confirmed by the mean of a TUNEL assay in DEV7 retinal explants. Although quantitative analyses of the number of TUNEL positive cell in control and LPS-treated retinal explants remains to be done, we did not observe obvious differences regarding the extent or the localization of TUNEL positive cells between LPS-treated and untreated retinal explants (data not shown). The upregulation of GFAP in Müller cells is considered as a hallmark of retinal stress (Bringmann et al., 2006). Analysis of GFAP mRNA expression after LPS-treatment showed an increase of retinal stress from 3 days of culture onwards compared to that observed in the control group (Figure 3B). Altogether, our results indicate that in our culture conditions, the amplified inflammatory response following LPS treatment does not result from enhanced neurotoxicity and does not increase the stress reactivity of Müller cells.

**LPS immune challenge induces Müller cell proliferation in retinal explants**

We next sought to determine the effect of LPS-induced proinflammatory context on Müller cell proliferation in retinal explants. To do this, we combined EdU incorporation assay with immunolabeling for SOX9, a known marker of Müller cells (Poché et al., 2008). EdU/SOX9 double labeled cells were detected in control retinal explants from DEV3 onwards, yet the overall number of proliferative Müller cells remained modest reaching a maximum of around 20 proliferative cells / field (Figure 4), as previously reported (Karl et al., 2008 ; Hamon et al., 2019a). Strikingly, LPS dramatically increased this number of proliferative Müller cells from DEV5 onwards (Figure 4). To determine whether this mitogenic effect was mediated by hyper-activated microglia or by direct activation of Müller cells by LPS, we analyzed Müller cell proliferative response to LPS treatment in microglia depleted explants (Figure 5A). We found that the number of EdU/SOX9 labelled cells was dramatically decreased, alongside the diminution of microglial cells (Figure 5B, C). In order to rule out the possibility that a toxic effect of PLX3397 underlies this decreased number of proliferative Müller cells, we quantified the density of Müller cells in retinal explants after both PLX3397 and LPS treatment. No differences in the number of Müller cells were observed between all the conditions (Supplementary Figure 1A). In addition, mRNA expression of GFAP remained unchanged in PLX3397, LPS or both PLX3397/LPS treated retinal explants (Supplementary Figure 1B). These results indicate

that PLX3397 does not promote Müller cell death not any retinal stress but specifically targets microglia cells. Altogether, our data therefore suggest that hyper-activated microglia trigger the re-entry of Müller cells into the cell cycle observed upon LPS treatment in retinal explants. Since we showed above that LPS treatment does not trigger enhanced neurotoxicity, which could have indirectly mediated Müller cell proliferative response, we propose that microglia can directly regulate Müller cell quiescence exit. It would be important to validate this model by the mean of a genetic approach, for instance using the *Cx3cr1*$^{CRE}$*DTR* mice which allow complete depletion of microglial cells (Bruttger et al., 2015; Goldmann et al., 2013; Han et al., 2017; Parkhurst et al., 2013). Although we found a significant increase in the level of Müller cell proliferation upon LPS treatment, the amplitude of the phenotype is far below the spectacular increase that we reported previously when explants were overexpressing the co-transcription factor YAP (Hamon et al., 2019a). On the other hand, it has been reported that although EGF treatment can trigger Müller cell proliferation in young mouse retinal explants, it cannot do so in adult one (Karl et al., 2008). This indicates that hyper-activated microglia are more potent to promote adult Müller cell proliferation than the mitogenic growth factor EGF.

**IL-6 is a potential mediator of microglia-dependent Müller cell proliferation**

Since hyper-activated microglia can induce Müller cell proliferation following LPS treatment, we reasoned that secreted proinflammatory factors secreted from these cells could be involved. We selected a potential candidate, the cytokine IL-6, which expression is strongly upregulated upon LPS treatment, with a pic at DEV1 followed by a progressive decrease (Figure 1D). We focused on this cytokine since it has been shown that administration of exogenous IL-6 induces the proliferation of Müller cells in undamaged zebrafish retina (Nelson et al., 2013; Zhao et al., 2014). Interestingly, we found that IL-6 exposure is indeed able to convert a subset of dormant Müller cells into proliferative ones (Figure 6). However, this effect appears largely dependent on the timing of IL-6 application on the explant. Although from DEV0 to DEV2, it leads to a significant increase in the number of EdU/SOX9-positive cells (Figure 6A), treatment from DEV0 to DEV6 does not have any effect (Figure 6B). Noticeably, when applied from DEV3 to DEV5, when Müller cell exhibit LPS-dependent proliferative activity, it promoted Müller cell proliferation as efficiently as the LPS treatment (Figure 6C). It is therefore tempting to speculate that IL-6 could be one of the cytokines secreted by hyper-activated microglia that mediate Müller glia cell cycle re-entry, and that its dynamics of expression is key for this process. Of note, these experiments have been performed only once and therefore need to be confirmed. Furthermore, to support our hypothesis, it would be useful to inhibit IL-6 pathway in explants exposed to LPS and assess whether this could be sufficient to prevent Müller cell

proliferation. Besides, another interesting candidate to focus on is TNFα. Indeed, as IL-6, we found that it is upregulated in LPS-induced hyper-activated microglia with a pic at DEV5, when Müller cells proliferate (Figure 1D). Moreover, as IL-6, TNFα has also been linked to the regeneration of several organs, including the retina (Conner et al., 2014; Elsaeidi et al., 2014; Fischer et al., 2014; Goldman, 2014; Iribarne et al., 2019; Nelson et al., 2013; Vannella and Wynn, 2017; Zhao et al., 2014). In addition, we found that microglia cell ablation in LPS-treated retinal explants, which prevents Müller cell proliferation, leads to a dramatic reduction of TNFα expression, whereas IL-6 downregulation was not significant. Finally, a recent preprint has reported that the addition of TNFα in a culture of Müller cells derived from mice exposed to light injury, stimulates the secretion of inflammatory factors and drives their proliferation (Niu et al., 2019). Altogether, all these data point to TNFα as an interesting candidate to study in order to assess whether it takes part to the inflammatory signaling mediating LPS-induced Müller cell proliferation. Combinatorial treatment may also prove to be more efficient than a single cytokine treatment. Simultaneous, or successive treatments of IL-6 and TNFα may thus be performed to reveal a potential "cytokine synergy" phenomenon.

To identify additional proinflammatory cytokines potentially involved in this process, it could also be very interesting to perform the explant culture experiments following other immune challenges than LPS, using for instance Zymosan (a fungal cell wall extract). The rationale is that LPS and Zymosan can induce distinct neuroinflammatory responses as they are recognized by different receptors (Dillon et al., 2006). We could determine the secretome of hyper-activated microglia from conditioned media prepared from microglia treated either with LPS or with Zymosan. We could then screen for potential candidates according to the efficiency of the proliferative effects of both immune challenges. This may help pointing to key cytokines underlying Müller cell proliferative response.

**LPS immune challenge inhibits the neurogenic potential of proliferating Müller cells**

In regenerative species, Müller cell proliferation following injury is accompanied by a reprogramming process, leading to the re-expression of several reprogramming and neurogenic factors, such as ASCL1, Lin28 and STAT3, that regulate the de-differentiation of Müller cells into neurogenic retinal progenitors (Hamon et al., 2016; Langhe and Pearson, 2019). In order to deepen our analysis of the effect of LPS immune challenge on Müller cell-dependent retinal regeneration, we investigated whether LPS-induced proliferative Müller cells undergo such de-differentiation event, as assessed by *Ascl1* expression levels. A significant *Ascl1* upregulation was detected from DEV5 (Figure 7A), coincident with the slight increase in Müller cell proliferation observed in control explants (Figure 4). This suggests that our culture conditions are sufficient to trigger an increase of *Ascl*1 expression in a

subset of Müller cells. Strikingly, this upregulation of *Ascl1* at DEV5 and DEV7 was inhibited in LPS-treated retinal explants (Figure 7A). This result was very surprising, as it indicates that although chronic LPS immune challenge awakes quiescent Müller cells and induce re-entry into the cell cycle *ex vivo*, it prevents, as least in part, their de-differentiation into retinal progenitors. The expression of other reprogramming/neurogenic factors, such as Lin28 or PAX6, should also be analyzed to support this conclusion. Besides, since the levels of *Ascl1* in LPS-treated explants is even lower than in control explants, it suggests that an inhibitory mechanism is in place in hyper-activated microglia. To confirm this inhibitory effect, coupling LPS and EGF treatments could be performed to assess whether the neurogenic potential of EGF is inhibited by hyper-activated microglia.

In the chicken retina, inhibition of STAT3 increases neurogenesis from reactive Müller cells in damaged retinas (Todd et al., 2016). Similarly, it has recently been reported that STAT3 signaling, triggered by neuronal injury, was potentially involved in the reduced *Ascl1*-mediated reprogramming of adult mouse Müller cells (Jorstad et al., 2020). We thus logically wondered whether the inhibition on *Ascl1* mRNA expression following LPS treatment could be due to an increase in STAT3 signaling. We found an increase in STAT3 signaling activation, assessed by the ratio of P-STAT3/STAT3 (Figure 7B), at all time points in control explants, with a pic at DEV1, coincident with the pic of expression of some pro-inflammatory factors, such as CCL2 and IL-6 (Figure 1D). The same P-STAT3/STAT3 ratio profile was observed in LPS-treated retinal explants with no statistically significant differences with the control at any time points (Figure 7B). This suggests that the absence of *Ascl1* upregulation observed in the presence of LPS is not related to an increase in STAT3 signaling. As a whole, although the mechanism preventing *Ascl1* upregulation in the presence of hyper-activated microglia remains to be determined, our data strongly suggest that LPS-induced proliferative Müller cells lack neurogenic potential. Interestingly, the absence of *Ascl1* upregulation in the mouse retina upon injury is seen as one of the main barriers for Müller cell-dependent retinal regeneration in mammals. It would thus be very interesting to determine the final fate of Müller cells that proliferate upon LPS immune challenge: if they cannot generate new neurons, do they stay in a proliferative state, can they self-renew and generate new Müller cells, do they undergo apoptosis to regulate their number? Unfortunately, culturing explants for longer time than 7 days may be technically challenging, and these questions may thus be difficult to address *ex vivo*.

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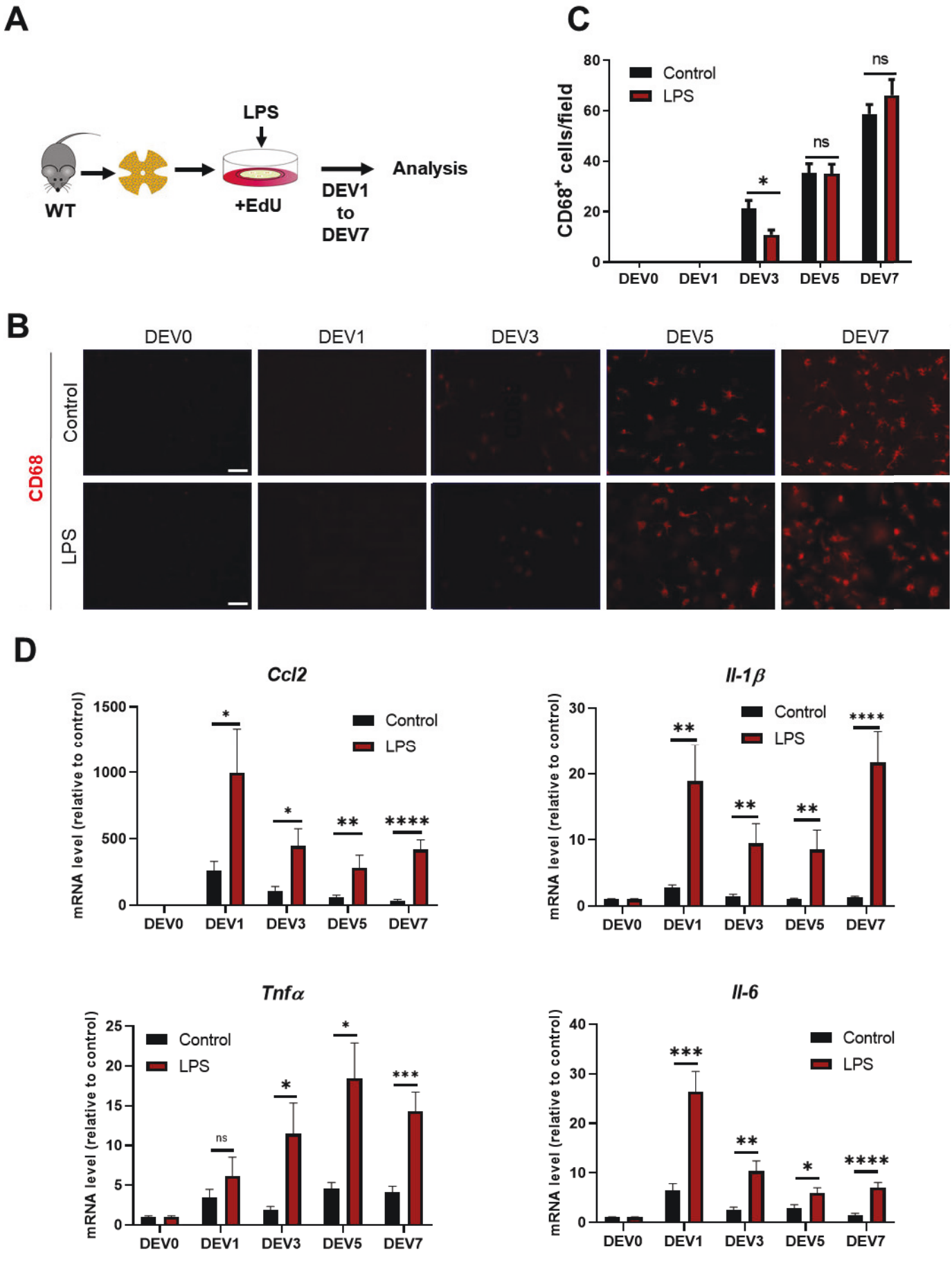
**Figure 1. LPS exposure leads to hyper-activated microglial cells in cultured retinal explants.** (A) Timeline diagram of the experimental procedure used in (B–D). Retinas from WT mice were flattened and cultured up to 7 days in the presence or not of LPS. EdU was added to the culture medium for immunostaining analysis. Analyses were performed every 2 days from DEV1 to DEV7. (B) Cd68 labeling in control and LPS-treated retinal explants at different culture time points. *Scale bar*: 50 µm. (C) Quantification of the total number of Cd68+ cells at each time point. Mean values +/- SEM from at least 5 independent retinas are shown. (D) Relative mRNA expression of *Ccl2*, *Il-1ß*, *Tnfα* and *Il-6* from control and LPS-treated retinal explants at different culture time points, obtained by RT-qPCR analysis and relative to uncultured condition (DEV0). Mean values +/- SEM from at least 7 independent retinas are shown. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤ 0.0001, ns: non-significant.

# Figure 1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Tnfα
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LPS

**Figure 2. LPS-induced immune response in retinal explants is mostly mediated by microglial cells.** (A) Timeline diagram of the experimental procedure used in (B–D). Retinas from WT mice were flattened and cultured for 7 days in the presence of LPS and/or the microglial inhibitor PLX3397. EdU was added to the culture medium for immunostaining analysis. (B) Cd68 labeling in control and LPS stimulated retinal explants treated with different doses of PLX3397 after 7 days of culture. (C) Quantification of the total number of Cd68+ cells for each condition. Mean values +/- SEM from at least 7 independent retinas are shown. *Scale bar*: 100 µm. (D) Relative mRNA expression of *Ccl2*, *Il-1β*, *Tnfα* and *Il-6* after 5 days of PLX3397 (10µM) and/or LPS treatment, obtained by RT-qPCR analysis and relative to control condition. Mean values +/- SEM from at least 7 independent retinas are shown. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ns: non-significant.

# Figure 2

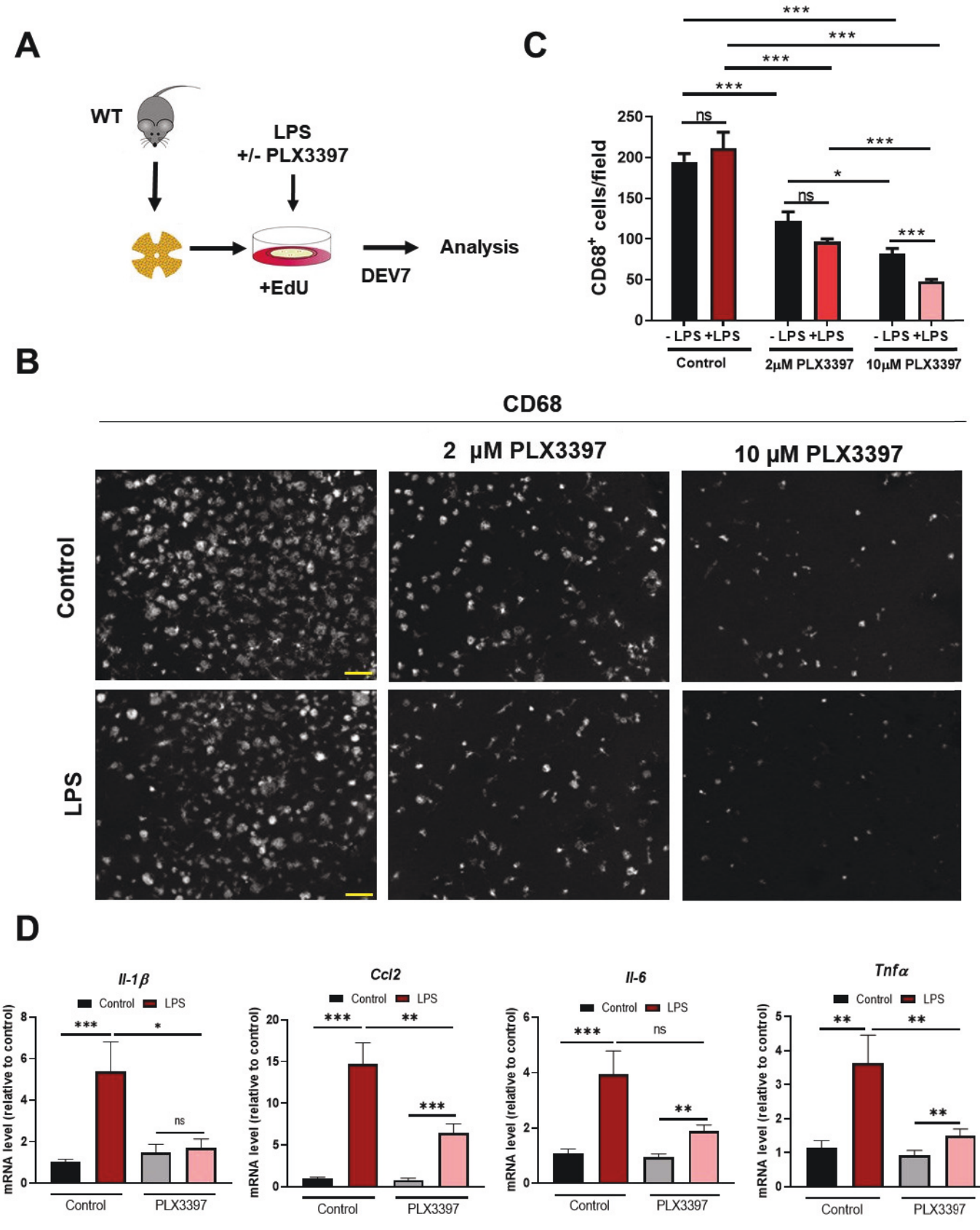
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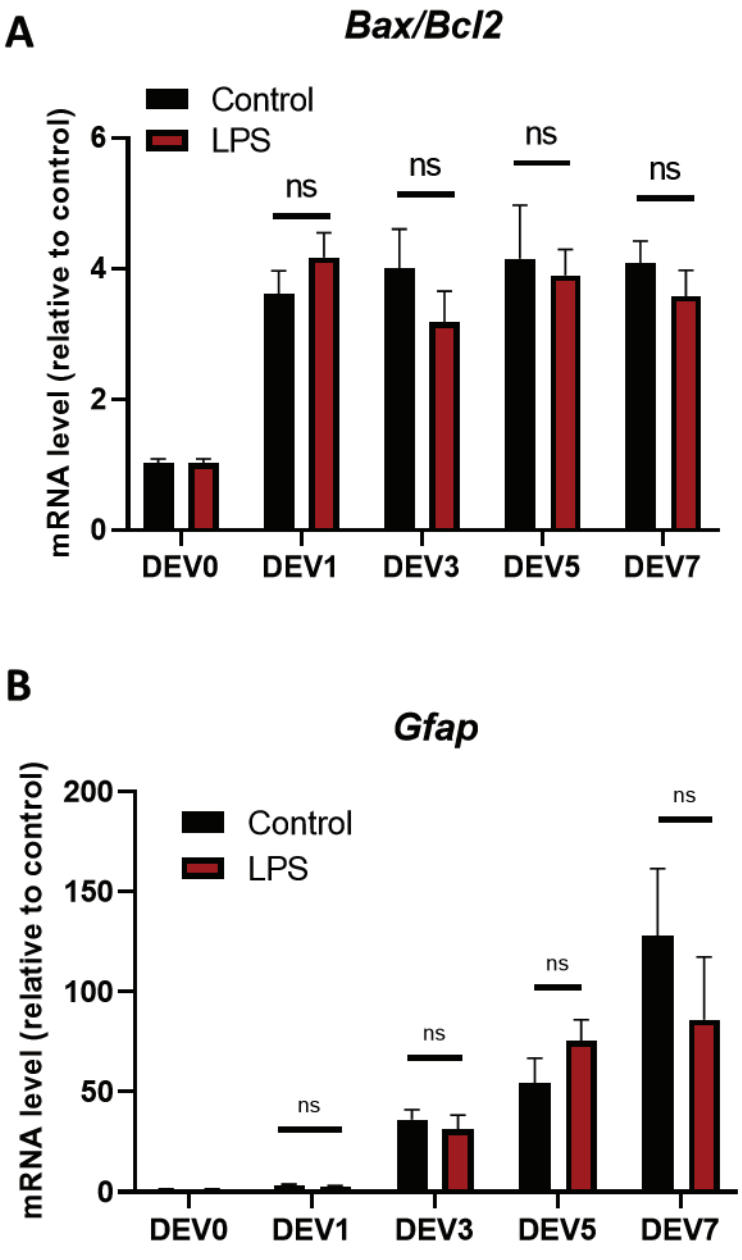
**Figure 3. LPS-induced inflammation does not increase neurotoxicity nor Müller cell stress response in cultured retinal explants**. (A) Relative *Bax/Bcl2* mRNA ratio in control and LPS-treated retinal explants at different culture time points, obtained by RT-qPCR analysis and relative to uncultured condition (DEV0). Mean values +/- SEM from at least 6 independent retinas are shown. (B) Relative mRNA expression of *Gfap* in control and LPS-treated retinal explants at different culture points, obtained by RT-qPCR analysis and relative to uncultured condition (DEV0). Mean values +/- SEM from at least 5 independent retinas are shown. Statistics: Mann-Whitney test, ns: non-significant.

# Figure 3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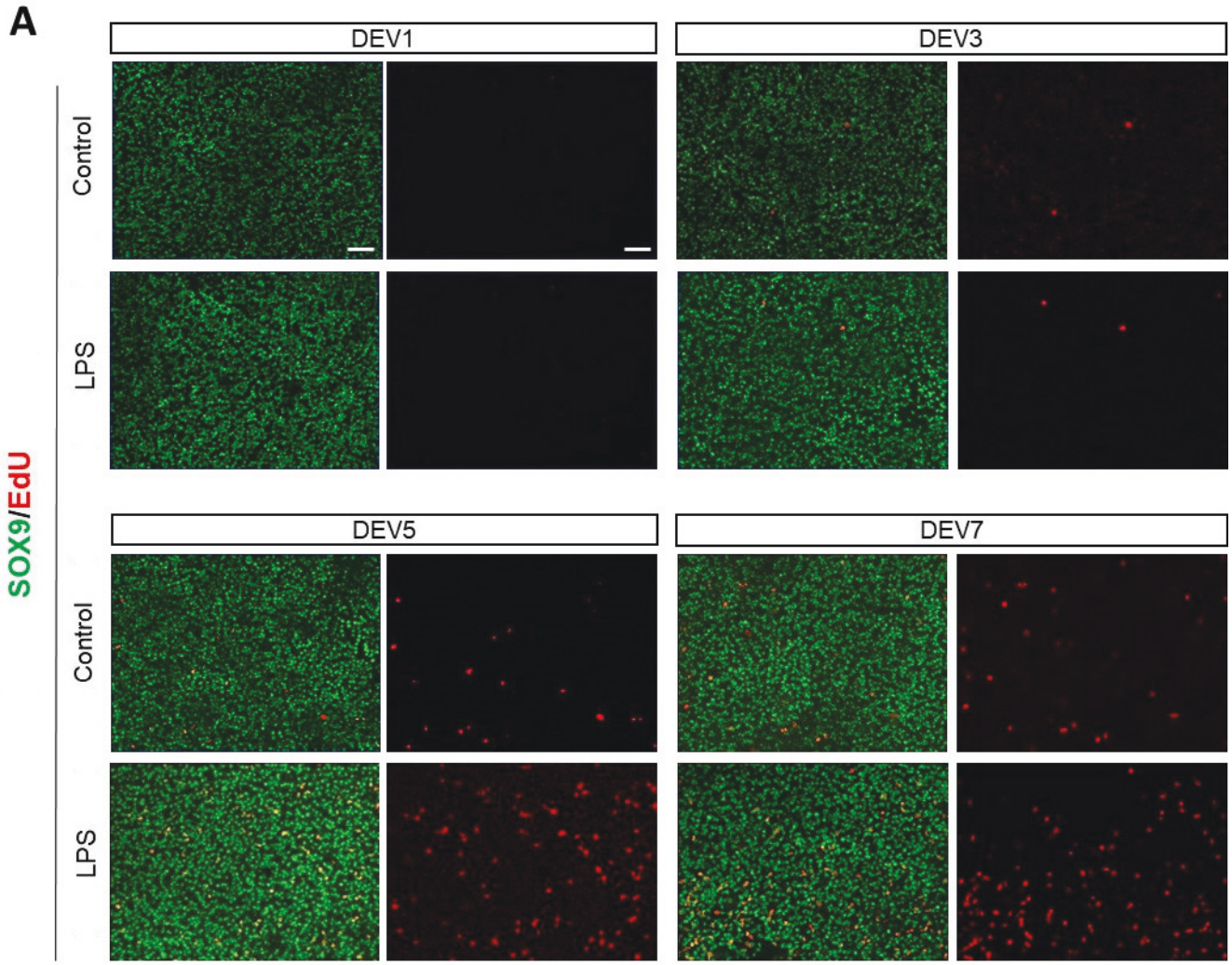
DEV5
DEV7
B
Gfap
Control
LPS
mRNA level (relative to control)
0
50
100
150
200
ns
ns
ns
ns
DEV0
DEV1
DEV3
DEV5
DEV7

**Figure 4. LPS-treatment increases the proliferative potential of Müller cells**. (A) EdU and Sox9 co-labeling in the inner nuclear layer of control and LPS-treated retinal explants at different culture time points. *Scale bar*: 50 µm. Quantification of the total number of double EdU+Sox9+ cells at each time point. Mean values +/- SEM from at least 5 independent retinas are shown. Statistics: Mann-Whitney test, ****p≤ 0.0001, ns: non-significant.

# Figure 4


A
DEV1
DEV3
DEV5
DEV7
Control
LPS
Control
LPS
SOX9/EdU

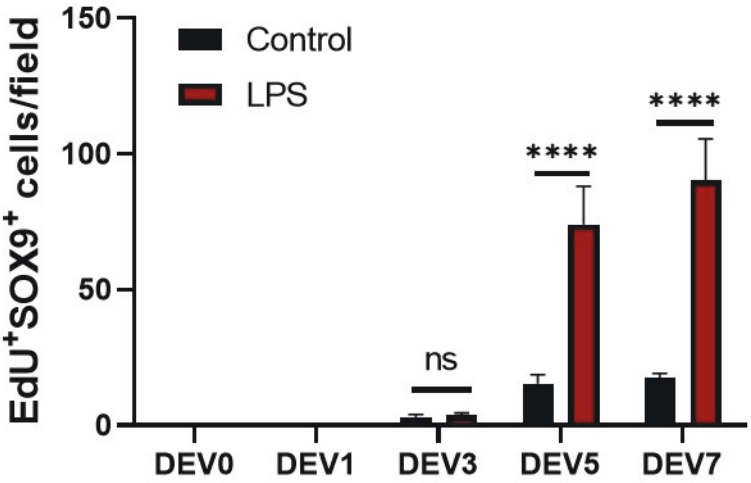
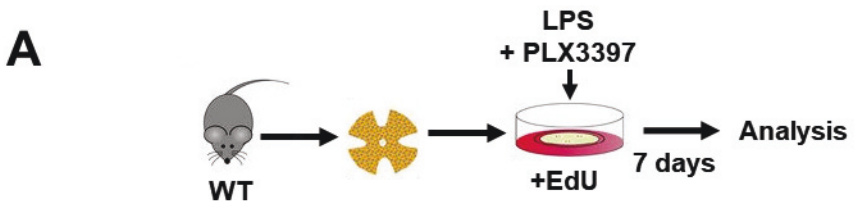
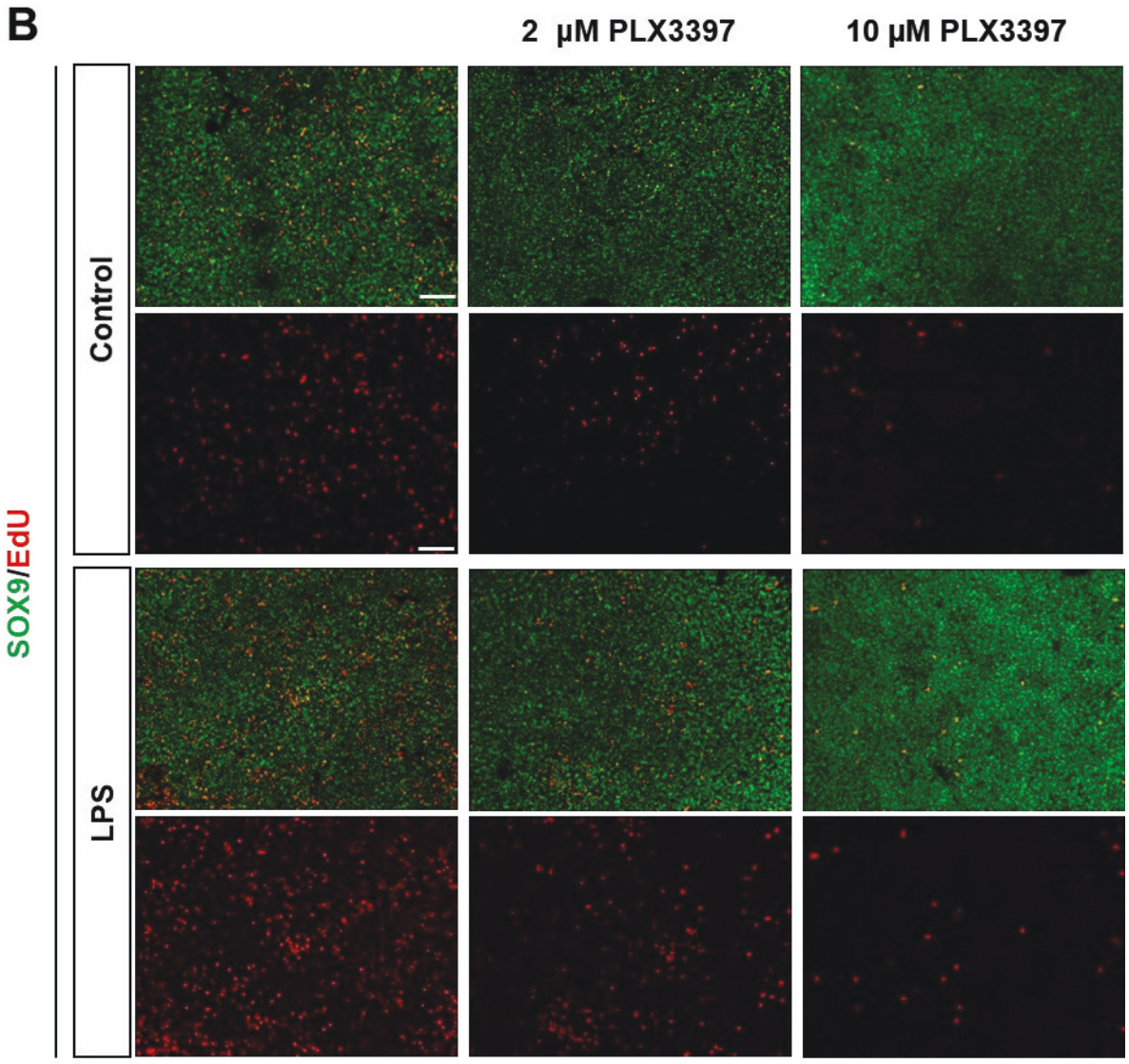

EdU+SOX9+ cells/field
150
100
50
0
Control
LPS
ns
****
****
DEV0
DEV1
DEV3
DEV5
DEV7

**Figure 5. Hyperactivated microglial cells trigger the re-entry in the cell cycle of Müller cells**. (A) Timeline diagram of the experimental procedure used in (B–C). Retinas from WT mice were flattened and cultured for 7 days in the presence of LPS and/or the microglial inhibitor PLX3397. EdU was added to the culture medium for immunostaining analysis (B-C). (B) EdU and Sox9 co-labeling in the inner nuclear layer of control and LPS stimulated retinal explants treated with different doses of PLX3397 after 7 days of culture. *Scale bar*: 100 µm. (C) Quantification of the total number of double EdU+Sox9+ cells for each condition. Mean values +/- SEM from at least 7 independent retinas are shown. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ns: non-significant.

# Figure 5


A
LPS
+ PLX3397
WT
+EdU
7 days
Analysis


B
2 μM PLX3397
10 μM PLX3397
Control
LPS
SOX9/EdU

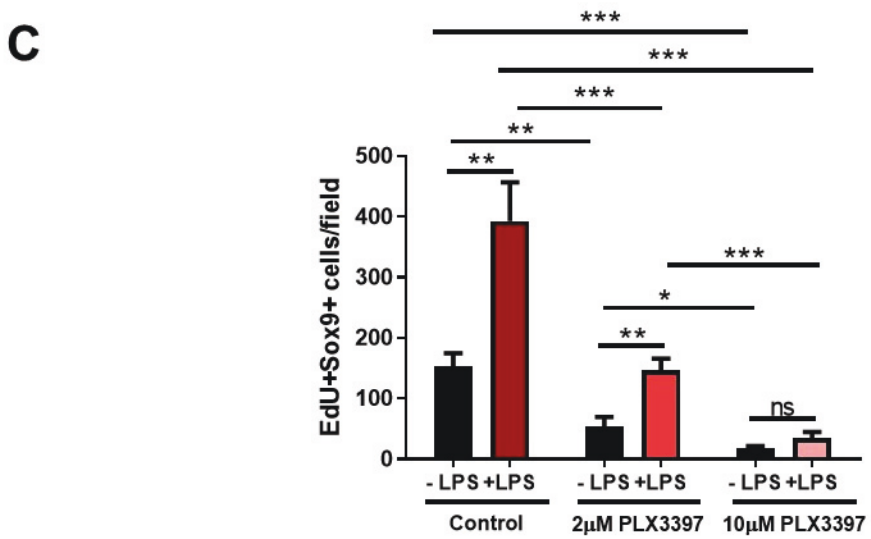
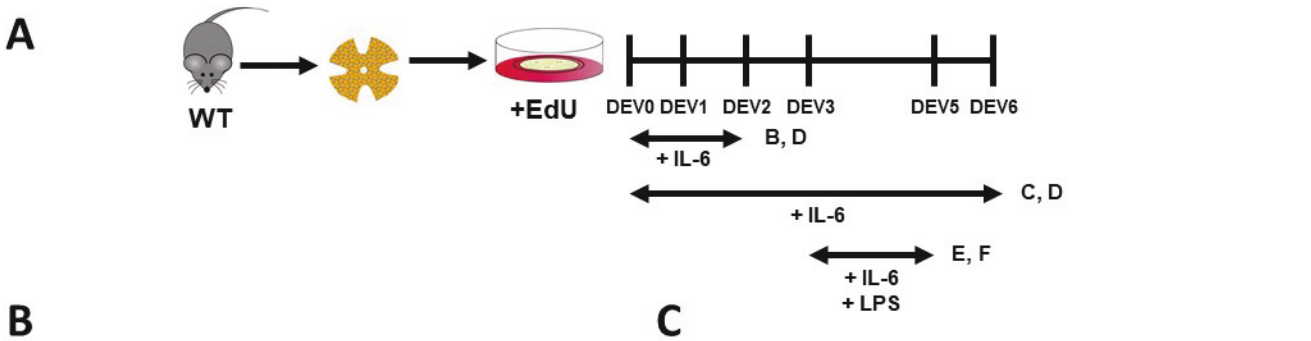
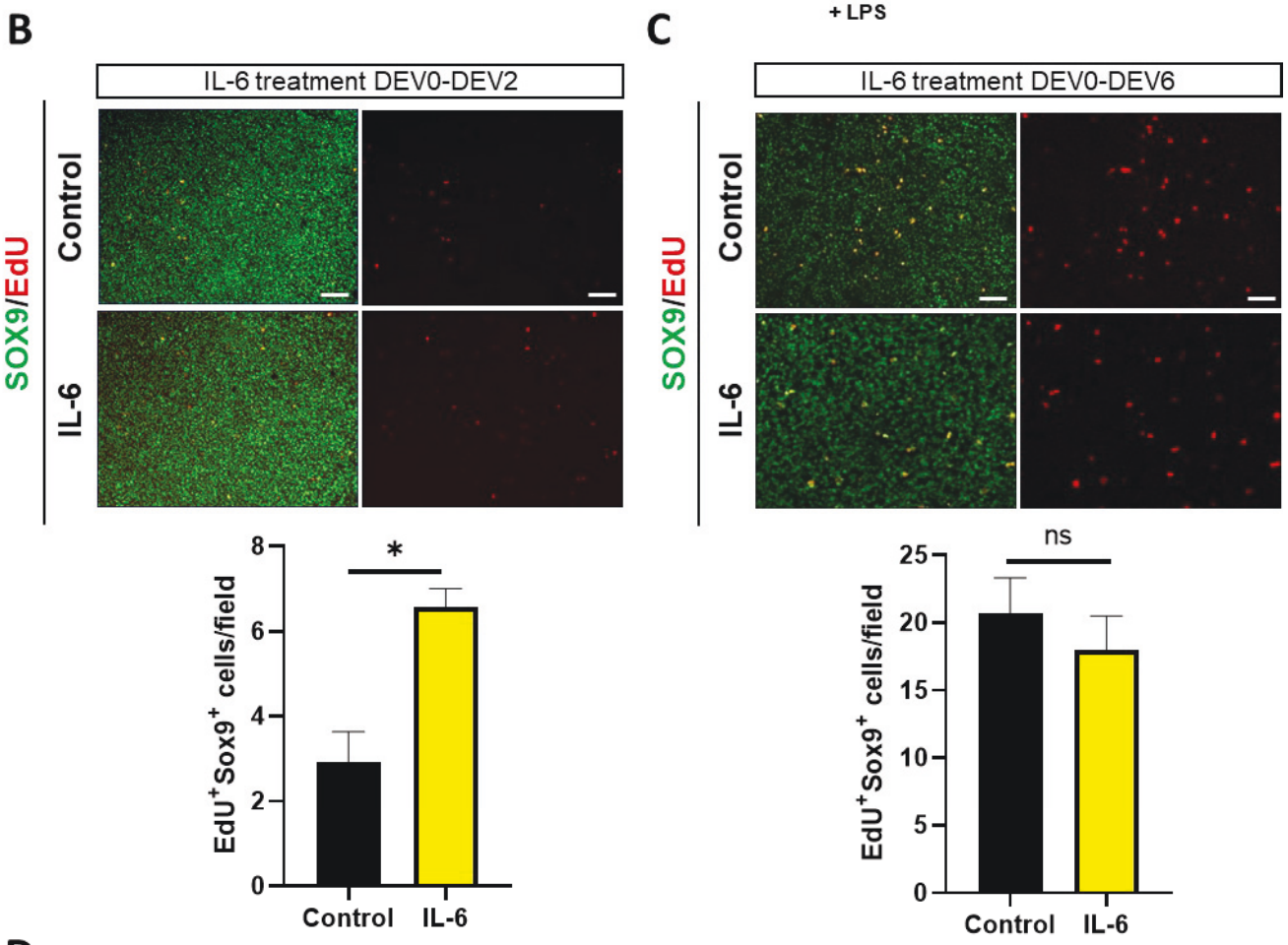

C
EdU+Sox9+ cells/field
500
400
300
200
100
0
***
***
***
**
**
***
*
**
ns
- LPS +LPS
- LPS +LPS
- LPS +LPS
Control
2μM PLX3397
10μM PLX3397

**Figure 6. IL-6 is a potential mediator of microglia-dependent Müller cell proliferation.** (A) Timeline diagram of the experimental procedure used in (B–D). Retinas from WT mice were flattened and cultured for 2 or 6 days in the presence of IL-6 and/or LPS. EdU was added to the culture medium for immunostaining analysis (B-D). (B) EdU and Sox9 co-labeling in the inner nuclear layer of control and IL-6 stimulated retinal explants treated from DEV0 to DEV2. The histogram corresponds to the quantification of the total number of double EdU+Sox9+ cells in B. Mean values +/- SEM from at least 4 independent retinas are shown. *Scale bar*: 100 µm. (C) EdU and Sox9 co-labeling in the inner nuclear layer of control and IL-6 stimulated retinal explants treated from DEV0 to DEV6. The histogram corresponds to the quantification of the total number of double EdU+Sox9+ cells in C. Mean values +/- SEM from at least 4 independent retinas are shown. *Scale bar*: 50 µm. (D) EdU and Sox9 co-labeling in the inner nuclear layer of control, LPS and IL-6 stimulated retinal explants treated from DEV3 to DEV5. *Scale bar*: 50 µm. The histogram corresponds to the quantification of the total number of double EdU+Sox9+ cells in D. Mean values +/- SEM from at least 4 independent retinas are shown. Statistics: Mann-Whitney test, *p≤ 0.05, ns: non-significant.

# Figure 6


A
WT
+EdU
DEV0 DEV1 DEV2 DEV3 DEV5 DEV6
B, D
+ IL-6
C, D
+ IL-6
E, F
+ IL-6
+ LPS


B
IL-6 treatment DEV0-DEV2
SOX9/EdU
Control
IL-6
EdU+Sox9+ cells/field
Control
IL-6
C
IL-6 treatment DEV0-DEV6
SOX9/EdU
Control
IL-6
EdU+Sox9+ cells/field
ns
Control
IL-6

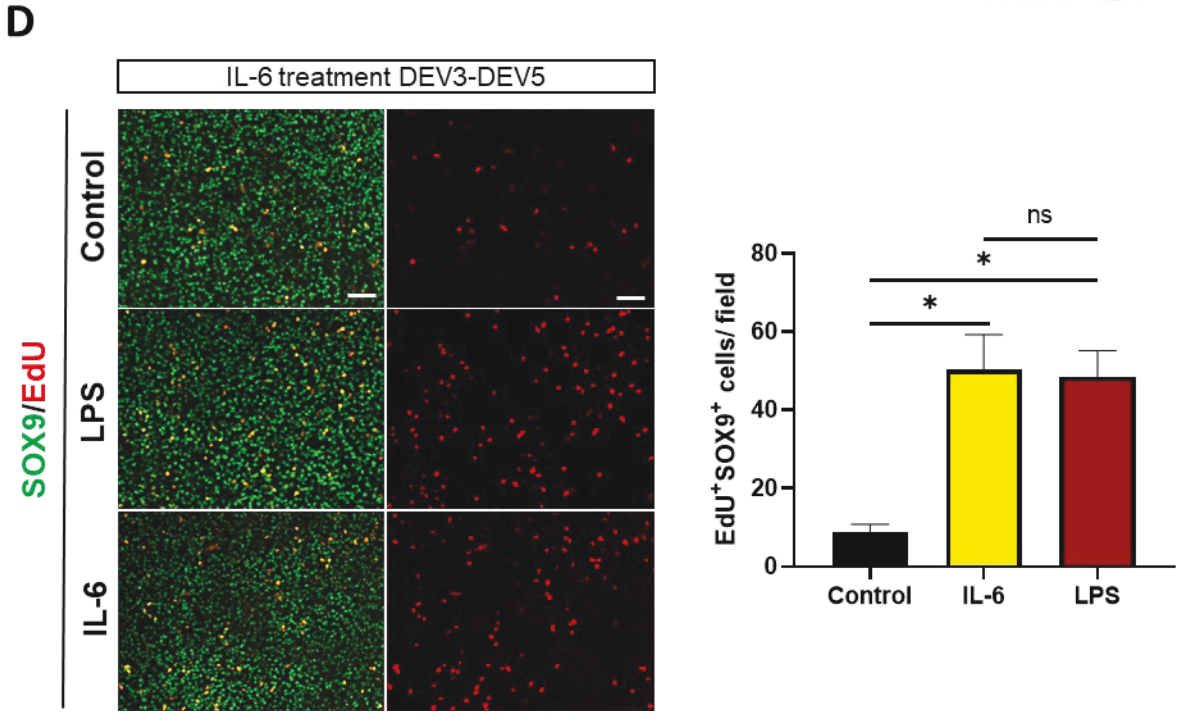

D
IL-6 treatment DEV3-DEV5
SOX9/EdU
Control
LPS
IL-6
EdU+SOX9+ cells/ field
ns
Control
IL-6
LPS

**Figure 7. LPS-treatment inhibits the neurogenic potential of Müller cells**. (A) Relative *Ascl1* mRNA levels in control and LPS-treated retinal explants at different culture time points, obtained by RT-qPCR analysis and relative to uncultured condition (DEV0). Mean values +/- SEM from at least 6 independent retinas are shown. (B) Western blots of retinal protein extracts from control and LPS-treated retinal explants at different culture time points were probed with anti-pSTAT3 and anti-STAT3. Anti-α-tubulin was used as a loading control. Quantification of the ratio pSTAT3/STAT3 signals obtained on the western blot. Mean values +/- SEM from at least 8 independent retinal explants are shown. Statistics: Mann-Whitney test, ns: non-significant; *p≤ 0.05.

# Figure 7

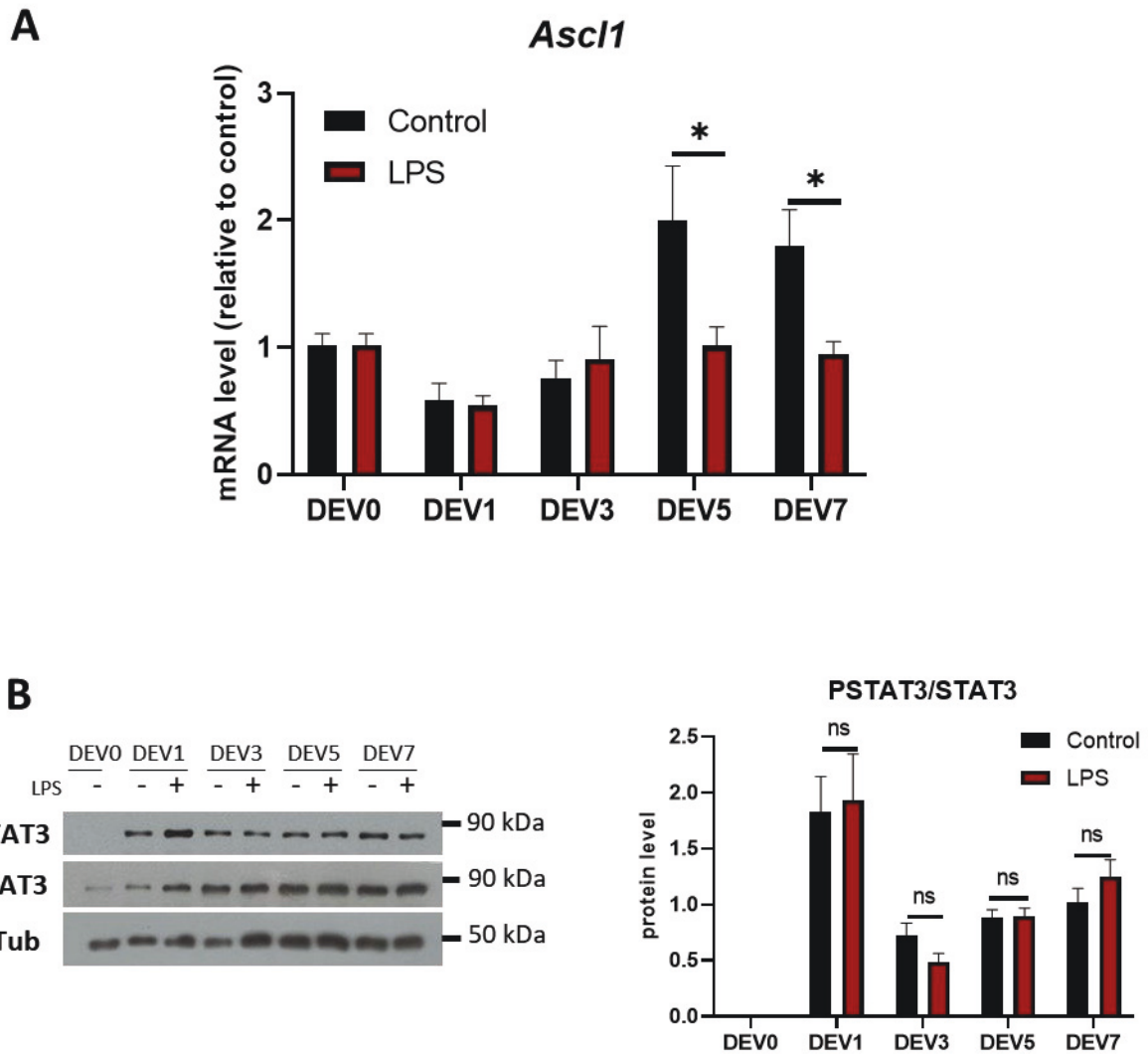

A
Ascl1
mRNA level (relative to control)
Control
LPS
DEV0
DEV1
DEV3
DEV5
DEV7
B
DEV0 DEV1 DEV3 DEV5 DEV7
LPS - - + - + - + - +
p-STAT3
STAT3
α-Tub
90 kDa
90 kDa
50 kDa
PSTAT3/STAT3
protein level
ns

**Figure 8. Schematic representation of a proposed model.** In case of retinal damage in species like zebrafish or Xenopus, Müller cells reactivate, exit from quiescence, proliferate, and reprogram themselves into retinal progenitors by the upregulation of several neurogenic factors such as ASCL1. This results in the generation of new retinal neurons and the regeneration of the retina. In the mouse retina this Müller cell reactivation is very limited and stopped after cell cycle re-entry. Here we reveal the existence of a positive impact of neuroinflammation on mammalian Müller cell reactivation. Our results demonstrated that *ex vivo* chronic stimulation of microglial-dependent inflammatory response boosts the usually very limited proliferative response of mouse Müller cells in case of retinal damage. Our results also suggest the possibility that inflammatory cytokines, such as IL-6, could underlie this mitogenic activity. Interestingly however, our data suggest that the inflammatory-induced proliferative Müller cells do not reprogram into neurogenic retinal progenitors.

# Figure 8

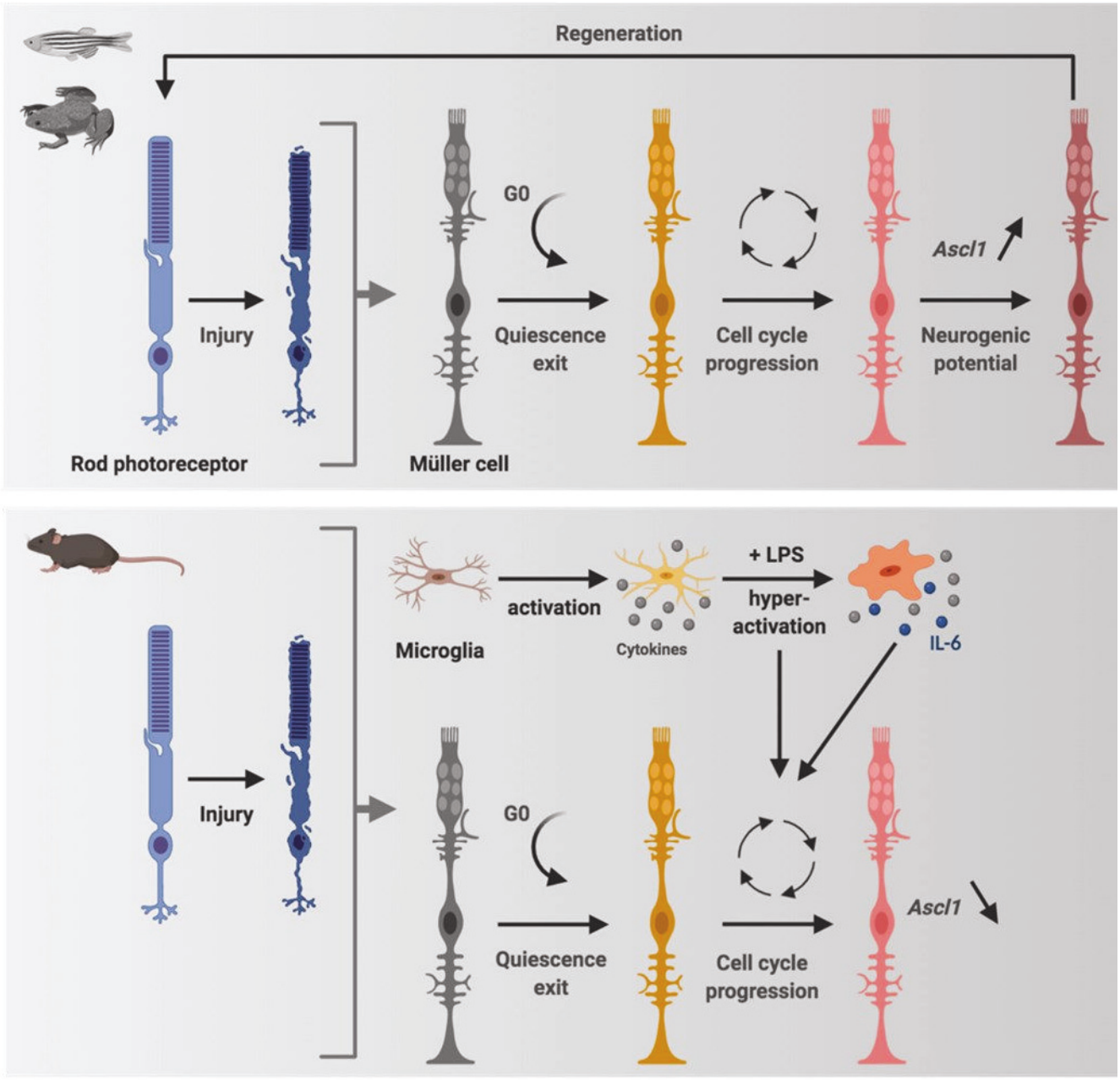
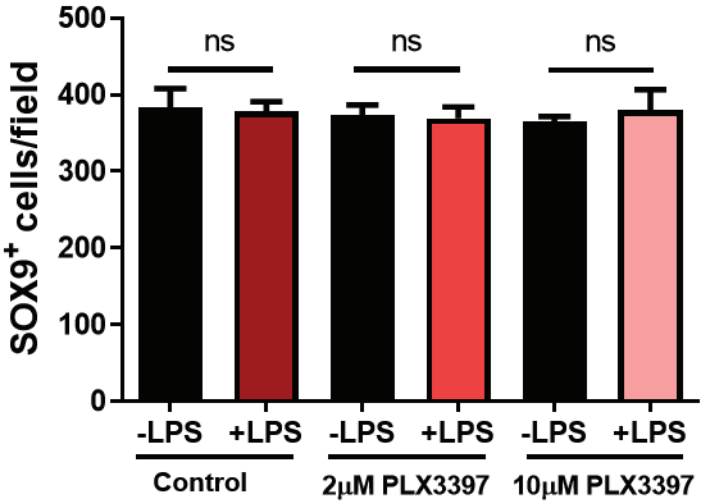

Regeneration
Injury
Rod photoreceptor
Müller cell
G0
Quiescence exit
Cell cycle progression
Ascl1
Neurogenic potential
Injury
Microglia
activation
Cytokines
+ LPS
hyper-activation
IL-6
G0
Quiescence exit
Cell cycle progression
Ascl1

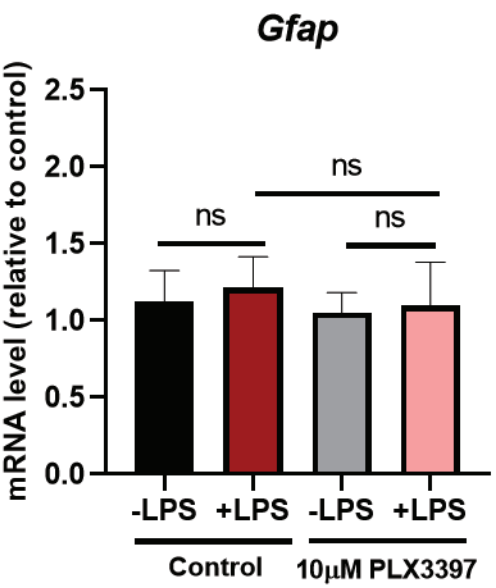
**Figure S1. Toxicity of PLX9337 on Müller cells**. (A) Quantification of the total number of $SOX9^+$ cells in the inner nuclear layer of retinal explants after 7 days of different doses of PLX3397 (2 and 10µM) and/or LPS treatment. Mean values +/- SEM from at least 7 independent retinas are shown. (B) Relative mRNA expression of *Gfap* after 5 days of PLX3397 (10µM) and/or LPS treatment, obtained by RT-qPCR analysis and relative to control condition. Mean values +/- SEM from at least 7 independent retinas are shown. Statistics: Mann-Whitney test, ns: non-significant.

# Supplementary Figure 1

A




B



| GENE NAME | SPECIES | FORWARD | REVERSE |
|---|---|---|---|
| *Ascl1* | *mus musculus* | TCTCCTGGGAATGGACTTTG | GGTTGGCTGTCGGTTTGTT |
| *Bax* | *mus musculus* | AGGCCTCCTCTCCTACTTCG | CTCAGCCCATCTTCTTCCAG |
| *Bcl2* | *mus musculus* | GGACTTGAAGTGCCATTGGT | AGCCCCTCTGTGACAGCTTA |
| *Ccl2* | *mus musculus* | GAAGGAATGGGTCCAGACAT | ACGGGTCAACTTCACATTCA |
| *Gfap* | *mus musculus* | CCAGTTACCAGGAGGCACTTG | CGATGTCCAGGGCTAGCTTAA |
| *Il-6* | *mus musculus* | GCCTTCTTGGGACTGATGCTGGT | TCCTCTGTGAAGTCTCCTCTCCG |
| *Il-1β* | *mus musculus* | GCCTCGTGCTGTCGG | TGTCGTTGCTTGGTTCTCCT |
| *Rps26* | *mus musculus* | CCCAAGGATAAGGCCATCAAG | AAGCACGTAGGCGTCGAAGA |
| *Srp72* | *mus musculus* | CACCCAGCAGACAGACAAACTG | GCACTCATGGTAGCGTTCCA |
| *Tbp* | *mus musculus* | CACAGGAGCCAAGAGTGAAGAAC | ATCACAGCTCCCCACCATGT |
| *Tnfa* | *mus musculus* | AAGTCAACCTCTCTGCC | TCCAAAGTAGACCTGCCCG |

**Table S1. List of primers**

| Antigene | Host | Supplier | Reference | Dilution (WB) | Dilution (IF-FM) |
|---|---|---|---|---|---|
| Alpha-tubulin | mouse | SIGMA | T9026 | 1:20000 | |
| CD68 | rat | Bio-Rad UK | MCA1957GA | | 1:1000 |
| Stat3 (124H6) | mouse | Cell Signaling | 9139S | 1:1000 | |
| Stat3, (Phospho-Tyr705) (3E2) | mouse | Cell Signaling | 9138S | 1:1000 | |
| SOX9 | rabbit | Abcam | ab185966 | | 1:300 |
| Alexa 488 anti-rabbit | donkey | Thermo Fischer Scientific | A21206 | | 1:500 |
| Alexa 647 anti-rat | goat | Thermo Fischer Scientific | A21247 | | 1:500 |

**Table S2. List of antibodies.** WB : western blot, IF-FM : immunofluorescence on flat mounted retinas.

# CONCLUSION AND PERSPECTIVES

## INFLAMMATORY SIGNALS PROMOTE THE PROLIFERATION OF MÜLLER CELLS BUT NOT THEIR NEUROGENIC POTENTIAL

In recent years, accumulated evidence has pointed to a positive effect of the immune system on retinal regeneration efficiency in species like zebrafish or chick. Here, we revealed the existence of a similar positive impact of neuroinflammation on mammalian Müller cell reactivation. We indeed demonstrated that *ex vivo* chronic stimulation of microglial-dependent inflammatory response boosts the usually very limited proliferative response of mouse Müller cells in case of retinal damage (Figure 8). Our results suggest the possibility that inflammatory cytokines, such as IL-6, could underlie this mitogenic activity. Interestingly however, our data suggest that the inflammatory-induced proliferative Müller cells do not reprogram into neurogenic retinal progenitors (Figure 8). In the future, it would thus be very interesting to explore whether coupling inflammatory and neurogenic signals could unlock both the proliferative and the neurogenic abilities of Müller cells.

## COULD IMMUNE CHALLENGE ALSO PROMOTE MÜLLER GLIA CELL CYCLE RE-ENTRY *IN VIVO* ?

The eye is one of the areas of the body considered to be a site of immune privilege. This means not only that it possesses physical barriers that prevent the entry and the exit of pathogens, immune cells or larger molecules; but also that the ocular microenvironment contains soluble immunosuppressive factors that activate the Treg cells and oppose the activity of immune-competent cells (Forrester and Xu, 2012; Zhou and Caspi, 2010). The benefit of the immune privilege is to avoid over inflammation to occur that would otherwise damage the retina as observed in many ocular pathologies. On the other hand, it is tempting to speculate that the dark side of the immune privilege would be to hinder Müller cell-dependent retinal regeneration in response to damage by limiting the pro-inflammatory response in a very efficient way. Our *ex vivo* data suggest indeed that Müller glia can reenter into the cell cycle if microglia are hyper-activated. As discussed above, retinal explants are a valuable model to study the interplay between microglia-dependent inflammation and Müller cells, since microglia are the only resident macrophages in *ex vivo* cultures. However, this could also be seen as a limitation compared to the *in vivo* situation where not only microglia, but also infiltrating immune cells, participate to the regulation of the inflammatory process (Bosak et al., 2018). Thus, it will be interesting to investigate whether an immune challenge, breaking the immune privilege of the retina, could also awake Müller cells *in vivo*.

## INFLAMMATION AS THE MECHANISM UNDERLYING REGENERATIVE DIFFERENCES BETWEEN SPECIES

The immune response has been reported as an important trigger of the central nervous system regenerative response (including the retina) in regenerative organisms such as zebrafish (Kizil et al., 2015; Kyritsis et al., 2012; White et al., 2017). Indeed, drug-mediated immunosuppression lessens the regenerative neurogenesis that normally occurs in the zebrafish brain upon injury, highlighting the importance of the inflammatory response in the regenerative process (Kyritsis et al., 2012). More importantly, the same study reported that induction of an inflammatory reaction was sufficient to stimulate brain neurogenesis in the absence of injury (Kyritsis et al., 2012). Strikingly, although in mammals several mouse studies imply that the inflammation has a negative effect on the proliferation of neural precursors cells and brain regeneration (Ekdahl et al., 2003; Monje et al., 2003), we show here that LPS-induced inflammation is sufficient to trigger Müller cell proliferation in the mammalian retina. Interestingly however, we also found that, contrary to regenerative species, where the inflammatory response trigger both Müller cell proliferation and reprogramming, in mammals the inflammatory response trigger Müller cell proliferation but inhibit their neurogenic potential. This suggests that hyper-activated immune cells secrete pro-proliferative inflammatory mediators that are not present, or present in insufficient amounts, upon retinal damage in mammals. Nevertheless, the factors conferring the reprogramming capacity would be still missing, insufficient or inhibited. On the contrary, retinal injury in regenerative species would trigger an inflammatory response leading to the secretion of such potential pro-regenerative inflammatory factors. Therefore, differences in the immune cell types involved, the level and the nature of cytokine expression, or even the timing of the inflammatory response could be responsible for the regenerative differences observed between species. Supporting this idea, it has been shown that the chick retina, but not mouse retina, contains an atypical glial cell type, the non-astrocytic inner retinal glia-like (NIRG) cells that participate to the inflammatory response (Fischer et al., 2010a; Fischer et al., 2010b). Along the same line, in case of injury in zebrafish, the inflammatory response is strong and quick, contrasting to mammals, where the pro-inflammatory context is often persistent (Alunni and Bally-Cuif, 2016; Bosak et al., 2018; Ceci et al., 2019). To determine if the nature or intensity of the inflammatory response may underlie the differential responses of Müller cells to injury between species, it would be interesting to perform comparative analysis between animal models with highly different regenerative properties. To this aim, my laboratory is currently comparing the inflammatory response between *Xenopus* and mouse. Overall, this novel research topic aimed at bridging the fields of inflammation and regeneration could help us to dig into the mechanisms that

underlie such differences and to increase our knowledge into the regenerative capacity of the mammalian retina.

# PART 4

## YAP MEDIATES MICROGLIA-MÜLLER CELL CROSSTALK

My results obtained during the third part of my PhD indicate the existence of a microglia-dependent regulation of Müller cell proliferation in case of retinal degeneration coupled to immune challenge. Our previous results regarding the role of YAP in Müller glia cell cycle re-entry, together with recent discoveries highlighting in multiple cell types a link between inflammation and YAP activity, prompted me to develop a project to explore the interplay between inflammation and YAP-dependent Müller cell proliferation in the retina. This study is still under progress, but the main data are presented here.

## 1. METHODOLOGY

In this study, I used both the loss and the gain of function tools described above, the ***Yap* cKO mouse** line and the **AAV-YAP5SA adenovirus**, respectively. In addition, I took advantage of retinal explant culture as a retinal degeneration model. The inflammation was induced or inhibited using **pharmacological tools**. To induce the inflammation, I used LPS, a bacterial lipopolysaccharide that is recognize by the toll-like receptor TLR4. To ablate microglia, I used PLX3397 (pexidartinib), a kinase inhibitor that blocks the colony stimulating factor receptor 1 (CRF-1R).

## 2. MAIN RESULTS

- YAP expression upregulation in Müller glia upon pathological conditions does not require microglia but can be fine-tuned by inflammatory signals
- YAP-induced Müller cell proliferation in degenerative retinal explants requires activated microglia
- YAP confers a pro-inflammatory environment in a degenerating retina
- LPS-induced Müller cell proliferation requires YAP activity
- YAP and inflammatory signals cooperate to enhance Müller cell proliferation

# Part 4

**Diana García-García**, Lorena Vidal-Gil, Catherine Hottin, Juliette Bitard, Annaïg Hamon, Jérôme Roger and Muriel Perron

## YAP mediates microglia-Müller cell crosstalk

*Ongoing work*

ABSTRACT

Vertebrate species exhibit tremendous differences in their regenerative abilities. Recently, there has been a growing appreciation of the contribution of inflammation to the regeneration process. Regarding the retina, microglia-dependent inflammation has been pointed as a trigger of the proliferative response of Müller cells, retinal glia cells endowed with regenerative potential. However, how immune cells and Müller cells interact and cooperate is largely unknown. Here, using retinal explants as a retinal degenerative *ex vivo* model, we discovered a positive regulatory feedback loop in which Müller cell expression of YAP, the effector of the Hippo pathway, was modulated upon immune challenge, while microglia-induced inflammatory signals were positively regulated by YAP. Moreover, we identified reciprocal functional interactions: Müller cell proliferative effects induced by YAP required inflammatory molecules released by microglia, while YAP activity was necessary for Müller glia cell cycle re-entry triggered by hyper-activated microglia. Finally, we discovered that overexpressing YAP in a pro-inflammatory context reinforces its Müller cell proliferative effects. This work thus highlights YAP as a molecular hub mediating the dialog between Müller cells and microglia in the retina and brings new insights into the complex interplay between the microenvironment and intrinsic signalling pathways underlying dormant stem cell reactivation.

# INTRODUCTION

Decoding the mechanisms underlying stem cell quiescence exit is expected to provide fundamental information in the field of regenerative medicine. The Hippo pathway emerged as a key signalling in controlling organ repair and regeneration (Moya and Halder, 2019). It consists of a core kinase cascade that regulates negatively the activity of the transcription coactivators YAP (Yes associated protein) and TAZ (WW domain–containing transcription regulator 1) (Moya and Halder, 2019). In case of damage, YAP/TAZ activity regulates genes involved in the expansion of tissue-resident progenitors and thereby promotes tissue regeneration (Del Re et al., 2013; Grijalva et al., 2014; Johnson and Halder, 2014). YAP is essential for intestinal or liver regeneration for instance (Barry et al., 2013; Gregorieff et al., 2015). Some other organs however do not regenerate in mammals, such as the retina. Müller cells are considered dormant retinal stem cells as they can re-enter into the cell cycle, reprogram into retinal progenitor and regenerate retina following damage in some species (Hamon et al., 2016; Langhe and Pearson, 2019). In mammals however, these cells reactivate (exit quiescence) upon injury but do not progress into the cycle and therefore are unable to regenerate retinal neurons. Several key signalling pathways have been shown to regulate Müller cell proliferation and able to awake mammalian Müller cells (Hamon et al., 2016; Langhe and Pearson, 2019). For instance, we have recently found that YAP is specifically expressed in Müller cells and is necessary for their cell cycle re-entry in a degenerating *Xenopus* retina (Hamon et al., 2019a). Besides, we and the team of R. Poché, have shown that its overactivation is sufficient to elicit mouse Müller glia proliferation, even in uninjured retinas (Hamon et al., 2019a; Rueda et al., 2019). The cellular microenvironment is also key to modulate a regenerative response, as it influences the activity of signalling pathways. For instance, inflammatory signals have recently been reported as important triggers for the regenerative response of Müller cells in the fish or chicken retina (García-García et al., 2020). In the mouse, we recently found that a pro-inflammatory context can favour Müller cell division (García-García 2020, in prep). However, molecular cues linking inflammation and Müller cell mitogenic activity upon retinal injury are still largely unknown. Interestingly, a growing number of studies in various tissues tend to suggest that the Hippo pathway orchestrates the interplay between the innate immune system and the regeneration process (Wang et al., 2020). In the mammalian liver or intestine for instance, it has been shown that YAP mediates inflammation-dependent regeneration (Su et al., 2015; Taniguchi et al., 2015). In the present study, we found that YAP plays a key function in the reciprocal interaction between Müller cells and microglia in the retina. Indeed, using retinal explants as a degenerative *ex vivo* model, we highlighted cross-regulations, inflammatory signals being able to modulate YAP expression while YAP being able to regulate pro-inflammatory signals. Moreover, we identified functional interactions, inflammatory

signals and YAP being dependent on each other to promote Müller cell proliferation. Finally, we discovered cumulative effects, making it possible to awake almost all Müller cells in a mouse retinal explant. This work thus brings new insights into the complex interplay between the microenvironment and intrinsic signalling pathways underlying dormant stem cell reactivation.

# MATERIAL AND METHODS

The majority of the material and methods used for determining the role of YAP in the microglia-Müller cell crosstalk has been already described in Part 3 (Western-blotting, RNA extraction and RT-qPCR, Histology and Immunofluorescence, TUNEL assay and EdU labelling, Imaging, Quantification and Statistical Analysis). Here, I describe the different/additional material and methods used.

**Mice**

Mice were kept at 21°C, under a 12-hour light/12-hour dark cycle, with food and water supplied *ad libitum*. *Yap*$^{flox/flox}$*;Rax-CreER*$^{T2}$ mice were obtained as previously described (Hamon et al., 2019b) by mating *Yap*$^{flox/flox}$ mice (Reginensi et al., 2013b) with heterozygous Rax-CreER$^{T2}$ knock-in mice (Pak et al., 2014). The Cre activity was induced through a double intraperitoneal injection of 4-hydroxy-tamoxifen (4-OHT; 1 mg/kg) at P10 and P14. Genotyping was done by PCR using genomic DNA prepared from mouse tail snips. Primers are provided in Supplementary Table S1. All experiments involving adult mice were performed with male or female mice that were 3 to 8 weeks. No difference between sexes was observed in any retinal phenotype.

**AAV production and retinal transduction**

Human YAP5SA cDNA was amplified by PCR from pCMV-flag-YAP2-5SA plasmid (a gift from Kunliang Guan, Addgene plasmid#27371; http://addgene.org/27371; RRID:Addgene_27371) and subcloned into an AAV transfer plasmid, where the expression is driven by the minimal cytomegalovirus (CMV) promoter (Klimczak et al., 2009). AAV vectors were produced as already described using the co-transfection method and purified by iodixanol gradient ultracentrifugation (Choi et al., 2007). AAV vector stocks were tittered by qPCR using SYBR Green (Thermo Fisher Scientific) (Aurnhammer et al., 2012). The previously engineered AAV-GFP (Klimczak et al., 2009) was used as a control. 10 µl (approx. $10^{11}$ vg) of AAV-GFP or AAV-YAP5SA were applied directly on mouse retinal explants for viral transduction at the beginning of the culture. Retinal explants were cultured as indicated before further western blot analysis or EdU labeling.

# RESULTS

**YAP upregulation in Müller glia upon pathological conditions does not require microglia but can be fine-tuned by inflammatory signals**

We previously found that YAP is specifically expressed in mouse Müller cells and that its expression in these cells, at both the mRNA and protein levels, is increased in a degenerative context (Hamon et al., 2017). Since retinal degeneration induces an inflammatory response, we wondered whether YAP upregulation in Müller glia could be controlled by signals released from activated microglia, the resident immune cells in the retina. To address this question, we took advantage of *ex vivo* retinal explants as a model of retinal degeneration and analyzed YAP expression by RT-qPCR and western blot following microglia ablation. This was performed by exposure to PLX3397 (pexidartinib) (Figure 1A), a kinase inhibitor that blocks colony stimulating factor receptor 1 (CRF-1R), a receptor necessary for microglia viability (Silverman and Wong, 2018a). As assessed by the expression of the activated microglia marker CD68, and as previously reported (García-García 2020, in prep), we validated the robust elimination of microglia cells upon PLX3397 treatment in explants cultured for 7 days *in vitro* (DEV) (Figure 1B). We found no difference in the amount of YAP levels in PLX3397-treated explants (Figure C-D), suggesting that YAP upregulation in Müller cells following retinal degeneration is not regulated by microglia-dependent inflammatory signals.

We next sought to investigate whether enhancing inflammation could impact YAP expression. To this aim, we used lipopolysaccharide (LPS), a bacterial endotoxin able to enhance microglia pro-inflammatory response (Hoogland et al., 2015; Lively and Schlichter, 2018; Lund et al., 2006) (Figure 2A). As we reported previously (Hamon et al., 2019a), YAP protein level increased in control retinal explants in a time-dependent manner. LPS was able to further enhance YAP expression at DEV1. This effect was transient as no differences were detected from DEV3 onwards between LPS and control conditions (Figure 2B). Interestingly, a similar profile, with a transient LPS-induced increase compared to controls at DEV1, was also detected for *Taz* mRNA level (Figure 2C). However, there was no significant change in the expression of the well-known YAP/TAZ direct target genes *Cyr61* and *Ctgf* (Figure 2D). Since the increase in YAP protein and *Taz* mRNA after LPS exposure is transient, we cannot exclude the possibility of an effect very restricted in time that we missed in-between DEV1 and DEV3. On the other hand, another known direct target of YAP/TAZ, *Ccdn1* (encoding the cell cycle factor CYCLIND1), that we previously identified as regulated by YAP in Müller cells (Hamon et al., 2019a), is also transiently increased at DEV1 in explants exposed to LPS (Figure 2E). Moreover, similarly, we have also recently identified HB-EGF (EGFR ligand) as a factor transcriptionally regulated by YAP in Müller glia (Hamon et al., 2019a). We found here that *Hb-egf* is as well upregulated specifically at DEV1 following LPS treatment (Figure 2E) and that this is associated with

a transient increase in the activation of the EGFR pathway, as assessed by the ratio of P-ERK/ERK (Figure 2F). In support with our results, short-term exposure to LPS has previously been shown to induce YAP expression and EGFR signalling (Lv et al., 2018; Taniguchi et al., 2015; Yi et al., 2016; Zhong et al., 2020; Zhou et al., 2019). Moreover, in lymphatic endothelial cells, a LPS-YAP-EGFR relationship has been recently described: LPS promotes YAP expression by activating the phosphorylation of ERK1/2 (Zhong et al., 2020). An activation of both YAP and EGFR pathways has also been reported *in vitro* in macrophages after short-term exposure to LPS (Qu et al., 2012; Qu et al., 2015; Zhou et al., 2019). Altogether, our data in the retina indicate that LPS-induced hyper-activated microglia promote a transient upregulation of YAP/TAZ, associated with an increased upregulation of *Ccnd1* and the enhancement of EGFR pathway activity. Since both *Ccnd1* and *Hb-egf* are not only expressed in Müller cells but also in microglia, it would be useful to analyze their expression following magnetic cell sorting of microglia and Müller cells (in collaboration with Florian Sennlaub team, Institut de la Vision) in order to undoubtedly attribute the cellular source of their upregulation. Regarding YAP, its expression in microglial cells is highly controversial. A recent study has reported the presence of YAP in a microglial cell line (Qing et al., 2020). However, RNAseq analysis and immunolabeling analysis in the central nervous system indicate that YAP is not present in microglial cells (Cheng et al., 2020; Huang et al., 2016). In addition, we have reported by immunostaining that in the retina, YAP is specifically expressed in Müller cells (Hamon et al., 2017). Therefore, it is tempting to speculate that LPS-induced YAP upregulation occurs in Müller cells and not in activated microglia.

Regarding the mechanism underlying YAP upregulation upon LPS treatment at DEV1, we cannot exclude the possibility that LPS directly induces YAP in Müller cells. Indeed, in addition to microglia, Müller cells also express LPS receptor, TLR4, and can respond to LPS stimulation *in vitro* (Lin et al., 2013). Therefore, it would be important to treat microglia-depleted retinal explants with LPS and analyse whether YAP is still upregulated at DEV1. If not, it would indicate that microglia are necessary, and it would then be important to identify which secreted molecules could be involved. Noteworthy, we previously found that LPS treatment in retinal explants promotes *Il-6* expression, with a pic at this particular time DEV1 (García-García 2020, in prep). Moreover, it has recently been reported in epithelial cells and in ovarian cancer cells, that a non-canonical IL-6 pathway leads to an increased stability and nuclear concentration of YAP, through the phosphorylation of the tyrosine Y357 and not the canonical serine S127 (Azar et al., 2020; Taniguchi et al., 2015). It would therefore be very interesting to test in our retinal explant model (*i*) whether IL-6 exposure also leads to an increased level of YAP, (*ii*) whether this cytokine inhibition prevents LPS-dependent increase of YAP protein levels at DEV1 and (*iii*) whether the status of YAP Y357 phosphorylation and the

nuclear/cytoplasmic ratio change upon LPS exposure. Together, this should allow to determine whether IL-6 could mediate LPS-induced YAP upregulation at DEV1. Regarding the timing of YAP upregulation upon LPS treatment, and given the known highly dynamic effects of inflammatory signals, one can wonder whether the maximal effect of LPS on YAP could take place very rapidly, before DEV1. It will thus be important to analyze IL-6 and YAP expression profiles at very short time points after LPS exposure. Finally, it would be interesting to get insights into the mechanisms underlying the transient upregulation of YAP after LPS immune challenge. Is this associated with a decrease of inflammatory signals such as IL-6 after DEV1, or is there any negative feedback loops taking place to restore YAP protein level to a control situation?

**YAP-induced Müller cell proliferation in degenerative retinal explants requires activated microglia**

We showed above that although LPS-induced inflammation can transiently trigger an enhanced YAP upregulation in retinal explants, YAP expression in Müller cells do not require the presence of activated microglia. We next wondered whether YAP activity could involve microglial inflammatory signaling. We thus induced microglial cell depletion with PLX3397 treatment in AAV-YAP5SA-infected explants. This AAV-YAP5SA adenovirus (Shh10 variant, (Klimczak et al., 2009)) allows the expression of a constitutively active form of YAP (YAP5SA) specifically in Müller cells (Hamon et al., 2019a). We previously reported that AAV-YAP5SA reprograms mouse Müller glia into highly proliferative cells (Hamon et al., 2019a). Unexpectedly, we found a remarkable inhibition of this proliferative activity in PLX3397-treated explants compared to controls (Figure 3). These results indicate that activated microglial cells are absolutely necessary for YAP-dependent proliferation of Müller cells. Whether this function is YAP specific or general for Müller glia cell cycle re-entry capacity remains to be addressed. For instance, it would be very interesting to test the mitogenic effect of several known Müller cell mitogenic factors, such as WNT or HB-EGF, on retinal explants depleted of microglial cells. Besides, in order to know which inflammatory factors are involved, it could be useful to undertake rescue experiments, adding back some cytokines, such as IL-6, on microglia-depleted explants infected with AAV-YAP5SA, and assess whether Müller cell proliferation can be restored.

**YAP is required for the pro-inflammatory response of a degenerating retina**

We showed that inflammatory signals can affect YAP expression and are required for its mitogenic activity in the retina. We next asked whether in turn, YAP could regulate the inflammatory response in a positive or negative feedback loop. Indeed**,** emerging discoveries these recent years highlighted the crucial regulatory roles of several Hippo pathway components in both innate and adaptive immunity (Taha et al., 2018; Wang et al., 2020; Zhou et al., 2018a). This includes several studies

pointing to central functions of YAP in the regulation of the inflammatory response (Wang et al., 2020) and in particular the regulation of IL-6 expression in endometrial cancer, breast cancer and hepatocellular carcinoma cell types (Emre and Imhof, 2014; Kim et al., 2015; Wang et al., 2019; Zhou et al., 2018b). In the retina, we did not observe any significant differences in the number, location, size of microglia cells in *Yap* CKO-derived retinal explants (data not shown). *Yap* CKO (*Yap*$^{flox/flox}$*; Rax-CreER*$^{T2}$) allow Cre-mediated *Yap* conditional gene ablation specifically in Müller cells (Hamon et al., 2019a). We next addressed the question on a transcriptomic large-scale basis. We previously performed whole transcriptome sequencing of *Yap* CKO retinas in a degenerating context (Hamon et al., 2019a) (Figure 4A). Retinal degeneration was triggered through *in vivo* methylnitrosourea (MNU) injections, a well-established paradigm for inducible photoreceptor cell death (Chen et al., 2014b). We identified 305 differentially expressed genes (DEGs) in MNU injected *Yap* CKO mice in comparison to MNU-injected control mice (Hamon et al., 2019a). Using Panther classification system based on Gene Ontology (GO) analysis, we obtained a list of biological processes significantly enriched in our dataset. Interestingly, one of the most relevant biological processes significantly overrepresented in the absence of YAP following MNU injection include the "inflammatory response" (GO:0006954), with 35 DEGs (Figure 4B). Hierarchical clustering analysis revealed that these 35 DEGs include key pro-inflammatory cytokines known to be secreted by activated microglia, such as IL-1α, IL-1ß, TNFα, NFκB, CCL2, CCL7, CXCL1, CXCL2, CXCL10 (Figure 4C). These inflammatory molecules are normally barely expressed in physiological conditions but are strongly upregulated upon retinal degeneration. Here we found that such increase is impaired in absence of YAP (Figure 4C). These results indicate that YAP is required for the proper expression of key pro-inflammatory signals in a retinal degenerative context. On the other hand, anti-inflammatory molecules, such as IL-4, IL-10, IL-11 or IL-13, do not appear deregulated, suggesting that YAP confers a pro-inflammatory response. The underlying mechanisms remain to be determined. One hypothesis is that YAP regulates key chemokines in Müller cells with microglia chemoattractant properties that are needed for their migration and pro-inflammatory response. This hypothesis is supported by the identification of *Ccl2* gene as a direct transcriptional target of YAP (Guo et al., 2017).

**LPS-induced Müller cell proliferation requires YAP activity**

We wondered whether, in addition to regulate the expression of inflammatory molecules, YAP could also be involved in inflammatory-dependent proliferative function. Indeed, we found recently that LPS immune challenge is sufficient to trigger Müller cell proliferation in retinal explants (García-García 2020, in prep). Considering the key role of YAP in Müller glia quiescence exit (Hamon et al., 2019a; Rueda et al., 2019), we logically wondered whether YAP takes part in this process. Therefore,

by the mean of EdU incorporation and immunostaining with SOX9, a nuclear marker of Müller cells, we examined at DEV7 the extend of Müller cell proliferation in *Yap* CKO retinal explants exposed to LPS (Figure 5A). We found that the number of $EdU^+/SOX9^+$ co-labelled cells upon LPS exposure was severely decreased in absence of YAP. This result suggests that the inflammatory-dependent Müller cell proliferative response requires YAP activity (Figure 5B). Our work thus highlights for the first time a link between YAP-regenerative ability and the inflammatory response in the nervous system. These data can be put in perspective with other studies performed in mammalian liver or intestine, which have reported that YAP acts as a downstream effector of inflammation-induced regeneration (Su et al., 2015; Taniguchi et al., 2015).

**YAP and inflammatory signals cooperate to induce Müller cell proliferation**

Considering the above identified crosstalk between YAP and inflammatory signals, we sought to determine whether overexpressing YAP in a hyper-activated inflammatory microenvironment could favor Müller cell mitogenic potential. Retinal explants were infected with AAV-YAP5SA and cultured in the presence of LPS for 7 days (Figure 6A). As expected from our previous work (Hamon et al., 2019a), YAP5SA overexpression resulted in a dramatic increase (~12-fold) in the number of $EdU^+/SOX9^+$ co-labelled cells compared to control explants (Figure 6B). Similarly, as we recently described, LPS is also able to trigger Müller cell proliferation although to a lesser extend (~4-fold) (Figure 6B). Strikingly, the combination of YAP5SA overexpression with LPS exposure led to an impressive increase (>30-fold) in the number of $EdU^+/SOX9^+$ co-labelled cells (Figure 6B). These data suggest that stimulating inflammation could provide a permissive environment that potentiates YAP-proliferative function in Müller cells. It would be interesting to known whether such cooperation is specific to YAP or could also take place with other mitogenic factors such as HB-EGF. Besides, it is widely accepted that after acute retinal damage induce by NMDA, the majority of induced proliferating Müller cells die and thus, only a very small subset of reactivated cells can differentiate into neurons (Ooto et al., 2004; Rueda et al., 2019; Yao et al., 2016). Interestingly, chronic LPS treatment has been shown to be required for an efficient clonal-expansion of YAP-overexpressing hepatocytes, since the increased proliferation of YAP-overexpressing hepatocytes was associated with an increase in the apoptotic response in the absence of inflammatory signals (Su et al., 2015). Thus, it would be interesting to assess whether the positive effect of inflammation observed in Müller cells overexpressing YAP could be due to an improved viability. Finally, I also would like to determine the effect of inflammation on the neurogenic potential of Müller cells overexpressing YAP. Indeed, I showed on one hand that the proliferative response of Müller cells triggered by YAP overexpression is accompanied by their reprograming into neurogenic progenitors (as inferred from

*Ascl1* up-regulation, (Hamon et al., 2019a)), and on the other hand that LPS-induced proliferative Müller cells repress *Ascl1* expression (García-García 2020, in prep, part 3). How stimulating simultaneously YAP and the inflammatory response influences Müller cell neurogenic potential is thus an entire open question.

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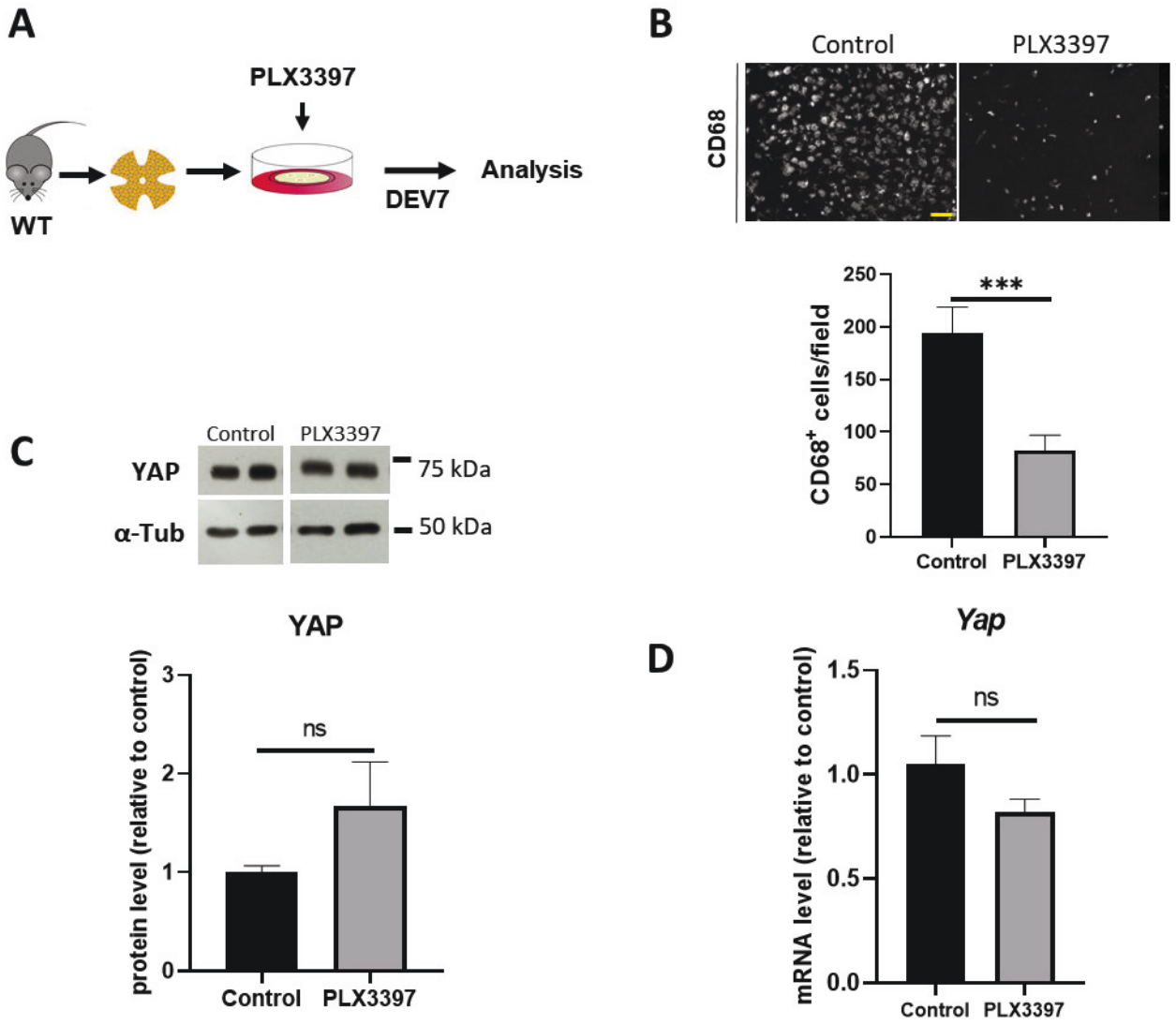
**Figure 1. Basal YAP upregulation in Müller glia upon damage does not require microglial cells.** (A) Timeline diagram of the experimental procedure used in (B–D). Retinas from WT mice were flattened and cultured for 7 days in the presence or not of 10 μM of PLX3397. (B) Cd68 labeling in control and PLX3397-treated retinal explants after 7 days of culture. *Scale bar*: 100 μm. Quantification of the total number of Cd68+ cells. Mean values +/- SEM from 8 independent retinas are shown. (C) Western blots of retinal protein extracts from control and PLX3397-treated retinal explants were probed with anti-YAP. Anti-α-tubulin was used as a loading control. Quantification of YAP western blot signals normalized to α-tubulin (α-tub) and relative to control. Mean values +/- SEM from at least 6 independent retinal explants are shown. (D) Relative mRNA expression of *Yaz* from control and PLX3397-treated retinal explants after 7 days of culture, obtained by RT-qPCR analysis and relative to control condition. Mean values +/- SEM from at least 8 independent retinas are shown. Statistics: Mann-Whitney test, ***p≤ 0.001, ns: non-significant.

# Figure 1


A
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DEV7
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CD68
CD68+ cells/field
250
200
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PLX3397
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YAP
α-Tub
75 kDa
50 kDa
YAP
protein level (relative to control)
3
2
1
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Control
PLX3397
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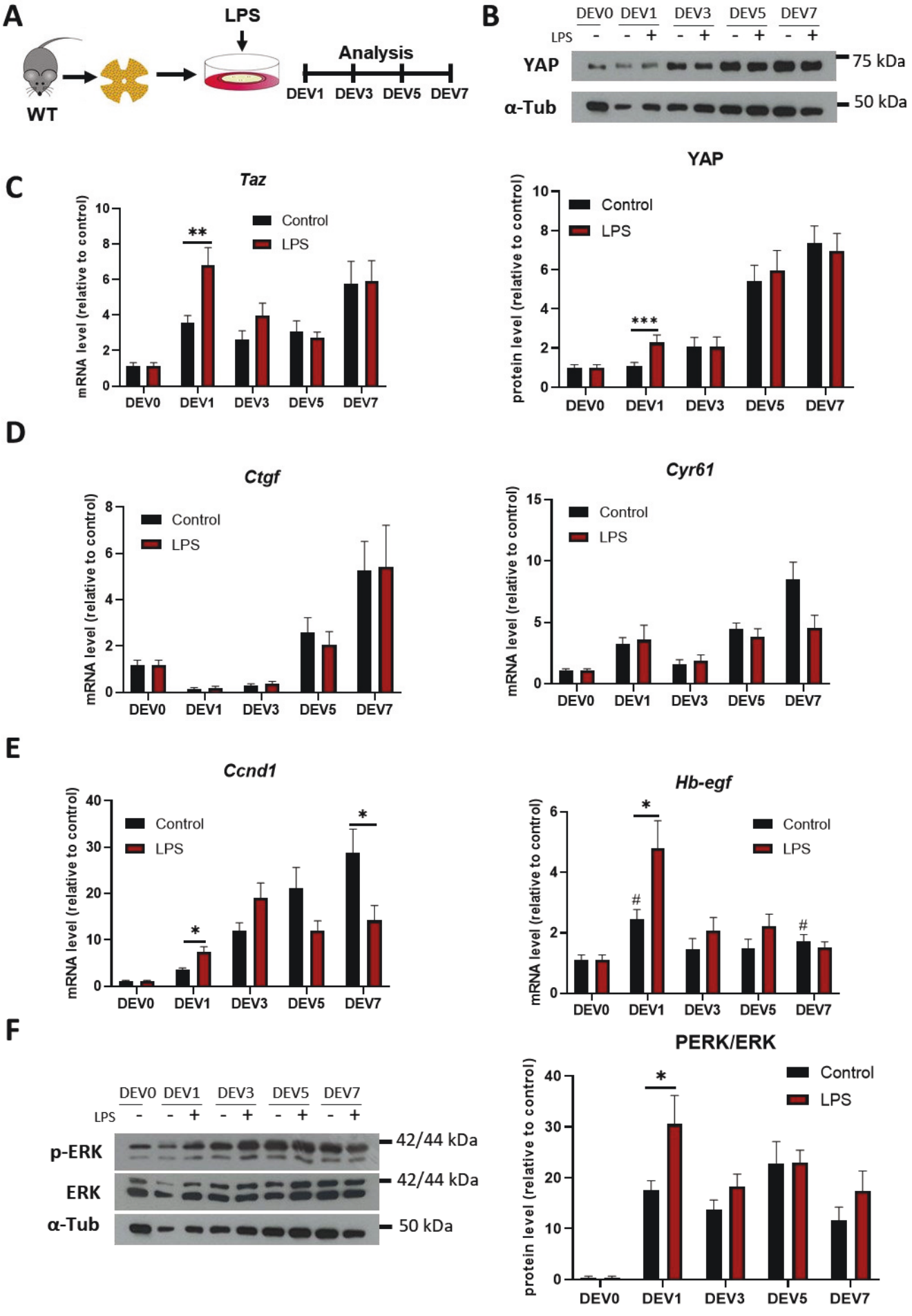
Yap
mRNA level (relative to control)
1.5
1.0
0.5
0.0
ns
Control
PLX3397

**Figure 2. LPS exposure induces an early transient upregulation of YAP.** (A) Timeline diagram of the experimental procedure used in (B–E). Retinas from WT mice were flattened and cultured up to 7 days in the presence or not of LPS. Analyses were performed every 2 days from DEV1 to DEV7. (B) Western blots of retinal protein extracts from control and LPS-treated retinal explants at different culture time points were probed with anti-YAP. Anti-α-tubulin was used as a loading control. Histogram representation corresponds to the quantification of YAP western blot signals normalized to α-tubulin (α-tub) and relative to uncultured control (DEV0). Mean values +/- SEM from at least 8 independent retinal explants are shown. (C-E) Relative mRNA expression of *Taz* (C) *Cyr61* and *Ctgf* (D), *Ccnd1* and *Hb-egf* (E) from control and LPS-treated retinal explants at different culture time points, obtained by RT-qPCR analysis and relative to uncultured condition (DEV0). Mean values +/- SEM from at least 5 independent retinas are shown. (F) Western blots of retinal protein extracts from control and LPS-treated retinal explants at different culture time points were probed with anti-pERK and anti-ERK. Anti-α-tubulin was used as a loading control. Quantification of the ratio pERK/ERK signals obtained on the western-blot. Mean values +/- SEM from at least 8 independent retinal explants are shown. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ns: non-significant.

# Figure 2


A
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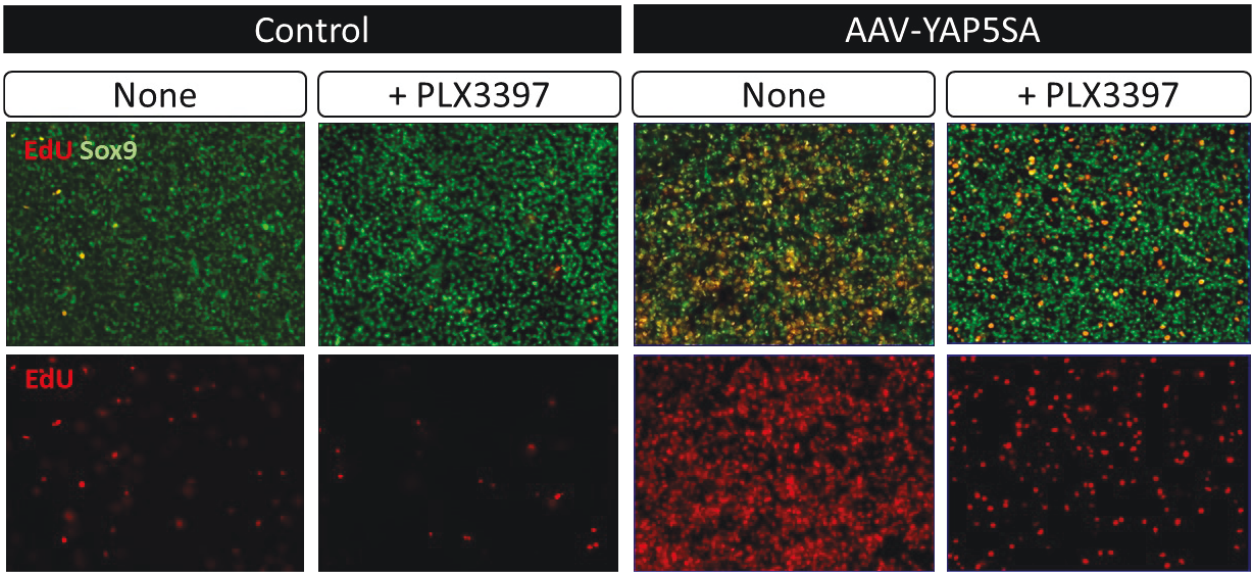
B
DEV0 DEV1 DEV3 DEV5 DEV7
LPS - - + - + - + - +
YAP
α-Tub
75 kDa
50 kDa
C
Taz
mRNA level (relative to control)
Control
LPS
**
YAP
protein level (relative to control)
***
D
Ctgf
Cyr61
E
Ccnd1
Hb-egf
*
#
F
p-ERK
ERK
42/44 kDa
PERK/ERK

**Figure 3. YAP-induced Müller cell proliferation requires activated microglia.** (A) EdU and Sox9 co-labeling in the inner nuclear layer of retinal explants transduced with AAV-GFP (control) or AAV-YAP5SA, treated or not with 10 µM of PLX3397 and cultured for 7 days. Quantification of the total number of double EdU+Sox9+ cells for each condition. Mean values +/- SEM from at least 6 independent retinas are shown. *Scale bar*: 50 µm. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤ 0.0001, ns: non-significant.

# Figure 3

**A**


Control
AAV-YAP5SA
None
+ PLX3397
None
+ PLX3397
EdU Sox9
EdU

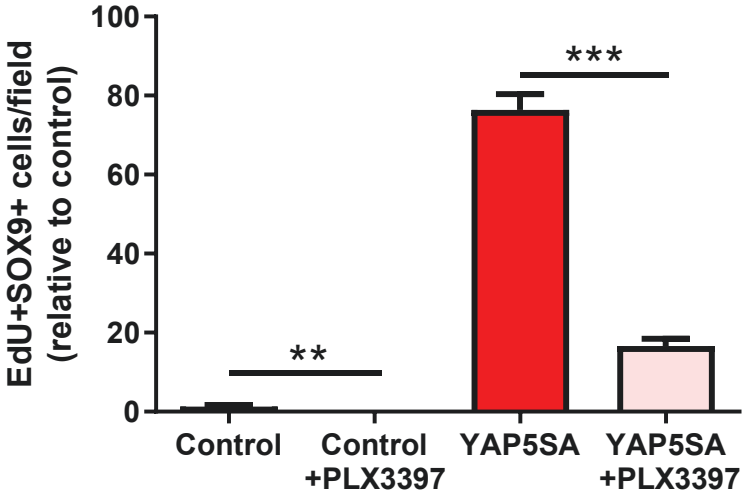

EdU+SOX9+ cells/field
(relative to control)
100
80
60
40
20
0
**
***
Control
Control
+PLX3397
YAP5SA
YAP5SA
+PLX3397

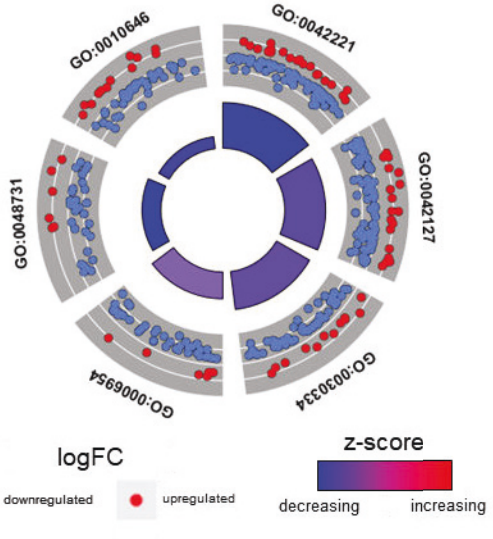
**Figure 4. *Yap* CKO retinas exhibit altered pro-inflammatory response upon injury.** (A) Timeline diagram of the experimental procedure used in (B–C). *Yap*$^{flox/flox}$ (control) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ (*Yap* CKO) mice received a single dose of 4-OHT at P10 and a single dose of MNU at 2 months. Retinas were then subjected to RNA-sequencing (RNA-seq) 16 h later. (B) Results of Gene Ontology (GO) enrichment analysis exemplifying six over-represented GO biological processes related to retinal response to injury. (C) Hierarchical clustering analysis of the 35 DEGs from the GO BP "inflammatory response" (GO:0006954) and the resulting heatmap representing the z-score expression values for each condition. Relative RNA expression (in fragments per kilobase of exon per million fragments mapped [FPKM]; data retrieved from the RNA-seq experiment) of *Ccl2*, *Cxcl2*, *Cxcl1*, *Tnf, Il-1α* and *Il-1β* in retinas from non-injected MNU WT mice or control and *Yap* CKO mice injected with 4-OHT and MNU as shown in (A).

# Figure 4

**A**


**B**

| ID | Biological process | P-Value |
|---|---|---|
| GO:0042221 | response to chemical | 1.32E-16 |
| GO:0042127 | regulation of cell proliferation | 5.50E-16 |
| GO:0030334 | regulation of cell migration | 1.20E-15 |
| GO:0006954 | inflammatory response | 3.65E-14 |
| GO:0048731 | system development | 3.80E-13 |
| GO:0010646 | regulation of cell communication | 2.86E-12 |




**C**

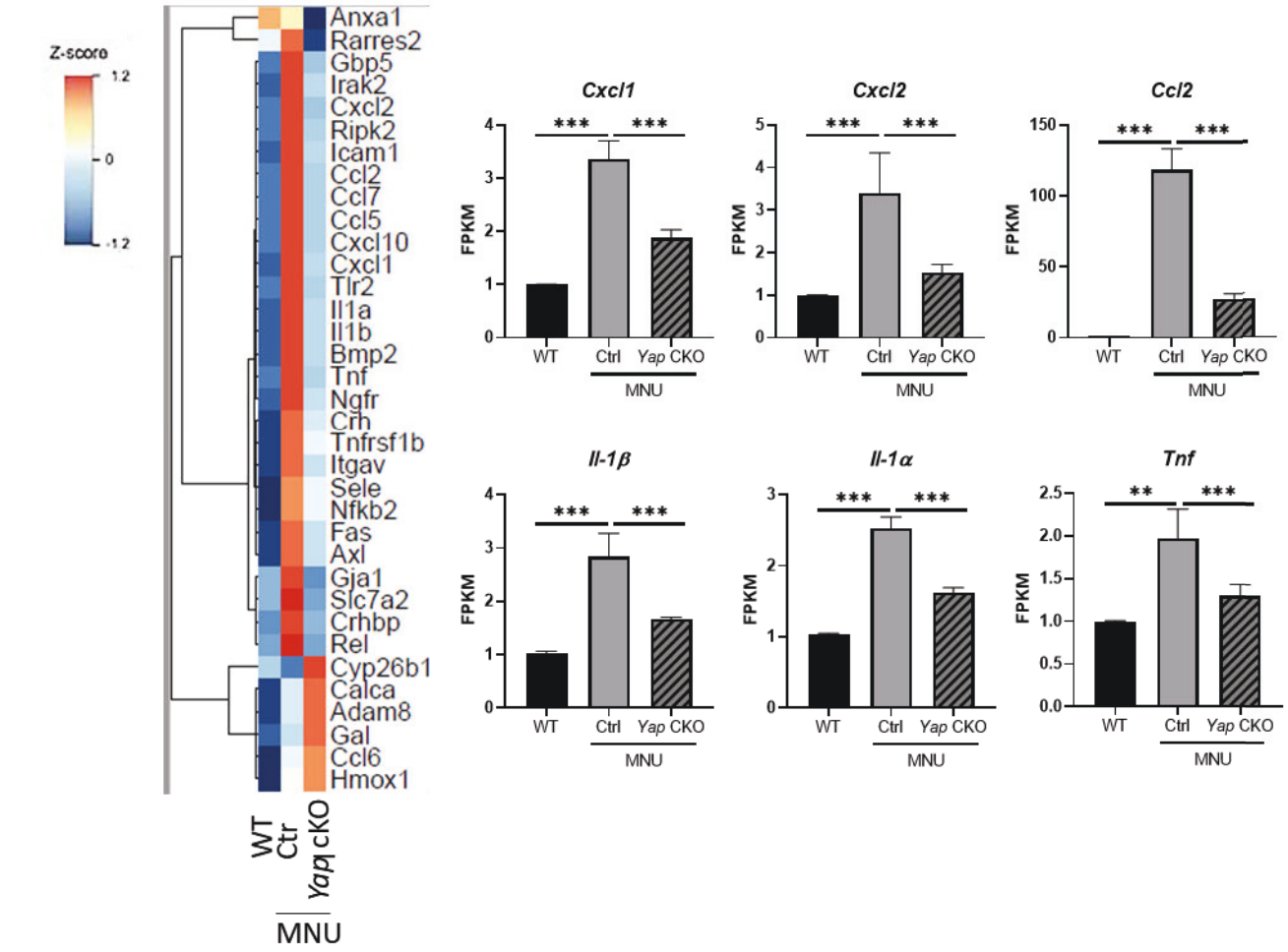


**Figure 5. LPS-induced Müller cell proliferation requires YAP activity.** (A) Timeline diagram of the experimental procedure used in (B). *Yap*$^{flox/flox}$ (control) and *Yap*$^{flox/flox}$;*Rax-CreER*$^{T2}$ (*Yap* CKO) mice received a single dose of 4-OHT at P10. After 2 months, retinas were flattened and cultured for 7 days in the presence or not of LPS. EdU was added to the culture medium for immunostaining analysis. (B) EdU and Sox9 co-labeling in the inner nuclear layer of control and *Yap* cKO retinal explants treated or not with LPS after 7 days of culture. Quantification of the total number of double EdU+Sox9+ cells for each condition. Mean values +/- SEM from at least 5 independent retinas are shown. *Scale bar*: 50 µm. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ****p≤ 0.0001, ns: non-significant.

# Figure 5

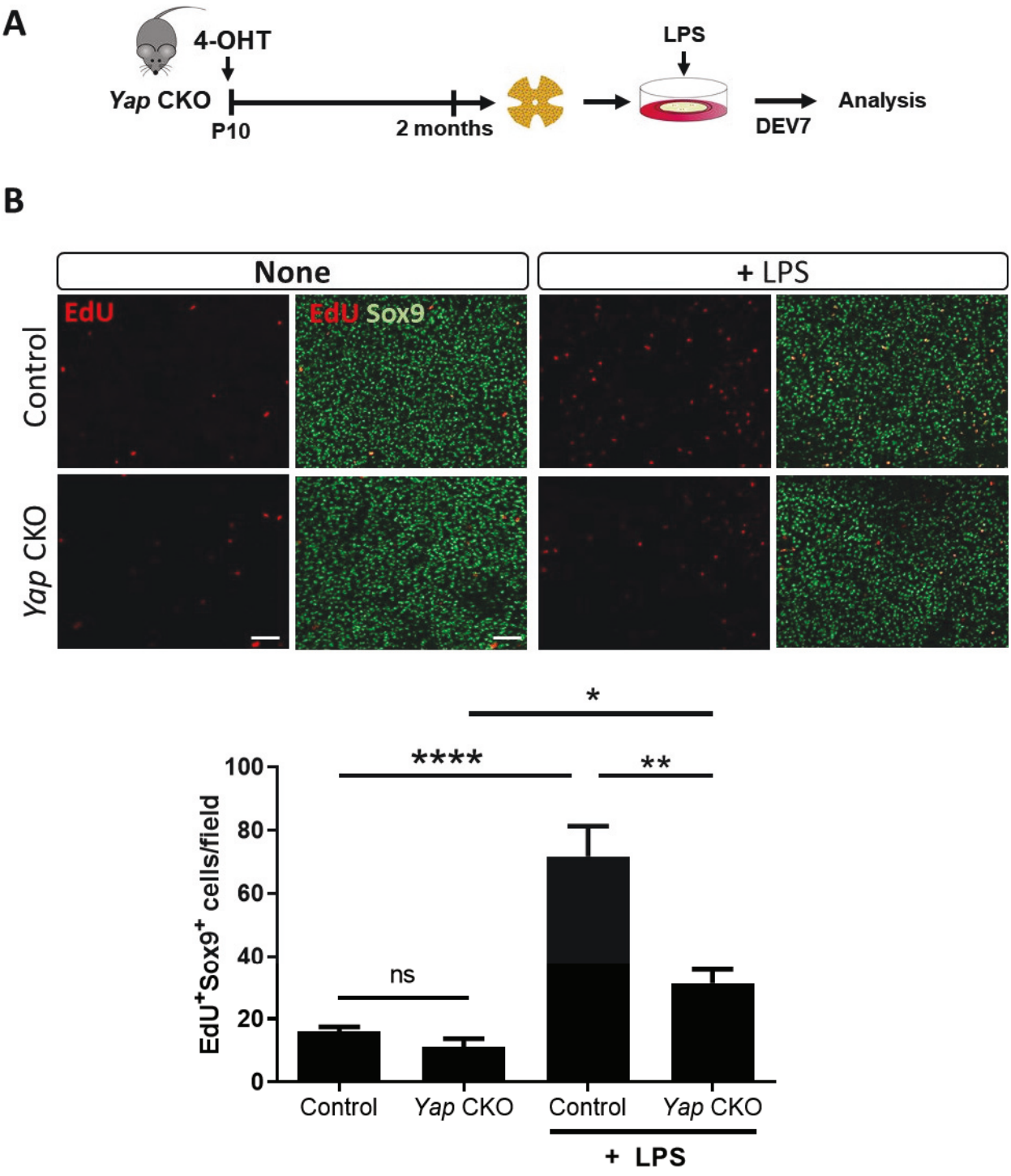
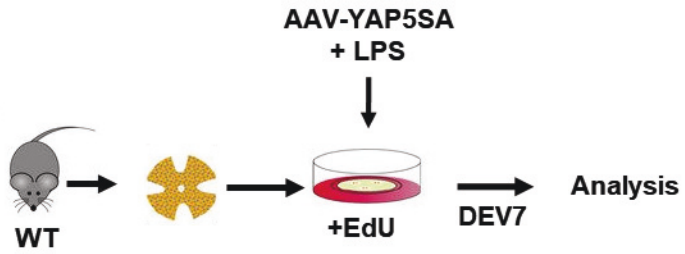

A
4-OHT
Yap CKO
P10
2 months
LPS
DEV7
Analysis
B
None
+ LPS
Control
Yap CKO
EdU
EdU Sox9
*
****
**
ns
EdU+Sox9+ cells/field
100
80
60
40
20
0
Control
Yap CKO
Control
Yap CKO
+ LPS

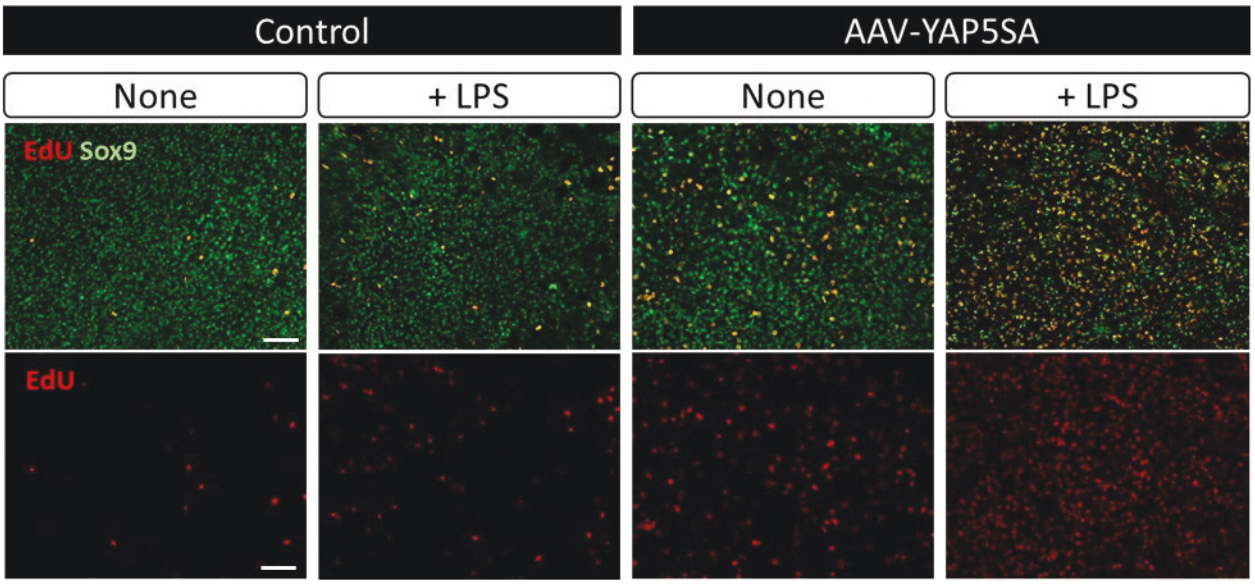
**Figure 6. YAP and inflammatory signals cooperate to induce Müller cell proliferation.** (A) Timeline diagram of the experimental procedure used in (B). Retinas from WT mice were flattened, transduced with AAV-GFP (control) or AAV-YAP5SA, and cultured for 7 days in the presence or not of LPS. EdU was added to the culture medium for immunostaining analysis. (B) EdU and Sox9 co-labeling in the inner nuclear layer of retinal explants transduced with AAV-GFP (control) or AAV-YAP5SA, treated or not with LPS and cultured for 7 days. Quantification of the total number of double EdU+Sox9+ cells for each condition. Mean values +/- SEM from at least 8 independent retinas are shown. *Scale bar*: 50 µm. Statistics: Mann-Whitney test, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤ 0.0001, ns: non-significant.

# Figure 6

**A**


AAV-YAP5SA
+ LPS
WT
+EdU
DEV7
Analysis

**B**


Control
AAV-YAP5SA
None
+ LPS
None
+ LPS
EdU Sox9
EdU

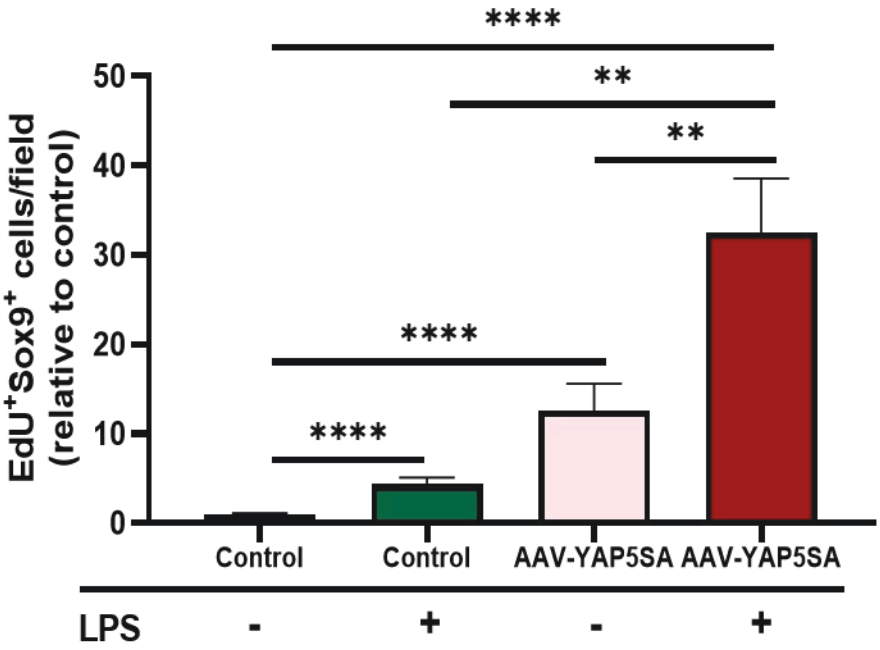
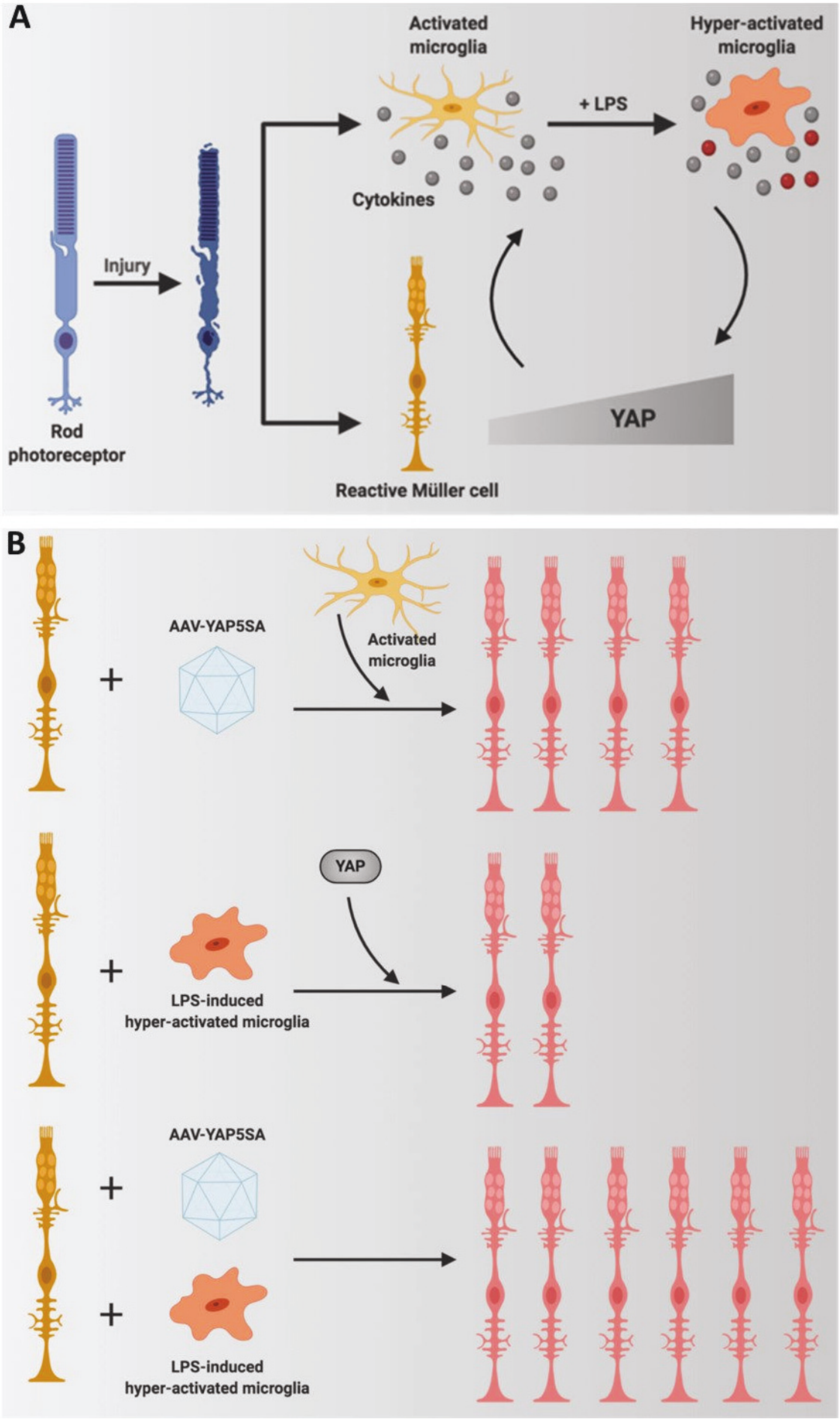

EdU+Sox9+ cells/field
(relative to control)
50
40
30
20
10
0
****
**
**
****
****
Control
Control
AAV-YAP5SA
AAV-YAP5SA
LPS
-
+
-
+

**Figure 7. Schematic representation of a proposed model.** (A) Our results support a role of YAP as a molecular hub coupling inflammatory signals and the regenerative process. Our data reveal that in case of retinal degeneration, inflammatory signals can modulate YAP expression, while YAP regulates the expression of key pro-inflammatory signals. (B) Our results support the existence of a crosstalk between the inflammatory and the YAP-mediated regenerative response in the mouse retina. More specifically, our results shown that chronic stimulation of the microglial-dependent inflammatory response results in a boost of the usually limited proliferative response of Müller cells in case of damage. This increased Müller cell proliferation results from the cooperation of the microglial activation and the co-transcriptional factor YAP. The microglial-secreted inflammatory factors and the Müller cell intracellular mechanisms that coordinates the cooperation LPS-YAP remains to be discovered.

# Figure 7


A
Activated microglia
Hyper-activated microglia
+ LPS
Cytokines
Injury
Rod photoreceptor
YAP
Reactive Müller cell
B
AAV-YAP5SA
Activated microglia
YAP
LPS-induced hyper-activated microglia
AAV-YAP5SA
LPS-induced hyper-activated microglia

| GENE NAME | SPECIES | FORWARD | REVERSE |
|---|---|---|---|
| *Ccnd1* | *mus musculus* | CCCAACAACTTCCTCTCCTG | TCCAGAAGGGCTTCAATCTG |
| *Ctgf* | *mus musculus* | GCCCTAGCTGCCTACCGACT | AGAACAGGCGCTCCACTCTG |
| *Cyr61* | *mus musculus* | TGAAGAGGCTTCCTGTCTTTGG | CGGCACTCTGGGTTGTCATT |
| *Hbegf* | *mus musculus* | GCATCCAAAGTGATCGCTGC | GACAACACTGCGGCCAGAAA |
| *Rax-CreETR$^2$ (genotyping)* | *mus musculus* | GGAATTGGTTTCCCGCAGAA | AAATGCCAGATTACGTATAT |
| *Rps26* | *mus musculus* | CCCAAGGATAAGGCCATCAAG | AAGCACGTAGGCGTCGAAGA |
| *Srp72* | *mus musculus* | CACCCAGCAGACAGACAAACTG | GCACTCATGGTAGCGTTCCA |
| *Taz* | *mus musculus* | AGACCCCAGGAAGGTGATGAA | TGTTGGTGATTCATTGCGAGAT |
| *Tbp* | *mus musculus* | CACAGGAGCCAAGAGTGAAGAAC | ATCACAGCTCCCCACCATGT |
| *Yap* | *mus musculus* | TCCTGATGGATGGGAGCAAG | CTCTGGTTCATGGCAAAACGA |

**Table S1. List of primers**

| Antigene | Host | Supplier | Reference | Dilution (WB) | Dilution (IF-FM) |
|---|---|---|---|---|---|
| Alpha-tubulin | mouse | SIGMA | T9026 | 1:20000 | |
| CD68 | rat | Bio-Rad UK | MCA1957GA | | 1:1000 |
| ERK1/2 (p44/42 MAPK) | rabbit | Cell Signaling | 9102 | 1:1000 | |
| P-ERK1/2 (Thr202/Tyr204) | rabbit | Cell Signaling | 4370 | 1:1000 | |
| SOX9 | rabbit | Abcam | ab185966 | | 1:300 |
| YAP | mouse | Abcam | ab56701 | 1:1000 | |
| Alexa 488 anti-rabbit | donkey | Thermo Fischer Scientific | A21206 | | 1:500 |
| Alexa 647 anti-rat | goat | Thermo Fischer Scientific | A21247 | | 1:500 |
| Alexa 647 anti-rabbit | goat | Thermo Fischer Scientific | A21244 | | 1:500 |

**Table S2. List of antibodies.** WB : western blot, IF-FM : immunofluorescence on flat mounted retinas.

# CONCLUSION AND PERSPECTIVES

## DEFINING THE MECHANISMS UNDERLYING YAP-DEPENDENT CROSSTALK BETWEEN MICROGLIA AND MÜLLER CELLS

The idea of YAP as a key player in the interplay between inflammation and regeneration is starting to emerge in the context of several mammalian organs, such as the liver, the skin or the intestine (Su et al., 2015; Taniguchi et al., 2015). Along this line, our results suggest a role of YAP as a molecular hub coupling inflammatory signals and the regenerative process in the mouse retina (Figure 7). In particular, we found reciprocal interactions: (i) inflammatory signals can modulate YAP expression while YAP regulates the expression of key pro-inflammatory molecules and (ii) the Müller cell proliferative effect of YAP requires microglia while the Müller cell proliferation effect of LPS-induced inflammation requires YAP. Moreover, their combined increased activity allows an impressive awakening of Müller cells, reaching almost 100% of Müller cells entering into the cell cycle. It will be interesting in the future to deepen our understanding of the molecular mechanisms underlying such microglia-YAP-Müller cell interactions. We can make some predictions about signalling pathways potentially involved. For instance, NFkB signalling pathway is a strong candidate. First, it has been described as an inducer of the proliferative response of Müller cells in regenerative organisms (Conner et al., 2014; Nelson et al., 2013). Second, many proliferation-related genes have TEAD (YAP transcription factor partner) as well as NFkB binding sites (Figure 42) (Su et al., 2015), suggesting a common regulation. Third, cytokines induced by LPS include TNFα, a well-known inducer of NFkB signalling pathway (Hayden and Ghosh, 2014). Fourth, along this line, I have shown that NFkB expression increases during chronic exposure to LPS in retinal explants. Fifth, we found that the expression of both TNFα and NFkB is reduced in absence of YAP. All these information warrant further investigation regarding TNFα and NFkB to figure out their position in the overall picture of YAP-mediated Müller glia-microglia reciprocal interactions.

## YAP-MEDIATED MÜLLER GLIA-MICROGLIA INTERPLAY *IN VIVO*

As mentioned in Part 3, retinal explants are a valuable model for the study of inflammation/microglia-Müller cell interactions but they present some limitations as not only microglia, but also infiltrating immune cells, participate to the regulation of the inflammatory process (Bosak et al., 2018). In order to know whether our results obtained *ex vivo* in retinal explants are biologically relevant, it would be important to validate my results *in vivo*. Modulating inflammation *in vivo* could be achieved by intraocular injection of PLX3397 or LPS. Although more laborious than intraperitoneal injections, we favor intraocular injections since systemic and local LPS

exposures produce different effects within the mouse retina. Indeed, intraocular injection of LPS is accompanied by a marked cellular infiltration, while systemic injection of LPS fails to produce a comparable effect (Rosenbaum et al., 2011). This work should provide novel insights into the potential of inflammation and YAP signalling as therapeutic targets for Müller cell-dependent regeneration.

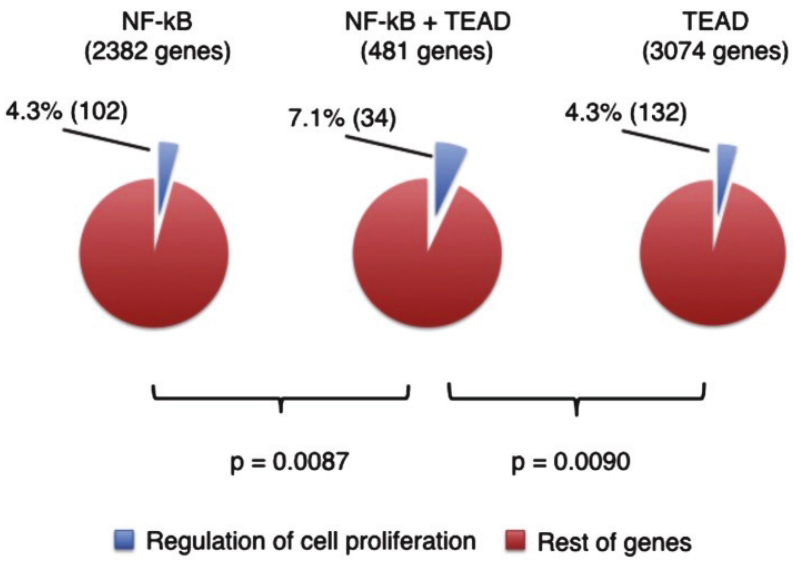



**Figure 42. TEAD and NFkB correlation regarding the binding sites in the promoters of genes regulating proliferation.** Schematic representation of the genes with DNA sequence motifs of NFkb and/or TEAD identified from mouse genome MM9 multiz30way using Motifmap (NFkB M00208, TEADs M01305). Genes related to cell proliferation were identified with David gene ontology GO:0042127 for regulation of cell proliferation. From (Su et al., 2015).

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# APPENDIX

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# Update on Müller glia regenerative potential for retinal repair

Diana García-García, Morgane Locker and Muriel Perron

Retinal regeneration efficiency from Müller glia varies tremendously among vertebrate species, being extremely limited in mammals. Efforts towards the identification of molecular mechanisms underlying Müller cell proliferative and neurogenic potential should help finding strategies to awake them and ensure regeneration in mammals. We provide here an update on the most recent and original progresses made in the field. These include remarkable discoveries regarding (*i*) unprecedented cross-species comparison of Müller cell transcriptome using single-cell technologies, (*ii*) the identification of new strategies to promote both the proliferative and the neurogenic potential of mammalian Müller cells, (*iii*) the role of the epigenome in regulating Müller glia plasticity, (*iv*) miRNA-based regulatory mechanisms of Müller cell response to injury, and (*v*) the influence of inflammatory signals on the regenerative process.

**Address**
Université Paris-Saclay, CNRS, Retina France, Institut des Neurosciences Paris Saclay, Orsay, France

Corresponding author:
Perron, Muriel (muriel.perron@universite-paris-saclay.fr)

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The absence of retinal regeneration in mammals results in vision loss upon damage or degeneration. In contrast, some vertebrate species can regenerate their retina following injury thanks to the reprogramming of Müller glia into retinal stem cells. Müller cells are the main type of glial cells in the vertebrate retina and they contribute to the maintenance of retinal homeostasis and visual function [1]. In case of retinal damage, they undergo gliosis, a process that includes cell-cycle re-entry. This occurs very efficiently in zebrafish or *Xenopus*, and this is accompanied by spontaneous neurogenesis and consequent regeneration of retinal neurons [2–4]. In post-hatched chicks, Müller cells also proliferate successfully in response to injury, but they possess a limited neurogenic competence, generating only inner retinal neurons [5]. In mammals, Müller glia have a very restricted proliferative capacity, leading to a negligible level of cell replacement in case of retinal degeneration [5,6]. Understanding why retinal self-repair varies tremendously across different species is essential to explore therapeutic strategies based on the stimulation of Müller cell regenerative capacity. This has been the subject of substantial investigations over the past decade. Our knowledge of the signalling network, and of the genetic or epigenetic events that either sustain or restrict Müller cell potential in different species, has recently increased spectacularly. In this review, we attempt to highlight the most recent and original progresses made in the field, opening new avenues for retinal repair.

## Species-specific Müller cell response to retinal injury

Identifying the mechanisms underlying the variability of Müller cell regenerative behaviour in different species has been the subject of intense investigation. Among species-specific differences that have been identified is the expression of Ascl1. This proneural transcription factor, which is essential for retinal regeneration, is indeed upregulated in fish and bird Müller cells upon injury but not in mammalian ones [7]. Notch signalling impact on Müller glia proliferation also seems to differ between fish and mammals. Its inhibition was reported to potentialize the pro-proliferative effects of reprogramming factors such as Ascl1 and lin28a in zebrafish, but not in mice [8]. In order to reach a more comprehensive and dynamic view of the intrinsic factors sustaining the regenerative ability of Müller cells across species, bulk and single-cell RNA sequencing (scRNA-seq) were recently applied [9••]. RNA levels were profiled in zebrafish, chick and mouse, in undamaged retinas or at different time-points following neuronal cell death induction. This study reveals in an unprecedented manner, species-specific transcriptional responses of Müller cells to retinal injury. Among new molecular cues identified are transcription factors of the NFI family. These are expressed in resting Müller cells and downregulated after retinal damage. While levels remain low in fish, an upregulation is observed at later stages in the mouse. NFI-deficient mouse Müller cells upregulate cell cycle regulators, along with the neurogenic factor Ascl1. This suggests that, upon acute retinal injury in mammals, NFI factors eventually repress proliferative and neurogenic competence, forcing reactive Müller glia to revert back to a resting state.

Distinct regenerative capacities in animals may thus rely, at least in part, on the differential activation of regulatory networks that restore quiescence of activated Müller glia (Figure 1a,b). This large-scale and cross-species analysis clearly opens new avenues for the functional characterization of additional identified candidate genes.

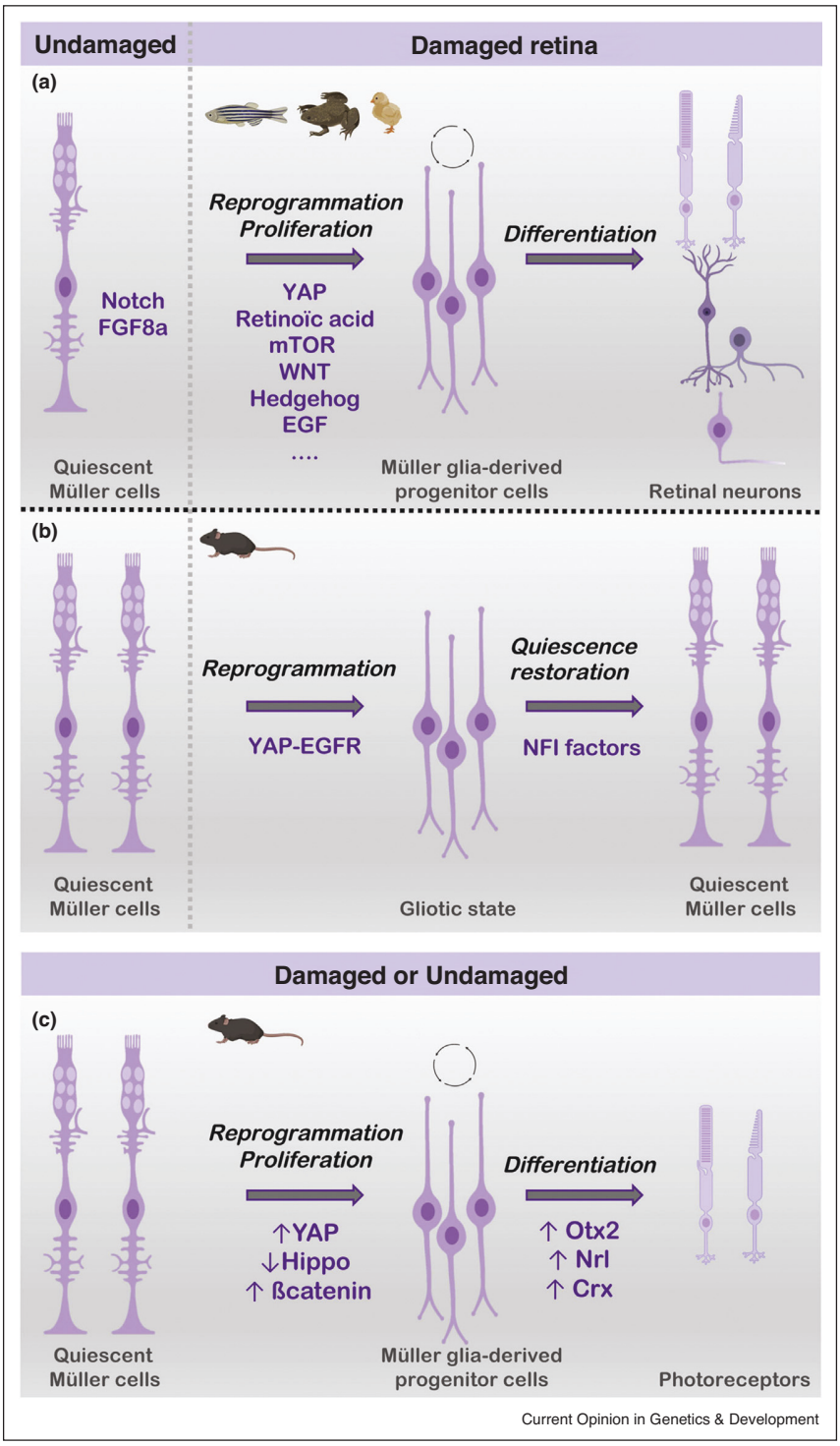
## The journey of a reactive Müller cell: reprogramming, proliferating and differentiating

What are the signals that trigger Müller cell response to injury? In the last decade, multiple extrinsic signalling pathways, including Wnt, BMP, TGFβ, EGF, Notch, or Sonic Hedgehog (all known to be involved in retinal development), were shown to trigger Müller glia cell cycle re-entry or conversely to maintain their quiescent state [2,3,6,10]. Additional signalling pathways were recently recognized as key regulators of these processes (Figure 1a). First, mTOR and retinoic acid signalling were shown to promote the generation of proliferative Müller glia-derived progenitor cells (MGPCs) in the injured chick and/or fish retinas [11–13]. Second, Fgf8a, which was considered as an inhibitor of proliferation, surprisingly revealed more complex stage-dependent functions, with a mitogenic impact on young Müller cells and an anti-proliferative effect on older ones [14]. Finally, recent studies including ours, identified the Hippo pathway as a key player for Müller glia cell cycle re-entry [15•,16•,17]. YAP, a downstream effector of the Hippo pathway, is expressed in *Xenopus* Müller cells and required for their division in response to injury [15•]. YAP is also expressed in mouse Müller cells, upregulated upon photoreceptor degeneration and involved in cell cycle gene upregulation in reactive glial cells [15•,16•,18]. Clearly, this is however not sufficient to trigger mammalian Müller cell proliferation following injury. However, turning dormant Müller cells into actively proliferative cells can be achieved through a genetic bypass of Hippo signalling (leading to YAP activation), or by overexpression of a constitutively active YAP variant, and this even in uninjured retinas [15•,16•] (Figure 1c). It is thus likely that failure to activate YAP at a sufficient level in reactive Müller cells constitutes a key barrier for these cells to proliferate in mammals. Besides, successful regeneration will only be achieved if MGPCs differentiate appropriately into retinal neurons. With the genetic tools used so far, this is only the case for a very limited subset of YAP-overexpressing proliferative Müller cells [16•], probably because the maintenance of a proliferative state counteracts the differentiation process. In addition, a two-step reprogramming approach, such as that recently developed by Chen's laboratory [19••], may also be necessary to drive the process of regeneration until completion (Figure 1c). In this study, induction of mammalian Müller glia dedifferentiation and proliferation was first obtained by stimulating Wnt signalling. Then, transcription factors essential for rod cell fate specification (Crx, Otx2 and Nrl) were overexpressed and likely forced activated Müller cells to differentiate into rod photoreceptors. Importantly, this could restore some visual responses in a mouse model of congenital blindness. Although many questions obviously remain regarding the feasibility in human, this might constitute a first laboratoryproof of concept that reprogramming endogenous Müller glia in mammals could restore some light sensitivity. Similar strategies may also be employed to direct the differentiation of proliferating Müller cells into other retinal cell types. NEUROG2 for instance recently proved sufficient to convert postnatal Müller cells into neurons exhibiting features of retinal ganglion cells [20] and could thus be an interesting candidate.

## Epigenetic basis of retinal regeneration

Müller cell-dependent regeneration involves a conversion of cellular identity, from a quiescent differentiated state to a multipotent retinal progenitor one. This is accompanied by significant changes in transcriptional programs which led researchers to investigate the remodelling of epigenetic marks, that is, DNA methylation and histone modifications [21,22]. Analysis of DNA methylation landscape indeed revealed dynamic changes during retinal regeneration: demethylation predominates just after injury while *de novo* methylation occurs at later timepoints [23] (Figure 2a). Contrasting with the situation observed during induced pluripotent stem cell formation, promoters of pluripotency-associated and regeneration-associated genes (such as *Ascl1a, Lin28, Sox2, Oct4*) were reported to be already hypomethylated in quiescent zebrafish Müller cells, likely contributing to their progenitor-like properties [23]. Surprisingly, a similar hypomethylated profile was found in mouse Müller glia as well. Based on these data, the authors proposed that DNA methylation of these genes may not be a barrier for these cells to reprogram [22–24]. This may however be more complex, as inferred from another study that examined *Oct4* methylation in the first exon, a region known to be important for its expression [25]. The authors here report that, in mouse, this *Oct4* region is demethylated shortly after injury, before returning back to a fully methylated state, similar to that observed in quiescent Müller cells. In terms of expression, this correlates with a rapid upregulation upon injury, followed by a subsequent silencing 24 hours later [25]. Importantly, *Oct4* was recently shown as essential for zebrafish Müller glia reprogramming through the regulation of several regeneration-associated factors such as Ascl1a and Lin28a [26]. Interfering with *Oct4* DNA methylation-mediated silencing may thus help enhancing mammalian Müller cell reprogramming. Another event of gene silencing has recently been identified in the injured medaka fish [27]. Medaka Müller cells, unlike their zebrafish counterparts, exhibit a restricted capacity to regenerate the retina and only give rise to photoreceptors. Lust and Wittbrodt found that *Sox2* expression is upregulated in the zebrafish damaged

**Figure 1**




Müller cell dependent retinal regeneration. **(a)** Upon retinal damage, zebrafish, *Xenopus laevis* or post-hatched chick Müller cells dedifferentiate, re-enter into the cell cycle and regenerate some types of neurons. Several signalling pathways were shown to be required for the reprogramming and proliferation events. The list has recently been broadened with the discovery that YAP, retinoic acid or mTOR are also key players in these processes [12,13,15•]. Notch and FGF8a, on the other hand, are required for maintaining the quiescence of adult Müller cells [14,51]. **(b)** In the mouse damaged retina, although Müller cells initiate reprogramming, an event that requires the YAP-EGFR axis [15•], they do not proliferate. Following acute retinal injury, these reactive Müller cells rapidly return to quiescence, a transition mediated by NFI factors [9••]. **(c)** It is possible to

retina [27], where it is necessary and sufficient for Müller cell proliferation [28]. In contrast, it is downregulated in medaka in response to injury [27]. Remarkably, the regenerative capacity of medaka Müller glia could be upgraded at a level comparable to that of zebrafish when *Sox2* expression was restored. This suggests that differential regulation of a single factor is responsible for the divergent regenerative capabilities of these two teleost species [27]. Besides, silencing of differentiation genes may also represent a barrier for efficient regeneration of fully functional neurons or for the production of specific cell types. In line with this, highly methylated DNA was found in promoter regions of several key mouse genes required for early born retinal neuron specification or phototransduction [22].

In addition to DNA methylation, histone modifications are epigenetic mechanisms that also have profound effects on gene regulation. Therefore, not surprisingly, chromatin modifying enzymes are emerging as regulators of retinal regeneration in zebrafish. For instance, both the histone methyltransferase Dot1l and the histone deacetylase HDAC1 were recently shown to be necessary for Müller cell-dependent regeneration [29,30•,31]. While Dot1l drives their dedifferentiation and cell cycle re-entry through canonical Wnt signalling activation [30•], HDAC1 seems to dynamically regulate MGPC formation through its action on the Her4.1/Lin28a/let-7 axis [29]. Of note, whether histone acetylation-independent effects of HDAC1 are involved remains to be determined. In the mouse, the limited capacity of adult Müller cells to reprogram correlates with reduced chromatin accessibility. Along this line, the use of HDAC inhibitors in conjunction with Ascl1 overexpression was shown to promote chromatin accessibility at key gene loci and thereby improve Müller cell regenerative potential [32•]. Thus, simultaneous manipulation of mitogenic/neurogenic factors and epigenetic modifiers could be envisaged to efficiently reprogram Müller cells and regenerate neurons in diseased retina.

## microRNA-driven mechanisms of gene regulation during retinal regeneration

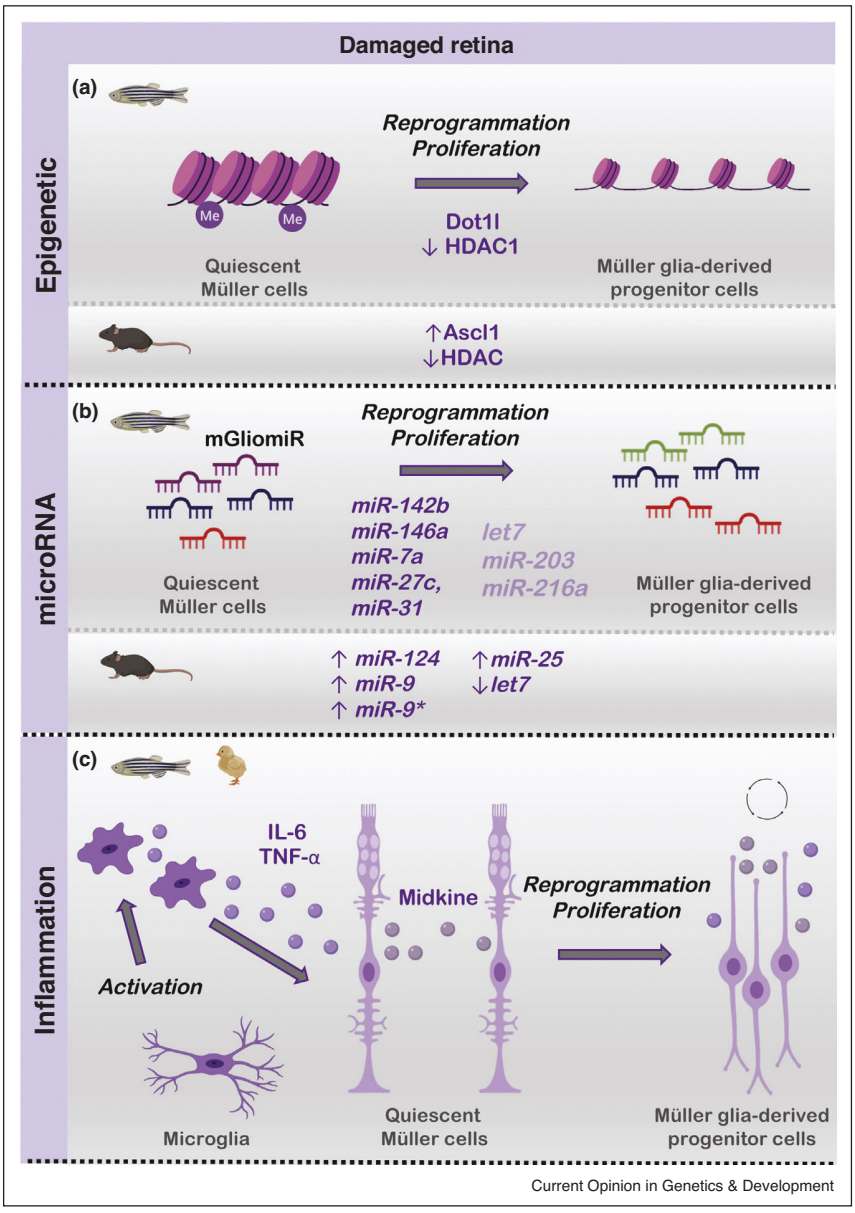
In recent years, several microRNAs (miRNAs) proved to play important roles during retinal regeneration [33]. Highlighting this fact is the finding that suppression of Dicer, a critical regulator of miRNA biogenesis, impairs zebrafish Müller glia cell cycle re-entry in response to injury [34]. High-throughput sequencing allowed identifying miRNA that exhibit differential expression in intact *versus* regenerating retinas. Some of them, *miR-142b*, *146a*, *7a*, *27c*, and *31* turned out to be required for the proliferation of MGPCs [34] (Figure 2b). Conversely, others miRNAs are downregulated following injury, and are therefore potentially involved in the maintenance of Müller cell quiescence and/or in the inhibition of their reprogramming. A well-known example is *let-7* miRNA, which needs to be inhibited to allow derepression of crucial dedifferentiation genes. Blockade of its maturation occurs through the action of the RNA binding protein Lin28, which is activated downstream Ascl1 [35], Wnt signalling [36] or Shh signalling [37]. Of note, *let-7* miRNA in turn regulates Shh pathway components, thereby participating to a complex regulatory loop [37]. *miR-203* and *miR-216a* are also repressed following injury, which contributes to Müller cell dedifferentiation through derepression of the transcription factor Pax6 and the histone methyltransferase Dotl1 [30•,38]. Together, this raised the question as to whether the manipulation of miRNA could promote retinal regeneration in mammals. Interestingly, overexpression of *miR-124*, *miR-9* and *miR-9** (alone or in combination with Ascl1) proved effective in stimulating the conversion of cultured murine Müller cells into MGPCs [40] (Figure 2b). To provide a more comprehensive view of miRNAs that differ between progenitors, neurons and glial cells in the mouse, miRNA expression profiling was performed, which revealed a Müller glia-specific miRNA signature, named mGliomiR [39,41,42•]. Functional assays revealed that antagonizing *let-7* while overexpressing *miR-25* and *miR-124* was sufficient to increase *Ascl1* expression and consequently to reprogram mouse Müller glia into neural progenitors *in vitro* [42•]. Subsequent single cell RNA-seq analysis of reprogrammed Müller glia allowed to identify potential targets of these miRNA [42•]. For instance, the top target of *miR-25* was the Wnt inhibitor *Dkk3*, which may partly explain its effect in stimulating Müller cell proliferation following overexpression. Thus, microRNAs are emerging as additional key targets for enhancing retinal regeneration.

## The influence of inflammatory signalling pathways on retinal regeneration

There has been a recent surge of interest in investigating the relationship between the immune system and the regenerative potential of neural tissue [43]. In the nervous system, microglia are the resident immune cells that can sense the microenvironment and rapidly respond to various insults by producing molecular mediators, in particular cytokines [44]. Compelling evidence points to the implication of microglia in shaping the responsiveness of Müller cells to injury (Figure 2c). Indeed, the ablation of microglia, either in the chick or fish retina, suppresses Müller cell proliferation, supporting a positive influence

**(Figure 1 Legend Continued)** awake the regenerative potential of mouse Müller glia. Overexpressing YAP or inhibiting the Hippo pathway in Müller cells is sufficient to induce their reprograming and proliferation, even in undamaged retina [15•,16•]. Following gene transfer of β-catenin to stimulate Müller cell proliferation, photoreceptors can be regenerated by forcing the expression of transcription factors promoting rod cell fate specification [19••].

**Figure 2**




Multiple regulatory mechanisms underlying Müller cell reprogramming. **(a)** In zebrafish, DNA demethylation predominates after injury, although some pluripotency-associated genes are already hypomethylated in quiescent Müller cells [23]. Changes in histone modifications also likely underlie Müller cell reprogramming. For instance, the histone methyltransferase Dot1l acts as an epigenetic modifier required for Müller cell dedifferentiation and MGPC proliferation [30•]. HDAC1 activity, which triggers condensed chromatin architecture that limits transcription, is decreased in reactive Müller cells, likely leading to the derepression of regeneration-associated genes. Yet, HDAC acts as a repressor of Her4 (an effector of Notch signalling, a pathway associated with Müller glia quiescence [51,55]), and as such is required for efficient proliferation of MGPCs [29]. Manipulation of epigenome modifiers in the mouse may be applied to remove the epigenetic barriers limiting Müller cell reprogramming. This was illustrated by the use of HDAC inhibitors in conjunction with Ascl1 overexpression [32•]. **(b)** Several miRNAs have been identified that are either up-regulated or repressed in response to injury and play important roles in the proliferation of MGPCs [33]. Manipulating miRNAs was shown to be sufficient to increase the proliferation of mouse Müller glia *in vitro* [39,40•,42•]. **(c)** Microglia become activated in damaged retina, producing inflammatory cytokines, including TNF-α and IL-6. Müller cells are also a source of cytokines, such as Midkine. These various inflammatory mediators are required for MGPCs proliferation in zebrafish and/or chick, highlighting the influence of inflammatory signalling pathways on retinal regeneration [45,50–53].

of inflammation on retinal regeneration [45,46••,47]. However, microglia likely have multifaceted effects, being either beneficial or detrimental for neural tissue repair, depending on the duration of their activation and on their polarization phenotypes (pro-inflammatory or anti-inflammatory) [48]. Along this line, White and coll. found that immune suppression performed before injuring the retina delays regeneration, while it accelerates it when performed after [46••]. The inflammatory response in zebrafish is thus necessary for Müller cell activation but becomes a brake to the regeneration process if resolution is delayed [46••,47]. This likely implies that immune-targeted therapeutic strategies should aim at adjusting the balance between microglia polarization states. In addition to resident microglia, infiltrating immune cells also appear to participate to the inflammatory response triggered by retinal injury [49]. The precise contribution of each immune cell types to the regeneration process remains to be investigated. Besides, cytokines/chemokines mediating the dialog between the inflammatory environment and Müller cells are just beginning to be explored. TNF-α and IL-6-family cytokines were for instance shown to promote injury-induced Müller glial cell reprogramming and proliferation in the chick and/or the fish retina [45,50–52]. A novel player impacting Müller cell cycle progression was recently identified as being the Midkine cytokine. Its loss-of-function in zebrafish mutants triggers reactive Müller glia to behave as in the mouse, initiating a reprogramming response following injury, entering the G1 phase of the cell cycle, but failing to progress further into S phase [53]. It is thus likely that comparing the cytokine repertoire between fish and mouse may help bringing new insights into the mechanisms that maintain Müller cells in a reactive state without cell cycle progression. As mentioned above, mTOR signalling is required for MGPC generation in chick and zebrafish injured retinas [11,12]. Interestingly, inflammation was recently shown to be necessary for mTOR signalling activation and to enhance retinal regeneration in a mTOR-dependent manner [12]. Finally, a recent study in chick revealed that pro-inflammatory signals from microglia induce NF-κB activation and consequent inhibition of Müller glia reprogramming [54]. Remarkably however, forcing NF-κB activation following microglial cell depletion (i.e. in a context when NF-κB levels are low), results in an opposite effect with increased formation of MGPCs. The authors thus propose that microglia-induced NF-κB activation is required to initiate Müller glia reprogramming, but becomes detrimental to the process if sustained. Such mechanism might be at work in the mouse retina where, in contrast to the fish situation, NF-κB signalling components are significantly upregulated following retinal damage [9••]. Differences in inflammatory signalling may thus contribute to different regenerative capacities in different species. A better understanding of the mechanisms underlying the bilateral communication between Müller cells and the innate immune system in regenerative and non-regenerative species thus awaits further investigation.

## Conclusion

Recent studies have shed new light onto the mechanisms underlying retinal regeneration and revealed potential strategies to stimulate Müller cell-dependent retinal repair in mammals. This review of the most recent literature in the field highlights the necessity of taking into account the different regulatory mechanisms that may affect expression of key regeneration genes. This includes genetic networks, epigenetic modifications and miRNA-mediated post-transcriptional gene silencing. The influence of Müller cell microenvironment is also starting to be scrutinized, in particular inflammatory regulatory molecules. Successful execution of retinal regeneration in mammals may thus rely on combined therapeutic strategies, comprising immune-targeted, genetic-targeted or epigenetic-targeted approaches. Deepening our comprehension of these various regulatory modules will thus be critical to translate this knowledge into therapeutic tools to restore vision in patients afflicted with degenerative retinal diseases.

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Signalisations et réseaux intégratifs en biologie (BIOSIGNE)

**Titre :** Les cellules de Müller et la régénération de la rétine : rôle de la voie de signalisation Hippo/YAP

**Mots clés :** Cellules gliales de Müller, Régénération rétinienne, Voie de signalisation Hippo/YAP, Inflammation

**Résumé :** Les maladies dégénératives de la rétine sont une des causes principales de cécité. Parmi les différentes stratégies thérapeutiques actuellement étudiées, notre équipe s'intéresse au potentiel régénératif de la rétine. Une source cellulaire d'intérêt sont les cellules de Müller, principal type de cellules gliales de la rétine capables de se réactiver en cas de dégénérescence, un processus appelé gliose réactive, et dans certaines espèces d'adopter des caractéristiques de cellules souches. Si un tel processus confère la capacité de régénérer la rétine chez les téléostéens, il est cependant largement inefficace chez les mammifères. Avoir une meilleure connaissance des mécanismes moléculaires sous-jacents pourrait aider à transformer leur potentiel de régénération en nouvelles stratégies thérapeutiques en condition pathologique de dégénérescence rétinienne. Dans ce contexte, mon laboratoire s'est focalisé sur l'effecteur terminal de la voie Hippo, le cofacteur de transcription YAP, dont il a été démontré qu'il stimule la régénération de plusieurs organes en cas de lésion. Dans la rétine, YAP est spécifiquement exprimé dans les cellules de Müller et son niveau d'expression augmente en cas de lésion. Cependant, sa fonction dans l'homéostasie rétinienne, et en particulier son rôle dans la régénération rétinienne, sont encore inconnus.
La première partie de ma thèse visait donc à décrypter la fonction de YAP dans les cellules de Müller de souris dans des conditions physiologiques et pathologiques. Nous avons révélé que YAP joue un rôle central dans l'homéostasie des cellules de Müller et en tant que tel, est un acteur clé de la survie des cônes au cours du vieillissement. En cas de lésion rétinienne, nous avons montré que YAP est essentiel pour la réactivation des gènes du cycle cellulaire qui accompagne normalement la gliose réactive. Dans ce contexte, nous avons également trouvé une interaction fonctionnelle entre YAP et la voie de signalisation EGFR, suggérant une fonction de YAP en tant qu'intégrateur des réseaux de signalisation mis en jeu dans le contexte régénératif. J'ai également constaté que la suractivation de YAP est suffisante pour induire la reprogrammation des cellules de Müller de souris en cellules hautement prolifératives, imitant ce qui se passe chez les poissons ou les amphibiens, où les cellules de Müller prolifèrent spontanément en cas de blessure. Dans l'ensemble, ce travail met en évidence le rôle critique de YAP dans la sortie de quiescence des cellules de Müller chez les mammifères et révèle ainsi une cible potentielle pour la médecine régénérative.
La deuxième partie de mon projet de doctorat naît des découvertes émergentes mettant en évidence les voies inflammatoires comme régulateurs du processus de régénération. Bien que l'inflammation soit considérée comme un obstacle à la régénération rétinienne chez les mammifères, il n'existe aucune étude concernant l'influence de l'inflammation sur le processus de régénération des cellules de Müller chez la souris. De plus, des découvertes récentes sur le rôle de YAP dans la régulation du processus inflammatoire m'ont conduit à faire l'hypothèse qu'il pourrait jouer un rôle dans la relation entre l'inflammation et la régénération rétinienne. J'ai donc cherché à étudier le rôle joué par l'inflammation sur le comportement des cellules de Müller de souris, et à comprendre comment YAP s'inscrit dans cette interaction. J'ai découvert de manière inattendue qu'un contexte pro-inflammatoire établi par les cellules microgliales stimule la prolifération des cellules de Müller de souris dans des explants rétiniens. De plus, mes résultats ont montré que cet effet mitogène se produit de manière dépendante de YAP. Par ailleurs, j'ai découvert que l'effet de la surexpression de YAP sur la prolifération des cellules de Müller peut être potentialisé par un environnement pro-inflammatoire et aboli en cas d'ablation des microglies. Enfin, nous avons constaté que, à son tour, YAP régule des cytokines inflammatoires clés. Dans l'ensemble, cette partie de mon projet permet non seulement d'approfondir nos connaissances concernant l'impact de l'inflammation sur le comportement des cellules de Müller de la souris, mais met également en évidence YAP en tant qu'acteur clé dans la connexion entre l'inflammation et la régénération rétinienne.

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Route de l'Orme aux Merisiers RD 128 / 91190 Saint-Aubin, France

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Signalisations et réseaux intégratifs en biologie (BIOSIGNE)

**Title :** Müller cells and retinal regeneration: role of the Hippo/YAP signaling pathway

**Keywords :** Müller glial cells, Retinal regeneration, Hippo/YAP signaling pathway, Inflammation

**Abstract :** Degenerative diseases of the retina are one of the main causes of blindness. Among the various therapeutic strategies currently being studied, our team is focusing on the regenerative potential of the retina. One cellular source of interest are Müller cells, the main type of glial cells in the retina capable of reactivating in case of degeneration, a process called reactive gliosis, and in some species adopting certain characteristics of stem cells. If such a process sustains powerful regeneration abilities in teleosts, it is however largely inefficient in mammals. Hence, increasing our knowledge of the molecular mechanisms underlying the behaviour of these cells under pathological conditions may help turning their regenerative properties into new therapeutic strategies. In this context, my laboratory focused on the terminal effector of the Hippo pathway, the co-transcriptional factor YAP, which has been shown to stimulate regeneration of several injured organs. In the retina, YAP is specifically expressed in Müller cells and upregulated in case of damage. However, its function in retinal homeostasis, and its role in retinal regeneration remained unknown.

The first part of my PhD aimed at deciphering YAP function in mouse Müller cells in both physiological and pathological conditions. In essence, we revealed a central role of YAP in Müller cell-dependent retinal homeostasis and as such, as a key player for cone survival during aging. In case of retinal damage, we showed that YAP upregulation is critical for cell-cycle gene reactivation that normally accompanies reactive gliosis. In this context, we also found a functional interaction between YAP and the EGFR signaling pathway, supporting a function of YAP as a hub within the complex signaling network of key regenerative signaling pathways. I also found that YAP overactivation is sufficient to induce mouse Müller cell reprogramming into highly proliferative cells, mimicking a fish or amphibian condition, when Müller cells spontaneously proliferate upon injury. As a whole, this work highlights the critical role of YAP in driving mammalian Müller cells to exit quiescence and thus reveals a potential target for regenerative medicine.

The second part of my PhD project stemmed from the emerging discoveries highlighting inflammatory pathways as regulators of the regenerative process. Although inflammation is considered to hamper retinal regeneration in mammals, there are no studies regarding the influence of inflammation on mouse Müller cell-dependent regenerative process. In addition, recent discoveries on the role of YAP in the regulation of the inflammatory process lead to the hypothesis that it could play a role in the relationship between inflammation and retinal regeneration. I thus aimed at investigating the role played by the injury-induced inflammation on mouse Müller cell behavior and how YAP fits in this interplay. I unexpectedly discovered that a microglial-dependent pro-inflammatory context stimulates mouse Müller cell proliferation in retinal explants. Importantly, my results showed that this mitogenic effect occurs in a YAP-dependent manner. Moreover, I uncovered that the effect of YAP overexpression on Müller cell proliferation can be potentiated by a pro-inflammatory environment, and abolished upon microglia depletion. Finally, we found that, in turn, YAP regulates key inflammatory cytokines. Altogether, this part of my project not only deepen our knowledge regarding the impact of inflammation on mouse Müller cell behavior, it also highlights YAP as a key player in the crosstalk between inflammation and retinal regeneration.

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