



Role of the Glycogen Synthase Kinase 3 for the Retinal Development and Homeostasis

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Role of the Glycogen Synthase Kinase 3 in Retinal Development and Homeostasis

Rôle de la Glycogène Synthase Kinase 3 dans le Développement et l'Homéostasie de la Rétine

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'Institut des Neurosciences Paris-Saclay
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Thèse présentée et soutenue à Orsay, le 22 mars 2018, par

Elena Braginskaja

Composition du Jury:

Pr. Simon Saule	Président
Pr. François Paquet-Durand	Rapporteur
Pr. Thomas Lamonerie	Rapporteur
Dr. Marie-Paule Felder-Schmittbuhl	Examinatrice
Dr. Gaël Orioux	Examineur
Dr. Muriel Perron	Directrice de thèse
Dr. Jérôme Roger	Co-Directeur de thèse

*To My Parents,
To Lutz Neumann*

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My 4-year adventure in France comes to the end with the accomplishment of my Ph.D. studies. Before heading for an “old-new life” in my home country, I would like to thank all the people, who accompanied and supported me in my journey that started with the Erasmus exchange in 2014, followed by my Master internship and finally my Ph.D. thesis.

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A Résumé détaillé

Le développement et le maintien des photorécepteurs sont principalement contrôlés par une combinaison de facteurs de transcription, nécessitant une régulation fine de leur activité selon les stades de développement ou le contexte environnemental. Outre la régulation du niveau de l'expression de ces facteurs, il existe une modulation fine de leur activité *via* des modifications post-traductionnelles (MPTs). Celles-ci ont la particularité de moduler rapidement la fonction des protéines dans la cellule. Ainsi, mon directeur de thèse et d'autres équipes ont montré par exemple que la SUMOylation de NRL et NR2E3, deux facteurs de transcription indispensables au développement des photorécepteurs, est nécessaire à leur fonction dans la différenciation des bâtonnets. La phosphorylation est un autre type de MPTs de première importance. Des expériences *in vitro* montrent que la phosphorylation de NRL est nécessaire à la régulation de l'expression de certains de ses gènes cibles comme la *Rhodopsine*, mais à ce jour la fonction des isoformes phosphorylées n'est pas connue *in vivo*. Cependant, et de manière intéressante, des mutations empêchant la phosphorylation de NRL conduisent à des rétinites pigmentaires chez l'homme. Plusieurs mutations conduisant à des rétinites pigmentaires chez l'homme ont été identifiées dans des gènes impliqués dans la régulation des MPTs ou bien altérant la capacité des protéines à être modifiées. Malgré l'importance de ces processus de régulation post-traductionnelle, les mécanismes sous-jacents et les conséquences fonctionnelles sont encore peu étudiés et en particulier *in vivo*. Dans ce contexte, mon projet de thèse se focalise sur des enzymes essentielles au développement et au fonctionnement du système nerveux, les Glycogène Synthase Kinases 3 (GSK3), qui modifient l'activité de leurs protéines cibles par phosphorylation. Ces kinases sont impliquées dans la régulation de nombreuses voies de signalisation et leur dérégulation a été observée dans de nombreuses pathologies neurodégénératives du système nerveux central. Ce lien potentiel entre les GSK3 et la neurodégénérescence, ainsi que l'importance des MPTs dans les photorécepteurs, nous a logiquement amené à nous interroger sur leurs rôles dans la survie des photorécepteurs.

L'objectif de ma thèse était d'étudier le rôle de ces kinases au cours du développement et de l'homéostasie rétinienne. En utilisant les souris invalidées de manière conditionnelle spécifiquement dans les progéniteurs rétiens (*Gsk3 α ^{fl/fl}Gsk3 β ^{fl/fl}; α -Cre*), j'ai montré que l'absence totale de *Gsk3 α* et de *Gsk3 β* très tôt au cours du développement rétinien entraîne une microphthalmie chez l'adulte, due à des défauts sévères de prolifération et de différenciation conduisant à la mort des progéniteurs. Ces données suggèrent que les kinases GSK3s sont essentielles au maintien des progéniteurs rétiens.

Les deux kinases jouent des rôles redondants puisque l'expression d'un seul allèle *Gsk3* est suffisante pour prévenir le phénotype de microphthalmie (*Gsk3α^{fl/+}Gsk3β^{fl/fl}; α-Cre* ou *Gsk3α^{fl/fl}Gsk3β^{fl/+}; α-Cre*). Cependant, une analyse phénotypique approfondie dans ce contexte génétique (un seul allèle *Gsk3*) a révélé une forte augmentation du nombre de cellules ganglionnaires déplacées (dRGCs) dans la couche nucléaire interne, qui apparaissent normalement en très faible nombre dans une rétine sauvage. Si la fonction de ces cellules reste encore à déterminer, j'ai pu montrer qu'elles sont produites en même temps que les autres cellules ganglionnaires. Le traçage des axones des cellules ganglionnaires par marquage antérograde a mis en évidence des projections dans un noyau ipsilatérale qui n'est pas visible chez les animaux sauvages. Dans l'ensemble, ces données suggèrent que les kinases GSK3s sont impliquées dans la genèse des dRGCs, dont les projections au cerveau sont particulières et dont la fonction est méconnue. Compte tenu du nombre important de dRGCs dans le modèle génétique que j'ai développé par rapport à la situation normale, ce modèle offre par conséquent un outil de choix pour étudier l'ontogenèse et la fonction de ces cellules.

Mes travaux de thèse se sont ensuite concentrés sur le rôle de GSK3 dans les photorécepteurs. En effet, des défauts de développement ou leur mort est l'une des principales causes de dégénérescence rétinienne. Afin de mieux comprendre la fonction de ces kinases dans la maintenance des photorécepteurs, j'ai utilisé des souris invalidées de manière conditionnelle pour *Gsk3α* et *Gsk3β* spécifiquement dans les précurseurs des photorécepteurs postmitotiques (*Gsk3α^{fl/fl}Gsk3β^{fl/fl}; Crx-Cre*). L'analyse phénotypique a révélé que l'absence de GSK3 dans les photorécepteurs conduit à une altération de leur maturation et de leur fonction dès 14 jours après la naissance (P14), suivie de leur dégénérescence progressive à partir de P18. Ces données suggèrent que les kinases GSK3 sont nécessaires à la différenciation et à la survie des photorécepteurs. J'ai alors combiné des analyses transcriptomiques et des approches *in vitro* et *in vivo* pour élucider les mécanismes sous-jacents. Les analyses d'expression des principaux facteurs de transcription spécifiques des photorécepteurs ont permis de mettre en évidence que l'expression de NRL (facteur de transcription nécessaire au développement des photorécepteurs de type bâtonnet) est inchangée au niveau transcriptionnel mais augmentée au niveau protéique. J'ai montré *in vitro* que GSK3 phosphoryle NRL et que cette phosphorylation régule sa stabilité en induisant sa dégradation rapide par le protéasome. Mes données m'ont donc conduit à proposer un modèle selon lequel l'absence de GSK3 dans les photorécepteurs conduit à des défauts de phosphorylation de NRL, augmentant sa stabilité. J'ai également montré que l'absence de GSK3 dans les photorécepteurs conduit à la diminution d'expression d'un sous-ensemble de gènes cibles

de *NRL*, co-régulés par *CRX*, et impliqués dans le développement et l'homéostasie des photorécepteurs. Mes travaux suggèrent donc que *GSK3*, *via* la régulation de la stabilité de *NRL*, joue un rôle essentiel dans le contrôle de l'expression de gènes nécessaires à la maturation et l'homéostasie des photorécepteurs. Une telle perte de régulation de *NRL* pourrait être à l'origine de la dégénérescence des photorécepteurs observée chez les patients atteints de rétinites pigmentaires et porteurs de mutations dans *NRL* connues pour bloquer sa phosphorylation.

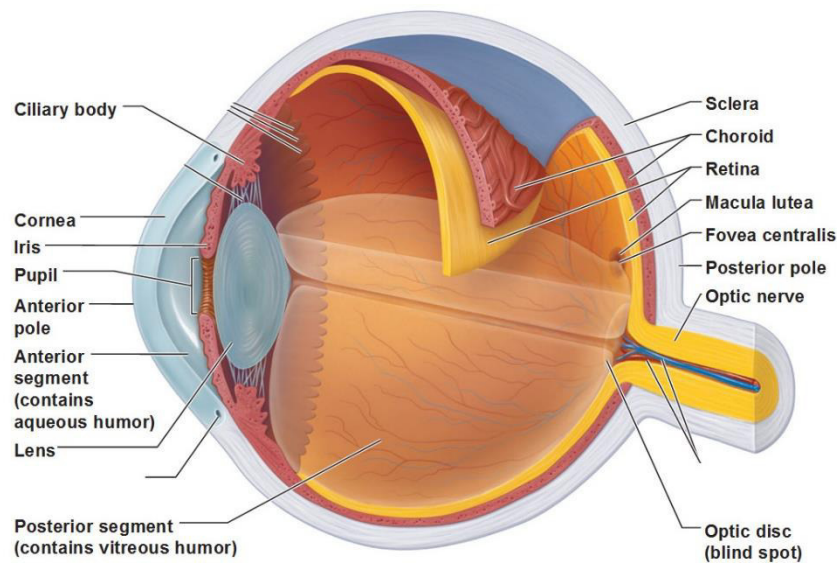


Figure 1. Anatomical organization of the human eye.

- The **cornea**, the most exterior part of the eye, is a protective barrier for the eye. It is mainly responsible for the refraction of the light. It is continuing with **sclera** in its posterior part.
- The second layer is the **uvea** that provides the eye with oxygen and nutrients. It contains the **iris** and the **ciliary body** in its anterior part and the **choroid** in its posterior part. The **iris** (*greek* rainbow) is a “pinhole” spanned around the pupil. It changes its diameter to regulate the amount of light reaching the retina. The ciliary body includes the ciliary muscle that controls the contraction of the lens and the ciliary epithelium that produces the aqueous humor.
- The **lens** plays a major role in the accommodation of the eye and changes its shape by contraction or relaxation of the ciliary muscles adjusting the refraction of light.
- The third layer at the back of the eye is the **retina**. The retina encompasses the **retinal pigment epithelium (RPE)** and the **neural retina**. The function of the retina is to receive the light and transform it into electrical signals that will be transmitted to the brain. These signals are then processed by brain areas dedicated to process vision information.
- Eye chambers embrace the anterior (between cornea and iris) and posterior (between iris, zonule fibers and lens) as well as the vitreous chamber (between the lens and the retina). Anterior and posterior eye chambers are filled with **aqueous humor** which flows from the posterior to the anterior eye chamber, whereas the **vitreous chamber** is filled with vitreous humor. The humor is able to refract the light and is therefore part of the optical system. Figure taken from www.eyephysicians.com.

B Introduction

B.1 Visual perception

The surface of our planet Earth is supplied with an abundance of light from the sun. Light is an essential source of energy, but also a major information carrier from our environment.

Vision is both a dominant and among the most valuable of our five senses, functioning primarily as a light perception system in animals. It allows us to integrate and process information from our environment, although we can only see “what the mind is prepared to comprehend”.

B.1.1 Anatomy of the eye

The eye is the organ that is responsible for mediating vision, which can be viewed as a connection between the brain and the outside world. It captures visual information and processes it before it is transferred via the optic nerve to the corresponding brain regions and transformed into an image. The eye is composed of three layers, the cornea, the uvea, and the retina, along with the lens and three chambers (Figure 1). This principal arrangement is similar between mice and humans (Figure 1). In the presented thesis manuscript, I will concentrate on the **retina**, the essential layer that lies at the back of the eye and is responsible for light capture, visual transduction, and processing. For a better understanding of the background and presented results, I will start with a description of the retina including the different retinal cells, with a focus on photoreceptors.

B.1.2 Organization of the retina

The **retina** is a multilayered tissue in the innermost part of the eye and encompasses the **retinal pigment epithelium (RPE)** and the **neural retina**.

The **RPE** consists of a monolayer of pigmented epithelial cells with photoreceptors at their apical side and the choroid at their basal side. Due to its melanin synthesis and light absorption, along with its absorption of lipofuscin and photooxidation products, it has a protective function (Strauss, 2005) (Sakina et al., 2013). Furthermore, it establishes tight junctions to contribute to the formation of blood-retinal barrier and secretes immunosuppressive factors such as pro- or anti-inflammatory cytokines and growth factors (Strauss, 2005), both of which are crucial for the immune privilege of the eye. The RPE helps maintain photoreceptor function: It contributes to selective exchange of ions, water and metabolites between the blood vessels of the choroid and the photoreceptors, thus “nourishing” photoreceptors.

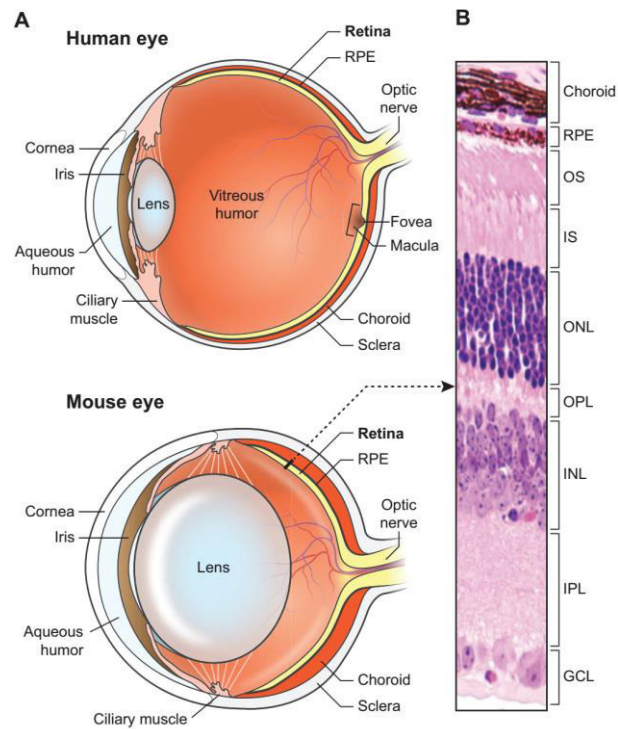


Figure 2. Structure of the human and the mouse eye and the neural retina.

(A) Scheme of the human and mouse eye. Light is focused by optical elements at the neural retina at the back of the eye. In contrast to the mouse retina, the human retina possesses the macula and the fovea. Relative to the eye size, the lens is much larger in mouse than in the human eye. **(B)** Hematoxylin and eosin photograph of a mouse retinal section indicating the laminar organization of the retina. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Figure reproduced from (Veleri et al., 2015).

It also sheds and phagocytizes outer segments (OS) of photoreceptors. In fact, around 10 % of OS discs are renewed at the distal end and added to the proximal end by disc shedding whereby a complete renewal of OS occurs in 10 - 15 days (Levine and Green, 2004). The RPE also plays a critical role in the visual cycle by recycling photopigment molecules of photoreceptors after they have been exposed to light. Due to the importance of the RPE for maintenance of photoreceptors, loss of any one of these functions can lead to retinal degeneration, which is associated with vision loss (Strauss, 2005).

The **neural retina** has a laminar structure comprising seven different types of neurons whose cell bodies are organized into three nuclear layers (**Figure 2B**).

Laminar organization of the neural retina

- The **outer nuclear layer (ONL)** incorporates photoreceptor cell bodies.
- The **outer plexiform layer (OPL)** encloses synaptic connections established between photoreceptors, bipolar and horizontal cells.
- The **inner nuclear layer (INL)** is composed of the bipolar, horizontal, amacrine and Müller glial cell bodies. Notably, close to the inner plexiform layer, a part of the INL displays nuclei with a larger diameter than the other INL. These cell types have been identified as displaced ganglion cells (Bunt et al., 1974) (Dräger and Olsen, 1980).
- The **inner plexiform layer (IPL)** encompasses synaptic connections established between bipolar, ganglion and amacrine cells. The IPL is much thicker than the OPL.
- The **ganglion cell layer (GCL)** includes ganglion cells and displaced amacrine cells.

B.1.2.1 Cellular organization of the neural retina

In the following section I will describe the different cell types present in the retina (**Figure 3**), with a particular focus on retinal photoreceptors, the input cells, as well as ganglion cells, the output cells of the retina. These cell types were the focus of my Ph.D. project.

B.1.2.1.1 Retinal photoreceptors

➤ **Photoreceptor subtypes**

Photoreceptors, the main cells for vision and most abundant in the mammalian retina, can be divided into two main subtypes, rods and cones. They each have distinct features in terms of shape, distribution, the type of photopigment they contain, and their pattern of synaptic connections.

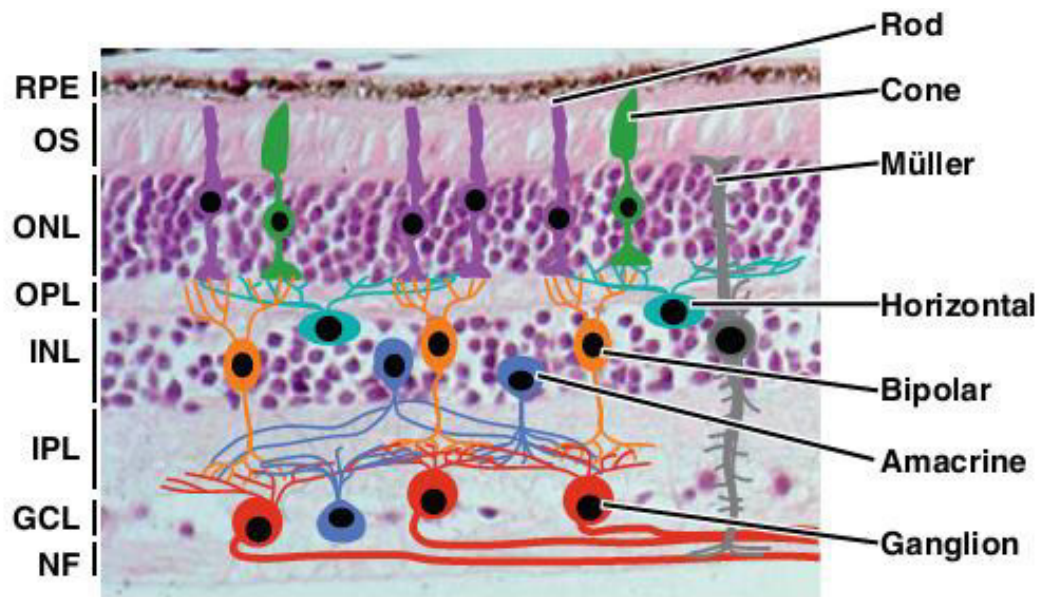


Figure 3. Schematic representation of the cellular organization in the neural retina.

Synaptic connections are established in the outer plexiform layer (OPL) and in the inner plexiform layer (IPL). RPE, Retinal pigment epithelium OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; NF, optic nerve fiber. Figure reproduced from (Bassett and Wallace, 2012).

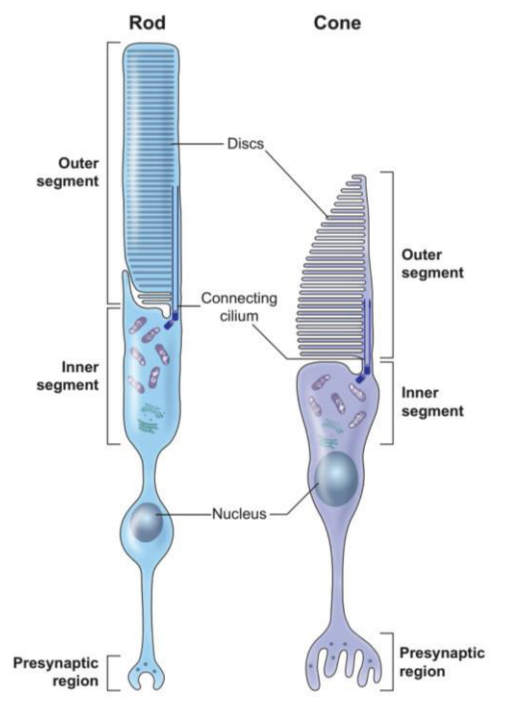


Figure 4. Schematic representation of the photoreceptor compartments.

Rod and cone photoreceptors have a distinct compartmentalized morphology, but both contain the same structure. Proteins are transported from the inner to the outer segment *via* the connecting cilium by intraflagellar transport. Figure taken from (Veleri et al., 2015).

- **Rod photoreceptors** are for scotopic vision under dim light conditions due to their high light sensitivity, but have low spatial resolution as well as a slow response and adaptation (Sato et al., 2015).
- **Cone photoreceptors** allow color vision and visual acuity under daylight conditions to distinguish features of visual environment at the expense of sensitivity (Sato et al., 2015).

➤ **Photoreceptor structure**

To sustain the high level of energy required for visual function, photoreceptors are highly differentiated and polarized neurons with a very high metabolic rate. They are composed of five structural and functional regions (Figure 4):

- The **outer segment (OS)** discs contact the RPE. They are rich in lipids and contain components of the phototransduction system. The OS structure differs between rods and cones. Rod OS have a stack of individualized coin-like membrane discs, whereas cone OS have stacked membranes resulting from continuous invagination of the cell membrane. The OS of both rods and cones consists of hundreds of stacked membranes discs carrying phototransduction proteins including opsins, the visual pigment that defines the photoreceptor subtype. Opsins are G-protein-coupled receptors. They are bound to the light-sensitive chromophore, 11-*cis*-retinal, that isomerizes to all-*trans* retinal upon photon capture, initiating the process of phototransduction, a cascade of biochemical events culminating in photoreceptor hyperpolarization and signal transmission. The well-organized OS is however devoid of any protein translation machinery and depends on the inner segment (IS) to synthesize the numerous components that are required for proper phototransduction in the OS.
- The **IS** are rich in cellular organelles necessary for protein synthesis, energy production and metabolism. In fact, the IS contains the endoplasmic reticulum, the Golgi apparatus and the mitochondria required for glycolysis, oxidative phosphorylation as well as ATP buffering.
- The **cilium** ensures intraflagellar transport (IFT), a high flow of membranes and proteins that have been synthesized in the IS for their utilization in the OS and is therefore crucial for the survival of photoreceptors. IFT is a bidirectional motility along axonemal microtubules.
- The **nucleus** is the control center localized in the ONL and surrounded by a very restrained cytoplasm. The different types of photoreceptors show specific chromatin

Rod nuclei are denser and have one large clump of heterochromatin, whereas cone nuclei display an irregularly shaped heterochromatin of one to three clumps (Carter-Dawson and LaVail, 1979). To improve light propagation through the retina and night vision, the rod chromatin in mice and other nocturnal mammals is inverted compared to conventional nuclei. Indeed, rod nuclei have heterochromatin centrally and euchromatin peripherally (Solovei et al., 2009).

- The **axonal terminals** develop a specialized structure called ribbon synapses. A synaptic ribbon is an electron-dense horseshoe-like organelle in the photoreceptor presynaptic cytoplasm and can be observed by electron microscopy. Axon terminals establish synaptic connections in the IPL with second order neurons, bipolar and horizontal cells to which they transmit signals in a graded fashion (tom Dieck and Brandstatter, 2006). Hereby, photoreceptors process large pools of fast-release vesicles tethered to the synaptic ribbons (Sterling and Matthews, 2005). Interestingly, rod photoreceptor terminals contain a single ribbon that has a horseshoe-shaped structure, called spherules (Rao-Mirotznik et al., 1995), whereas cone photoreceptor terminals are larger and contain multiple smaller synaptic ribbons in a single cone photoreceptor axonal terminal, called pedicles.

- **Photoreceptor distribution and visual pigments organization in the retina**

In mammals, photoreceptors account in total for more than 70 % of retinal cells. The distribution or relative density of rods and cones within the retina is adapted to the “lifestyle” of species (Peichl, 2005). The density ratio of cones to rods ranges from 1:200 in the most nocturnal to 20:1 in a few diurnal species. The diurnal to nocturnal repartition of rods versus cones is not the only segregation criteria, as nocturnal rodents, such as mice, possess 97 % of rods and diurnal humans possess 85 % of rods (Jeon et al., 1998) (Swaroop et al., 2010).

Visual pigments are transmembrane proteins, G protein-coupled receptors localized in the OS of photoreceptors. Opsin is composed of a protein moiety with seven α -helices and its ligand, the aldehyde form of vitamin A, referred to as chromophore retinal. Light rays must cross through non-light sensitive elements of the retina including the retinal vasculature and all retinal layers to reach visual pigments of photoreceptors. The latter are in charge of converting electromagnetic radiation of light photons into neuronal signals and stimulating all the machinery to conduct phototransduction. The vertebrate retina has only one type of rod photoreceptor, carrying the rhodopsin visual pigment with a maximum absorption wavelength of 500 nm.

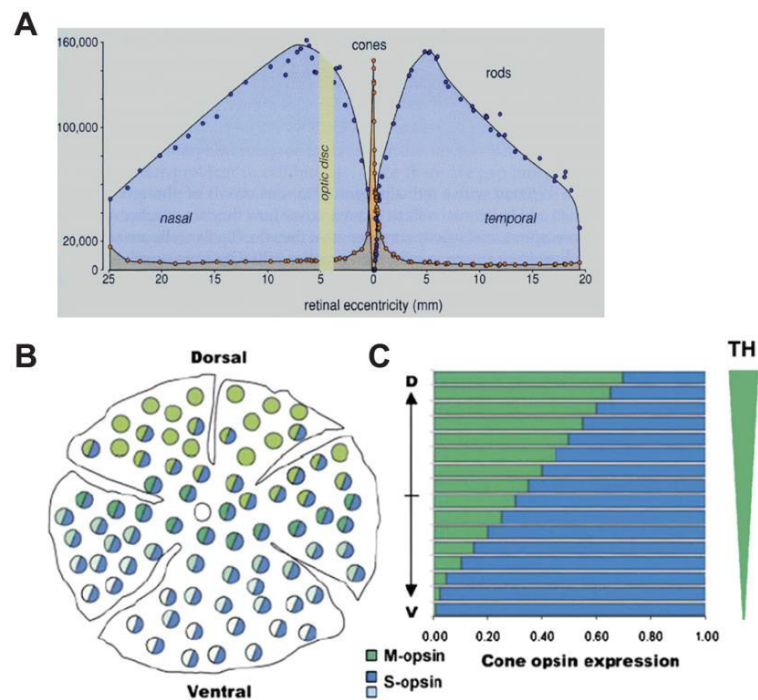


Figure 5. Spatial photoreceptor distribution and opsin expression in human and mouse retina.

(A) In human retina rods (blue) predominate cones (orange) except for the central region, corresponding to the macula. The fovea, adjacent to the optic disc, marks position 0. (B) In mouse retina, M-cones (green) predominate in the dorsal retina and S-cones (blue) in the ventral retina. (C) M- and S-opsin expression follow opposite gradients, established by the action of the thyroid hormone (TH). Figure reproduced from (Hennig et al., 2008).

The type of cones varies between species due to the spectral sensitivity of different opsins. Most birds, fish, some insects, and reptiles are at least **tetrachromatic**, with at least four types of cones (Goldsmith, 2006) (Hofmann and Carleton, 2009) (Scholtyssek and Kelber, 2017). The fourth cone photoreceptor allows these animals to perceive UV light. (Hisatomi et al., 1996). Humans and Old-World monkeys are **trichromats** with three distinguishable opsins: L- (long, 564 nm), M- (medium, 533nm) and S- (short, 437 nm) wavelength cones to discriminate between red, green and blue colors, respectively (Hunt et al., 1998) (Lucas et al., 2003). New World monkeys and most mammals, including rodents are **dichromats** and possess two different cone types: M/L- and S- cones, with the exception of howler monkeys that are uniformly trichromatic (Kainz et al., 1998).

Both Humans and Old-World monkeys, but also New-World monkeys possess a central retinal region, the **macula lutea** (Hunt et al., 1998). In the center of the macula, cones are concentrated in a cone-enriched area, containing in its center a thin, pit-like, cone-only region, the **fovea centralis**, the region conferring the highest visual acuity (**Figure 2A**) (**Figure 5A**). Rods are excluded from this region and evenly distributed predominating cones elsewhere in the retina (**Figure 5B**).

In rodents, cones account for 3 % of retinal neurons. Diurnal nil grass rats which possess a cone-rich retina with 35 - 40 % of cones and a higher spatial activity than usual laboratory rodents, are an exception (Gaillard et al., 2008). In contrast to primates, the cone distribution generally differs between rodents. Mice do not possess a cone-rich area, the macula lutea, but their two cone subtypes are organized following inverse dorso-ventral gradients in the retina. Most cones co-express both S- and M-opsins throughout the retina, however S-cones predominate in the ventral retina, and M-cones are denser in the dorsal area (**Figure 5B**) (Applebury et al., 2000) (Lukats et al., 2005).

B.1.2.1.2 Neurons of the inner nuclear layer

- **Bipolar cells** receive signals from rods (thus named rod bipolar cells) or cones (thus named cone bipolar cells) and transfer them to cells in the inner retina or directly to the RGCs *via* their synaptic ribbon terminals (Euler and Schubert, 2015). They comprise at least 13 different subtypes and are excitatory neurons which use glutamate as a neurotransmitter (Euler and Wässle, 1995) (Helmstaedter et al., 2013). The signal from bipolar cells is modulated by other interneurons comprising amacrine and horizontal cells (Diamond, 2017) before being processed by ganglion cells whose axons bundle to form the optic nerve for transmission to the brain.

Horizontal cells regulate signal transmission from photoreceptors to bipolar cells by releasing the inhibitory neurotransmitter γ -aminobutyric acid (GABA). This mechanism is known as dendritic lateral inhibition, and ensures a better contrast (Kramer and Davenport, 2015).

Amacrine cells constitute the most diversified retinal cell class with at least 28 subtypes. These subtypes can be classified into two subgroups depending on the neurotransmitter they release: GABAergic or Glycinergic (Wassle et al., 1998) (Kolb et al., 2002). The majority of amacrine cells are located in the proximal part of the INL, and displaced amacrine cells, which encompass 10 different subtypes, are found in the GCL (Galvez et al., 1977) (Perry and Walker, 1980) (Perez De Sevilla Muller et al., 2007).

In contrast to dendritic lateral inhibition of horizontal cells, amacrine cells are responsible for lateral inhibition at axon terminals (Kolb et al., 2002).

Retinal ganglion cells (RGCs) convey visual information from the neural retina to the brain. Their axons form the optic nerve to transfer signals to the visual cortex in the brain. RGCs have soma with a larger diameter and larger axons than most preceding neurons. More than 20 different subtypes have been identified which differ morphologically in their soma size, dendritic field size, dendritic ramification and projection level. A recent study revealed even more subgroups of RGCs, at least 32, distinguished based on their light responses and basic anatomical criteria (Baden et al., 2016). To implement binocular vision, RGCs project both to the ipsilateral and contralateral sides of the brain in mammals. In humans, the 45 % of RGCs that are ipsilateral ensure efficient binocular vision. In contrast, mice have very poor binocular vision because of the low amount of ipsilateral-projecting RGCs (3 %) residing exclusively in the ventrotemporal (VT) retina, whereas contralateral RGCs are distributed across the entire retina. Interestingly, the retina also contains **displaced retinal ganglion cells (dRGC)** located in the INL. These cells are very rare, representing only 2 % of RGCs in the retina (Buhl and Dann, 1988) (Nadal-Nicolas et al., 2014). It is believed that dRGCs are misplaced due to an ontogenic aberration rather than representing an independent class of RGCs (Doi et al., 1994) (Buhl and Dann, 1988). However, their exact origin, time of production, projection to the brain, and function have never been described in mammals in the literature due to the absence of suitable models.

B.1.2.1.3 Glial cells

Microglia are the principal resident immune cells of the central nervous system with macrophagic function. Under physiologic conditions, microglia are required for the maintenance of synaptic structure (Wang et al., 2016). Under pathological conditions, such as in glaucoma, microglia become activated and redistributed within the retina (Neufeld, 1999). **Müller cells** represent the main glial cells in the mammalian retina. They are the only glial cells that share a common embryonic origin with retinal neurons (Turner and Cepko, 1987) (Holt et al., 1988). In response to stress, injury or in retinal diseases, Müller cells and microglia become activated (de Hoz et al., 2016).

B.2 Retinal dystrophies and available treatments

B.2.1 Photoreceptor dystrophies

Retinal neurodegenerative diseases are mainly due to progressive dysfunction of photoreceptors, such as their inability to detect or transmit light to the brain. Photoreceptor degenerative diseases are genetically and clinically heterogeneous and constitute the major cause of blindness. The onset of degeneration can directly affect photoreceptor function and can originate from the RPE.

Retinal degenerative diseases comprise two main forms:

- monogenic (inherited, Mendelian) retinal diseases, such as Retinitis Pigmentosa
- multifactorial (complex) diseases such as Age-related Macular degeneration (AMD)

Monogenic retinal diseases can be in turn subdivided into non-syndromic types including Retinitis pigmentosa, Leber Congenital Amaurosis (LCA) and Macular Degeneration or syndromic diseases. The latter are characterized by pleiotropic clinical symptoms and affect multiple organs or functions. Examples are the Bardet-Biedl syndrome (affecting retinal function and many other functions) or the Usher Syndrome (affecting retinal and auditory function) (Berger et al., 2010) (Veleri et al., 2015). Up to date, around 300 genes have been mapped and 250 have been identified to cause retinal diseases (Figure 6). They can alter photoreceptor and/or RPE development and key functions such as the visual cycle, phototransduction, gene regulation or transport (Wright et al., 2010).

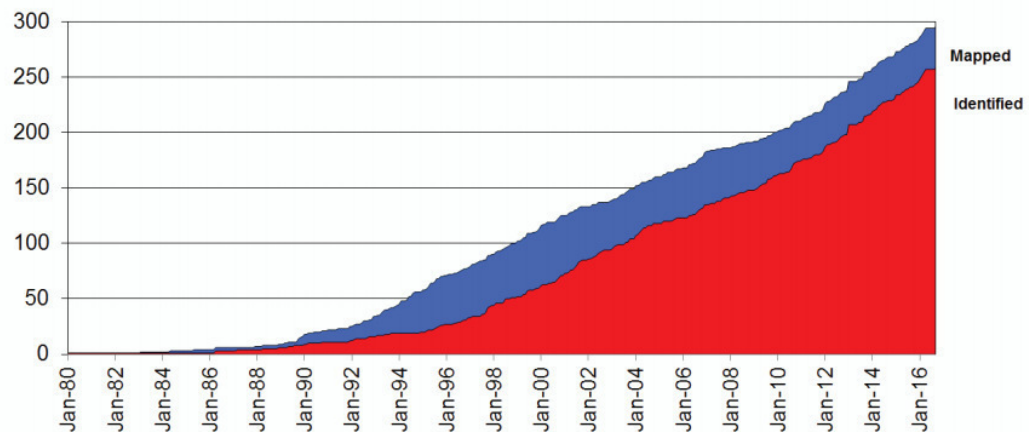


Figure 6. Mapped and identified Retinal Disease Genes between 1980 and 2017.

Progress in the discovery of causal genes for retinal degenerative diseases. In 1996, 19 genes were identified to cause retinal degenerative diseases, in 2017 the gene number reached 256. Figure reproduced from <https://sph.uth.edu/retnet/sum-dis.htm>.

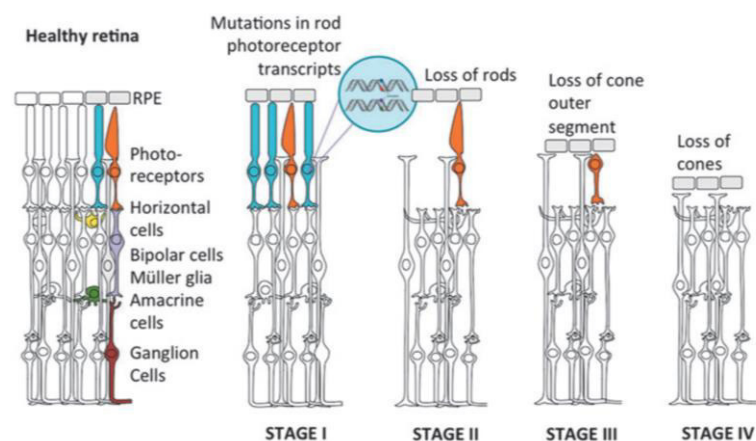


Figure 7. Retinitis pigmentosa as an example of retinal degeneration progression.

Representation of the progressive loss of the photoreceptors occurring in a retina with *retinitis pigmentosa*. Mutations mostly concern rod photoreceptors (blue) leading to a primary loss of rods (Stage I), followed by progressive cone death (orange, Stages II, III and IV). Figure taken from (Dalkara et al., 2016).

Retinitis Pigmentosa, the most common subtype of inherited photoreceptor degeneration, has a prevalence of 1:3000 to 1:7000 people. There is a large genetic heterogeneity with over 50 genes for which mutations have been identified to cause the disease (Daiger et al., 2013), with autosomal dominant, recessive or X-linked inheritance (Wright et al., 2010). Some genes are mutated very frequently such as *Rhodopsin* mutations, which occur in 30 % of all cases (al-Magthteh et al., 1993). The pathology is often characterized by an initial loss of night vision due to the loss of the rod photoreceptors. During disease progression, secondary loss of cone photoreceptors occurs as well, even when the mutation is in a rod-specific expressed gene (**Figure 7**) (Narayan et al., 2016). Consequently, the daylight vision also becomes affected and the visual field progressively decreases from the periphery to the center, resulting in tunnel vision and ultimately in complete blindness (Nash et al., 2015).

A specific form of Retinitis Pigmentosa is the **Cone-rod dystrophy** which concerns 1:40000 patients. This form is characterized by an initial loss of cone photoreceptors followed by, or with a concomitant, loss of rod photoreceptors.

Leber Congenital Amaurosis (LCA) and early-onset severe retinal dystrophy (EOSRD) are the most severe congenital or early-onset diseases. LCA patients present clinical features such as involuntary eye movements, called nystagmus, photophobia, abnormal pupillary response and undetectable or severely abnormal electroretinogram. LCA has prevalence between 1:33,000 to 1:80,000 and accounts for more than 5 % of all inherited retinal degenerations. For instance, mutations in the major ciliary component gene nephrocystin-6 *NPHP6*, also known as *CEP290* are responsible for up to 25 % of Leber Congenital Amaurosis forms and mutations in the *RPE65* gene, encoding the enzyme in charge of chromophore recycling, occurs in around 15 % of cases (Morimura et al., 1998) (den Hollander et al., 2008) (Kumaran et al., 2017).

Macular Degeneration is characterized by a loss of the central vision caused by the progressive death of cone photoreceptors located in the central, cone-only macula, mainly caused by the loss of RPE cells. It can be inherited in a Mendelian manner, and mutations in several genes are associated with the disease (Stone, 2007). The most common is Stargardt disease with a prevalence of 1:10000 patients and mostly caused by mutations in the *ABCA4* gene (Allikmets et al., 1997). The *ABCA4* gene encodes the cone and rod photoreceptor Rim protein which is a transmembrane transporter of vitamin A intermediates. *ABCA4* malfunction leads to the accumulation of yellow-brown pigment granules composed of lipid-containing residues of lysosomal digestion in and around the macula. The retina of Stargardt patients displays cholesterol deposits from unshed photoreceptor OS in the macular region (Molday and Zhang, 2010).

Macular Degeneration can also manifest as a complex, multifactorial late-onset disease, then termed **Age-related Macular Degeneration (AMD)**. AMD is the major cause of blindness in industrial countries, with a prevalence of 25 to 30 million people worldwide, and an incidence of 500,000 patients a year. As such, twice as many patients are suffering from AMD than from Alzheimer's disease (Fletcher et al., 2014). AMD is characterized by formation of lipid- and protein- rich extracellular deposits named drusen beneath the macula (Wang et al., 2010) (Zarubina et al., 2016) (Neely et al., 2017), inflammation with macrophage and microglia infiltration accompanied by complement activation (Parmeggiani et al., 2012) and ultimately degeneration in the macula.

Importantly, since the pioneering discovery of a common polymorphism in the complement factor H gene (Klein et al., 2005), new genetic AMD-associated risk variants are steadily being identified with a total of 20 susceptibility loci found. Together they account for 40 - 60 % of the disease heritability (Zhan et al., 2013) (Fritsche et al., 2014).

In addition to genetics, environmental risk factors such as age, cigarette smoking, blood hypertension, high lipid levels, obesity, and low physical activity contribute to the disease progression. Therefore, AMD patients uniformly suffer from loss of cone photoreceptors in the central macula area, but can exhibit phenotypic heterogeneity (Fritsche et al., 2014). At advanced stages, the central vision is impaired because of the cone loss impairing the visual field.

There are two types of late AMD:

- The wet or exudative AMD (choroidal neovascularization) affects a minority of AMD patients. In wet AMD, abnormal blood vessels grow underneath the retina in the macula zone. The blood vessels leak and eventually damage the retina resulting in vision loss.
- The dry or atrophic AMD affects 80 - 90 % of AMD patients, which is, unlike wet AMD, a slowly progressing AMD form leading in its late stage to so-called geographic atrophy, typically defined as a round or oval area of atrophy of 175 μm or more (Sacconi et al., 2017).

B.2.2 Available treatments

In total, blinding diseases affect a steadily increasing patient population, currently 34 million people worldwide. Therefore, effective treatment strategies are constantly being developed. They include cell replacement, antibody therapy, gene therapy, neuroprotection, optogenetics and retinal prosthesis (Santos-Ferreira et al., 2016). The most common therapy forms are briefly presented here (Figure 8):

- **Antibody-based therapy** against vascular endothelial growth factor (VEGF) is widely used to slow down the wet neovascular form of AMD (Krzystolik et al., 2002). Conversely, no existing therapy has been approved for geographic (dry) atrophy (Bandello et al., 2017).
- **Cell-based therapy** consists of replacing the dysfunctional or degenerated retinal cells, primarily RPE and photoreceptor cells using stem cells. The advantage of this therapeutic approach is its disease independence. Encouraging results have been obtained by transplanting human embryonic stem cell-derived (hESC) RPE and induced pluripotent stem cells (iPSC) RPE (Nazari et al., 2015) (Schwartz et al., 2016). Clinical trials in phase I/II have been initiated to transplant hESC-derived RPE showing medium-term to long-term safety in patients with AMD and Stargardt disease (Schwartz et al., 2015). iPSC-derived RPE has also been transplanted into AMD patients in 2014 and their safety seemed to be confirmed (Takahashi, 2016). However, a serious adverse event in a clinical trial participant, who received iPSC-based therapy, was reported in January 2018. This patient developed retinal edema requiring hospitalization for treatment. A recent study in rats with retinal degeneration demonstrated a better effect in terms of photoreceptor rescue and visual acuity when hESC-RPE cell sheets were grown on a human amniotic membrane compared to hESC-RPE delivered via cell suspension (Ben M'Barek et al., 2017). These findings could influence the production and delivery of hESC-RPE in future clinical trials. One of the special cases of cell therapy is the use of the **neurogenic potential of endogenous cells** located in the RPE, the ciliary body or the Müller cells to regenerate degenerative cells. However, such neurogenic potential and the stem cell properties are extremely limited and inefficient in mammals, in contrast to fish, *Xenopus*, and other non-mammalian vertebrates (Jayakody et al., 2015) (Hamon et al., 2016).

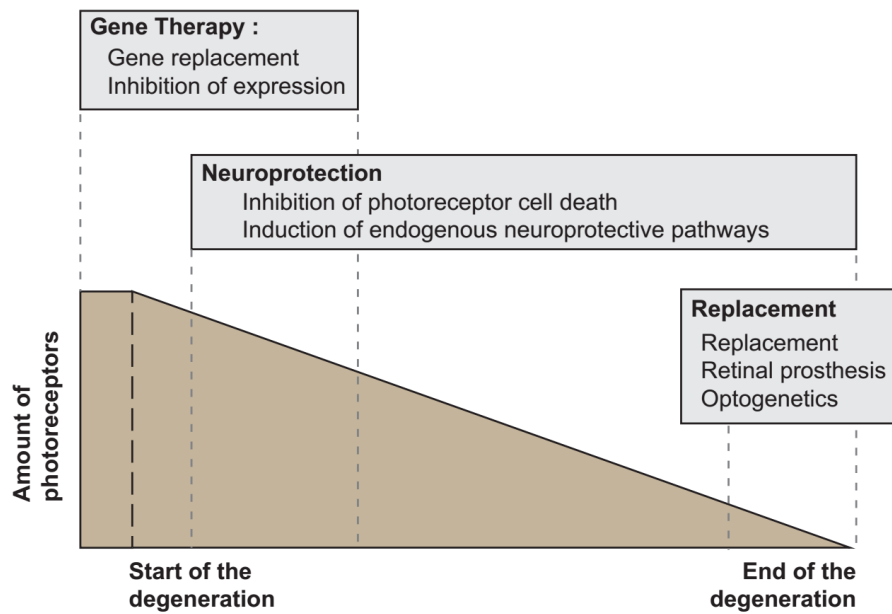


Figure 8. Main therapeutic approaches to treat retinal dystrophies.

Gene therapy has to be used at early stage of the degeneration since it requires viable photoreceptors. Conversely, retinal prosthesis and optogenetics aim at restore vision at later stage when most or all photoreceptors are lost. Neuroprotection has a broader application efficiency window. Figure adapted after (Trifunovic et al., 2012).

- **Gene therapy** uses viral vectors, including in most cases the adeno-associated virus (AAV) vectors, to enable delivery of a functional copy of a gene that is mutated in patients. Most frequently, gene therapy is applied *via* subretinal injections as most inherited retinal degenerations are caused by mutations in photoreceptors. Cells need to be alive for this approach and therefore an early diagnosis is required (Jayakody et al., 2015) (Dalkara et al., 2016). This approach has causal treatment effect, but, success has been limited to few patients due to the large genetic heterogeneity of retinal disorders. Hence, it cannot be applied to a large number of patients. The first gene therapy started with clinical trials in 2007 with the replacement of the RPE specific protein RPE65 to treat LCA, that accounts for 5 - 10 % of all LCA cases, and was a big success in subsequent clinical trials (Jacobson et al., 2006) (Maguire et al., 2008) (Amado et al., 2010) (Bennett et al., 2016) (Le Meur et al., 2018). RPE65 gene therapy has just been approved in December 2017 by the U.S. Food and Drug Administration under the name of Luxturna™ to treat patients with confirmed biallelic *RPE65* mutation. While the price of the treatment has been announced at \$425,000 per eye, the scientific breakthrough is undeniable.
- **Neuroprotection** aims to administer pro-survival, anti-apoptotic and anti-inflammatory factors to stop or delay photoreceptor cell death. It can also be achieved by stimulating pro-survival pathways upregulated in degenerative retina but whose endogenous potential is not sufficient to rescue photoreceptors. Structural and functional rescue of retinal cells has been demonstrated for neurotrophic factors such as ciliary neurotrophic factor (CNTF), pigment epithelium-derived factor (PEDF) and glial cell line-derived neurotrophic factor (GDNF). In contrast to gene therapy and similar to cell-based therapy, neuroprotective approaches are mutation independent and can be applied to treat a wide range of retinal degenerative diseases. A pioneering clinical trial to deliver CNTF using encapsulated cell implants is currently being pursued in phase III (Tao et al., 2002) (Sieving et al., 2006). This promising approach may have applications beyond diseases caused by genetic mutations. In addition, it avoids secondary effects due to repetitive intraocular injections. Gene therapy approaches aiming to deliver neurotrophic factors using viral vectors have similar advantages (Cuenca et al., 2014) (Dalkara et al., 2016). A prominent candidate is the rod-derived cone viability factor (RdCVF). It has been administered in the form of AAV-RdCVF and prolonged cone survival and function in Retinitis Pigmentosa mice (Byrne et al., 2015).

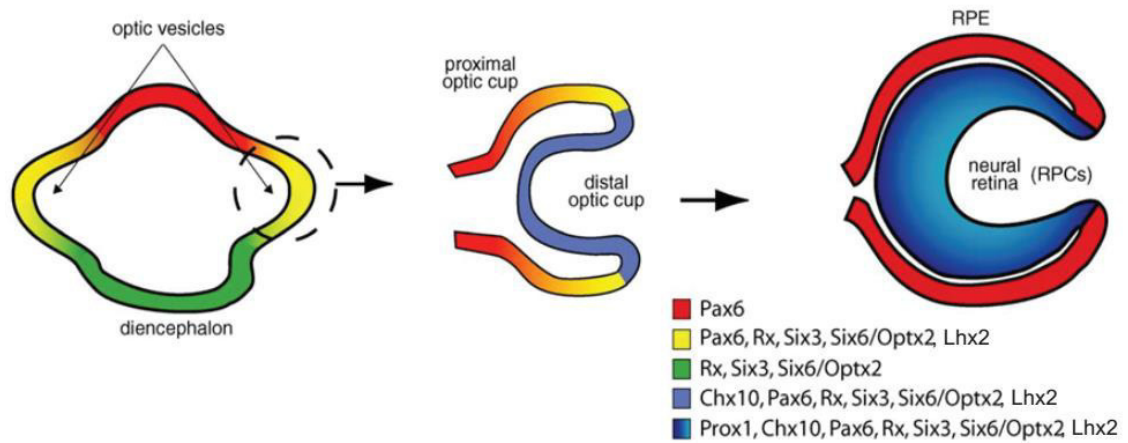


Figure 9. Eye and retina specification involving homeobox gene expression.

The eye field separates bi-laterally from the diencephalon to form the double-layered optic cup: The distal optic cup gives rise to the neural retina and proximal optic cup to the RPE. All transcription factors with the exception of *Chx10* and *Prox1* are broadly expressed before being restricted to RPCs. *Pax6* is the only transcription factor expressed both in RPCs and in the RPE. Figure adapted after (Levine and Green, 2004).

Interestingly, additional drug candidates can originate from drug libraries conceived for other indications. For example, olaparib, commonly used for cancer treatment, has shown promising neuroprotective effects in models of retinitis pigmentosa by inhibiting the activity of poly-ADP ribose polymerase (PARP) (Sahaboglu et al., 2016) (Jiao et al., 2016).

- **Optogenetics** and **prosthetic devices** constitute an approach to treat severe photoreceptor degeneration with almost no functional cells remaining. For optogenetics, this is achieved *via* genetically encoded light sensors to photosensibilize retinal cells by expressing one or several genes such as *channelrhodopsin* from the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2002). Promising results have been obtained in neurodegenerative mouse models (Cuenca et al., 2014). As for optogenetics, prosthetic devices (epiretinal or subretinal **implants**) use an artificial photosensor to capture light. The current is used to stimulate retinal cells within the remaining retinal circuit (Mills et al., 2017).

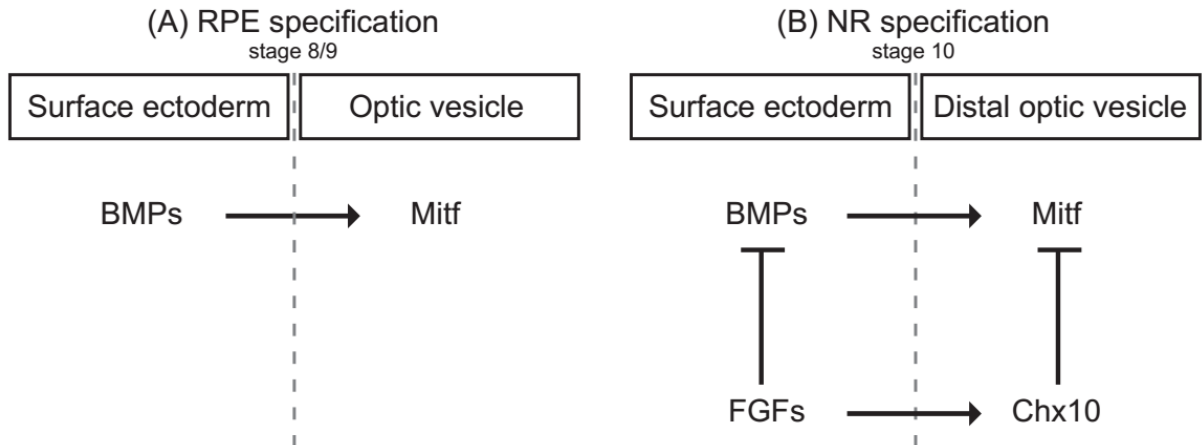


Figure 10. Proposed model for the regulation of the RPE and NR specification.

(A) To achieve RPE specification, BMP induces *Mitf* expression in the underlying optic vesicle. (B) NR specification is initiated by FGF induction of *Chx10* in the distal portion of the optic vesicle that inhibits *Mitf* expression. RPE: retinal pigment epithelium; NR: neural retina. Figure adapted after (Muller et al., 2007).

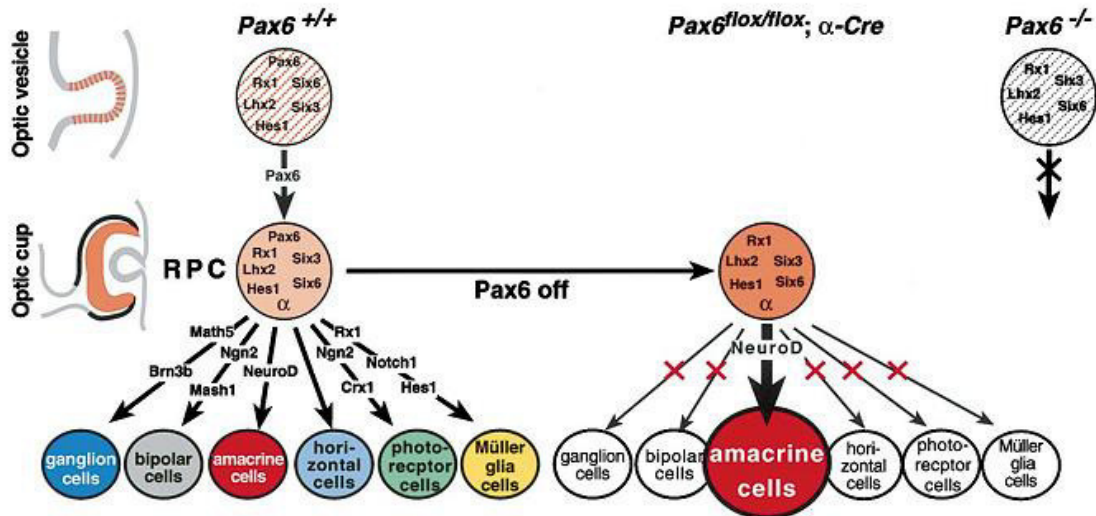


Figure 11. RPCs require Pax6 to exert their retinogenic potential.

Pax6 is required for the expression of different cell type specific transcription factors and generation of different retinal cell types (left). Upon conditional deletion of *Pax6* in RPCs, only *NeuroD* is expressed, leading to an exclusive generation of amacrine cells (middle). Homozygous *Pax6* loss-of-function (*Pax6*^{-/-}) results in anophthalmia (right). Figure reproduced from (Marquardt et al., 2001).

B.3 Development of the vertebrate retina

B.3.1 Retinal specification

Eye formation is initiated after the onset of gastrulation. The eye identity, named “eye field”, is formed from the neuroepithelium within the midline of the anterior neural plate. The eye field separates bi-laterally to produce the **optic primordia** that evaginate to form the **optic vesicles (diencephalic evagination)**. They extend toward the surface ectoderm where the lens placode develops as well as the primordia of the anterior structures of the eye, such as the cornea. At the same time, the optic vesicles invaginate to form the double-layered **optic cup** (Figure 9). The inner layer of the optic cup gives rise to the **neural retina** and the non-pigmented region of the ciliary body, while the outer layer becomes pigmented and forms the **RPE** as well as the pigmented region of the ciliary body (pigmented neuroepithelium) (Chow and Lang, 2001) (Sinn and Wittbrodt, 2013).

Specific transcription factors are decisive in neural retina *versus* RPE specification (Figure 10). Microphthalmia-associated transcription factor (**MITF**) triggers gene expression that is important for RPE cell differentiation (Mochii et al., 1998). MITF is itself induced by the bone morphogenetic protein (**BMP**) (Steinfeld et al., 2017). Another gene important for the RPE melanogenesis is **Otx2** (Figure 10) (Martinez-Morales et al., 2003) (Beby and Lamonerie, 2013) (Fuhrmann et al., 2014).

In contrast, fibroblast growth factor (**FGF**) from the surface ectoderm is decisive for the formation of the neural retina and represses MITF (Pittack et al., 1997) (Nguyen and Arnheiter, 2000). This inhibition is mediated by the expression of the paired-like homeodomain transcription factor **Chx10 (Vsx2)** (Figure 10) (Horsford et al., 2005).

Interestingly, the homeodomain transcription factor Pax6 holds a dual role in retina *versus* RPE specification. In combination with MITF it acts as an anti-retinogenic, whereas in combination with retinogenic genes as a pro-retinogenic factor (Bharti et al., 2012).

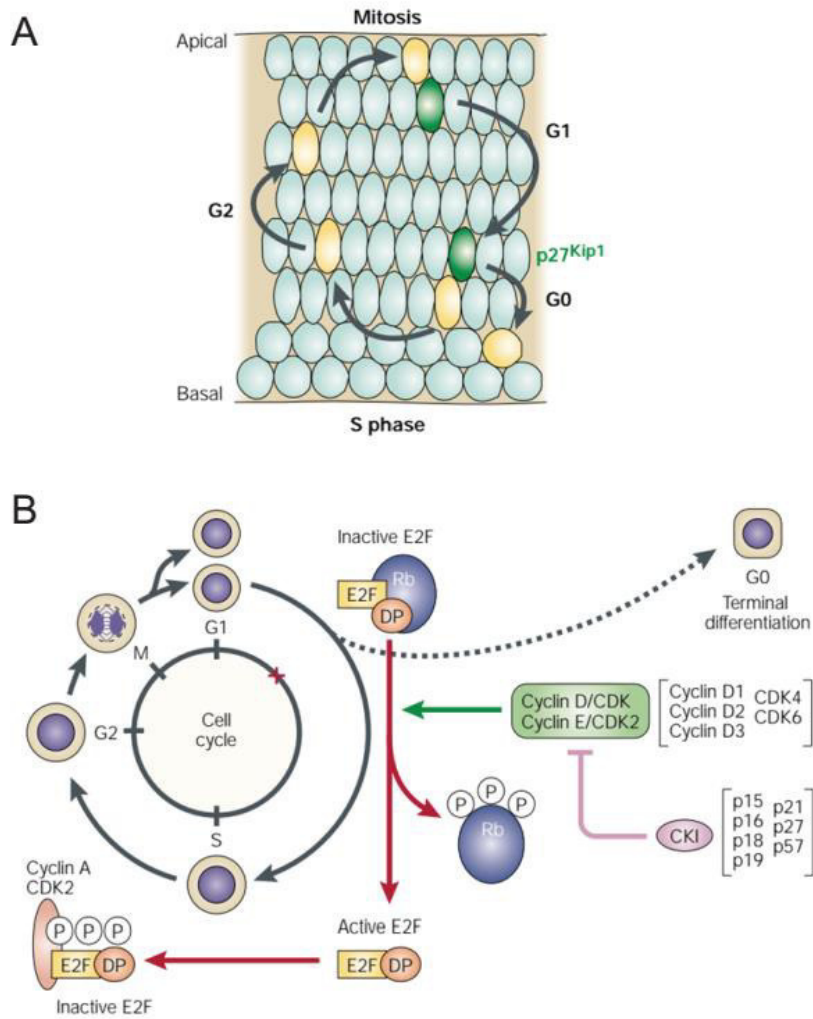


Figure 12. Cell cycle of RPCs.

(A) RPCs nuclei localization changes during the different cell cycle phases; mitosis occurs near the apical side and S-phase near the basal side. P27/Kip1 is expressed in most RPCs that are exiting the cell cycle. **(B)** Different proteins intervene at cell cycle progression. The phosphorylation state of Rb dictates whether a RPC will pass the G1/S checkpoint for another cell cycle round or exit the cell cycle and differentiate. If a progenitor remains proliferative, Rb is phosphorylated by a cyclin-dependent kinase (CDK) complex, and thus becomes inactive allowing cell cycle progression. If the cell is going to exit the cell cycle and undergo differentiation, this phosphorylation is blocked by cyclin-kinase inhibitor proteins (CKIs). Hypophosphorylated Rb binds to the E2F/DP heterodimer to prevent transcription of E2F-regulated genes. Figure adapted after (Dyer and Cepko, 2001c).

B.3.2 Retinal proliferation maintenance of progenitors

B.3.2.1 Intrinsic factors controlling retinal proliferation

The homeobox factors Pax6, Rax, Six3, Optx2/Six6 are crucial for proliferation and multipotency of RPCs within the developing optic cup (Figure 9) (Levine and Green, 2004).

To exemplify the importance of these transcription factors, I will focus on the homeodomain transcription factor **Pax6**, probably the most studied in eye development and highly conserved across species (Morgan, 2004). Throughout retinogenesis, it is expressed in RPCs (Remez et al., 2017) and has an essential role to regulate transcription factors to generate all retinal cell types (Marquardt et al., 2001). Conditional deletion of *Pax6* in RPCs leads to exclusive generation of amacrine cells. However, recent work suggests that Pax6 also inhibits photoreceptor differentiation (Figure 11) (Remez et al., 2017)

During the cell cycle, nuclei of RPCs migrate within the retina, and this phenomenon is referred to as interkinetic nuclear migration (Del Bene, 2011). During the M-phase of the cell cycle, they are located at the apical site of the retina. The S-phase of the cell cycle occurs at the basal site of the retina, and the nuclear migration through the apico-basal axis occurs during the phases G1 and G2 (Figure 12A) (Dyer and Cepko, 2001c) (Del Bene, 2011).

The regulation of the cell-cycle and the participating molecules are evolutionarily conserved, highly coordinated, and precisely regulated. This accurate regulation is necessary to avoid dysplasia or any kind of malignancy (Dyer and Cepko, 2001c). Typically, the **cyclins** are responsible for cell cycle progression at different stages of the cell cycle and therefore promote cell proliferation. **Cyclin D1** forms a complex with **CDK4** to phosphorylate and inhibit Rb protein and thus regulates the cell cycle during G1/S transition. Cyclin D1 deficiency slows down the cell cycle time and enhances the cell cycle exit (Dyer and Cepko, 2001c).

Three of seven **cyclin inhibitors** described in literature (**p27**, **p19**, **p57**) are implicated in retinal development (Dyer and Cepko, 2001a) (Dyer and Cepko, 2001b) (Cunningham et al., 2002). They control the exit from the cell cycle of retinal progenitors in the phase G0/G1. In mouse retina, the main representative **p27/Kip1** is expressed throughout retinogenesis in cells that exit the cell cycle and differentiate (Dehay and Kennedy, 2007).

Altogether, the interplay between cyclin D1 and p27 is tightly involved in retinal proliferation and cell cycle exit regulation of RPCs (Figure 12B).

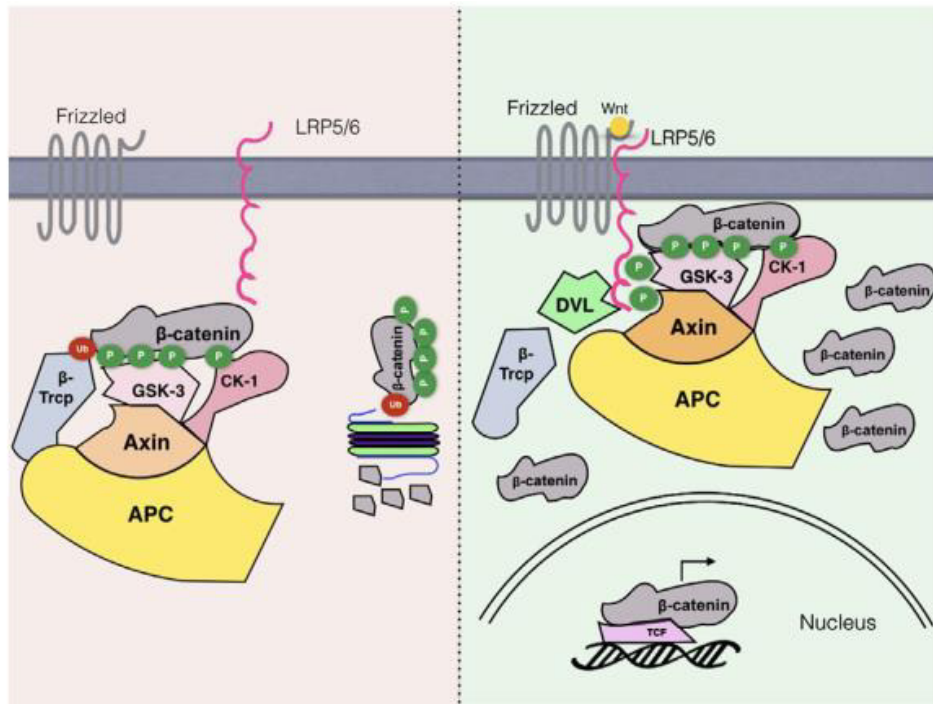


Figure 13. The Wnt signaling pathway.

In absence of Wnt ligands under basal conditions (**left**), a “destruction complex” of APC, Axin, CK and βTrCP and GSK3 is formed. **GSK3** becomes activated in this complex and **phosphorylates β-catenin** at the N-terminal “destruction box” on Thr41 and Ser 37 and 33 after priming phosphorylation of β-catenin by casein-kinase 1 (CK1) at Ser 45. Phosphorylation of β-catenin creates a recognition site for the E3 ubiquitin ligase βTrCP **triggering its degradation by the ubiquitin proteasome system**. Upon Wnt ligand stimulation that binds to Frizzled receptors, Frizzled associates with low-density lipoprotein receptor 5/6 (LRP5/6) (**right**). Phosphorylation of LRP5/6, achieved by GSK3 and CK1, activates Dishevelled (DVL) and creates high-affinity binding sites for Axin so that the entire complex including GSK3 but excluding βTrCP is recruited to the cell membrane. Thus, β-catenin is stabilized and accumulates in the cell. Subsequently, β-catenin translocates to the nucleus where it binds to TCF/LEF DNA-binding factors facilitating target gene expression involved in cell fate regulation. Figure taken from (Patel and Woodgett, 2017).

B.3.2.2 Extrinsic pathways controlling retinal proliferation

RPCs repeat several rounds of division to produce a pool of progenitors before they exit the cell cycle to give birth to different retinal cell types. Pathways to maintain this RPC pool mostly include Wnt, Shh, Notch as well as growth factor signaling. All these signaling pathways involve the regulation by a central node molecule, the Glycogen Synthase Kinase 3, that will be discussed in detail further in the manuscript.

➤ Wnt signaling

The Wnt signaling cascade, involving Wnt ligands (Wnts), evolutionary conserved glycoproteins, occurs in all phyla of the animal kingdom. It is among the most important signaling pathways controlling a large number of neurodevelopmental processes including cell proliferation, polarity, differentiation and neurogenesis during brain development (Figure 13) (Logan and Nusse, 2004).

There are approximately 20 different Wnt ligands binding to 15 different Frizzled receptor subtypes. Wnt binding to Frizzled receptors leads to the accumulation of β -catenin and its nuclear translocation. In the nucleus, β -catenin interacts with the TCF/LEF family of transcription factors and regulates their target genes. In absence of Wnt, β -catenin is phosphorylated by a “destruction complex” composed of multiple proteins, including Axin2 and GSK3 β , and targeted for degradation (Loh et al., 2016).

The pro-proliferative role of Wnt, which helps neural progenitors to maintain their undifferentiated state, is widely accepted. However, there is also evidence that Wnts can promote differentiation of stem cells (Otero et al., 2004) (Muroyama et al., 2002) (Inestrosa and Varela-Nallar, 2015). As such, Wnt ligands are involved in many steps and the use of reporter line and genetic tools allowed the identification of some of their function or expression pattern. The Wnt signaling pathway is particularly active in the peripheral embryonic and adult retina, including the CMZ in various species, such as zebrafish, *Xenopus*, chicken and mice (Denayer et al., 2008) (Kubo and Nakagawa, 2008). The use of transgenic mice reporting Wnt activity (TCF/Lef-LacZ) demonstrated that the highest levels of expression of the reporter gene was in the CMZ (Liu et al., 2003). Similar results were obtained in zebrafish and chicken (Dorsky et al., 2002) (Cho and Cepko, 2006). Specifically, Wnt activity in the retina is particularly well mirrored by *Wnt2b* expression whose expression is similar to the mentioned reporter gene expression in mice (Liu et al., 2003). Overexpression of *Wnt2b* inhibits neuronal differentiation and leads to the maintenance of retinal progenitors. *Wnt2b* loss-of-function suppresses proliferation in the CMZ triggering premature differentiation (Kubo et al., 2003) (Kubo et al., 2005).

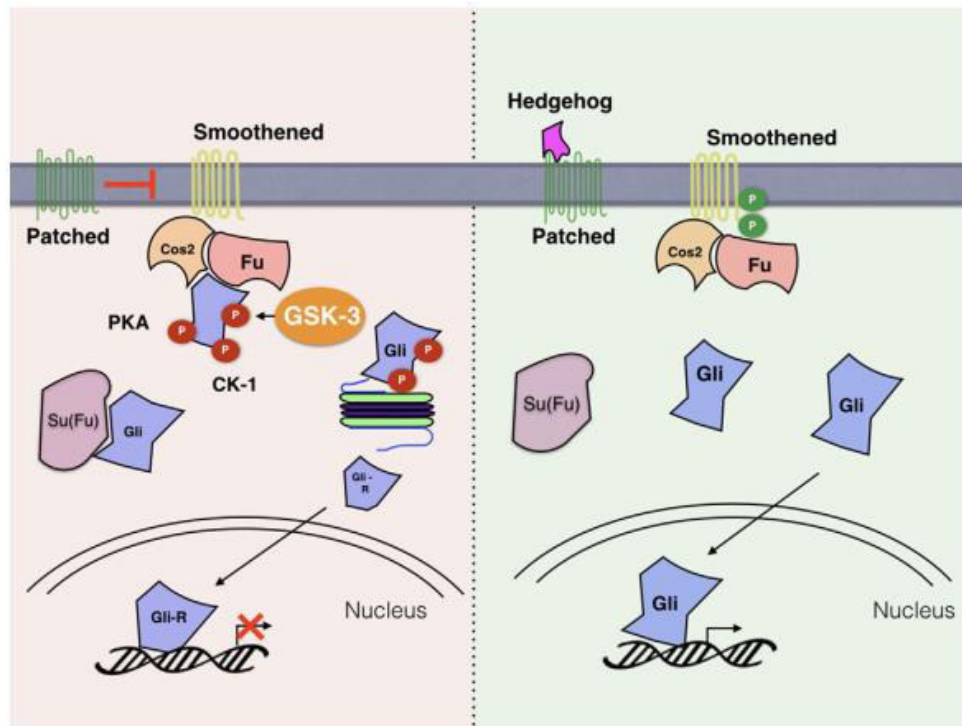


Figure 14. The Hedgehog signaling pathway.

In absence of Hedgehog ligands (**left**), under basal conditions, the 12-pass transmembrane receptor Patched represses another 7-pass G-protein coupled receptor-like protein Smoothened. The role of Smoothened is to inhibit the proteolytic cleavage of the effectors of the Hedgehog pathway, the zinc-finger proteins Gli (cubitus interruptus, Ci in flies). **Under basal conditions** though, Smoothened is repressed allowing **sequential phosphorylation of Gli** proteins are within their carboxy-terminal domain **by** PKA, **GSK3** and CK which target them **for cleavage** when bound to Kif7/Cos and SuFu **via** the protein E3-ligase βTrCP. The cleaved Gli transcription factors are transcriptional repressors. The binding of Hedgehog ligand to its receptor Patched releases the inhibition of Smoothened by Patched (**right**). In its active state, Smoothened promotes dissociation from Kif7/Cos and SuFu releasing full length Gli proteins that translocate to the nucleus where they act as transcriptional activators of cell cycle-related target genes. Figure taken from (Patel and Woodgett, 2017).

Based on these results, Wnt2b seems to represent an important factor for neural proliferation.

Strikingly, results obtained in mouse RPCs upon an activation or inactivation of members of the canonical Wnt signaling pathway suggested an inhibitory function of the Wnt signaling on differentiation, without affecting cell cycle exit or proliferation (Ouchi et al., 2005) (Ouchi et al., 2011). Elimination of β -catenin or ectopic activation did not alter the proliferation rate of RPCs (Fu et al., 2006).

In summary, Wnt seems to be crucial for maintaining stem cell potential in the CMZ, while it does not appear to have major pro-proliferative function in RPCs.

➤ **Hedgehog signaling**

Similar to Wnt, the Hedgehog signaling pathway, conserved across species, is a critical regulator of cell fate determination during development. In mammals, Hedgehog ligands include Indian Hedgehog, Desert Hedgehog, and Sonic Hedgehog (Shh). The binding of Hedgehog ligand to its receptor Patched releases the inhibition of Smoothened by Patched. Smoothened activation releases the Gli proteins that translocate to the nucleus where they act as transcriptional activators of cell cycle-related target genes (Figure 14). Most studied Hedgehog target genes are involved in cell cycle regulation, such as cyclins D and E, N-myc, Bcl2, Tbx2, but also its receptor Patched in a negative feedback loop (Patel and Woodgett, 2017).

Several studies have identified Shh signaling as implicated in proliferation of RPCs. For instance, in the embryonic mouse retina, *Smoothened* ablation in proliferating RPCs dramatically reduces RPCs since RPCs exhibit aberrant expression of cell cycle regulators and a delayed G1/S transition (Sakagami et al., 2009). Therefore, Shh has a profound impact on cell proliferation and progression of the cell cycle. On the contrary, mice with a constitutively active Hedgehog pathway exhibit persistent progenitors in peripheral retinal regions until 3 months of age, reminiscent of the ciliary marginal zone of lower vertebrates (Moshiri and Reh, 2004).

Hedgehog activation during early development in *Xenopus* or in chicken has been associated with CMZ stem cell proliferation, while inhibition of Hedgehog signaling *via* cyclopamine, a Smoothened antagonist, results in the opposite effect (Locker et al., 2006) (Moshiri et al., 2005). In summary, Hedgehog signals promote progenitor proliferation in the developing central nervous system.

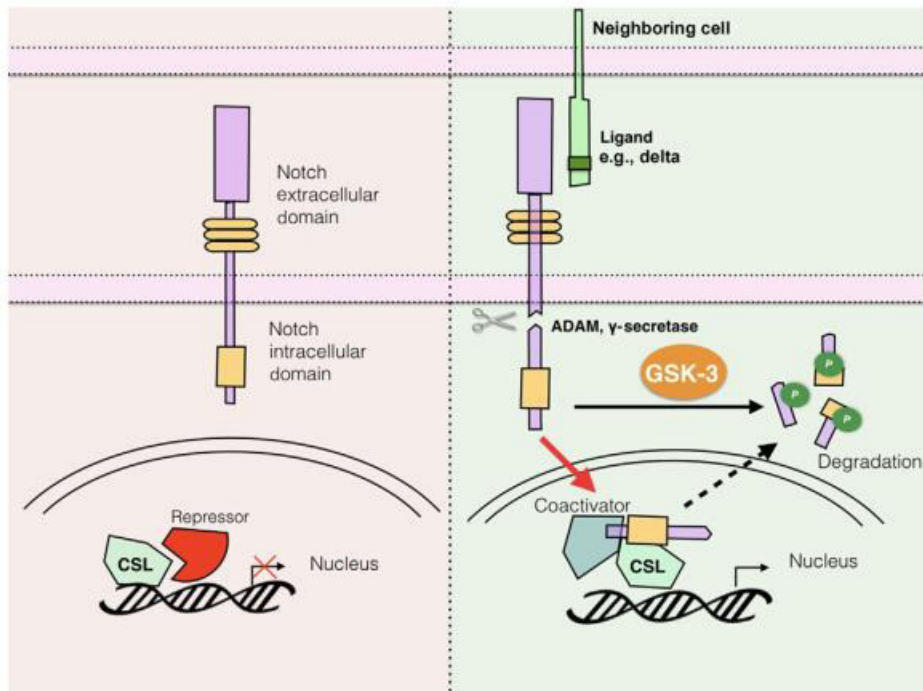


Figure 15. The Notch signaling pathway.

Notch is a ligand-activated transmembrane receptor, expressed on the surface of cells (**left**), and triggered by direct juxtacrine physical cell-cell contacts, acts as a transcription factor (**right**). Notch ligands are transmembrane proteins themselves and include Delta-like 1 (Dll1), Jagged and Serrate from neighbouring cells bind. Upon ligand binding (**right**), two intracellular enzymes ADAM and γ-secretase are activated to cleave and release notch intracellular domain (NICD). NICD translocates to the nucleus where it binds to the CSL transcription factor to regulate gene expression including the bHLH transcriptional repressors Hes1 and Hes5. These repressors inhibit transcription of proneural genes, and thus promote proliferation of progenitors while inhibiting differentiation into neurons. **GSK3 has been shown to bind and phosphorylate NICD probably leading to its degradation.** Figure taken from (Patel and Woodgett, 2017).

➤ Notch signaling

Similar to the Wnt and Shh, the Notch signaling pathway is evolutionarily conserved across species. It regulates tissue patterning, cell division, polarity, fate and death. Upon ligand binding, intracellular enzymes cleave and release notch intracellular domain (NICD). NICD translocates to the nucleus where it binds to the CSL transcription factor to regulate gene expression including the bHLH transcriptional repressors Hes1 and Hes5 (Figure 15). In the nervous system, components of the Notch signaling pathway promote proliferation of neural progenitor cells and *via* Hes1 and Hes5 and suppress their differentiation into neurons (Furukawa et al., 2000) (Imayoshi et al., 2010).

Since Notch expression forms an apico-basal gradient in the neuroepithelium (Murciano et al., 2002), the hypothesis arose that progenitor cells receive variable Notch signals depending on their migration. Therefore, Notch seems to be implicated in interkinetic nuclear migration and cell cycle regulation (Buchman and Tsai, 2008) (Baye and Link, 2008).

In contrast to the brain, the role of the Notch signaling in a developing retina is less studied. Few studies have depicted the function of Notch during early retinal development in mammals. Activation of Notch signaling pathways revealed the necessity of the Notch for the maintenance of RPCs, while inactivation is associated with increased neuronal differentiation (Alexson et al., 2006) (Nelson et al., 2007). In line with this, recent studies in mice demonstrated that Notch signals originating from the RPE are required to maintain the RPC pool in the neural retina, whereas a subthreshold level of Notch signals triggers differentiation into a neuron (Ha et al., 2017).

In addition, components of the Notch signaling pathway are expressed in the CMZ of zebrafish and *Xenopus* (Raymond et al., 2006) (Perron et al., 1998) indicating that Notch is active in the zone of retinal stem cell proliferation.

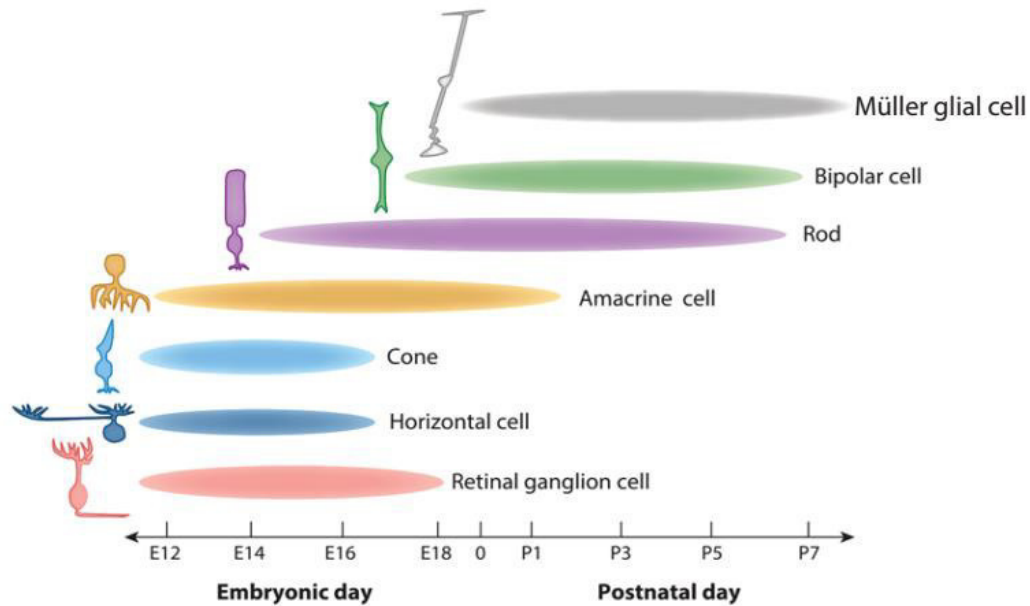


Figure 16. Birth of retinal cells is conserved across species.

Ganglion cells are produced during the early retinogenesis, followed by horizontal cells, cone photoreceptors and amacrine cells during intermediate neurogenesis. Finally, rod photoreceptors, bipolar cells and Müller glia cells differentiate during late neurogenesis. Figure adapted after (Cepko, 2015).

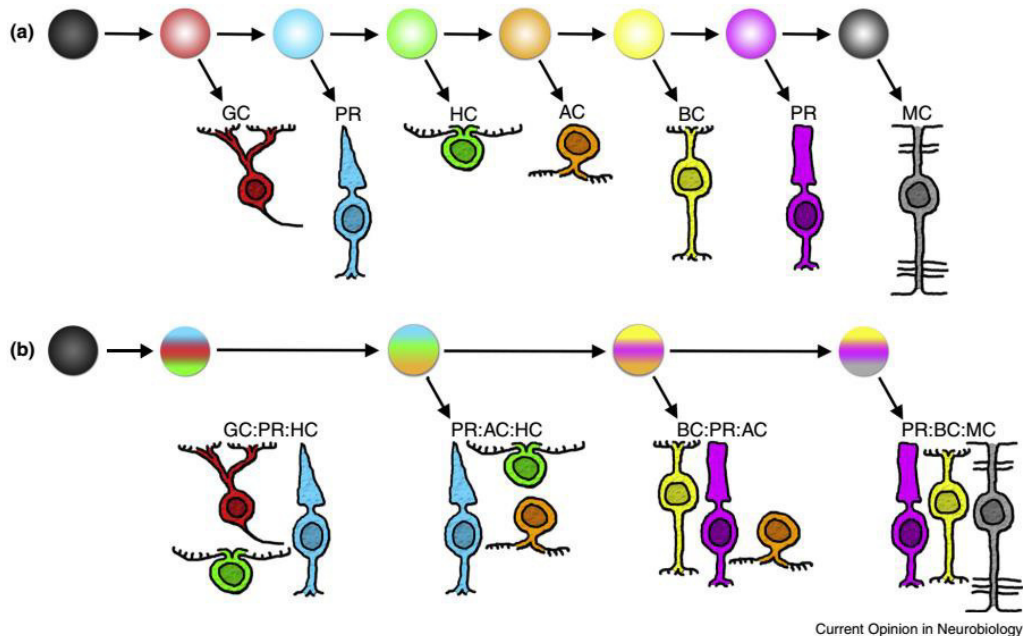


Figure 17. Comparison between the “classical” and the “updated” competence model.

(A) The upper panel depicts the “classical” competence model. At every competence state, one particular cell type is generated. (B) The “updated” competence model suggests that progenitor cells can have multiple cell fates at any given time, which is mainly due to stochastic events. Figure reproduced from (Boije et al., 2014).

B.3.3 Retinal differentiation and the competence model

During development, at eyecup stage, a wave of neurogenesis progresses from the central retina to the periphery and from the basal to the apical side. The generation time windows of retinal cell type are very well conserved across species and follow three successive waves of differentiation (Cepko, 2014). The first cells that differentiate in all vertebrates are the ganglion cells, referred to as **early neurogenesis**, followed by horizontal cells, cone photoreceptors and amacrine cells that are produced during **intermediate neurogenesis** and lastly rod photoreceptors, bipolar interneurons and Müller glia cells that are born during **late neurogenesis** (Kahn, 1974) (Figure 16) (Marquardt and Gruss, 2002) (Marquardt, 2003) (Livesey and Cepko, 2001).

In the linear classical view of the competence model, RPCs give birth to a particular cell type while losing their capacity to give birth to the previously generated retinal cell type (Figure 17). However, recent studies suggest that despite the unidirectional transition of competence, RPCs are multipotent and can choose from multiple fates at any one time (Boije et al., 2014). Despite the importance of extrinsic factors during retinal development, gain- and loss-of-function experiments performed in late 1990's have brought evidence on the critical role of intrinsic factors in RPC cell fate determination. Recently, it has been proposed that in addition to the regulatory function of intrinsic factors and extrinsic cues, stochastic events are also determinant during retinal development (Gomes et al., 2011) (He et al., 2012) (Cepko, 2014). As such, transcription factors expression pursues the well-established competence model. In addition, stochastic events allow the production of more than one different cell type at a given developmental time point, however without the possibility to “return back in competence” and generate earlier-born cell types at a later developmental stage (Figure 17) (Boije et al., 2014).

Hence, I will provide an overview about the current knowledge on extrinsic and intrinsic factors in the context of retinal cell fate determination. With combined actions of extrinsic, intrinsic and stochastic factors, RPCs will first acquire the competence to become one or another retinal precursor cell type (e.g. a photoreceptor precursor). Second, each precursor cell type will be specified to become a specialized precursor (e.g. a cone or a rod precursor). Finally, the differentiation program of each specialized precursor will allow the generation of terminally differentiated retinal cell types (e.g. an S-cone, an M-cone, or a rod photoreceptor).

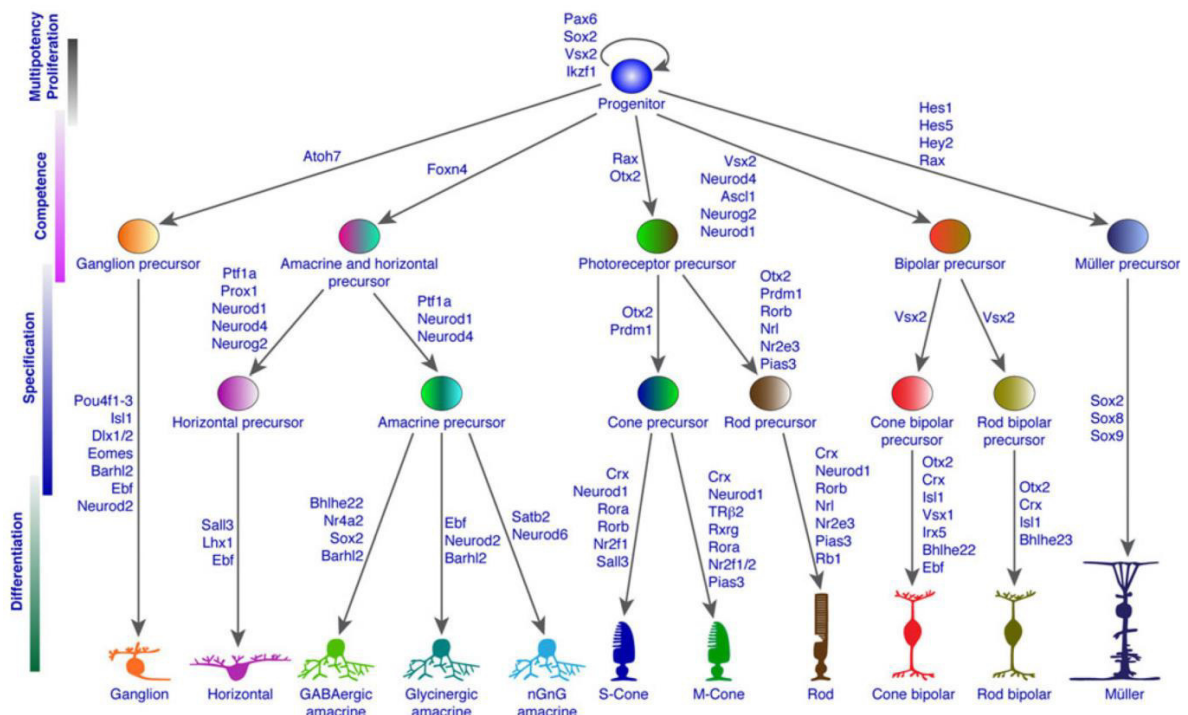


Figure 18. Transcription factors involved in multipotency of RPCs and generation retinal cell types.

Distinct transcription factor combinations are decisive a) to maintain **multipotency** and keep RPCs in proliferation, b) for the **competence** of retinal progenitors to generate retinal precursors, e.g. a photoreceptor precursor, c) for **specification** of retinal precursors, e.g. into a cone or a rod precursor, d) for **differentiation** of specified precursors, e.g. a cone into a S-cone or an M-cone. Figure reproduced from (Xiang, 2013).

Extrinsic factors for retinal cell fate determination and survival comprise fibroblast growth factor (FGFs), epidermal growth factor (EGFs), CNTF, Shh, thyroid hormone and Notch/Delta 1 (Harris, 1997) (Cepko, 1999) (Livesey and Cepko, 2001) (Yang, 2004). Constitutively activated Notch maintains a pool of proliferative retinal progenitor cells and suppresses early neuronal differentiation, but promotes Müller glial cell fate at the expense of photoreceptors (Furukawa et al., 2000) (Mizeracka et al., 2013). Conversely, Notch1 deficient RPCs initiate photoreceptor specific transcription program and differentiate into rod or cone photoreceptor fate, dependent on developmental stage (Jadhav et al., 2006) (Yaron et al., 2006). In addition, cell extrinsic cues from postmitotic retinal neurons can alter the retinal microenvironment exerting positive or negative influences on RPCs mitosis, cell cycle exit, differentiation, and thus the cell fate. This was demonstrated for ganglion cells secreting Shh and controlling cell fate and differentiation of adjacent cells (Waid and McLoon, 1998). Importantly, the ability of the developing retina to react to these growth factors changes over time. Earlier progenitors are more sensitive towards FGF signals, whereas later progenitors display a higher proliferative response to EGF changes (James et al., 2004) (Lillien, 1995). These observations concerning distinct reactivity of RPCs to different extrinsic factors are encompassed in the competence model (Cepko, 1996) (Desai and McConnell, 2000) (Boije et al., 2014).

The intrinsic factors comprise mainly basic helix-loop-helix (bHLH) and homeobox-type transcription factors (Figure 18) (Cepko, 2014). The important transcription factors for competence, specification and differentiation will be described in the following with a focus on retinal photoreceptors.

- **Ganglion cell differentiation**

RGCs are the first cells to differentiate (Young, 1985). The regulation of RGCs fate determination implies a hierarchical gene-regulatory network of transcription factors (Isenmann et al., 2003) (Mu and Klein, 2004), mainly through the actions of Math5 (Atoh7), Brn3b (Pou4f2) and Islet1 (Isl1) transcription factors (Figure 18).

Math5 is the first transcription factor regulating the competence state of RGCs. It is regulated by Pax6 and starts to be expressed at E11 at the onset of RGCs differentiation, but its expression is inhibited at postnatal stages (Brown et al., 1998) (Hufnagel et al., 2007). Lack of *Math5* expression in mice leads to a complete lack of RGC differentiation (Brown et al., 2001).

Additional loss- and gain-of-function studies in different species, including *Xenopus*, zebrafish and chicken, definitively established *Math5* as the master regulator of RGC genesis (Kanekar et al., 1997) (Kay et al., 2001) (Liu et al., 2001) (Brown et al., 1998) (Sakagami et al., 2009) (Prasov and Glaser, 2012a).

Math5 controls RGC competence in part by directly activating the expression of ***Brn3b*** (*Pou4f2*) and *Isl1*, two homeobox transcription factors involved in RGC specification and differentiation (Gan et al., 1996) (Wang et al., 2001) (Gao et al., 2014) (Wu et al., 2015). *Brn3b* not only promotes RGC differentiation but also inhibits non-RGC differentiation programs. It suppresses the expression of transcription factors involved in the specification and differentiation of amacrine, horizontal, and late-born ganglion cells (Xiang, 2013). From a functional point of view *Brn3b* is required for RGC specification from RPC stage and induces *Brn3a* and *Brn3c* expression for terminal RGC differentiation (Xiang, 1998). The different combinations of these three transcription factors (*Brn3a*, *Brn3b* and *Brn3c*) define distinct RGC subtypes and regulate RGC production, axonal and dendritic formation (Badea et al., 2009) (Shi et al., 2013).

Other transcription factors specifying the ganglion cells comprise Eomesodesmin (*Tbr2*), *Ebf1*, *Ebf3*, *Onecut1* and *Onecut2* (Badea et al., 2009) (Badea and Nathans, 2011) (Mao et al., 2008) (Sapkota et al., 2014) (Shi et al., 2013).

In mammals, there are two types of RGCs projecting either to the contralateral or ipsilateral side of the brain. The generation of contra- and ipsilateral RGCs is sequential with first birth of contralateral RGCs upon expression of *Isl1* and *Brn3b* at E11 (Rachel et al., 2002). Conversely, *Zic2*-positive ipsilateral RGCs are generated around E13 - 15 (Herrera et al., 2003) (Escalante et al., 2013).

- **Bipolar cell differentiation**

Bipolar cells are generated during the last differentiation wave, mainly based on the combination of the transcription factors ***Chx10*** (*Vsx2*), ***Math3*** and ***Mash1*** (Figure 18) (Liu et al., 1994) (Rowan and Cepko, 2004) (Hatakeyama et al., 2001).

Interestingly, bipolar precursor cells also have the potential to differentiate into rods or cones. Therefore, additional transcription factors are required for bipolar cell fate specification including *Otx2*, *Crx*, *Isl1* and *Vsx1*. ***Otx2*** plays a crucial role in bipolar cell maturation (Koike et al., 2007). Importantly, in photoreceptor precursors, it induces ***Blimp1*** (***Prdm1***) which is decisive in the binary choice between photoreceptor and bipolar cell genesis by repressing *Chx10* (Katoh et al., 2010).

Upon conditional *Blimp1* deletion in the developing retina, *Chx10* was upregulated and bipolar-like cells and proliferating cells were increased at the expense of photoreceptors (Brzezinski et al., 2010) (Katoh et al., 2010) (Brzezinski et al., 2013). In line with this, overexpression of *Blimp1* by *in vivo* electroporation suppressed bipolar cell genesis (Katoh et al., 2010). Altogether, *Blimp1* and *Chx10* favor photoreceptor specification restricting their competence to differentiate into bipolar cells, whereas *Chx10* promotes bipolar cell fate by inhibiting transcription of photoreceptor specific genes (Dorval et al., 2006). In the mouse retina, a higher degree of differentiation will lead to only one type of rod bipolar cells and at least nine types of morphologically and physiologically distinct cone bipolar cells (Ghosh et al., 2004). At the present time, little is known about how each of these subtype identities is specified and differentiated from the bipolar precursors.

- **Amacrine and horizontal cell differentiation**

The two interneuron subtypes, amacrine and horizontal cells, are produced during the second intermediate differentiation wave. A common set of transcription factors including *Foxn4*, *NeuroD* (*NeuroD1*), and *Math3* (*NeuroD4*), is involved in the specification of both amacrine and horizontal cells suggesting the existence of a shared precursor cell (Figure 18).

Foxn4 is required for the competence and genesis of amacrine and horizontal cells (Li et al., 2004). It activates expression of *NeuroD*, *Math3*, and *Prox1* (Li et al., 2004) (Perez de Sevilla Muller et al., 2017). ***NeuroD*** is required for amacrine cell genesis (Cherry et al., 2011) (Morrow et al., 1999). Notably, *NeuroD* is also expressed in RPCs and photoreceptor precursors in rodents governing photoreceptor genesis through Delta/Notch signaling (Taylor et al., 2015). ***Math3*** is temporally expressed in amacrine cells until P14, but it remains constantly expressed in horizontal cells (Morrow et al., 1999) (Inoue et al., 2002). ***Prox1*** is also crucial for differentiation of horizontal cells (Dyer et al., 2003).

A major transcription factor to define GABAergic/Glycinergic *versus* glutamatergic cell specification in *Xenopus* retina is ***Ptf1a*** (Dullin et al., 2007). Results obtained in our laboratory in *Xenopus* retina demonstrated the role of transcription factors ***Mash1*** and ***Prdm13*** acting downstream of *Ptf1a*, to regulate specifically GABAergic cells (Mazurier et al., 2014) or glycinergic amacrine cells (Bessodes et al., 2017), respectively. Studies performed in the mouse retina have shown an essential role of *Ptf1* both for GABAergic and glycinergic amacrine and horizontal cell differentiation (Nakhai et al., 2007).

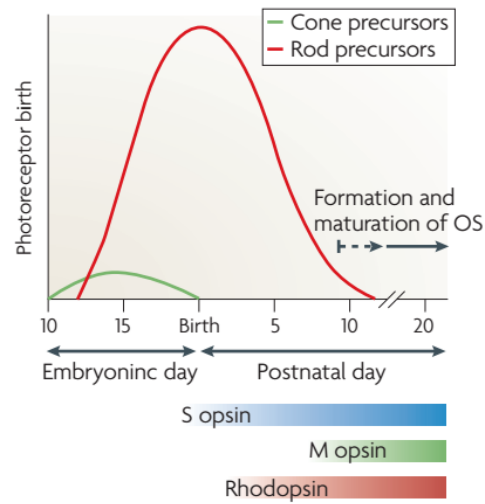


Figure 19. Photoreceptor genesis and maturation in mice.

In rodents, cone photoreceptors are generated between E11.5 and E18.5 and rod photoreceptors between E12.5 and P7, with a peak at P0. *Opsin* expression begins around E18 for *S-opsin*, P6 for *M-opsin* and P2 for *Rhodopsin*. Formation of OS and synapses occurs later during photoreceptor maturation. Figure adapted from (Swaroop et al., 2010).

Recently, the factor Bcl-2-associated transcription factor (**Bclaf1**) was also identified in early-born postmitotic cells to trigger their differentiation into ganglion, amacrine or horizontal cells, to inhibit cone photoreceptor differentiation (Orieux et al., 2014).

- **Müller cell differentiation**

It is not well understood why Müller cells are the last cells to differentiate during development. Their expression profile is close to RPCs (Hatakeyama et al., 2001) (Hatakeyama and Kageyama, 2004) (Roesch et al., 2008). Thus, they keep the molecular signature of undifferentiated neurons until differentiation into the glial cell type is possible. This could explain on the one hand their inherent timing of differentiation, and on the other hand their stem cell-like properties.

- **Photoreceptor differentiation**

In rodents, cones birth starts with other early born neurons around E11.5 and continues until E18.5 (**Figure 19**) (Swaroop et al., 2010). The window to generate rod photoreceptors is wider and lies between E12.5 and P7, with a peak at P0 (Akimoto et al., 2006). Interestingly, there is a delay that accounts for several days for newly born photoreceptor precursors to begin expressing the specific opsin characteristics for their mature phenotype in rodents (**Figure 19**) (Watanabe and Raff, 1990) (Cepko, 1996). During this period, photoreceptor precursors can be induced to differentiate into rods or cones depending on the combination of several intrinsic transcription factor expression (Nishida et al., 2003) (MacLaren et al., 2006) (Roberts et al., 2006).

In total, different combinations of six key photoreceptor transcription-regulatory factors are responsible for mammalian rod and cone cell fate determination, development and maturation: Ror β , Otx2, Nrl, Tr β 2, Crx, and Nr2e3 (**Figure 20**) (Swaroop et al., 2010).

- **Otx2** decides upon **photoreceptor cell fate determination**.
- **Ror β** and **Nrl** are responsible for **committing photoreceptors into rods or cones**.
- **Crx**, **Nr2e3**, **Tr β 2** as well as other transcription factors are important for **photoreceptor terminal differentiation**, including synaptogenesis and OS formation.

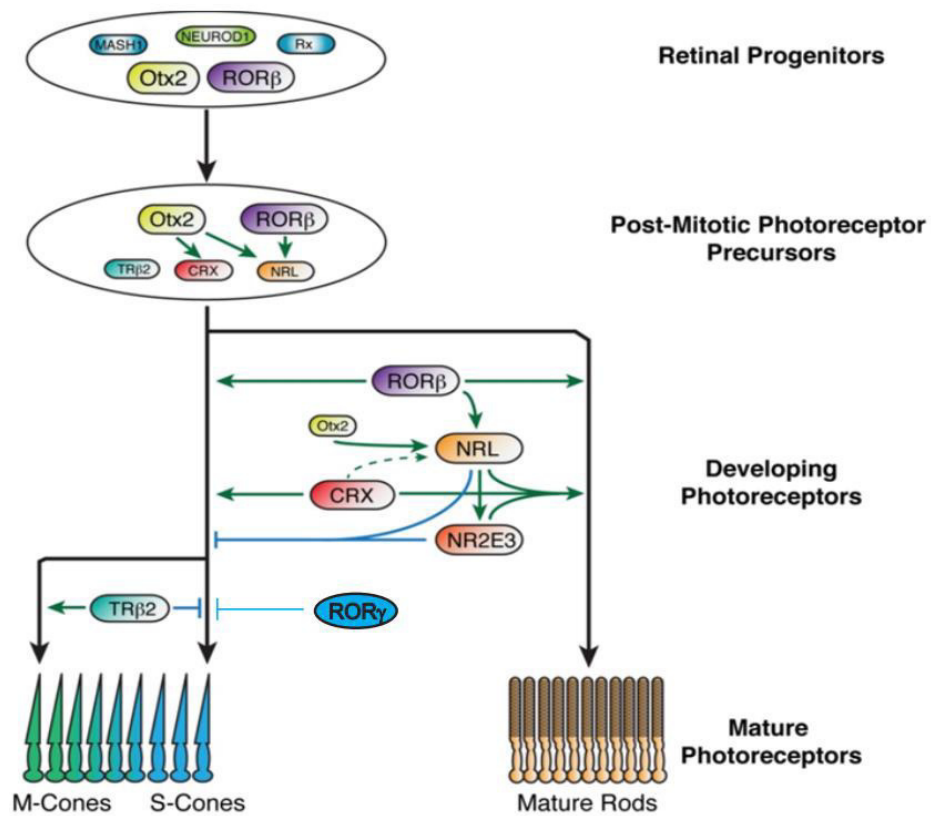


Figure 20. Transcription factors network required for photoreceptor differentiation and maintenance.

Otx2 is the key transcription factor to commit retinal progenitors to photoreceptor precursor cell fate. Photoreceptor precursors will differentiate into a default S-cone state unless Nrl reaches a certain threshold to promote rod cell fate. Cones will differentiate into M-cones mainly in the dorsal retina dependent on Thr β 2 activity. Figure adapted after (Roger et al., 2014).

Transcription factors for photoreceptor cell fate determination:

- **Orthodenticle homeobox 2 (Otx2)**

In the developing retina, *Otx2* is expressed during the terminal cycle in retinal progenitors and early postmitotic photoreceptor precursors (Figure 20) (Beby and Lamonerie, 2013). More precisely, its expression starts to increase around E10.5 and peaks at E17.5 in the outer part of the neuroblastic layer, where photoreceptor precursors are located. Conditional knockout of *Otx2* in immature RPCs results in an almost complete loss of rods and cones, as well as horizontal and bipolar cells, and generates an increased number of amacrine-like cells in mice (Nishida et al., 2003). Conditional deletion of *Otx2* in mature retina leads to dramatic photoreceptor degeneration until their full disappearance within 4 months (Beby et al., 2010) (Housset et al., 2013). Forced *Otx2* expression in P0 rat retina, on the contrast, results in an increased photoreceptors number at the expense of bipolar, amacrine and Müller cells (Nishida et al., 2003). Altogether these results define *Otx2* as a key transcription factor for photoreceptor cell fate determination (Koike et al., 2007) (Bernard et al., 2014).

Transcription factors for rod versus cone cell commitment:

The use of animal models led to the identification of two transcription factors required for rod photoreceptor cell fate. These two factors are the Retinoid related orphan receptor beta (*Rorβ*) that induces the rod-specific Neural Retina Leucine Zipper (*Nrl*) (Figure 20). The presence of only cone photoreceptors in mice lacking one of these two genes led to the hypothesis that cones are the default photoreceptor fate.

- **Retinoid related orphan receptors (Rorβ)**

Ror is a family of orphan nuclear receptors, related to receptors for retinoids that are involved in photoreceptor specification and maturation.

In *Rorβ*^{-/-} retina, exclusively cone-like cells are produced at the expense of rods (Jia et al., 2009) (Fu et al., 2014) demonstrating its crucial function to specify rods. However, these S-cone-like photoreceptors are poorly differentiated, lack OS and are not functional due to the lack of phototransduction protein expression, and mainly *S-opsin* expression (Srinivas et al., 2006). Hence, *Rorβ2* has a dual function and is important both for rod differentiation and S-cone maturation; however, it does not seem to influence M-opsin expression (Hennig et al., 2008).

In summary, synergistic action of *Rorβ* and *Otx2* is required to produce rod photoreceptor precursors but additional factors are required for further rod development and maturation.

- **Rod-specific transcription factor Neural Retina Leucine Zipper (Nrl)**

Nrl is a bZIP transcription factor belonging to the Maf family proteins (Swaroop et al., 1992). It is highly expressed in the neural retina, and transcripts were detected as early as E12.5 in cells that will become rod photoreceptors after their terminal cell cycle (Akimoto et al., 2006). Immunostaining of Nrl in human and mouse retinas showed its expression in rod nuclei (Swain et al., 2001), suggesting its role for rod development and maintenance as its expression is also maintained in adults.

In *Nrl*^{-/-} mice, all photoreceptor precursors differentiate into cones, especially S-cones, leading to a cone-only (or rodless) retina with no detectable expression of rod-specific genes (Mears et al., 2001). These in excess produced S-cones exhibit shorter outer segments and share many molecular and functional features with normal cones, such as an increased sensitivity and cone specific gene expression, including *Cone Transducin* and *Cone Arrestin* (Daniele et al., 2005). Conditional *Nrl* expression later during S-cone differentiation generates photoreceptor hybrids that co-express both *Rhodopsin* and *S-opsin* (Oh et al., 2007). In addition, ectopic *Nrl* expression in all postmitotic photoreceptors results in a rod-only retina with a complete loss of cone gene expression (Oh et al., 2007). Based on these studies, **Nrl function is to determine rod cell fate from photoreceptor precursors** by activating numerous rod-specific genes as well as by suppressing cone-specific genes.

Interestingly, in early adulthood, *Nrl*^{-/-} retina undergoes a rapid, but temporal degeneration including cone apoptosis. Unexpectedly, cone degeneration reaches a plateau by 4 months of age and the remaining cones expressing S- and M-opsins preserve photopic electroretinogram (Roger et al., 2012). Ablation of *Nrl* in adult rods leads to a loss of rod-specific genes with a concomitant increase of cone-specific genes (Montana et al., 2013). Thus, besides its role in photoreceptor development, **Nrl is essential to maintain rod differentiation and homeostasis in mature retina**.

Based on these studies, the idea then arose that *Nrl* knockdown could be used as a therapy approach to prevent secondary cone loss in retinal disease. Indeed, secondary cone loss, such as it is the case in *retinitis pigmentosa*, has a major impact on vision loss and is therefore clinically more significant. To achieve this, a tamoxifen-inducible *Cre* recombinase was used to specifically delete *Nrl* in mature rod photoreceptors. As expected, rods were reprogrammed into cone-like cells. However, the therapeutic effects of such an approach has only been evaluated in *Rho*^{-/-} mice (Montana et al., 2013). Whether it could be extended to other photoreceptor mutations remained to be clarified until a very recent new study. A genome editing approach in mature photoreceptors was performed using AAV-mediated

CRISPR/Cas9 to inactivate *Nrl* gene. The **protective effect of transforming rods into cones** was demonstrated in three different models of retinal degeneration thus adding a new therapeutic strategy (Yu et al., 2017).

At the molecular level, it has been demonstrated that *Rorβ* is upstream of *Nrl* by binding directly to regulatory consensus elements within *Nrl* gene. Moreover, *Nrl* sustains its own expression by activating the *Rorβ2*-specific promoter of *Rorβ* and therefore reinforces photoreceptor commitment into rod cell fate (Fu et al., 2014). In addition, *Rorβ* acts in combination with other transcription factors, such as *Otx2* and *Crx* to sustain *Nrl* expression in developing and mature photoreceptors (Montana et al., 2011) (Kautzmann et al., 2011). Therefore, the lack of rod photoreceptor occurring in *Rorβ*^{-/-} retina originates from the absence of *Nrl* expression induction (Jia et al., 2009).

In addition to regulations at the transcriptional level, *Nrl* activity could also be modulated at the protein level by posttranslational modifications (PTMs). Indeed, *Nrl* has multiple differentially phosphorylated isoforms with a molecular weight ranging from 29 to 35 kD (Swain et al., 2001) (Kanda et al., 2007). Mutations in codon 50 (Ser to Thr) and 51 (Pro to Ser) altering *NRL* phosphorylation have been associated with *Autosomal Dominant Retinitis Pigmentosa* (ADRP) in humans (Kanda et al., 2007) (Bessant et al., 1999) (Martinez-Gimeno et al., 2001) (DeAngelis et al., 2002) (Swain et al., 2007). *In vitro* experiments identified them as gain-of-function mutations. They restrain *Nrl* phosphorylation but lead to its enhanced transcriptional activity on its target genes, including *Rhodopsin*, when combined with *Crx in vitro* (Bessant et al., 2003) (Nishiguchi et al., 2004) (Kanda et al., 2007). On the contrary, recessive *Nrl* mutations are likely to be loss-of-function mutations as demonstrated by *in vitro* function studies (Nishiguchi et al., 2004). They were identified in patients with pigmentary retinal degeneration that exhibited a rod loss, but normal cone function (Nishiguchi et al., 2004). Thus, differential *Nrl* phosphorylation seems to be important to fine tune its transcriptional activity and control its target gene expression.

In addition to phosphorylation, *NRL* activity is also fine-tuned by sumoylation (Roger et al., 2010) as shown *in vivo* and *in vitro*. In contrast to phosphorylation, *NRL* sumoylation mutants have a lower transcriptional activity with downregulation of its target genes, including *Rho* and the nuclear factor *Nr2e3* (Figure 20), known for its inhibitory function on cone genes in rod photoreceptors. In line with these *in vitro* data, *in vivo* electroporation experiments in newborn mice demonstrated that lack of *NRL* sumoylation leads to the differentiation of hybrid photoreceptors expressing both rod and cone markers due to impaired *Nr2e3* expression.

Around 300 direct *Nrl* target genes have been identified by coupling chromatin immunoprecipitation followed by ChIP-Seq, including approximately 20 genes that are associated with retinal diseases involving photoreceptor degeneration (Hao et al., 2012).

Transcription factors for rod and cone terminal differentiation:

- **Cone-rod homeobox (Crx) for rod and cone maturation**

Before being restricted to bipolar cells, *Otx2* induces ***Crx*** expression in photoreceptors (Nishida et al., 2003). *Crx* is expressed in postmitotic photoreceptor precursors derived from cells that have just exited the cell cycle and express *Otx2* (Figure 20) (Glubrecht et al., 2009). Hence, *Crx* expression coincides with cone cell birth and starts at E12.5, peaks at P5 in mice with the onset of photoreceptor maturation and is maintained in adulthood (Hennig et al., 2008). Lack of *Crx* in mice leads to an absence of photoreceptor outer segment formation with no rod and cone visual function causing congenital blindness and subsequent rapid retinal degeneration (Furukawa et al., 1999). Similar to *Rorβ*^{-/-} mice, *Crx*^{-/-} mice lack both rod and cone photoreceptor maturation (Srinivas et al., 2006). Forced expression of *Crx* in P0 rat retinas increases rod cell number and reduces the amacrine and Müller glial cells although less potent than *Otx2* (Furukawa et al., 1997). Therefore, ***Crx*** is essential for maturation but not specification of both rod and cone photoreceptors.

Serial analysis of gene expression and RNA-Seq analyses on *Crx*^{-/-} mice combined with chromatin immunoprecipitation (ChIP) assay revealed that most of the photoreceptor specific genes are *Crx*-dependent proving that *Crx* is required to induce the transcription of most rod and cone-specific genes (Blackshaw et al., 2001) (Roger et al., 2014) (Peng and Chen, 2005) (Corbo et al., 2010) (Chen et al., 1997). To regulate photoreceptor expressed genes, *Crx* needs to interact with other transcription regulators, including rod-specific transcription factors such as *Nrl* (Mitton et al., 2000a), *Qrx* (present in bovine and human, but absent in mouse genome) (Wang et al., 2004), the transcription-splicing protein *NonO* (Yadav et al., 2014), *Rorβ* (Srinivas et al., 2006) and *Otx2* (Fant et al., 2015). Interestingly, *Nrl* and *Crx* ChIP-Seq analysis revealed that most genes involved in photoreceptor function, including most rod phototransduction genes (*Rho*, *Gnat1*, *Pde6a*), encompass overlapping binding sites for both *Nrl* and *Crx* (Hao et al., 2012).

Consistent with its major role in photoreceptor maturation, more than 50 autosomal dominant mutations have been identified in the human *Crx* gene. They lead to a variety of retinal degenerative diseases such as autosomal dominant cone-rod dystrophy, LCA, and *retinitis pigmentosa* (Sohocki et al., 2001) (Huang et al., 2012) (Freund et al., 1998) (Freund et al., 1997).

Many of the Crx disease-associated mutations are missense or deletion mutations (Chen et al., 2002) (Tran and Chen, 2014) acting as dominant negative mutations interfering with normal Crx function (Roger et al., 2014) (Ruzycki et al., 2017), mostly altering the binding capacity of Crx to the DNA. The consequence is impaired target gene expression leading to absence of photoreceptor maturation. This is, for instance, the case for the *Crx^{Rip}* dominant negative mutation that leads to congenital blindness and non-recordable response, reflecting the phenotype of LCA (Roger et al., 2014). As *Crx^{-/-}* mice, *Crx^{Rip/+}* are characterized by arrested photoreceptor differentiation. However, in contrast to *Crx^{-/-}* mice, the retina of *Crx^{Rip/+}* contains only immature cone-like photoreceptors with long-term photoreceptor preservation. Thus, photoreceptors remain very primitive in *Crx^{Rip/+}* retina, with a complete absence of rod *versus* cone cell fate determination, while they differentiate in *Crx^{-/-}* retina but remain non-functional due to the lack of maturation. In both cases, *Otx2* expression is largely upregulated but in the case of *Crx^{Rip/+}* retina it is unable to compensate the dominant negative function of mutant Crx. *Nrl* expression is normal up to P2 in *Crx^{Rip/+}* retina but decreases rapidly after to be null in adult retina. Further analysis revealed that *Crx^{Rip}* mutant protein inhibits the action of the remaining *Crx^{WT}* protein, and also impairs compensatory function of *Otx2* explaining the phenotypic differences observed in *Crx^{Rip/+}* and *Crx^{-/-}* retina (Roger et al., 2014). Such compensatory effects occur since *Otx2* and Crx act synergistically *via* the same *cis*-regulatory motif to activate photoreceptor specific genes (Chen et al., 1997) (Furukawa et al., 1997). Altogether, these results demonstrated that Crx takes over *Otx2* function in wildtype postnatal retina when *Otx2* expression decreases in order to sustain the expression of rod and cone photoreceptor expressed genes required for terminal differentiation.

- **Nr2e3 or photoreceptor-specific nuclear receptor (PNR) transcription factor for terminal rod differentiation**

Nr2e3 transcription factor is expressed from E18 onwards (Chen et al., 2005) (Peng et al., 2005), whereby its expression peaks during rod differentiation and is maintained in adulthood (Bumsted O'Brien et al., 2004) (Kobayashi et al., 1999) (Haider et al., 2000). *Nr2e3* is a direct target of *Nrl* (Figure 20) (Hao et al., 2012) (Mears et al., 2001).

The naturally occurring *rd7* mouse mutant, carrying a spontaneous null allele of the *Nr2e3* locus (*rd7*) does not produce the *Nr2e3* protein (Peng et al., 2005) (Haider et al., 2006).

Phenotypically, their retina shows morphological defects with whorls and rosettes. Photoreceptors overexpress *S-opsin* and progressively degenerate (Haider et al., 2001). The majority of these photoreceptor cells are hybrid cells expressing both rod and cone genes (Chen et al., 2005) (Corbo and Cepko, 2005). They are characterized by a low ERG response (Akhmedov et al., 2000) (Haider et al., 2000). In contrast, ectopic *Nr2e3* expression under *Crx* promoter in *Nrl*^{-/-} retinas suppresses cone differentiation and results in non-functional, morphologically rod-like photoreceptors (Cheng et al., 2006).

The phenotype observed in *rd7* mice mimics the enhanced S-cone syndrome (ESCS), an autosomal recessive human retinal dystrophy caused by *NR2E3* mutations. These patients show a large clinical heterogeneity (Audo et al., 2008) with hyperfunction of S-cones, but not always defective rods with progressive loss of sight (Haider et al., 2000) (Wright et al., 2004) (Kinori et al., 2011). However, *NR2E3* mutations are not systematically associated with ESCS (Schorderet and Escher, 2009) (Fradot et al., 2007).

Notably, as for *Nrl*, *Nr2e3* requires sumoylation to exert its ability and repress cone gene expression for rod specification. It is sumoylated by E3 SUMO ligase Pias3 that is selectively expressed in developing photoreceptors (Onishi et al., 2009). As determined by ChIP assays, *Nr2e3* associates with both rod and cone gene promoters. This interaction is dependent on interaction with *Crx* (Peng et al., 2005) (Peng and Chen, 2005), or on *Crx/Nrl* to potentiate rod gene expression, such as *Rhodopsin* (Cheng et al., 2004) (Peng et al., 2005), and repress activity of cone gene promoters (Chen et al., 2005) (Peng and Chen, 2005).

The molecular mechanisms underlying these dual functions of *Nr2e3* in rod *versus* cone gene expression may rely on interactions with other co-activators and co-repressors. As such, *Nr2e3* interacts with another nuclear receptor *Nr1d1* (Rev-Erba), a member of circadian clock, which forms a complex with *Crx/Nrl* to potentiate *Rhodopsin* expression (Cheng et al., 2004). Interestingly, *Nr1d1* can rescue *Nr2e3* loss in the *rd7* mouse mutant described above with clinical, histological, functional and molecular restoration, demonstrating the functional redundancy of the two factors (Cruz et al., 2014).

Altogether, these results established *Nr2e3* as an important factor in enhancing rod development by activating rod-expressed genes and inhibiting cone-expressed genes in rod photoreceptors (Figure 20).

Factors for cone terminal differentiation:

S-cones first differentiate in the mouse retina shortly after birth, and it is believed that they represent the default photoreceptor state. This hypothesis is supported by the fact that developing mouse rods have a molecular footprint of S-cones. Indeed, rod photoreceptors transiently express *S-Op sin*, but not *M-Op sin* during development. It is diminished in rods when *Nrl* is expressed (Kim et al., 2016).

M-cones begin differentiating about a week after, mostly in the dorsal retina, at the expense of S-cones (Cepko, 1996) (Roberts et al., 2006). At the completion of retinal development, a dorso-ventral gradient is observed with high expression of *M-opsin* at the dorsal part whereas *S-opsin* is more expressed at the ventral part of the retina (Figure 5). The establishment of such a precise gradient involved the interplay of extrinsic signals (growth factors or hormones) and intrinsic factors.

The retinoic acid receptor (Rar) and retinoid-related receptor (Rxr) families are ligand-dependent nuclear receptors consisting of three genes: A, B, and C, and consequently produce the three receptor isoforms α , β , and γ expressed in the retina (Mori et al., 2001) (Cvekl and Wang, 2009). Of particular interest for cone development is the cone specific **Rxry** receptor. Lack of Rxry in mice results in an exclusive *S-opsin* expression in all cones in the retina (Roberts et al., 2006). The authors concluded an important role for Rxry to suppress *S-opsin* expression in developing M-cones, however no effect on *M-opsin* expression was detected (Figure 20).

Thyroid hormone (TH) is most likely responsible to establish the M-cone gradient (Figure 5C) (Forrest et al., 2002). At birth, TH is symmetrically distributed across the retina as *S-opsin* expression begins. During postnatal development, TH forms a gradient and becomes more elevated in dorsal than in ventral regions, at the time of *M-opsin* expression onset at P10 (Roberts et al., 2006).

The nuclear TH receptors are ligand-activated transcription factors produced by two genes (Forrest et al., 2002). The TH receptor **TR β 2** is a splice variant from the thyroid hormone receptor B (*Thrb*) gene expressed in retinal progenitors and developing photoreceptors starting at E16. Its expression peaks at E18, coinciding with cone differentiation and decreases thereafter (Ng et al., 2011) (Yanagi et al., 2002). In contrast to TH, its expression is even across the retina (Ng et al., 2001) (Roberts et al., 2006) (Ng et al., 2011). *TR β 2*^{-/-} in photoreceptors abolishes M-cones at the expense of S-cones and the dorso-ventral gradient *in vivo* (Ng et al., 2001).

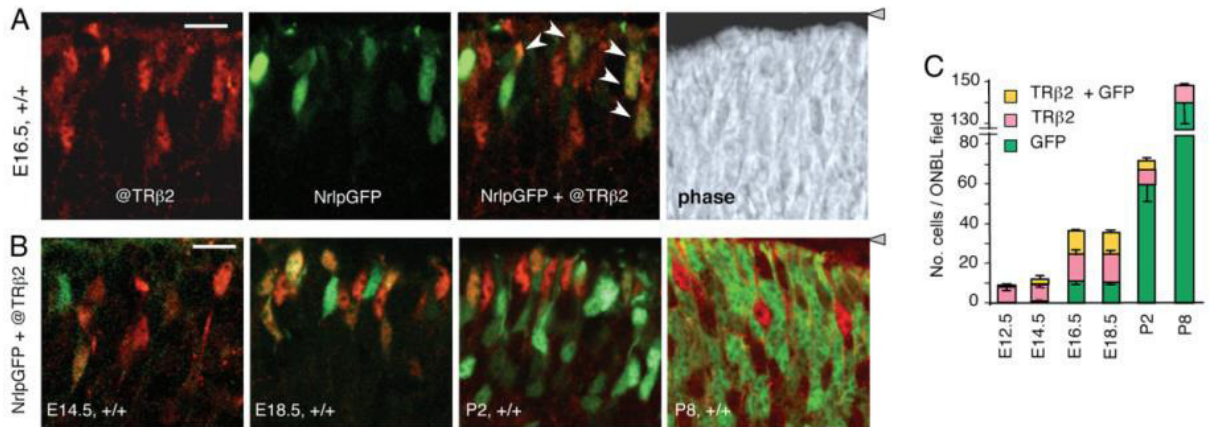


Figure 21. Temporal coexpression of *TRβ2* and *Nrl* in developing photoreceptor precursors.

(A) Analysis of *TRβ2* (red) and *Nrl*-GFP (green) expression in the outer neuroblastic layer in mouse retina at E16.5. Arrows (yellow or orange) indicate colocalization of *TRβ2* and *Nrl*-GFP. Grey arrowhead indicates RPE localization. (B) Analysis of *TRβ2*- and *Nrl*-GFP-positive cells positive at E14.5, E18.5, P2 and P8. (C) Cell counting: *TRβ2* and/or *Nrl*-GFP cells are observed predominantly at E16.5 and E18.5. Scale bar: 14 μ m. Figure reproduced from (Ng et al., 2011).

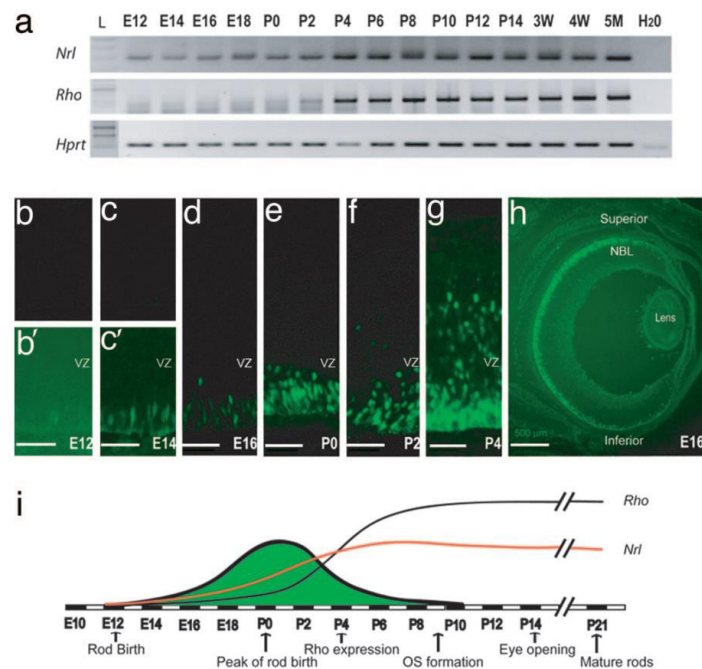


Figure 22. The time gap between *Nrl*-GFP and *Rhodopsin* expression in the developing mouse retina.

(a) RT-PCR shows the expression of *Nrl* and *Rhodopsin* (*Rho*) transcripts. *Nrl* is detected at E12, but *Rho* only at P4. (b', d, e, f, g) *Nrl*-GFP is first detected at E12 at long exposure. It increases from E16 to P4. (h) Low-magnification at E16 demonstrates dorsoventral gradient of *Nrl*-GFP expression. (i) Time course of rod photoreceptor genesis (green area) as well as kinetics of *Nrl* and *Rho* expression. As in (a) there is a delay of *Rho* expression. VZ, ventricular zone; NBL, neuroblastic layer. Scale bars: 25 μ m (b-g) and 25 μ m (h). Figure reproduced from (Akimoto et al., 2006).

In addition, expression of *TRβ2* at the *Nrl* locus in mice induces a conversion of rods into M-cones (Ng et al., 2011) revealing the **plasticity of a photoreceptor precursor** to become a rod, S-cone, or M-cone **depending on *TRβ2* and *Nrl* expression**.

To conclude, TH binding to Trβ2 promotes *M-opsin* and suppresses *S-opsin* expression triggering cone precursors to differentiate into M-cones (Figure 20) (Yanagi et al., 2002). Upon TH binding, Trβ2 preferably forms homodimers to induce *M-opsin* expression, and most probably, Trβ2 heterodimerizes with Rxr receptor to exert its function as a suppressor of *S-opsin* expression (Szanto et al., 2004).

Interestingly, posttranslational modifications also contribute to M- versus S-cone specification. In this context, *Pias3*^{-/-} retina exhibits reduced photopic b-wave amplitude following green light stimulation. This suggests a compromised visual response of M-cones. In contrast, no change in S-cones response was detected until 7 months (Campla et al., 2017). As such, Pias3 functions to activate expression of *M-Opsin* and represses *S-Opsin* expression by modulating the cone-transcription factors Rxrγ, Rorα and Trβ1 by sumoylation. Therefore, Pias3 contributes to establish the dorso-ventral cone gradient (Onishi et al., 2010) (Campla et al., 2017).

In summary, the commonly accepted photoreceptor specification model corresponds to a **two-step transcriptional switch**, which can direct three photoreceptor fates: first, ***Nrl* decides between rods and cones**, and second, if *Nrl* fails to act, ***TRβ2* dictates either M-cone or S-cone identity** (Yanagi et al., 2002) (Swaroop et al., 2010).

As delineated, in addition to transcription factor expression, their activity needs to be regulated by posttranslational modifications (PTMs).

B.3.4 Posttranslational modifications

The expression of a cone or rod specific factor in postmitotic photoreceptors is not mutually exclusive. Indeed, a transitory expression of both *TRβ2* and *Nrl* has been observed in postmitotic photoreceptor precursors (Figure 21) revealing the plasticity of a photoreceptor precursor to become a rod, S- or M-cone depending not only on the expression, but also on the **activity *TRβ2* and *Nrl*** (Ng et al., 2011). As described previously, *Nrl* can also be regulated posttranslationally. These PTMs influence the activity of transcription factors and control the balance required for rod photoreceptor differentiation and target genes expression (Onishi et al., 2009) (Roger et al., 2010) (Onishi et al., 2010). We hypothesize that by regulating *Nrl* activity and/or stability they also play a decisive role in the choice of rod versus cone cell fate.

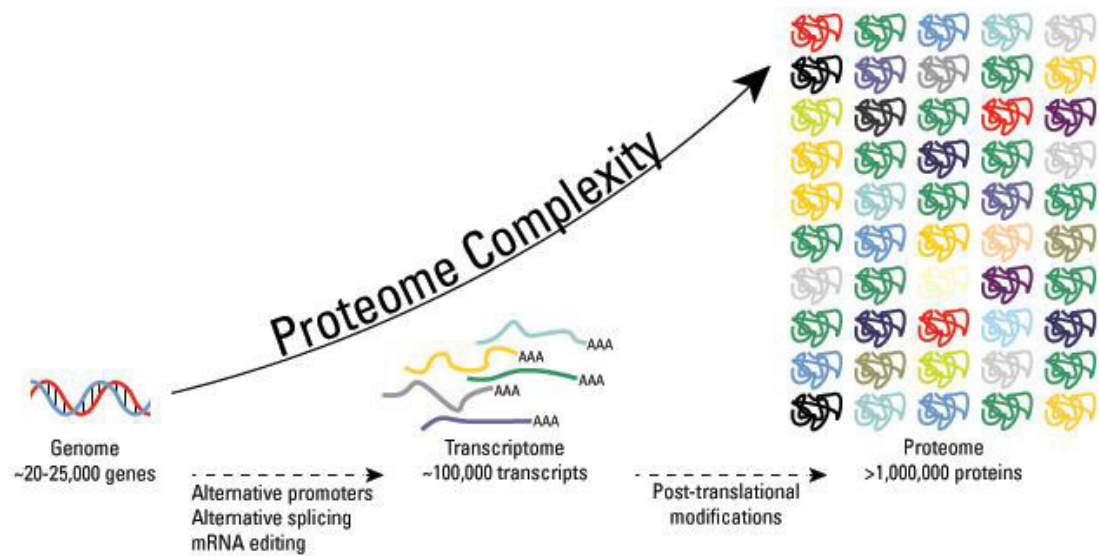


Figure 23. Posttranslational modifications exponentially increase proteome diversity.

The genome comprises 20,000 to 25,000 genes, but the proteome is estimated to contain over 1 million proteins. This increase is due to changes at the transcriptional and translational levels, and especially to posttranslational modifications. Figure taken from <https://www.thermofisher.com>.

Another fact supporting the significance of PTMs in retinal development is that despite the presence of key activators such as Nrl, Crx and Nr2E3 early in development, their target gene expression is delayed. Indeed, Nrl is detected at E12, but *Rhodopsin* transcript not before P2 with a peak at about P14 (Figure 22). This observation suggests that key factors to trigger this target gene expression are still missing or that activity of these transcription factors Nrl, Crx and Nr2e3 is regulated on an additional level, presumably also involving PTMs (Swaroop et al., 2010).

Generally speaking, PTMs are rapid and mostly reversible protein modifications to ensure a quick adaptation of the cell in response to a changing microenvironment, e.g. in response to cell stimuli. Notably, their different combinations of phosphorylation, sumoylation, acetylation, ubiquitinylation and other PTMs greatly increase the proteome complexity (Figure 23). They can play a pivotal role in a large number of physiological and pathological signaling pathways in the cell. Consequently, the identification and functional characterization of such modifications is critical to decipher the complexity of gene regulatory networks and their importance during the development and homeostasis of the system, and in our specific case for photoreceptors. In addition, there is rising evidence for their involvement in the context of neurodegenerative and psychiatric diseases of the brain as well (Ehrnhoefer et al., 2011) (Kontaxi et al., 2017) (Narayan and Dragunow, 2017) (Thomas, 2017).

Despite the importance of the PTMs in regulating key proteins activity and/or stability in retinal photoreceptors (Bessant et al., 1999) (Martinez-Gimeno et al., 2001) (DeAngelis et al., 2002) (Kanda et al., 2007) (Swain et al., 2007), the underlying mechanisms and functional consequences are still understudied.

In this context, my project concentrates on two essential and central enzymes introducing such PTMs, the Glycogen Synthase Kinases 3 (GSK3s). These kinases are important in regulating many signaling pathways and their deregulation has been observed in numerous pathologies of the nervous system, in particular neurodegenerative diseases. This potential link between GSK3 and neurodegeneration as well as the significance of PTMs in retinal photoreceptors has inspired us to delve deeper into the role of GSK3 in the retina, with a focus on photoreceptor development, maturation and survival.

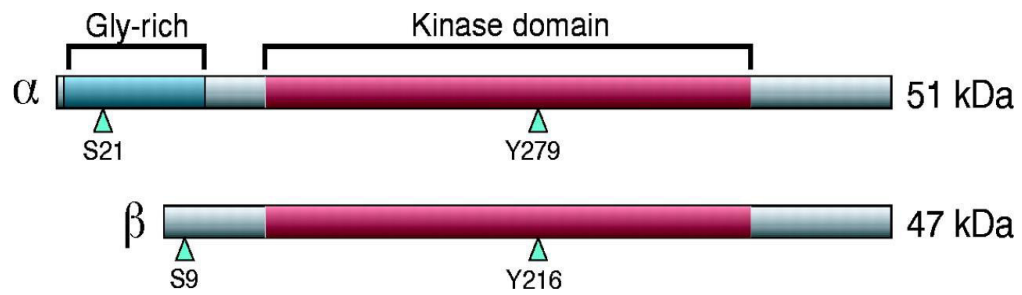


Figure 24. Schematic representation of the mammalian GSK3α and GSK3β.

The conserved kinase domain is shared by both isoforms. Sites of inactivating serine (S) and activating tyrosine (Y) phosphorylation are indicated with blue arrowheads. The glycine-rich N-terminal domain unique to GSK3α. Figure reproduced from (Doble and Woodgett, 2003).

B.4 Glycogen synthase kinase 3 alpha and beta

Among the central protein kinases are the Glycogen Synthase Kinases 3 (GSK3s). They were originally discovered in the late 1970s as key regulator of glucose metabolism. They are central node enzymes that phosphorylate the key rate-limiting metabolic enzyme in the glycogen pathway, the glycogen synthase, which catalyzes the last step of the glycogen synthesis upon insulin stimulation (Woodgett and Cohen, 1984) (Wang and Roach, 1993). Molecular cloning in the 1990s retrieved two isoforms, GSK α (51 kDa) and GSK3 β (47 kDa) encoded by two separate genes sharing 95 % homology in the catalytic kinase domain, however differing significantly in their N- and C-terminal domains (**Figure 24**) (Woodgett, 1990). GSK3s are ubiquitously expressed and conserved Ser/Thr kinases and belong to the CMGC family (**CDK**, **MAPK**, **GSK3** and **CLK** kinases) (Cole, 2012). Both kinases are highly expressed in the brain (Woodgett, 1990) (Yao et al., 2002) in both neurons and glial cells (Ferrer et al., 2002). Rodents and humans possess an alternative splice variant of GSK3 β , named GSK3 β 2 (49 kDa) carrying a 13-amino acid insertion near the catalytic domain. GSK3 β 2 is specifically expressed in the central nervous system compared to ubiquitously expressed GSK3 β (Mukai et al., 2002).

The physiological role GSK α and GSK3 β as multi-tasking kinases and node molecule involves coordination of multiple intracellular pathways activated by Wnt/ β -catenin, Sonic Hedgehog, Notch, growth factor/receptor tyrosine kinase (RTK), and G protein-coupled receptor signals. All these pathways are involved in central processes crucial for the central nervous system such as neurogenesis, neuronal migration and polarization, as well as axon growth and guidance (Hur and Zhou, 2010) (Sutherland, 2011).

B.4.1 GSK3 regulation and mode of action

Unlike most other kinases, GSK3 has a basal activity in resting cells and requires extracellular signaling for its inactivation.

➤ Mechanisms of GSK3 activity regulation

- **Phosphorylation of Ser21 in GSK3 α and Ser9 in GSK3 β** by other kinases within the phosphate binding pocket will impair the interaction of GSK3 with its substrate. Indeed, this binding pocket usually interacts with the primed Ser/Thr substrate and positions it for phosphorylation by GSK3. This inhibitory phosphorylation acts as a “pseudo-substrate” impairing substrate binding (**Figure 25**) (Dajani et al., 2001).

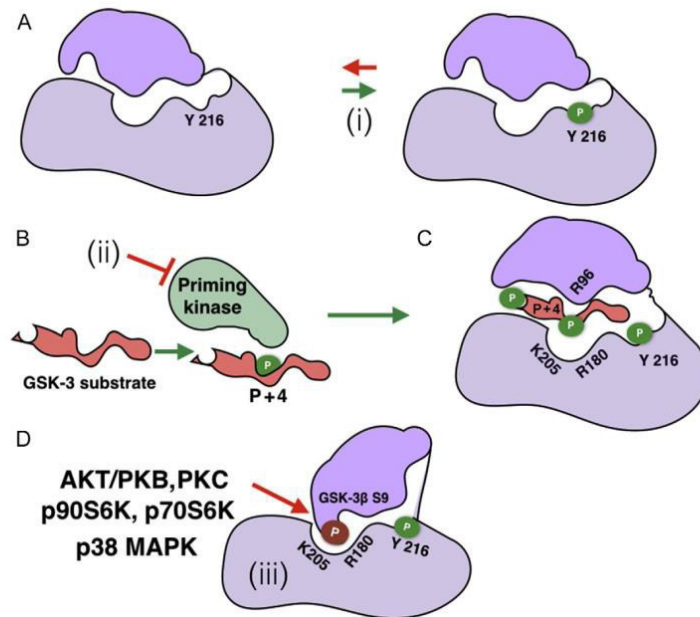


Figure 25. GSK3 regulation.

(A) GSK3β is constitutively phosphorylated at tyrosine 216 to facilitate substrate accessibility (i) Inhibition of tyrosine (Y) 216 phosphorylation may prevent efficient access of substrates to the GSK3 substrate binding site. (B, C) Priming is required for the majority of GSK3 substrates 4 amino acids after the first GSK3-phosphorylated residue (P+4) for proper substrate positioning. (ii) Inhibition of the priming site can prevent phosphorylation of GSK3 substrates. (D) Phosphorylation on S9 of GSK3β leads to the kinase folding into its own priming site binding pocket preventing substrate entry (ii) Kinases involving AKT/PKB introduce this inhibitory phosphorylation. Figure taken from (Patel and Woodgett, 2017).

This kind of inactivation is promoted by growth factors signaling, including insulin signaling pathway, or certain amino acids through other kinases of the AGC family (**PKA**, **PKG**, **PKC**) (Cross et al., 1995) (Eldar-Finkelman et al., 1995).

- Changes in GSK3 subcellular localization involving complex formation, such as is the case in the Wnt signaling pathway where the kinase activity is inhibited by **protein-protein interactions** and recruitment of GSK3 to the plasma membrane (**Figure 25**) (Thomas et al., 1999) (Frame et al., 2001).
- **Inactivation of the priming kinases of GSK3** which varies for each substrate. For example, for the microtubule-associated protein Tau, the priming kinase is CDK5 (**Figure 25**) (Li et al., 2006).
- Changes in **activating tyrosine phosphorylation**, Tyr279 in GSK3 α and Tyr216 in GSK3 β , required for proper folding and GSK3 activity. This is an autophosphorylation and most likely occurs constitutively (Cole et al., 2004) (Lochhead et al., 2006). For example, phosphorylation at Tyr216 in GSK3 β increases its activity to fivefold (**Figure 25**) (Hughes et al., 1993).

➤ **Criteria for a potential GSK3 substrate according to Sutherland**

Consistent with its implication in numerous signaling pathways, GSK3 has nearly 100 substrates (Sutherland, 2011) and many more have been predicted (Linding et al., 2007) (Shinde et al., 2017).

According to these studies, a putative GSK3 target has to meet the following criteria:

- Highly purified GSK3 should phosphorylate the substrate at a significant rate *in vitro* at residues on the substrate that are phosphorylated *in vivo*.
- Manipulation of GSK3 activity in cells and *in vivo* should alter the phosphorylation of the specific residue targeted by GSK3 *in vitro*. For instance, GSK3 inhibition should specifically reduce phosphorylation of its target in cells.
- Finally, a function of the substrate should change in parallel to alteration of phosphorylation and cellular GSK3 activity. Mutations of the GSK3 target residue to Ala, such as mutations within the priming phosphorylation site should render the target insensitive to GSK3 manipulation.

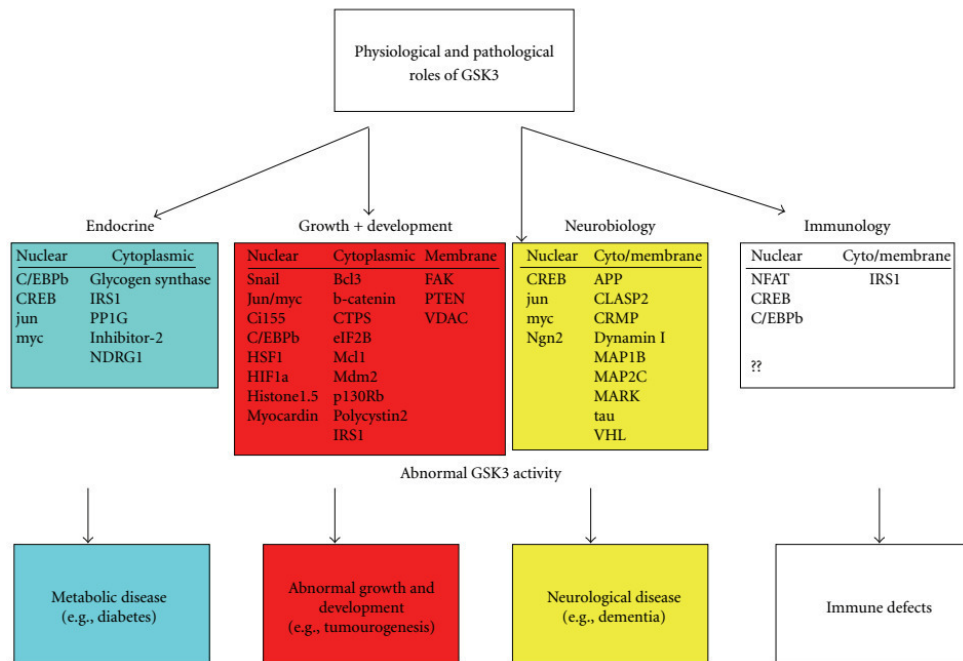


Figure 26. GSK3 mode of action.

Numerous GSK3 targets exert endocrine, growth and development as well as neurobiological and immunological physiological functions. Accordingly, GSK3 dysfunction is related to various pathological effects affecting multiple organs or tissues. Figure reproduced from (Sutherland, 2011).

These two kinases have a preference for substrates that have already been primed by phosphorylation before they can be efficiently phosphorylated by GSK3. The general GSK3 substrate consensus sequence is Ser/ThrXXX(pSer/pThr), where X is any amino acid. Additionally, a Pro is required prior to the priming kinase site. The priming kinase site is at a proximal Ser/Thr located 4 or 5 amino acids downstream of the residue to be phosphorylated by GSK3 (Thomas et al., 1999) (Cohen and Frame, 2001) (Doble and Woodgett, 2003).

Several kinases can function as priming kinases such as CDK5 (Sengupta et al., 1997) (Noble et al., 2003), casein kinases 1 and 2 (Amit et al., 2002) (Picton et al., 1982), PKA (Singh et al., 1995) and ERK (Woodard et al., 2015). For instance, c-myc, c-jun, Klf5, Cyclin E1, Gli3, and Snail are among the nuclear factors requiring phosphorylation by a priming kinase before the subsequent GSK3 action (Figure 26) (Sutherland, 2011) (Cole, 2012). There are, however, putative GSK3 substrates that do not need such a consensus sequence. They have a priming site much further from the consensus sequence, or do not require any priming (Sutherland, 2011).

GSK3 sequentially phosphorylates its target proteins, and often GSK3 phosphorylation occurs motif every fourth residue in a string of sequential sites (Beurel et al., 2015). For example, GSK3 phosphorylates Ser 652, 648, 644 and 640 in glycogen synthase, and residues 41, 37, and 33 in β -catenin.

The outcome of GSK3 phosphorylation can couple apparently antagonistic processes, ubiquitinylation/degradation, but also activation potential of target proteins, such as it has been demonstrated for MAFA (Rocques et al., 2007) (Herath et al., 2014). However, most of GSK3 substrates are targeted for ubiquitinylation and proteasomal degradation such as has been demonstrated for β -catenin of the Wnt signaling pathway.

B.4.2 GSK3 function in the nervous system

B.4.2.1 GSK3 at the crossroad of signaling pathways

➤ GSK3 and Wnt signaling

Activation of the Wnt pathway has been shown to preferentially promote neuronal proliferation and repress differentiation (Haegeler et al., 2003) (Kielman et al., 2002). Indeed, GSK3 is constitutively active in the Wnt signaling pathway to negatively regulate the transcriptional effector β -catenin (Figure 13).

In absence of Wnt, GSK3-triggered proteolysis of β -catenin and inhibition of the Wnt signaling favors differentiation (Aubert et al., 2002) (Cajane et al., 2009). Accordingly, upon *Gsk3* deletion, proliferation is promoted and differentiation is inhibited (Doble et al., 2007). Similar effects are observed upon pharmacological GSK3 inhibition and Wnt-induced inhibition of GSK3 activity, for example with previously mentioned Wnt3a and GSK3 inhibitor SB216763 (Sato et al., 2004) (Inoue et al., 2006) (Ying et al., 2008).

➤ **GSK3 and Hedgehog signaling**

Similar to the Wnt signaling pathway, GSK3 is constitutively active in the Hedgehog signaling pathways so as to negatively regulate the key transcriptional effector Gli triggering its degradation. Hence, GSK3 antagonizes the Hedgehog signaling pathway (Figure 14).

In addition, GSK3 can phosphorylate and stabilize the protein Sufu, a negative regulator of Hedgehog signaling, thus indirectly provoking degradation of Gli proteins (Chen et al., 2011).

➤ **GSK3 and Notch signaling**

GSK3 has been shown to bind and phosphorylate notch intracellular domain (NICD) and Notch2 *in vitro* and *in vivo*. However, in contrast to the Wnt and the Hedgehog signaling pathways, the cellular outcomes in terms of activation and inactivation await clarification (Figure 15) (Han et al., 2012) (Espinosa et al., 2003). While GSK3 α has been reported as a negative regulator of Notch1/NICD (Jin et al., 2009), findings concerning GSK3 β function on Notch are contradictory. Some studies reveal a protective function of GSK3 β on NICD from proteasomal degradation, whereas others show an inhibitory function of GSK3 on Notch target genes, including Hes1 (Foltz et al., 2002) (Cole, 2012).

➤ **GSK3 and insulin signaling**

In general, growth factors, such as EGF (Craig et al., 1996), FGF (Kuhn et al., 1997), Transforming growth factor (TGF) (Craig et al., 1996), and CNTF (Emsley and Hagg, 2003), contribute to neurogenesis in the developing and the adult brain by supporting the survival of neurons and protecting them from cell death (Yao and Cooper, 1995) (Kauffmann-Zeh et al., 1997) (Kennedy et al., 1997). The commonly used signal transduction pathway for growth factors is the phosphatidylinositol 3-kinase (PI3K) and Protein Kinase B (PKB or AKT) signaling pathway, referred to as PI3K/AKT. AKT inhibits GSK3 by phosphorylating it on Ser residues (Frame et al., 2001). PTEN is the major antagonist of the PI3K/AKT pathway.

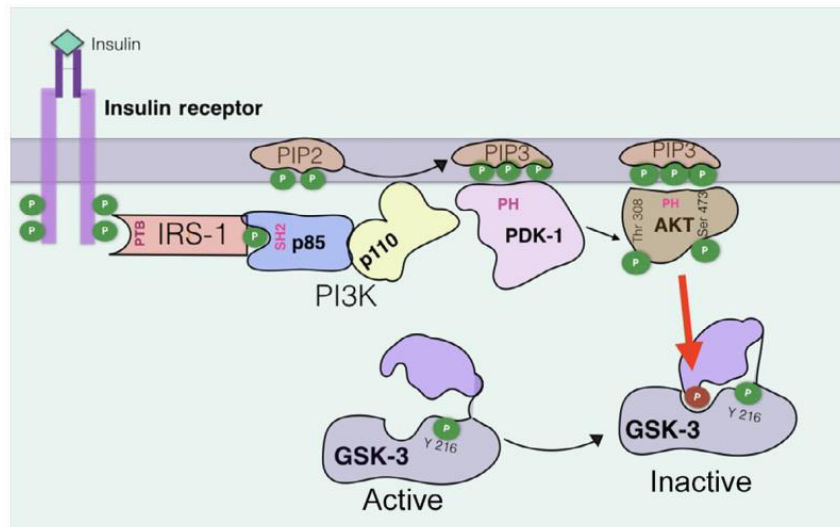


Figure 27. Insulin signaling pathway.

Insulin binding to insulin receptor (IR) phosphorylates insulin receptor substrates (IRS) on tyrosine residues. IRS interacts with phosphatidylinositol 3' kinase (PI3K) via its phosphotyrosin binding module that binds the Src Homology (SH2) domains of the regulatory subunit P85 of the PI3K. This interaction increases the activity of the catalytic subunit of PI3K, p110 so that PI3K can phosphorylate its substrate phosphatidylinositol biphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3). PIP3 recruits to the plasma membrane the serine kinases PDK-1 as well as PKB/AKT via their pleckstrin homology (PH) domains. PDK-1 phosphorylates and activates PKB/AKT that, in turn, **phosphorylates and inactivates GSK3 α and β** . This results in a reduced inhibitory phosphorylation of the glycogen synthase by GSK3 and **increased glycogen accumulation**. In addition, GSK3 can have an indirect inhibitory effect on protein phosphatase (PPI), a key regulator of insulin signaling, which converts PIP3 to PIP2, a feedback mechanism releasing the chain reaction that inactivates GSK3. GSK3 phosphorylates Inhibitor-2, a regulator of PP1 and antagonizes Inhibitor-2 function thereby inducing PPI activity. GSK3 also phosphorylates (IRS)-1 at Ser332, following priming at Ser336 and its subsequent degradation. Figure taken from (Patel and Woodgett, 2017).

To exemplify the growth factor signaling, I have chosen to focus on the insulin signaling pathway. It is a crucial metabolic pathway in many, if not all organs within an organism, including the brain and the retina. Defects in the insulin signaling pathway are related to glucose metabolic disorders (**Figure 27**) (Porte et al., 2005). In the central nervous system, insulin receptors (IR) as well as insulin signaling proteins are widely distributed (Havrankova et al., 1978). In retina photoreceptors, IR are present in photoreceptors with a higher basal level of phosphorylation than in other organs (Rajala and Anderson, 2010). Importantly, IR ablation in rod photoreceptors results in stress-induced photoreceptor degeneration. Indeed, IR contributes to the maintenance of rod photoreceptors during bright-light exposure (Rajala and Anderson, 2010) by preventing apoptosis of the retinal neurons through PI3K/AKT cascade (Barber et al., 2001) (Rajala et al., 2007) (Li et al., 2008). AKT activation inhibits opening of the mitochondrial permeability transition pore through the inactivation of GSK3 β by phosphorylation on Ser9 (Feng et al., 2005). However, whether GSK3 α also becomes inactivated, remains unclear.

All these results demonstrate that **GSK3 are clearly at the crossroad of several signaling pathways** and act as molecular switches mediating their output to regulate numerous cellular processes. As such, GSK3 has a major role in central nervous system development and homeostasis (Hur and Zhou, 2010).

➤ **Interaction of Wnt, Notch and growth factor signaling pathways involving GSK3**

Studies have demonstrated an association of FGF-mediated inhibition of GSK3 in the cell nucleus and a simultaneous activation of TCF/LEF and Notch transcriptional complexes, favoring proliferation and inhibiting differentiation (Shimizu et al., 2008), although other findings were contradictory. Interestingly, FGF signaling seems also to be required to promote proliferation of retinal stem cells following Wnt3a and SB216763 application to inhibit GSK3 (Inoue et al., 2006).

Interference of the Transforming growth factor β (TGF β) signaling with Wnt and FGF signaling has also been observed: Wnt signaling inhibits GSK3, preventing TGF β effector Smad4 degradation and, in cooperation with FGF signaling, extends the TGF β signaling (Demagny et al., 2014). This contributes to the survival of newly generated neurons and their differentiation (Kandasamy et al., 2014). In summary, GSK3 is often implicated in interactions between Wnt, Notch, and growth signaling pathways.

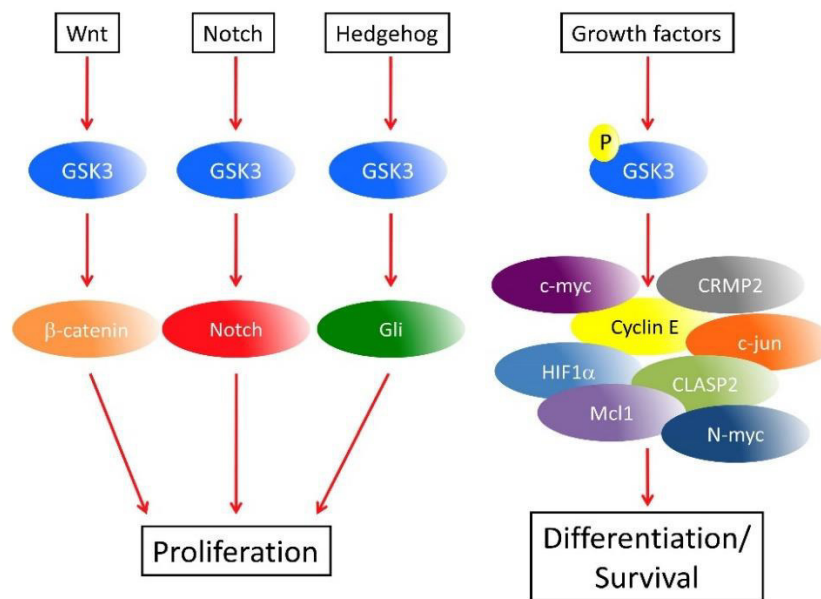


Figure 28. Comparison between developmental and growth factor signaling pathways on GSK3 regulation.

Wnt, Notch and Hedgehog signaling disrupt complexes involving GSK3, however without introducing inhibitory Ser phosphorylation on GSK3. This kind of GSK3 inhibition only has consequences on a single substrate. Conversely, growth factor-mediated inhibition of GSK3 occurs *via* phosphorylation of the inhibitory Ser reducing its action on many substrates. Figure reproduced from (Cole, 2012).

Generally speaking, activation of all these pathways commonly suppresses GSK3 activity and this inhibition appears to be a key mechanism to promote either proliferation or survival of newly-generated neurons during neurogenesis (Hur and Zhou, 2010) (Cole, 2012). However, the mechanisms of GSK3 inactivation differ between the Wnt, Notch, and Hedgehog signaling on one hand and growth factor signaling on the other hand. In the Wnt, Notch, and Hedgehog signaling pathways, ligand-mediated activation does not directly inhibit GSK3 activity by phosphorylation of inhibitory Ser within the N-terminus. Instead, these pathways disrupt the respective signaling/scaffolding complexes. Therefore, they exclusively reduce phosphorylation of a specific substrate. In contrast, growth factor signaling pathways directly inhibit GSK3 activity *via* phosphorylation of the respective residues within the N-terminus. Hence, growth factor signaling pathways globally affect GSK3 activity and therefore have an impact on a wider range of substrates (Figure 28).

B.4.2.2 GSK3 function in development and homeostasis of the nervous system

Due to its implication in several various targets, alterations of GSK3 expression have mostly severe consequences. Through the years, several animal models have been developed to decipher GSK function, but also to determine how much functional redundancy occurs between the isoforms α and β . Despite the high degree of structural similarity, the proposed redundancy between GSK3 isoforms in the Wnt signaling pathway (Doble et al., 2007) is largely refuted in other signaling pathways. Complete *Gsk3 β* , but also systemic *Gsk3 β* overexpression is embryonically lethal (Hoeflich et al., 2000) (Brownlee et al., 1997). On the contrary, *Gsk3 α ^{-/-}* are viable, but show enhanced glucose and insulin sensitivity and enhanced glycogen deposition in liver (MacAulay et al., 2007) (Patel et al., 2011).

➤ GSK3 in the control of neuronal proliferation and differentiation

To circumvent the systemic effects upon systemic *Gsk3 β* knockout or overexpression, conditional *Gsk3* mouse models have been developed, especially to study its function in brain development. *Gsk3* deletion in brain progenitors was achieved by crossing *Gsk3 α ^{-/-}* mice and *Gsk3 β* floxed mice (*Gsk3 β ^{fl/fl}*) carrying a *Nestin Cre*-recombinase (*Nestin-Cre*). Complete ablation of *Gsk3* in the developing brain resulted in a late embryonic lethal phenotype (Kim et al., 2009). During embryonic development, lack of GSK3 led to the hyperproliferation of neural progenitors along the entire neural axis.

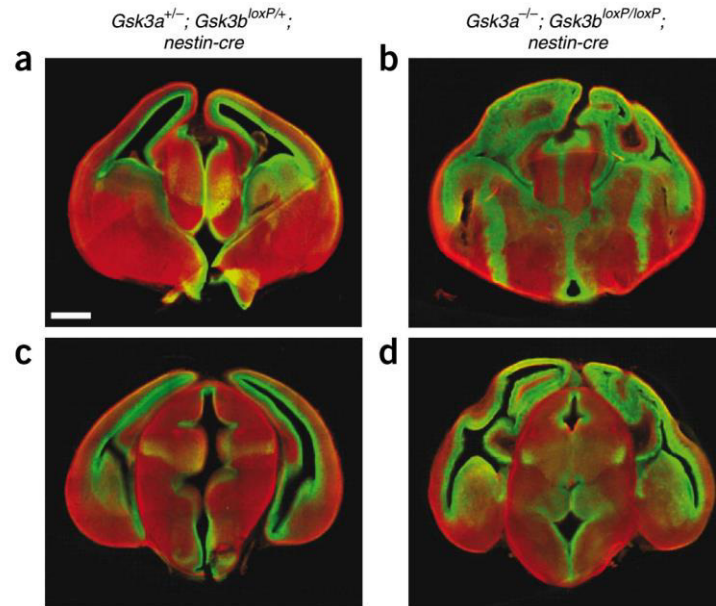


Figure 29. Conditional *Gsk3* ablation in the brain using *nestin-Cre* recombinase inhibits neurogenesis. (a-d). Progenitors stained by Sox2 (green) are extremely expanded, whereas maturing neurons, stained by Tuj1 (red) reduced throughout the whole brain in *Gsk3α*^{-/-}; *Gsk3β*^{loxP/loxP}; *nestin-cre* mice. a, b shows rostral and c, d caudal brain areas. Scale bar: 100 μm Figure reproduced from (Kim et al., 2009).

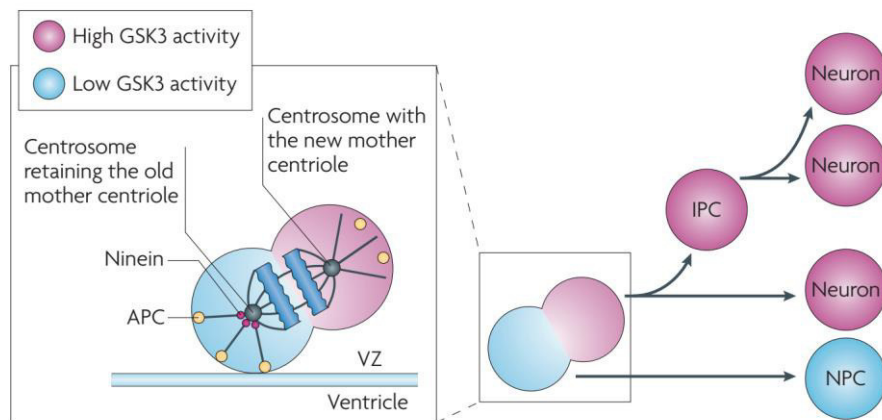


Figure 30. Proposed model for the role of GSK3 signaling during neurogenesis.

During asymmetric cell division, daughter cells have different GSK3 activities due to upstream regulators, such as disrupted in schizophrenia (DISC) and under the control of extrinsic factors, for example Wnt or FGF. **The daughter cell with lower GSK3 activity accumulates pro-proliferative factors**, such as β -catenin, Gli and c-myc. The microtubule-associated proteins adenomatosis polyposis coli (APC) and ninein organize the microtubules to attach it to the ventricular zone (VZ) surface and adopt a progenitor cell fate. This daughter cell **becomes a neural progenitor cell (NPC)**. **The daughter cell with higher GSK3 activity**, in contrast, **removes pro-proliferative proteins** and proteins for microtubule assembly presumably through ubiquitin proteasome system degradation. In this daughter cell, APC is unable to bind to microtubule. Therefore, the cell detaches from the VZ surface and becomes an **intermediate progenitor cell** or a **neuron**. Figure reproduced from (Hur and Zhou, 2010).

However, the cortex was thinner than in controls as generation of both intermediate neural progenitors and postmitotic neurons was suppressed. This severe phenotype was accompanied by a loss of radial polarity and neuronal migration (Figure 29) (Kim et al., 2009). The observed severe effects were correlated with a dysregulation of Hedgehog, Notch, Wnt, and FGF signaling and the associated effectors of the respective pathway. These results support the idea of GSK3 signaling as an essential mediator of homeostasis that regulates neural progenitors during mammalian brain development (Kim et al., 2009). GSK3 control of proliferative neural precursors could be explained by the fact that GSK3-phosphorylation inactivates cyclin D1 and cyclin E and leads to their proteolysis. As such, GSK3 has an inhibitory role on the cell cycle progression (Diehl et al., 1998) (Welcker et al., 2003).

These and further studies supported the function of GSK3 as a key regulator of neural precursor cell proliferation and maintenance of stem cells, such as it is the case in the subventricular zone of the brain, one of the two regions containing neural stem cells able to generate neurons in adults. Indeed, GSK3 controls the balance of progenitor proliferation and differentiation (Cartwright et al., 2005). GSK3 is highly active in maturing cells, but less active in undifferentiated proliferating neuronal cells mainly due to Wnt, Notch, and Hedgehog, all of which maintain these cells in proliferation until signals for differentiation are provided (Figure 30).

➤ **GSK3 in the control of axon formation and neural polarization**

In the hippocampus, a constitutively active GSK3 β mutant inhibited **axon formation and neuronal polarization**. In contrast, multiple axons were formed upon reduction of GSK3 β activity by pharmacological inhibitors. Mechanistically, AKT kinase and PTEN phosphatase upstream and CRMP2 downstream of GSK3 β balance GSK3 β activity and favors axon versus dendrite formation (Jiang et al., 2005) (Yoshimura et al., 2005).

In cortex and the hippocampus, conditional mouse mutants lacking both *Gsk3 α* and *Gsk3 β* in newly-born cortical excitatory neurons showed migration failure (Morgan-Smith et al., 2014). Hence, these mouse models reveal the function of GSK3 in neuronal polarization in the hippocampus as well as radial migration and dendritic orientation of cortical neurons.

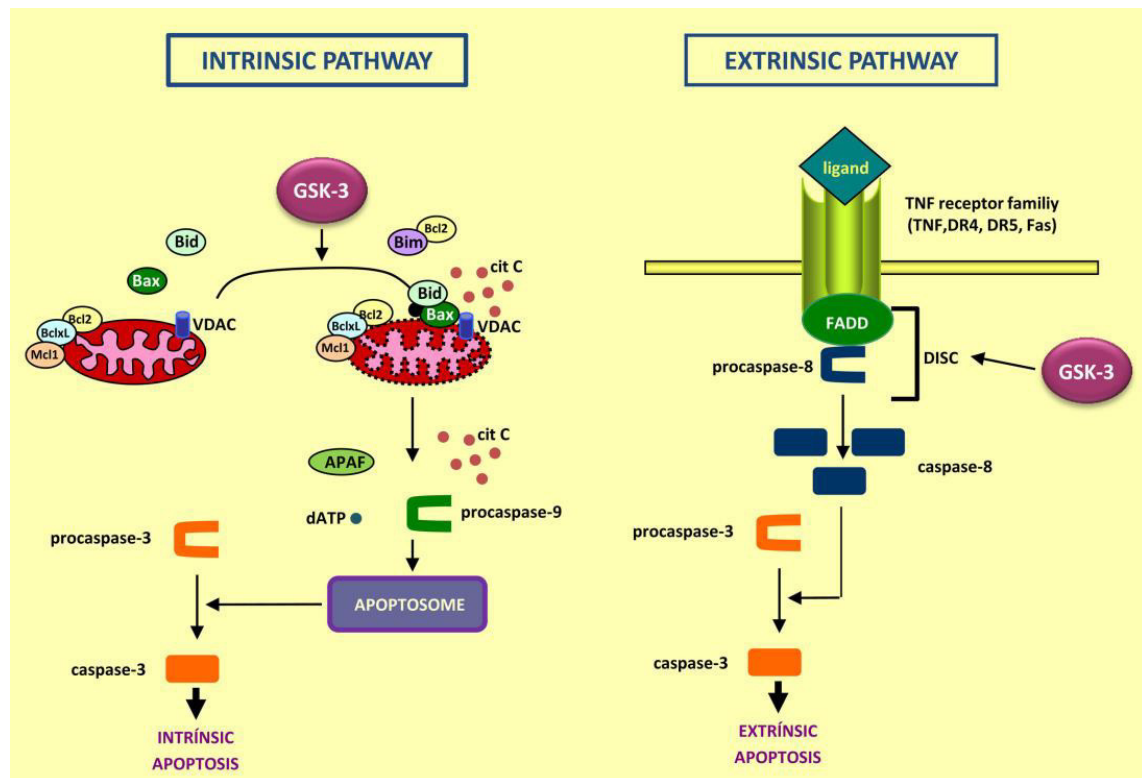


Figure 31. GSK3 involvement in intrinsic and extrinsic apoptotic pathways.

In the intrinsic apoptotic pathway, the role of GSK3 is pro-apoptotic (left). In the intrinsic apoptosis pathway, cytochrome c and other proteins of the mitochondrial intermembrane space are regulated by Bcl-2 protein family. The permeabilization of the outer mitochondrial membrane is mediated by the pro-apoptotic Bcl-2 protein Bax leading to cytochrome C (cit C) release into the cytoplasm. In the cytoplasm, cytochrome C forms, together with APAF, ATP and procaspase 9 the complex named apoptosome. This leads to a caspase activation that finally induces cell death. Bax activation requires direct phosphorylation by GSK3. GSK3 can also target other proteins of the apoptosome in a similar way, such as Bax, Bim or VDAC and thus promote permeabilization of mitochondria and cell.

In contrast, the role of GSK3 is anti-apoptotic in the extrinsic apoptotic pathway (right). In the extrinsic apoptotic pathway, death receptors of the TNF receptor family, located in the plasma membrane are activated. They involve TNF-R, Fas (CD95), DR4 and DR5. The stimulation by the ligand results in receptor trimerization allowing the binding of the protein Fas-associated death domain (FADD) and procaspase-8. This, in turn, triggers the formation of the death-inducing signaling complex (DISC). Caspase-8 becomes activated in the DISC complex leading to activation of effector caspases and initiation of apoptosis. GSK3 associates with the death receptor and forms an anti-apoptotic complex preventing the formation of DISC. When activated, the death receptor induces changes in the complex suppressing GSK3 and triggering extrinsic apoptosis. Figure taken from (Gomez-Sintes et al., 2011)

B.4.2.3 GSK3 at the balance of cell death and survival

The fact that PI3K inhibits cell death and inhibits GSK3 suggests a role of GSK3 in cell death regulation. For example, it has been shown that GSK3 overexpression induces apoptosis in Rat-1 fibroblasts, neuronal PC12, and human SH-SY5Y neuroblastoma cells. Cell death could be prevented in these cells using a dominant-negative *Gsk3* mutant, following inhibition of PI3K (Pap and Cooper, 1998) (Bijur et al., 2000). In most of these studies, the use of small molecule inhibitors of GSK3 such as SB-216763 and SB-415286 or lithium protected neurons from death, following PI3K inhibition, supporting the action of GSK3 inhibitors as neuroprotective in this context (Cross et al., 2001) (Cohen and Goedert, 2004) (Bijur et al., 2000). These and similar findings showed the pro-apoptotic role for GSK3 in neuronal cells, and the importance of GSK3 suppression in these cells for the pro-survival effect of PI3K signaling pathway.

This anti-apoptotic role of GSK3 is less studied. In this context, GSK3 inhibitors could be used to suppress tumor growth, including glioblastoma. Indeed, studies conducted in tumor cell lines show potentiation of the extrinsic apoptosis pathway by selective inhibitors of GSK3 (Liao et al., 2003) (Ougolkov et al., 2007). Importantly, inhibition of GSK3 β could be beneficial for glioblastoma treatment (Furuta et al., 2017). In 2004, GSK3 was reported as a component of the extrinsic apoptosis pathway in neuronal cells. In this context, apoptosis induced by the extrinsic pathway was enhanced by GSK3 inhibitors, such as lithium (Song et al., 2004).

Based on these studies, **GSK3 seems to have opposite roles in apoptosis**. In fact, the form of apoptosis is determinant: GSK3 promotes cell death caused by the mitochondrial intrinsic apoptotic pathway (Type I) but inhibits the death receptor-mediated extrinsic apoptotic pathway (Type II). Consequently, inhibitors of GSK3 protect from intrinsic apoptotic signaling but potentiate extrinsic signaling (**Figure 31**) (Beurel and Jope, 2006).

B.4.2.4 GSK3 implication in diseases of the nervous system

The dual function of GSK3 in cell death presented in the previous paragraph is illustrated by its ambiguous role in disease contexts.

A significant role of GSK3 in neuroscience and neurodevelopment has emerged with the discovery that GSK3 phosphorylates the microtubule-associated protein Tau at sites relevant for Alzheimer's disease (Takahashi et al., 1994).

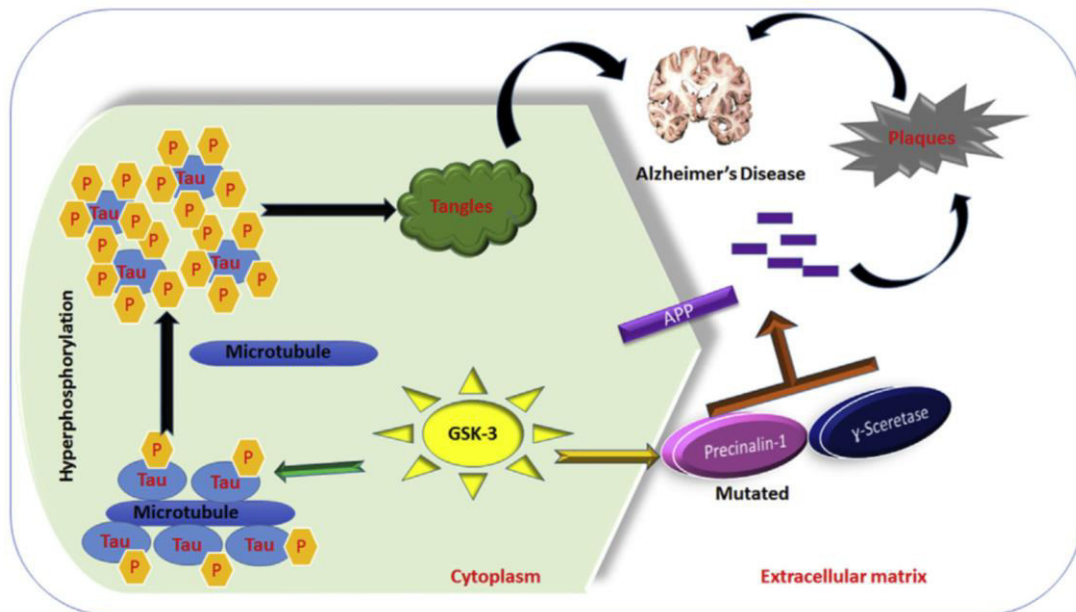


Figure 32. GSK3 involvement in Alzheimer's disease progression.

GSK3 phosphorylates the tau protein and contributes to the β -amyloid peptide ($A\beta$) production from the amyloid precursor protein (APP). These are the two main mechanisms causing Alzheimer's disease. Figure reproduced from (Maqbool et al., 2016).

Since then, several studies have shown GSK3 implication in the pathogenesis of the central nervous system, including neurodegenerative diseases, such as Alzheimer's (Kremer et al., 2011) (Maqbool et al., 2016), and Parkinson's (Li et al., 2014a) (Golpich et al., 2015) or psychiatric diseases (O'Leary and Nolan, 2015) (Jope et al., 2017) including schizophrenia (Emamian, 2012), and brain tumors (Atkins et al., 2013) (Majewska and Szeliga, 2017). Several independent studies have also shown the involvement of GSK3 in the serotonin and dopamine neurotransmitter action for antidepressant and antipsychotic drugs (Beaulieu et al., 2011) (Polter and Li, 2011) (Polter et al., 2012), suggesting that GSK3 may represent an important target for treating mental illness.

➤ **Neurological diseases**

In Alzheimer's disease, GSK3 promotes every major pathological process, including β -amyloid peptide production (Phiel et al., 2003) (Serenio et al., 2009) and tau phosphorylation, promoting its aggregation into neurofibrillary tangles (Figure 32) (Himmelstein et al., 2012). β -amyloid plaques together with neurofibrillary tangles are the hallmarks of Alzheimer disease. However, β - amyloid peptide production triggered by GSK3 has been disputed (Jaworski et al., 2011). In addition, GSK3 also promotes apoptosis by modulating proteins in the apoptosis signaling pathway, which likely contributes to cell loss during Alzheimer's disease progression (Mines et al., 2011).

Transgenic mice with an increased GSK3 activity exhibit hyperphosphorylated Tau and develop subsequent neurological defects, whereas lithium-mediated treatment reduces the number of tangles in the brain (Lucas et al., 2001) (Noble et al., 2005). GSK3 inhibitors are also used to improve cognitive functions and improve dementia in rodent models of Alzheimer's disease (King et al., 2014). However, clinical trials using lithium to inhibit GSK3 in Alzheimer's diseases revealed contradictory results. Some studies clearly demonstrated its beneficial effect on cognitive function (Forlenza et al., 2011) (Nunes et al., 2013), whereas others failed to do so (Hampel et al., 2009).

New GSK3 inhibitors, including specific small molecule inhibitors, have been generated to protect against GSK3-mediated progressive cell death (Noh et al., 2013) (Tell et al., 2016). Nonetheless, defects in GSK3 are not a direct cause of Alzheimer's disease as no mutations or polymorphisms have been consistently detected in patients. Instead, GSK3 acts as an "environmental sensor", reacting to stimuli such as growth factors and relaying them as cell signals to influence cell fate.

Altogether, there is abundant evidence that inhibition of GSK3 can be therapeutic for a number of neurological disorders, particularly for Alzheimer's disease (Medina and Avila, 2010) (King et al., 2014) (Maqbool et al., 2016). Nevertheless, more work is needed to understand how GSK3 is regulated and its mode of action. In particular, there is a need to design specific inhibitors to target specific cellular functions of GSK3.

GSK3 inhibitors are also expected to have a beneficial effect for other tauopathies, pathologies presenting a tau hyperphosphorylation and aggregation, including Parkinson's. In fact, the role of GSK3 β has also been demonstrated in Parkinson's disease progression, a neurodegenerative disease characterized by neuronal apoptosis in the substantia nigra and depletion of dopamine in the striatum (Golpich et al., 2015). Therefore, GSK3 inhibitors may counteract neuronal loss by reducing neurotoxicity in Parkinson's disease (Golpich et al., 2015). GSK3 inhibitors such as lithium have been shown to stop α -synuclein-mediated Tau phosphorylation and aggregation in Parkinson's diseases models making some of them potent therapeutic candidates. One example is SC001 which is able to cross the blood-brain barrier and exhibits a protective function on dopaminergic neurons and neuroinflammation reduction (Duka et al., 2009) (Morales-Garcia et al., 2013). So far, only few genetic variants within the *Gsk3* gene have been linked to neurodegenerative diseases of the brain. For Alzheimer's disease, so far only one genetic variant in *Gsk3 β* , which is under others associated with cognitive function deficits (Kettunen et al., 2015). For Parkinson's disease, a polymorphism has been identified to alter *Gsk3 β* transcription and splicing. Surprisingly, the identified polymorphism may increase or decrease the risk for Parkinson's disease (Kwok et al., 2005).

➤ Neuropsychiatric diseases

Lithium, developed by Australian psychiatrist John Cade in 1949, has been used for over fifty years for treatment of bipolar disorders. It has shown benefits in approximately 60 - 80 % of patients (Jope, 1999) (Phiel and Klein, 2001). However, the mechanisms of its action have been unknown until recently. Indeed, lithium inhibits GSK3 activity directly or indirectly (Klein and Melton, 1996). Therefore, GSK3 can be a putative therapeutic target for bipolar disorder (Dandekar et al., 2017).

GSK3 dysfunction is also associated with other psychiatric disorders because of its modulation of dopamine and serotonin levels (Beaulieu et al., 2009) (Valvezan and Klein, 2012).

However, unlike bipolar disorder patients, schizophrenic patients do not respond to lithium although elevated levels of GSK3 have been detected in schizophrenic patients. This may be due to alterations in upstream molecules regulating GSK3 activity, such as Disrupted in Schizophrenia 1 (DISC1) and Akt1, which are genetically linked schizophrenia.

Under normal conditions, DISC1 directly binds GSK3 and reduces its β -catenin phosphorylation. Reduced β -catenin stabilizes the protein to activate proliferative genes. Conversely, inactivating DISC1 mutations or DISC1 deletion abolishes the inhibitory effect on GSK3 rendering GSK3 hyperactive. Hence, GSK3 exerts a stronger inhibitory function on Wnt signaling. This is likely to impair neurogenesis and may affect both the developing and the adult brain function contributing to schizophrenia progression. This has been determined in mouse models. The effects could be abolished using GSK3 pharmacological inhibitors (Mao et al., 2009) (Singh et al., 2011). Other schizophrenic patients demonstrate reduced Akt1 levels, with concomitantly decreased GSK3 phosphorylation and inactivation, and thus increased GSK3 activity (Emamian et al., 2004).

Unlike with neurodegenerative diseases, several *Gsk3* polymorphisms have been associated with psychiatric diseases (Cole, 2013), such as schizophrenia and bipolar disorder as well as major depressive disorder (Inkster et al., 2009) (Liu et al., 2017). Therefore, a better understanding of GSK3 functions and targets could provide important insights for the development of new pharmaceutical drugs for a large spectrum of brain disorders. With this objective, several studies identified new potential targets and developed new algorithms to predict GSK3 phosphorylation sites and clearly demonstrated the large number of substrates (Linding et al., 2007). Other selective and potent GSK3 inhibitors are currently in development (Gentile et al., 2012) (Maqbool et al., 2016).

In the context of neuropsychiatric diseases, it is of great importance to identify measurable indicators or biomarkers to distinguish physiological and pathological processes and assess the outcome of a therapeutic intervention and, understand the underlying mechanism. An interesting link between neuropsychiatric diseases and the retina involving the central molecule GSK3 has been recently provided. Lavoie and colleagues found that rods' ERG b-wave amplitude was lower in young asymptomatic offspring of patients with schizophrenia or bipolar disorder than in controls. The ERG changes were also found in different mouse models reflecting neuropsychiatric diseases, i.e. the dopamine 1 receptor knockout mice. The role of dopamine on ERG b-waves is known in the retina and therefore the authors suspected that the observed b-wave changes might reflect the dopaminergic dysfunction in the brain suggesting the use of ERG as a strong biomarker for neuropsychiatric diseases (Lavoie et al., 2014b).

Using three genetically altered mouse strains, the authors demonstrated that reduced GSK3 β expression increased the b-wave amplitude under scotopic conditions, suggesting a negative correlation between GSK3 levels and ERG amplitude. Conversely, reduced GSK3 α expression increased b-wave amplitude under both scotopic and photopic conditions, demonstrating the non-redundancy of both isoforms in this context. Taken together, the data suggest that ERG b-wave alterations in high-risk offspring of patients with schizophrenia or bipolar disorders reflect genetically predisposed changes in GSK3. Thus, GSK3 functions as a link, and could provide a molecular explanation, between the observed phenotype and the ERG b-wave amplitude defects (Lavoie et al., 2014b) (Lavoie et al., 2014a).

B.4.2.5 GSK3 function in the retina

The retina and the brain share physiological and pathological molecular pathways. Therefore, diseases affecting the brain and the retina show similarities. For instance, neuroinflammation is a symptom for brain pathologies, including mood disorders (Felger, 2017) (Garcia-Alvarez et al., 2017) and Alzheimer's disease (Ardura-Fabregat et al., 2017), but also retinal pathologies, such as retinitis pigmentosa and diabetic retinopathy (Tang and Kern, 2011). In the addressed example, the implication of GSK3 in neuroinflammation provides a link for all the mentioned pathologies (Joje et al., 2007).

As depicted, the use of conditional knockout mice for both *Gsk α* and *Gsk3 β* in the developing brain has clearly contributed to a better understanding of GSK3 function during CNS embryonic development. Nonetheless, since the animals lacking both *Gsk*s genes did not survive until adulthood, there are clear limitations in delineating GSK3 function in mature neurons of the brain (Kim et al., 2009) (Morgan-Smith et al., 2014). Therefore, and as will be presented in the results section, the retina as a part of the brain with high similarities in physiological and pathological molecular pathways offers an advantageous and versatile approach to delineate GSK3 function in the nervous system. Up to date, there are only few studies depicting GSK3 function in the retina.

The subcellular localization of both Glycogen Synthase and its regulating enzyme GSK3 β in the retina was reported suggesting their involvement in glycogen content control in the retina (Perezleon et al., 2013). However, in this study, the localization of GSK3 β in photoreceptors and Müller cells is not convincing and requires further analyses. Subsequent investigations revealed GSK3 function as a key node in neuronal death both in ischemic injury associated with glaucoma and in diabetic retinopathy (Russo et al., 2015) (Li et al., 2014b).

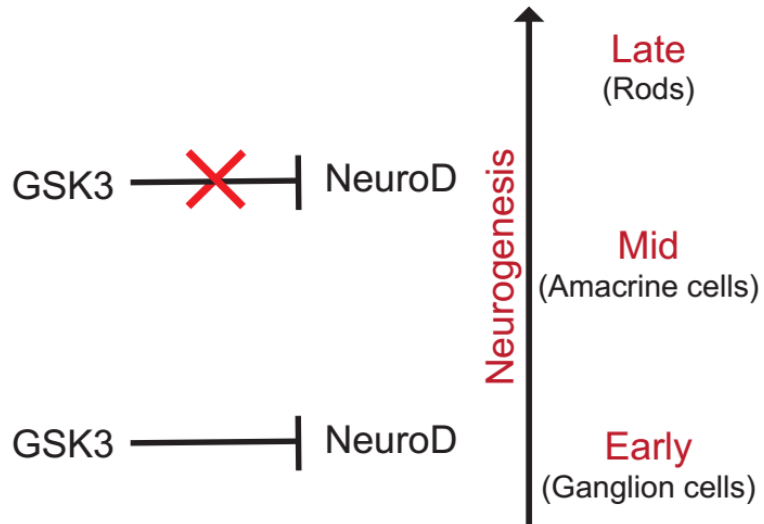


Figure 33. GSK3 phosphorylation of NeuroD regulates the timing of its activity.

At early phases of retinal development, GSK3 inhibits NeuroD activity, thus triggering generation of RGCs. During further development, GSK3 inhibitory function on NeuroD is released so that NeuroD can promote generation of later-born cell types, such as amacrine cells. Figure adapted after (Moore et al., 2002).

Importantly, a recent study reported an absent or reduced Ser phosphorylation of both GSK3 α or GSK3 β in peripheral blood mononuclear cells of AMD patients, accompanied with increased phosphorylation of GSK3 substrates, such as insulin receptor substrate 1, and TAU, indicative of enhanced GSK3 activation. In addition, PI3K activation was reduced contributing to increased GSK3 activation (Liu and Yao, 2017).

There have been attempts to use GSK3 inhibitors to treat retinal degenerative diseases. This is the case for lithium which has been used to decrease the elevated ocular pressure, a hallmark of glaucoma, in a rat glaucoma model (Sun et al., 2014). Furthermore, valproic acid has been used as a direct inhibitor of GSK3 to treat retinitis pigmentosa patients but showed only a short-time beneficial effect (De Sarno et al., 2002) (Kumar et al., 2014) (Iraha et al., 2016). The mechanism for this protective effect remains unclear.

Recently, a direct neuroprotective effect of GSK3 inhibitors in retinal disease context has been demonstrated in retinal explants. Two models of retinal degeneration were used: the first one to mimic retinitis pigmentosa (using the *rd10* mouse model, a homozygous recessive mutant for phosphodiesterase 6b) and the second one to mimic glaucoma (wildtype retina treated with 50 mM NMDA). Three different small molecules with different potency and binding to GSK3 were used as pharmacological agents including tideglusib, a compound used in clinical trials for autism spectrum disorders (Marchena et al., 2017). The authors found that GSK3 inhibition reduced cell death of RGCs and photoreceptors. This suggests that GSK3 inhibitors are a potential therapeutic strategy for a broad number of retinopathies.

There are only few studies depicting the direct GSK3 function, and in general the role of PTMs, in the retina. In *Xenopus* retina, GSK3 has been reported to control retinal differentiation by inhibiting the major neurogenic bHLH transcription factor NeuroD at early stages of development (Marcus et al., 1998) (Moore et al., 2002). NeuroD promotes determination of later born cell types, such as amacrine cells (Figure 33). Upon expression of a dominant negative form of GSK3 β , RPCs adopt early born cell fate to differentiate into RGC at the expense of later born cell types. In line with that, NeuroD mutants, which are insensitive to GSK3 β , also generate earlier-born RGC. Hence, GSK3 controls the timing of NeuroD action. During early stages of retinal development, GSK3 phosphorylates and inhibits NeuroD, and this inhibition is abrogated in later stages of development allowing NeuroD to exert its activity. This form of inhibition is poorly understood in mammalian retina but highlights the importance of PTMs such as GSK3-mediated phosphorylation (Moore et al., 2002).

Consistent with this idea, GSK3 has been shown to regulate the stability of the bHLH transcription factor NeuroAB, involved in maturation and maintenance of bipolar and GABAergic amacrine cells in the chick retina (Ohkawara et al., 2004). These results are in line with data obtained in the brain, reporting that GSK3 controls the bHLH transcription factor Neurogenin 2 (Neurog2), involved in cell fate decision (Yan et al., 2001). Its proneural activity declines in late corticogenesis concomitantly with its phosphorylation by GSK3 whose activity increases during corticogenesis (due to a decreasing Wnt signaling) (Li et al., 2012). Altogether, these studies provide evidence for the direct implication of GSK3 kinases to control activity of proneural bHLH transcription factors in both the brain and the retina. To delve deeper into the mechanisms underlying cell fate specification in physiological and pathological conditions, it is therefore crucial to identify other potential candidates regulated in a similar fashion by GSK3.

Another example of GSK3 β function in the retina has been described on the arborization field and maturation of RGCs in zebrafish. The expression of the dominant negative form of GSK3 β was associated with an inhibition of the arborization field of retinal ganglion cells axon terminals (Tokuoka et al., 2002).

Interestingly, in zebrafish retina, the Wnt signaling pathway activation and consequently GSK3 β inhibition was found to stimulate dedifferentiation of Müller cells generating multipotent retinal progenitors that could differentiate in all retinal cell types (Ramachandran et al., 2011). Therefore, GSK3s may be promising drug targets for neural regeneration.

Apart from these few studies, the function of GSK3 is poorly described in a retinal context, and especially in mammalian retina. Therefore, the objective of my Ph.D. thesis is to decipher the function of GSK3 both in developing and mature retina.

B.5 Objective of my Ph.D. project

GSK3s are major players in the progression of many neurological and psychiatric diseases. Drugs targeting GSK3s hold a lot of promises to treat such diseases. However, due to its involvement in numerous signaling pathways, direct GSK3 inhibition in patients may result in detrimental side-effects. Therefore, selective targeting of GSK3 target proteins rather than the global suppression of GSK3 kinases would offer a better therapeutic approach. Hence, the global aim of my project was to identify such critical GSK3 targets and pathways that are implicated in disease progression. However, despite excessive studies in the nervous system, the vital role of both GSK3 kinases in the brain has impaired further investigation in mature neurons and, consequently, identification of its targets (Kim et al., 2009).

The retina, as part of the nervous system provides a perfect model to investigate the role of GSK3 kinases in developing and mature neurons and should also allow for identification of GSK3 targets. The physiological and pathological functions of GSK3 in the retina are largely unknown. Thus, it remains to be clarified whether GSK3 kinases are important for retinal development and implicated in retinal diseases.

In this context, the general objective of my Ph.D. work was to delineate the function of GSK3 kinases during development of retinal progenitors and focused on its role in photoreceptor development and homeostasis.

At the time I initiated my Ph.D., my supervisor Jérôme Roger just finished his postdoc at the NIH in the US in the laboratory of Anand Swaroop where he performed preliminary experiments on GSK3 function in the retina. He found severe eye developmental defects in mice associated with a total absence of GSK3 in the retina. However, and in contrast to the phenotype in the brain, the animals survived until adulthood. These preliminary data were very encouraging to pursue this project studying the role of GSK3 in the retina. In addition, during his postdoc, Jérôme Roger was focusing on transcription factor regulation by posttranslational modifications during retinal development and homeostasis. Based on the central role of GSK3 in development and disease, he generated a mouse line to specifically elicit GSK3 function in photoreceptors, the main cells for vision, whose dysfunction is often linked to retinal disease. The characterization of this mouse line started in France.

C Results

C.1 GSK3 function in retinal progenitors

We first investigated the function of GSK3 during early retinal development. To circumvent mouse lethality, we opted for the conditional knock-out strategy and generated a mouse line with a specific deletion of both *Gsk3α* and/or *Gsk3β* in retinal progenitors. Using this mouse model, we studied the consequences of GSK3 loss during development and in adult murine retina.

Associated publication (to be submitted in Spring 2018)

Braginskaja E, Vigouroux RJ, Thomas L, Shah P, Chédotal A, Perron M, Swaroop A, Roger JE. Glycogen Synthase Kinase 3 maintains the pool of progenitors and restrains ganglion cells production in the inner nuclear layer during early retinal development.

Used animal models

To achieve conditional deletion of *Gsk3α* and/or *Gsk3β* specifically in retinal progenitors, we used the α -Cre mouse line carrying the retina-specific regulatory element of murine *Pax6* driving the Cre recombinase (α -Cre) in retinal progenitors as early as E10.5 (Marquardt et al., 2001). These mice were mated with *Gsk3α* and/or *Gsk3β* floxed mice (*Gsk3α^{fl/fl}* and/or *Gsk3β^{fl/fl}*), which were generously provided by Dr. Jim Woodgett (University of Toronto, Canada) (MacAulay et al., 2007) (Patel et al., 2008). We generated mice carrying either

- a complete deletion of both *Gsk3α* and *Gsk3β* (*Gsk3α^{fl/fl}β^{fl/fl}*; α -Cre) or
- a partial $\frac{3}{4}$ *Gsk3* deletion (*Gsk3α^{fl/+}β^{fl/fl}*; α -Cre or *Gsk3α^{fl/fl}β^{fl/+}*; α -Cre) or
- a deletion of either *Gsk3α* or *Gsk3β* (*Gsk3α^{fl/fl}β^{+/+}*; α -Cre or *Gsk3α^{+/+}β^{fl/fl}*; α -Cre)

Phenotypic analysis

The mouse phenotypes were studied using classical laboratory methods such as EdU labelling, immunohistochemistry, immunoblotting but also electroretinography. Based on the obtained exciting results, we started very recently a collaboration with the team of Alain Chédotal at the Vision Institute in Paris to investigate potentially altered axonal projections RGCs to the brain.

C.1.1 Main results

- Loss of both *Gsk3 α* and *Gsk3 β* in retinal progenitors impairs retinal development, leading to cell death and microphthalmia in adults.
- Loss of *Gsk3* signaling blocks neuronal differentiation.
- Partial *Gsk3* loss leads to an increased number of displaced ganglion cells (dRGCs).
- These dRGCs are produced at the same time as other ganglion cells.
- Partial *Gsk3* loss alters axonal projections of RGCs, which is presumably due to the increased number of dRGCs.

Altogether, our results demonstrate an essential role of GSK3s for both retinal proliferation and differentiation during development. Importantly, we demonstrate an essential role of GSK3s in dRGC genesis. Our model thus offers a great tool to further investigate dRGC specification and function in the retina.

C.1.2 Article n°1

Glycogen Synthase Kinase 3 maintains the pool of progenitors and restrains ganglion cells production in the inner nuclear layer during early retinal development.

Elena Braginskaja, Robin J Vigouroux, Leah Thomas, Parth Shah, Alain Chédotal, Muriel Perron*, Anand Swaroop* and Jerome E Roger*

Glycogen Synthase Kinase 3 maintains the pool of progenitors and restrains ganglion cells production in the inner nuclear layer during early retinal development

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Running Title: **GSK3 regulates ganglion cell genesis**

ABSTRACT

Glycogen Synthase Kinase 3 (GSK) proteins are key mediators of multiple signaling pathways, with crucial roles in coordinating fundamental biological processes during neuronal development. GSK3 alpha (GSK3 α) and beta (GSK3 β) are encoded by two different highly homologous genes and control the balance between neural progenitor proliferation and differentiation in the central nervous system. Here we show the impact of complete or partial deletion of *Gsk3 α* and *Gsk3 β* alleles during early retinal development. Complete loss of GSK3 signaling in retinal progenitors leads to microphthalmia with broad retinal morphological defects. At the cellular level, lack of GSK3 impairs proliferation of retinal progenitors, resulting in defects in neuronal differentiation and enhanced cell death. A single allele of *Gsk3 α* or *Gsk3 β* is able to rescue these phenotypes. Nonetheless, we detected a dramatic increase in the number of Brn3a-positive retinal ganglion cells in the inner nuclear layer in the presence of only one *Gsk3* allele. These ganglion cells exhibited characteristics of displaced retinal ganglion cells (dRGCs), for which fate determinants are currently unknown. It is unclear whether dRGCs, that are produced concomitantly with orthotopic ganglion cells, represent a rare subtype or whether they originate from migration defects during early retinal development. Finally, we demonstrated that *Gsk3 β* function is critical for the inhibition of RGC axonal projection to the ipsilateral medial terminal nucleus. Our studies thus reveal an unsuspected role of GSK3 in controlling the genesis of dRGCs during retinal development and represent an opportunity to unravel the origin and function of dRGCs in vision.

Key words: Retinal development, Glycogen Synthase Kinase 3, Posttranslational modifications, Displaced ganglion cells, Cell death, Proliferation

INTRODUCTION

Glycogen Synthase Kinase 3 alpha (GSK3 α) and beta (GSK3 β) are serine/threonine kinases encoded by two different genes, sharing 95 % of identity in their kinase domain and well-known functional redundancy (1). Among the different signaling pathways regulated by GSK3 kinases, Wnt canonical pathway is the most well described, with GSK3 β inhibition triggering an increase of β -catenin protein levels and its nuclear translocation to activate target gene expression (2). GSK3 kinases are inhibited by FGF signaling *via* phosphatidylinositol-3-kinase (PI3K) (3). These kinases are also modulator of the Notch and Hedgehog signaling pathways in vertebrates (4-6). Therefore, GSK3 exists at the crossroad of multiple signaling pathways and acts as molecular switch to mediate their output and regulate distinct cellular processes. GSK3 function is mediated by phosphorylation of a wide range of substrates associated with transcription, proteosomal degradation, microtubule dynamics, signaling molecules, and intracellular transport (2, 7).

GSK3 is a key regulator of neural precursor cell proliferation and maintains stem cells in the subventricular zone during brain development (8-10). In addition to the pharmacological approach where the degree of inhibition is not easily controlled, the use of genetic models with complete loss of *Gsk3 α* and/or *Gsk3 β* has allowed a better understanding of their function as master regulators of neuronal development by coordinating proliferation and differentiation signals (11). Conditional deletion of both *Gsk3 α* and *β* early during brain development results in overexpansion of neural progenitor cells at the expense of differentiated neurons. GSK3 activity has to be precisely controlled for appropriate timing of cell cycle exit and differentiation of neural progenitors.

GSK3 regulates the activity of the proneural factor, Neurogenin 2, during corticogenesis (12). GSK3 signaling participates in neuronal polarization in hippocampus as well as radial migration and dendritic orientation of cortical neurons (13-16). GSK3 also contributes to fine-tuning the balance between cell death and survival, and alteration of its function is associated with neurodegenerative pathologies such as Alzheimer's diseases, bipolar disorders or Parkinson's disease (17-19). The conditional knockout mice for *Gsk3 α* and *Gsk3 β* have clearly contributed to a better understanding of their function during central nervous system (CNS) development; however, high lethality early in life has restricted their usage (11, 14).

The retina is an attractive model for elucidating complex aspects of the central nervous system, including cell fate determination and lineage restriction, because of a limited number of neuronal cell types that are organized in a well-organized laminar pattern. The mature retina contains six types of neurons and one glial cell type that are generated from a limited pool of progenitors, which differentiate during three waves of neurogenesis with ganglion cells being the first born (starting at embryonic day (E) 10.5) and bipolar and Müller cells being the last produced postnatally in mice (20, 21). In the retina, both GSK3 kinases are ubiquitously expressed (22). GSK3-dependent phosphorylation is critical for regulating the activity of transcription factors in order to delineate the competence and the precise timing of cell fate determination and terminal differentiation of neural progenitor cells. Clonal analysis in the developing *Xenopus* retina showed that inhibition of GSK3 signaling by a dominant negative form of GSK3 β leads to enhanced RGC differentiation (early-born cell type) at the expense of Müller glia (late-born cells) (23).

GSK3 kinases are shown to actively control the timing of proneural bHLH transcription factor activity, such as NeuroD, as reported for Neurogenin 2 during corticogenesis (12).

To better delineate GSK3 function during retinal development, we generated conditional loss-of-function *Gsk3 α* and *Gsk3 β* alleles using *α -Cre* mouse line allowing recombination as early as E10.5 in retinal progenitors (24-26). Complete loss of both GSK3 kinases revealed extensive deleterious impact on retinal morphology with microphthalmia. The severe phenotype could be rescued by expressing one *Gsk3 α* or *Gsk3 β* allele, resulting in laminated and functional retina that included a large number of Brn3a-positive ganglion cells, named displaced retinal ganglion cells (dRGCs), in the inner nuclear layer (INL). Our study, for the first time, identifies a possible fate determinant of dRGCs, a poorly-investigated rare cell subtype in the retina.

MATERIALS AND METHODS

Animals and tissue collection

All animal experiments have been carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EEC), European Union guidelines effective and with the Association for Research in Vision and Ophthalmology statement for the Use of animals in ophthalmic and visual Research. All animal care and experimentation were also conducted in accordance with guidelines, under the license APAFIS#1018-2016072611404304 v1 by the Institutional animal care committee n°059 in France and by Animal Care and Use Committee at the National Institutes of Health (ASP#650). *Gsk3 α* and *Gsk3 β* floxed mice were generously provided by Dr. Jim Woodgett (University of Toronto, Canada) (24, 26). Floxed *Gsk3* mice were mated with those carrying the retina-specific regulatory element of murine *Pax6* driving the Cre recombinase (α -*Cre*) in retinal progenitors as early as E10.5 (25). Mice from either sex were used for experimental procedures. All mouse genotyping was performed as described (24-26). Animals were killed by CO₂ inhalation followed by cervical dislocation, and all efforts were made to minimize suffering. Eyeballs of pups were enucleated and proceeded for immunohistochemistry (IHC).

Hematoxylin & eosin (H&E) staining, and immunostaining

Methacrylate sections were used for H&E staining as described (27). For IHC on frozen sections, eyeballs were fixed at the required stage in 4 % PFA for 60 min on ice and incubated in an increasing concentration of sucrose (10 %, 20 % and 30 %), then embedded in OCT. Eyeballs were serially cut to 12 μ m sections using a cryostat. For embryonic stages, pregnant females were euthanized and whole heads of pups were harvested in paraffin. IHC was performed as described (28).

Primary and secondary antibodies are listed in Supplementary Table 1. Sections were counterstained with 1:1000 4',6-diamidino-2-phenylindole (DAPI) (1 mg/mL (Thermo Scientific)).

EdU labeling and TUNEL assay

For EdU labelling, females were injected intraperitoneally with 10 mM of 5-ethynyl-20-deoxyuridine (EdU) (Life Technology). EdU incorporation was detected on paraffin sections or frozen sections using the Click-iT EdU Imaging Kit per manufacturer's recommendations (Life Technology) (29). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays using in situ cell death detection kit (Promega). All images were acquired using a Zeiss LSM710 confocal microscope and Zen software (Zeiss).

Immunoblotting

Frozen retinas were lysed by sonication in lysis buffer (20 mM Na₂HPO₄, 250 mM NaCl, 30 mM NaPPi, 0.1 % NP40, 5 mM EDTA, 5 mM DTT) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates concentration was determined using a Lowry protein assay kit (Bio-Rad) following sonication and centrifugation. The supernatant proteins were separated under denaturing conditions by SDS-PAGE, transferred onto nitrocellulose membrane and probed with various antibodies, as described (28). Proteins were visualized using enhanced chemiluminescence kit (Bio-Rad). α -tubulin was used as the loading control. Quantification was performed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain at NIH).

Electroretinography

Electroretinogram (ERG) recordings were performed using a focal ERG module attached to Micron IV (Phoenix Research Laboratory). Briefly, mice were dark-adapted overnight and prepared for the experiment under dim-red light. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and received topical proparacaine hydrochloride (0.5 %, Alcon) via eye drops. Pupils were dilated with tropicamide (1 %, Alcon) and phenylephrine (2.5 %, Alcon) and lightly coated with GONAK hypromellose ophthalmic demulcent solution (2.5 %, Akorn). Lens of the Micron IV was placed directly on the cornea, and a reference electrode was placed on the mouse head. Scotopic responses were elicited with a series of flashes of increasing light intensities from -1.7 to 2.2 cd.s/m². Photopic responses were elicited under rod-desensitizing background light with a series of flashes of increasing light intensities from -0.5 to 2.8 cd.s/m². Values of a- and b-wave were extracted and plotted for comparisons between groups of interest.

Anterograde and Retrograde labeling of retinal ganglion cells

For retrograde labeling eyes were enucleated with optic nerve and fixed in PFA for 30 min. Rhodamine B isothiocyanate–Dextran (Sigma-Aldrich) was applied on the top of the optic nerve and incubated for 60 min. Eyes were flat mounted after the remaining dye was washed out for 48 hours in PBS at 4 °C. Z series images were acquired using SP5 confocal microscope (Leica Biosystems), and 3D reconstruction was performed using Volocity (Perkin Elmer). For anterograde tracing of retinal projections, a Cholera Toxin beta subunit (CTB) was used.

Animals were anesthetized using a cocktail of ketamine (60 mg/kg) and xylazine (10 mg/kg) and a subsequent bilateral injection of 1.2 μ l CTB at 1 mg/ml coupled to either an Alexa-555 or Alexa-647 (Lifesciences) were performed intravitreally. Three days following the injection, mice were perfused with 4 % PFA.

Tissue Clearing and 3D imaging

For 3D imaging of CTB-labelled brains, a methanol clearing protocol was carried out using modification from the iDISCO protocol (30, 31). Briefly, brains were dehydrated by immersion in progressive baths of methanol (20 %, 40 %, 60 %, 80 %, 100 %, 100 %) for 2 hours each at room temperature on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 ml centrifuge tube (TPP, Dutcher) protected from light. Following these baths, samples were immersed overnight in 2/3 Dichloromethane (DCM; Sigma-Aldrich) and then a 30-min bath in 100 % DCM before being transferred in Di-Benzyl Ether (DBE; Sigma-Aldrich) for 2 hours prior to imaging.

3D imaging and Image processing for all samples was performed as previously described (Belle M et al. Cell Rep. 2014; Belle M et al. Cell. 2017). Acquisitions were done using an ultramicroscope I (LaVision Biotec) with the ImspectorPro software (LaVision Biotec). The step size between each image was fixed at 2 μ m. For image analysis, 3D volumes and movies were generated using Imaris x64 software (Version8.0.1, Bitplane). Movie reconstruction with .tiff series were done with ImageJ (1.50e, Java 1.8.0_60, 64-bit) and iMovie (version 10.1.1).

Statistical analysis

Results are reported as mean \pm SEM. Nonparametric Mann-Whitney U test was used to analyze cell counting. *P* value \leq 0.05 was considered significant.

RESULTS

Retina-specific deletion of *Gsk3 α* and *Gsk3 β* results in nuclear accumulation of β -catenin and morphological defects with microphthalmia

To investigate the role of GSK3 during early retinal development, we crossed the floxed *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$* (24) with the *α -Cre* mouse lines (25) to generate *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl};\alpha$ -Cre* line in which *Gsk3* deletion occurs only in retinal progenitors as early as E10.5 (25). We first validated our model by assessing the efficacy of *Gsk3 α* and *Gsk3 β* deletion as early as E12.5, a couple of days after Cre expression begins (Fig. 1A) (25). IHC using an antibody recognizing both GSK3 showed ubiquitous expression of both kinases in the control retina (Fig. 1A). In the peripheral retina of *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl};\alpha$ -Cre* mice, both GSK3 kinases were efficiently deleted with preserved expression in the central retina following Cre expression pattern, as previously described (25). We then investigated the subcellular localization of β -catenin, an established GSK3 target mediating the activation of the *Wnt*-canonical pathway. In the control retina, β -catenin expression was mostly cytoplasmic (Fig. 1B). Upon *Gsk3 α* and *Gsk3 β* deletion, β -catenin was still expressed in the cytoplasm but it was also translocated to the nucleus in some cells as early as E12.5. *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl};\alpha$ -Cre* retina at E14.5 revealing a well-defined boundary between the Cre-positive area (GSK3-negative cells) at the periphery with nuclear translocation and large accumulation of β -catenin in the nucleus and the Cre-negative area (GSK3-positive cells) in the central area. Such expression pattern supports the predicted strong activation of the *Wnt*-canonical pathway in the absence of both *Gsk3 α* and *Gsk3 β* .

One-month old *Gsk3α^{ff/ff}β^{ff/ff};α-Cre* mice exhibited microphthalmia (Fig. 2A). Based on the severe phenotype observed in the adult, we focused the histological analysis during early development. H&E staining revealed major morphological defects in *Gsk3α^{ff/ff}β^{ff/ff};α-Cre* retina as early as E12.5, with the loss of radial arrangement of retinal progenitors and profound disorganization with folds and aggregates of retinal cells (Fig. 2B). In addition, blood cells were detected inside the retinal neuroblastic layer. All these defects worsened during retinal development although the central part of the retina retained a normal structure, consistent with the absence of *α-Cre* expression in this region. Starting at E16.5 and up to P2, the retina inside the eyeball was largely reduced although the size of the eye itself was comparable to littermate controls (Fig. 2B). Altogether, these results demonstrate the critical role of GSK3 in retinal progenitors for proper retinal development.

Lack of *Gsk3α* and *Gsk3β* leads to cell cycle aberrations and retinal progenitor gene deregulation during early development

We next sought to investigate the cellular defects occurring in the retina of *Gsk3α^{ff/ff}β^{ff/ff};α-Cre* mice. Due to extensive defects observed after E14.5 in the mutant retina, we focused our analysis on early developmental stages (E12.5 and E14.5) to better apprehend the cause of the microphthalmia phenotype. We thus investigated whether proliferation was altered in *Gsk3α^{ff/ff}β^{ff/ff};α-Cre* mouse retina. A single dose of EdU, to label retinal progenitors in S-phase, was injected 16 hours before harvesting embryos at E12.5 and E14.5. Although EdU-positive cells were observed in *Gsk3α^{ff/ff}β^{ff/ff};α-Cre* retinas at both stages (Fig. 3A), their total number remained unchanged in contrast to the controls where it largely increased between E12.5 and E14.5 (Fig. 3B).

As a result, the number of retinal progenitor in S-phase was significantly lower at E14.5 in the absence of GSK3 compared to controls, suggesting retinal progenitor proliferative defects. We further analyzed the impact of *Gsk3* knock-out on cell proliferation by labeling late-G2/M-phase retinal progenitors by phospho-histone 3 (pH3) antibody. In controls, strongly labelled cells, likely M-phase cells, were positioned in the apical surface of the retina where mitosis occurs. Cells with less intense labeling, presumably late-G2 cells, were present in the outer part of the neuroblastic layer. At both stages, all pH3-positive cells in *Gsk3 $\alpha^{f/f}\beta^{f/f};\alpha$ -Cre* retina were positioned distant from the apical surface (Fig. 3A). Moreover, despite a slight increase of pH3-positive cells at E12.5 at the periphery compared to control retinas, their number subsequently decreased at E14.5 (Fig. 3B). In *Gsk3 $\alpha^{f/f}\beta^{f/f};\alpha$ -Cre* animals, 48.3 % \pm 4.4 and 37.3 % \pm 2.3 of pH3-positive cells were also EdU-positive in the absence of GSK3s, at both E12.5 and E14.5, respectively, compared to 15.5 % \pm 1.2 and 18.9 \pm 2.3 in controls (Fig. 3B). Such results indicate aberrant cell cycle kinetics in the absence of GSK3 kinases that could impair the maintenance of the pool of retinal progenitors. To address this question, we used an anti-HES1 antibody (32). At E12.5 and E14.5, HES1 labeled all retinal progenitors throughout the control retina with the exception of the basal side where differentiating cells are located. In *Gsk3 $\alpha^{f/f}\beta^{f/f};\alpha$ -Cre* retina, HES1 expression was similar to controls at E12.5 indicating the maintenance of a pool of retinal progenitors at this stage (Fig. 4). In contrast, E14.5 retina showed less HES1 positive cells at the periphery where *Gsk3* expression was missing, although they were still detectable in the control central area (Fig. 4). The reduction of HES1 expression in the periphery correlates with the decreased number of EdU- and pH3-positive cells observed previously at the same stage (Fig. 3B).

Other progenitor markers, such as Pax6 and Sox2 exhibited similar decrease in *Gsk3α^{fl/fl}β^{fl/fl};α-Cre* retina compared to controls (data not shown). Thus, our results show that GSK3 kinases are required for cell cycle progression and for maintaining the pool of proliferative retinal progenitors.

Loss of *Gsk3α* and *Gsk3β* impairs retinal progenitor differentiation and leads to cell death

We wondered whether neuronal differentiation is impacted in the absence of GSK3. We thus performed IHC using two neuronal markers, Doublecortin (Dcx) and Brn3a, to label neuronal precursor cells and ganglion cells, respectively. At E12.5 and E14.5, Dcx- and Brn3a-positive cells were localized in the inner part of the neuroblastic layer where retinal differentiation occurs. Neuronal differentiation was completely abolished in *Gsk3α^{fl/fl}β^{fl/fl};α-Cre* retina compared to controls, except in the most central part, corresponding to the non-Cre area (Fig. 5). Lack of neuronal differentiation in the absence of both *Gsk3* was confirmed at later stages using Calbindin, an amacrine cell marker (data not shown).

The absence of retinal differentiation at E14.5, together with the decreased expression of retinal progenitor markers, prompted us to investigate whether the absence of both *Gsk3* in retinal progenitors could trigger cell death. Apoptosis assay showed a significant increase in the number of TUNEL-positive cells in *Gsk3α^{fl/fl}β^{fl/fl};α-Cre* retina (Fig 6). These results suggest that microphthalmia observed in the absence of both *Gsk3α* and *Gsk3β* is due to impaired proliferation and differentiation, with concomitant increase in cell death.

Expression of one *Gsk3α* or *Gsk3β* allele is sufficient to generate a functional retina

The deleterious effect of the deletion of both *Gsk3α* and *Gsk3β* in retinal progenitors during early development prevents the analysis of late retinal histogenesis. To circumvent this problem, we generated animals with different degrees of *Gsk3* deletion (loss of only one *Gsk3* gene: *Gsk3α^{fl/fl}β^{+/+};α-Cre* or *Gsk3α^{+/+}β^{fl/fl};α-Cre* or $\frac{3}{4}$ deletion: *Gsk3α^{fl/fl}β^{fl/+};α-Cre* or *Gsk3α^{fl/+}β^{fl/fl};α-Cre*). Immunoblot analysis using anti-GSK3 antibody (recognizing both isoforms), in 2-months old animals with different combination of *Gsk3α* and *Gsk3β* floxed alleles (*Gsk3α^{fl/fl}β^{+/+}*, *Gsk3α^{+/+}β^{fl/fl}*, *Gsk3α^{fl/+}β^{fl/fl}* or *Gsk3α^{fl/fl}β^{fl/+}*) demonstrated the efficacy of *Gsk3α* or *Gsk3β* deletion (Fig. 7A). In addition, IHC analysis using anti-GSK3β showed ubiquitous expression of *Gsk3β* in adult retina and the complete loss of this isoform in *Gsk3α^{fl/+}β^{fl/fl};α-Cre* retina (Fig. 7B) (22). Histological analysis showed the formation of a normal retina in the presence of only one allele of *Gsk3α* or *Gsk3β*, revealing the ability of a single wild type allele of *Gsk3α* or *Gsk3β* to rescue the phenotype described earlier and highlighting the redundancy between the two kinases. Although distinct combination of *Gsk3* mutated alleles ($\frac{1}{2}$ or $\frac{3}{4}$ deletions) allowed the formation of an apparent normal retina, we performed a detailed phenotypic analysis in 2 months old animals using antibodies to label different retinal cell types. Rod and cone photoreceptor staining was similar between *Gsk3α^{fl/+}β^{fl/fl};α-Cre* and controls using anti-Rhodopsin and anti-cone Arrestin antibodies, respectively (Fig. 7C). Interneurons (amacrine and horizontal cells) were also similarly labeled using anti-Calbindin and anti-Calretinin antibodies (Fig. 7D).

Notably, slight activation of Müller cells could be detected using anti-GFAP antibody, indicating a potential retinal stress though we did not detect retinal degeneration even in older mice (data not shown). The rescue was confirmed at a functional level since scotopic and photopic ERG recordings did not show any differences between *Gsk3α^{f/+}β^{f/f};α-Cre* and control retina (Fig. 7E, F).

Loss of either *Gsk3α* or *Gsk3β* leads to an increase in displaced Retinal Ganglion Cells during early retinal development

Although restoring a single copy of either *Gsk3α* or *Gsk3β* restored retinal function, we wondered whether all retinal cell types were unaffected in these mutants. The total number of Brn3a-positive RGCs in the GCL remained unchanged between *Gsk3α^{f/+}β^{f/f};α-Cre* and controls. However, we observed an increase in the number of Brn3a-positive cells in the inner nuclear layer (INL) in *Gsk3α^{f/+}β^{f/f};α-Cre* retina compared to the controls (Fig. 8A), likely corresponding to dRGCs (33). The increase in dRGCs was seen in retina with varying combinations of *Gsk3* deletions (*Gsk3α^{f/f}β^{+/+}*, *Gsk3α^{+/+}β^{f/f}*, *Gsk3α^{f/+}β^{f/f}* or *Gsk3α^{f/f}β^{f/+}*), with the highest number observed in *Gsk3α^{f/+}β^{f/f};α-Cre* mice (22 % ± 0.6) compared to the controls (2 % ± 0.8) (Fig. 8B). This increase of dRGCs in the INL was not at the expense of RGCs located in the GCL, called orthotopic RGCs (oRGCs) (Fig. 8B). Due to their low number in the WT retina, dRGCs have been poorly characterized and no markers has yet been identified. Unfortunately, despite the high proportion of dRGCs in mutant retina, we also could not further characterize these cells at the molecular level since they do not express melanopsin, a marker of photosensitive retinal ganglion cells (ipRGCs), or markers of amacrine and horizontal cells, including Cholin-Acetyltransferase (CHAT), Calretinin and Calbindin (data not shown).

While dRGCs were negative for potential RGCs subtype markers, the morphology of these Brn3a-NF68-positive dRGCs was unique among INL cells as observed by H&E staining (Fig. 8C). To confirm that these Brn3a-positive cells in the INL were RGCs with extended projections into the optic nerve, we performed retrograde labeling with Rhodamin-Dextran applied onto the optic nerve. Subsequent 3D reconstruction demonstrated that axons of dRGCs indeed reached the optic nerve (Fig. 8D), further validating their RGC identity.

We then reasoned that the observed increase in dRGCs in *Gsk3* mutant mice could result from defects in RGC birthdate. To test this hypothesis, we performed EdU pulse chase experiments. EdU was injected at E12.5 corresponding to the peak of RGC birthdate, and retinal sections from one-month old animals were immunolabelled using anti-Brn3a antibody (Fig. 9A). Between 40 % to 50 % of RGCs were EdU-positive in all layers examined (GCL and INL) in control and *Gsk3 $\alpha^{f/+}\beta^{f/f};\alpha$ -Cre* retina, indicating that both dRGCs and oRGCs differentiate at the same time (Fig. 9B). We next wondered whether the difference in the number of dRGCs found between *Gsk3 $\alpha^{f/+}\beta^{f/f};\alpha$ -Cre* and control retinas could result from a difference in dRGCs survival. Indeed, massive developmental cell death occurs postnatally in the GCL of wild type retina during the two weeks of life (34, 35). We therefore immunolabelled P0 retina from *Gsk3 $\alpha^{f/+}\beta^{f/f};\alpha$ -Cre* and control retina with anti-Brn3a. At this stage, a larger proportion of Brn3-positive cells, presumably dRGCs, are located in the inner part of the neuroblastic layer in *Gsk3 $\alpha^{f/+}\beta^{f/f};\alpha$ -Cre* retina (30 % \pm 1.4) compared to the control retina (6 % \pm 0.1) (Fig. 9C and D). No massive dRGC death was detected postnatally in control retina since their number observed at P0 is similar to the one found in adults.

Thus, the increased number of dRGCs in mutant mice does not seem to result from a difference in cell survival compared to a control situation. Since the number of oRGC was unchanged in *Gsk3 $\alpha^{f/+}\beta^{f/f};\alpha$ -Cre* retina, we propose that the increase observed in dRGCs is due to an aberrant production of the dRGC subtype during the early wave of retinogenesis. Altogether, these data suggest that GSK3 plays a role in controlling the number of dRGCs produced in the retina.

dRGCs play a critical role in accessory visual system circuitry

As the increased number of dRGCs in *Gsk3* mutant mice persisted into adulthood, we questioned whether this increased pool of dRGCs projected into specific visual nuclei in the brain. Previous studies in birds and reptiles have reported that dRGCs could be responsible for optokinetic nystagmus, as they mostly project to the accessory optic nuclei (36). To test whether this observation is true in mammals and whether the increased dRGCs pool in GSK3 mutants could lead to an increased volume of accessory optic nuclei, we traced the total pool of RGCs and dRGCs. Bilateral injection of CTB coupled to either an alexa-555 or -647 together with 3D imaging, allowed us to trace both ipsi- and contra-lateral projecting axons of RGCs as well as dRGCs (Fig 10). Interestingly, GSK3 mutants displayed a specific increase in ipsilateral projecting RGCs in the accessory optic nucleus, the Medial Terminal Nucleus (MTN). This increase in RGC projections was restricted to the ipsilateral MTN, as revealed by a comparison of volume analysis between major nuclei in the brain (Lateral geniculate nucleus (LGN), Superior colliculus (SC), and MTN). To confirm that CTB injections labelled dRGCs, the retinas of the injected eyes were flat mounted after immunolabelling with anti-Brn3a antibody (data not shown).

The large increase of RGC projections into the ipsilateral MTN was observed only in complete absence of *Gsk3 β* . Interestingly, complete loss of *Gsk3 β* led also to the highest number of dRGCs (Fig. 8B), suggesting that labeling of the ipsilateral MTN mostly originates from dRGCs. Altogether, our results strongly suggest that *Gsk3 β* is a critical regulator of dRGC production and suppresses their projections to the ipsilateral MTN.

DISCUSSION

Our study reports, for the first time, on the function of *Gsk3 α* and *Gsk3 β* during early retinal development. Complete loss of GSK3 activity in retinal progenitors results in severe morphological defects with progressive death of the pool of proliferative retinal progenitors and lack of neuronal differentiation, leading to microphthalmic eyes in adult mice. Such phenotype was completely rescued by the expression of only one *Gsk3 α* or *Gsk3 β* allele confirming the functional redundancy of these two kinases (1). However, further analysis of mouse retinas with only one allele of *Gsk3* revealed the presence of an excessive number of dRGCs, a specific RGC subtype located in the INL. Our data thus identified GSK3 as the first dRGCs determinant during retinal histogenesis.

The lack of neuronal differentiation that we observed in the retina in double *Gsk3* knockout mice, was similar to the phenotype described in the developing brain of *Gsk3* mutant mice (11). Therefore, in the whole central nervous system, GSK3 signaling appears essential for progenitors to differentiate into neurons. Surprisingly however, we found that the absence of GSK3 impairs the maintenance of the pool of retinal progenitors leading to increased cell death and subsequently to microphthalmia in adults.

Our results are in striking contrast with the “big head phenotype” due to a large expansion of the pool of neural progenitors by hyperproliferation and the absence of cell death at early stages observed in *Gsk3 α ^{-/-};Gsk3 β ^{f/f}*; nestin-cre embryos (11). Thus, we suggest that the retinal progenitors are more sensitive to the lack of GSK3 activity compared to the neural progenitors in the brain. GSK3 kinases are established regulators of the Wnt canonical pathway and inhibit Wnt ligand binding to Frizzled receptors, leading to stabilization and nuclear translocation of β -catenin (2). Alteration of Wnt signaling during early retinal development due to the absence of Frizzled 5 receptors (*Fz5*) and/or Frizzled 8 (*Fz8*) leads to microphthalmia and incomplete closure of the optic fissure causing coloboma (37, 38). In humans, an autosomal dominant mutation in *FZD5* that antagonizes both canonical (involving GSK3 regulation) and non-canonical pathways leads to coloboma (39). Interestingly, in the absence of Wnt signaling, β -catenin remains cytoplasmic in contrast to our model where lack of GSK3 largely increases its amount. Based on these results, it is likely that *Gsk3* knockout phenotype results from more than just Wnt/ β -catenin pathway activation, which may explain the more severe phenotype. In contrast, the effect of *Gsk3* loss of function in the retina mimics the effect of β -catenin gain of function. Indeed, constitutive retinal activation of β -catenin does not elicit an overproliferation of retinal cells. Instead, it leads to decreased cell proliferation and to a loss of neural differentiation, resulting in a small eye phenotype (40, 41). Besides, β -catenin has been recognized as a key regulator of cell adhesion (40). The lamination defects observed in *Gsk3* knockout mice may thus also result from altered cell adhesion due to β -catenin activation. However, unlike *Gsk3* knockout, β -catenin gain of function does not lead to increased retinal cell death (41).

GSK3 regulates the stability of a large range of proteins (9), including Microtubules Associated Proteins (MAPs) (42-45). Alterations of microtubule dynamic may be at the origin of the loss of radial organization and morphogenesis defects when GSK3 activity is completely abolished. This may also contribute to cytokinesis defects, resulting in the observed mislocalization of M-phase cells distant from the apical surface. Ectopic mitosis has indeed been associated with altered mitotic spindle orientation (46, 47). Along these lines, inhibition of GSK3 has been shown to disrupt centrosome regulation generating mitotic catastrophe and apoptosis (48). Therefore, such microtubule associated cell division defects may also contribute to the increased cell death observed in GSK3 mutant mice.

Over 20 % of dRGCs among the total RGCs in the mutant retina is a remarkable phenotype when a single copy of either *Gsk3 α* or *Gsk3 β* is present in retinal progenitors. Indeed, dRGCs in the INL are a very rare and poorly-described type of cells representing only 2 % in WT mice (33, 49). To our knowledge, while a reduction of dRGCs in the retina has previously been observed in albino rodents (33, 50-52), an increased number of dRGCs compared to WT has never been reported in the literature. Although it remains to be demonstrated, dRGCs are believed to be misplaced in the INL due to an ontogenic aberration rather than representing an independent class of RGCs (49, 51). One can therefore hypothesize that enhanced dRGCs in $\frac{3}{4}$ *Gsk3* mutant mice are the consequence of increased aberration events. Indeed, differential cell adhesion plays a key role in sorting and migration of retinal cells in their appropriate layers, especially of RGCs (40). Increased dRGCs in the $\frac{3}{4}$ *Gsk3* mutant retina may result from impaired RGC migration; however, a conundrum in our studies is that the oRGC number in GCL is unaltered in mutant mice.

As β -catenin functions in cell adhesion during retinogenesis (40), it would be interesting to investigate whether this dRGC phenotype could be associated with overactivation of the Wnt/ β -catenin pathway. Multiple mechanisms are reported to tightly regulate RGCs abundance during retinal development (53, 54) and could contribute to equalize the number of RGCs in the GCL between GSK3 mutants and controls.

Augmented dRGC in $\frac{3}{4}$ GSK3 mutant mice, without a concomitant decrease in oRGCs, suggests that dRGCs represent a specific subset of RGCs and that their increase is a consequence of cell fate specification defects. Our hypothesis is supported by topographic and quantitative analysis of RGCs in albinos and pigmented rats, suggesting that Brn3-positive dRGCs are indeed not misplaced by ontogenic mistakes but represent a subset of RGCs (33). GSK3 is involved in neural cell fate decision by controlling the timing of the activity of bHLH transcription factors, such as NeuroD or Neurog2 (12, 23). If dRGCs are not produced following ontogenic aberrations but are instead determined by a proper genetic program, it would be interesting to identify the involved transcription factors and seek for any regulation by GSK3 kinases.

Anterograde labeling clearly demonstrated a large increased of signal in the MTN in absence of *Gsk3 β* , whereas it was absent or very weak in control animals. So far, it is unclear if the absence of signal in control retina is due to the low number of dRGC and if such difference observed in absence of *Gsk3 β* originates from dRGCs projections or from oRGCs or from both, although the intensity of the signal followed the increased number of dRGCs (Fig. 8B). Our results strongly suggest that these displaced cells may primarily project into the MTN.

In mice, it has been shown by anterograde labeling from the superior colliculus (SC), which receive large amount of RGC projections, that dRGCs/oRGCs project to one or both SCs (33, 55-58). Same experiments with injection into the ipsilateral MTN may allow to discriminate whether the increased signal in absence of *Gsk3 β* originates from dRGCs and whether these cells also project into this area in WT retina.

Given the very low percentage of dRGCs in the control retina, their function has not been studied in mammals. In contrast, dRGCs function, brain projections and topography have been extensively investigated in bird and reptile retina (59). In birds, cryptochrome-expressing dRGCs are used as a magnetic compass for orientation (60). In European Robin birds, *Erithacus rubecula*, a low number of dRGCs have been identified but specifically express Cryptochrome 1b only during nocturnal migration period (61). In rodents, different dRGCs are proposed to exist with independent function based on their projection to the brain and their topography in the retina (33). As such, it has been suggested that dRGCs are in a good position to quickly treat visual information coming from above their head, thereby allowing a quick detection of the predators. Such hypothesis could be tested in our mouse models using suitable behavior tests. Therefore, *Gsk3* mutant mice displaying enhanced dRGCs might offer a powerful system to further study the embryonic origin, synaptic connections and visual function of dRGCs.

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FIGURE LEGENDS

Figure 1. Aberrant nuclear translocation of β -catenin, a key effector of the Wnt canonical pathways, with complete loss of *Gsk3* expression. (A) Immunohistochemistry (IHC) of E12.5 retina using a pan-Gsk3 antibody shows efficient deletion at the periphery where the Cre expression has been previously reported (delimited by dashed-line) Scale bar: 100 μ m. (B) β -catenin accumulation (red) at E12.5 and E14.5 in the Cre-expressing area of *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; α -Cre* animals especially at E14.5. Magnification on the right panel shows the squared delimited area. L, Lens; NR, neural retina; + Cre, Cre-expressing area; - Cre, area without Cre expression Scale bar: 100 μ m, magnification area scale bar 40 μ m.

Figure 1.

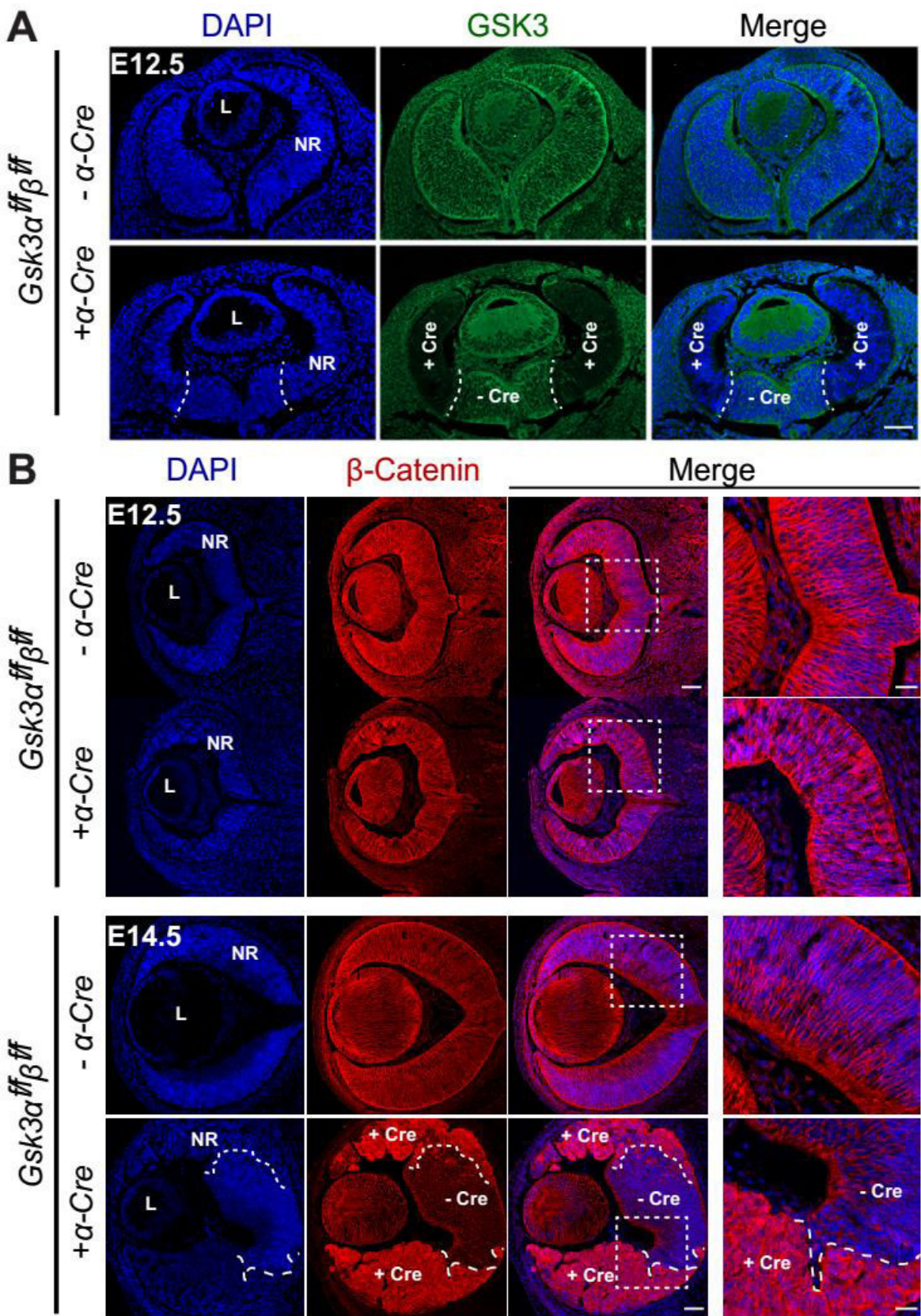


Figure 2. Developmental defects in *Gsk3*-deficient retina. (A) Microphthalmia in one-month old *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$; α -Cre* adult animals compared to controls. (B) Hematoxylin and eosin (H&E) stainings on methacrylate sections from E12.5 to P2 reveal large retinal morphogenesis defects in *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$; α -Cre* with blood cells invasion into the eyeball (showed by *). L, Lens; NR, neural retina. Scale bar: 100 μ m at E12.5, E14.5 and E16.5. 500 μ m at P2.

Figure 2.

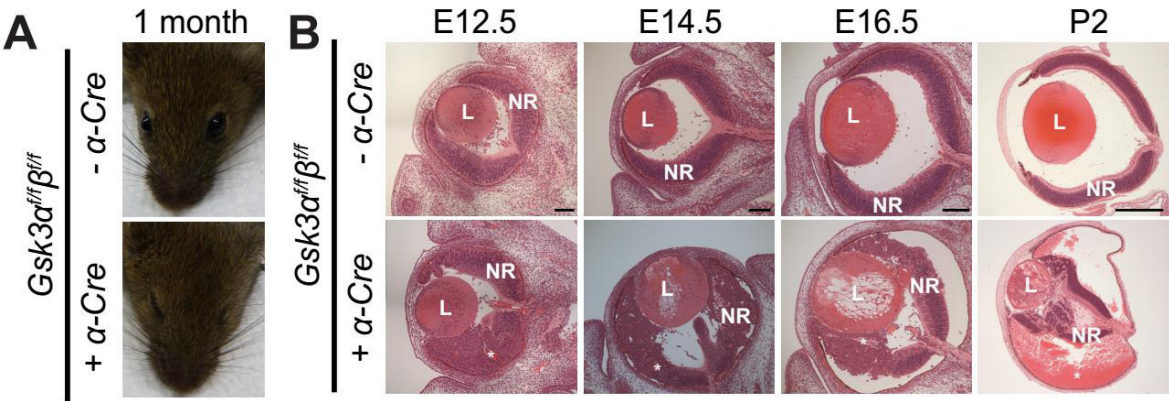


Figure 3. Lack of GSK3 signaling alters cell cycle progression of retinal progenitor cells. (A) Mislocalisation of pH3-positive cells in the neuroblastic layer of *Gsk3α^{ff}β^{ff};α-Cre* retina. E12.5 and E14.5 retina stained for pH3 (late-G2/M-phase) and EdU (marker of S-phase) following a 16H chase. Edu-positive cells are observed in the absence of GSK3 at both stages. Scale bar: 100 μm, magnification 40 μm. (B) Lack of GSK3 signaling alters cell cycle progression of retinal progenitors. Quantification at E12.5 and E14.5 of the number of EdU-positive cells (*upper panel*), pH3-positive cells (*middle panel*) and double positive cells for EdU and pH3 among pH3-positive cells (*lower panel*) in *Gsk3α^{ff}β^{ff};α-Cre* animals and controls. Mean ± SEM values are presented from 4 to 6 biological replicates, * indicates $P \leq 0.05$.

Figure 3.

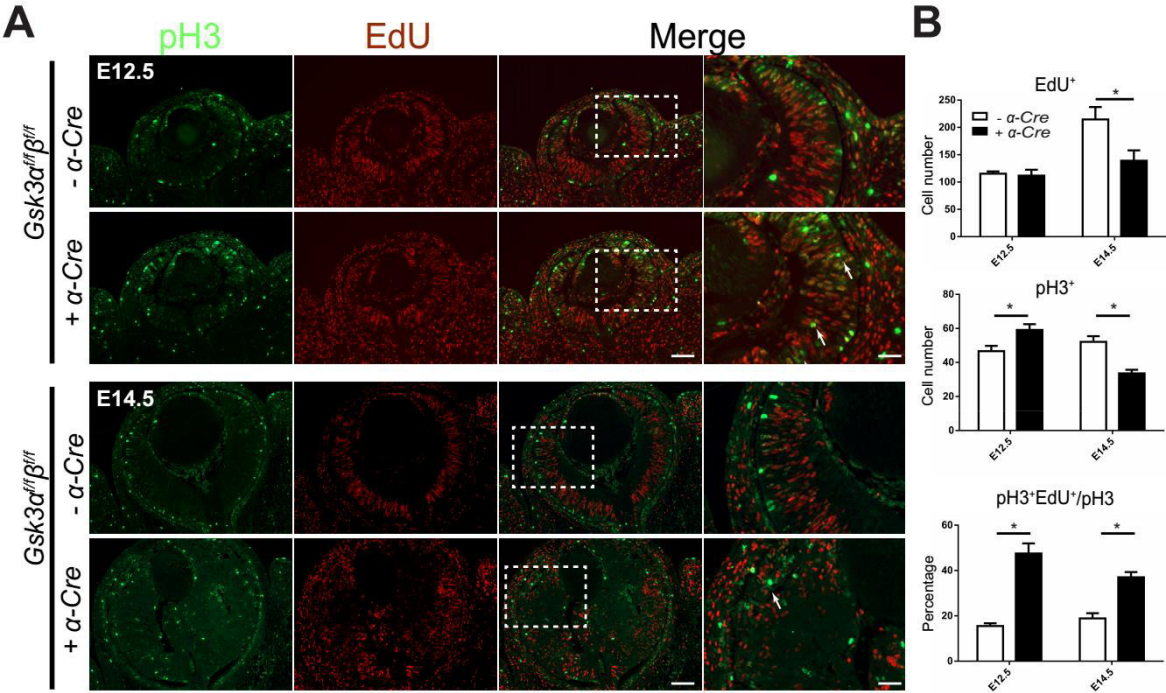


Figure 4. Lack of *Gsk3 α* and *Gsk3 β* expression impairs the maintenance of the pool of retinal progenitors. IHC on *Gsk3 $\alpha^{f/f}\beta^{f/f}$* and *Gsk3 $\alpha^{f/f}\beta^{f/f};\alpha$ -Cre* retina using HES1 (*red*) and GSK3 (*green*) indicates a loss of retinal progenitors at E14.5. *Dashed lines* delimit the central *Gsk3*-expressing area and the *Gsk3*-deleted area at the periphery Scale bar: 100 μ m, magnification 40 μ m.

Figure 4.

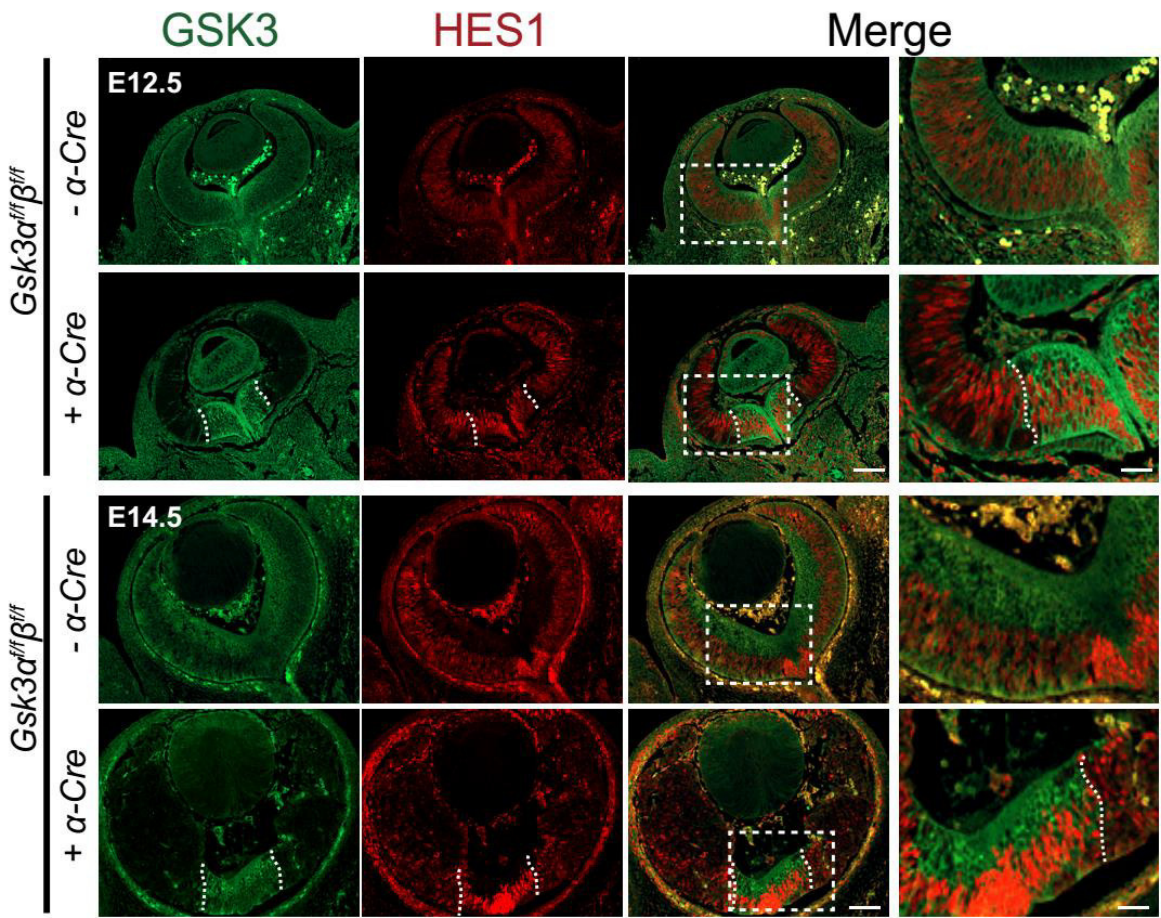


Figure 5. Lack of *Gsk3 α* and *Gsk3 β* expression in retinal progenitors blocks retinal progenitor differentiation. IHC using Doublecortin (Dcx) (*green*) and Brn3a (*red*) antibodies shows the localization of neuronal precursor cells and RGCs, respectively, at the central and basal part of the neuroblastic layer of E12.5 control retina. In contrast, neural differentiation is not observed in *Gsk3 $\alpha^{fl/fl};\beta^{fl/fl};\alpha$ -Cre* retina, with the exception of a small central area where Cre is not expressed. At E14.5, Dcx- and Brn3a-positive cells are distributed across the whole retina in controls, whereas they are absent in periphery in *Gsk3 $\alpha^{fl/fl};\beta^{fl/fl};\alpha$ -Cre* mice. Magnification on the right-hand side depicts a representative area at the boundary between a Cre-positive and a Cre-negative area. Scale bar: 100 μ m, magnification 40 μ m.

Figure 5.

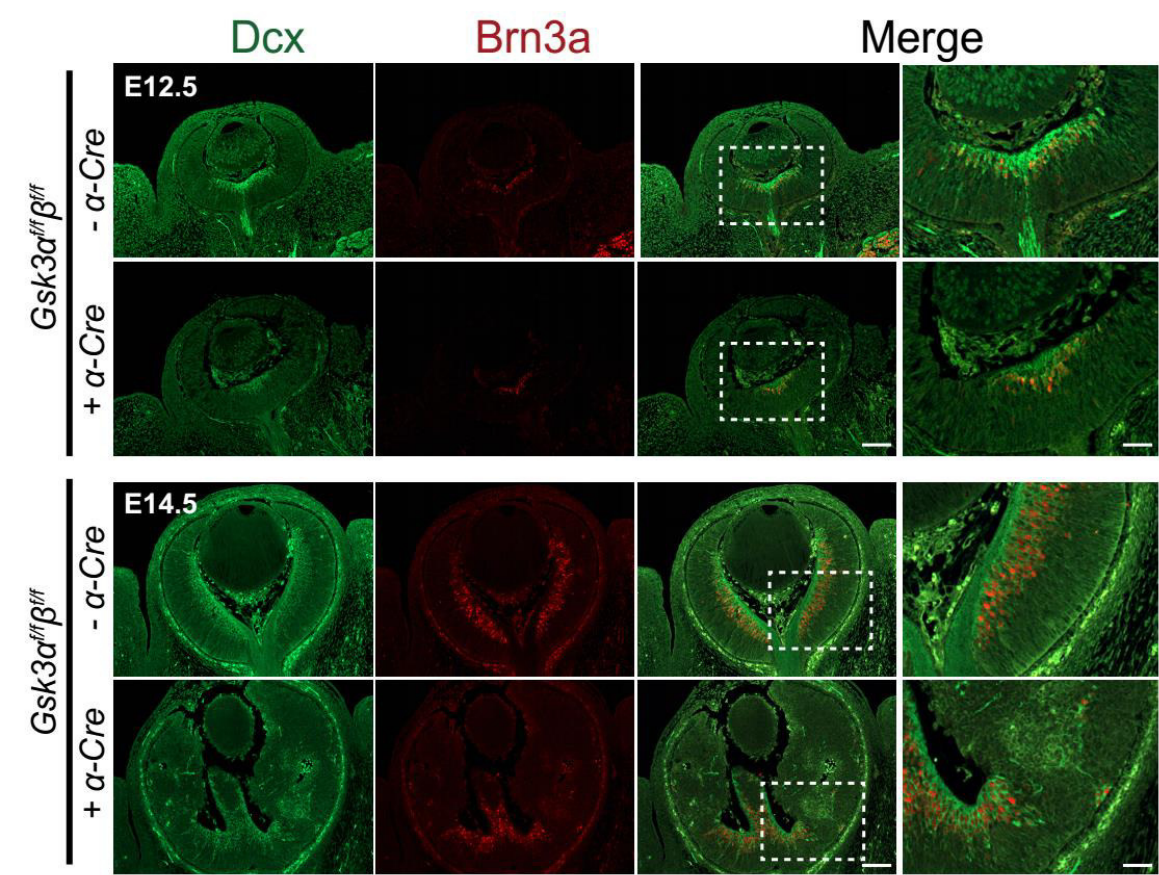


Figure 6. Lack of *Gsk3α* and *Gsk3β* expression in retinal progenitors leads to increased cell death. (A) TUNEL assay on E12.5 and E14.5 *Gsk3α^{fl/fl}β^{fl/fl};α-Cre* and control animals reveals an increase of cell death (TUNEL-positive cells, *green*) in *Gsk3α^{fl/fl}β^{fl/fl};α-Cre* animals compared to littermate controls. (B) Quantification of the number of TUNEL-positive cells per retinal section. Mean ± SEM values are presented from 5 to 6 biological replicates for E12.5, 4 biological replicates for E14.5, * indicates $P \leq 0.05$.

Figure 6.

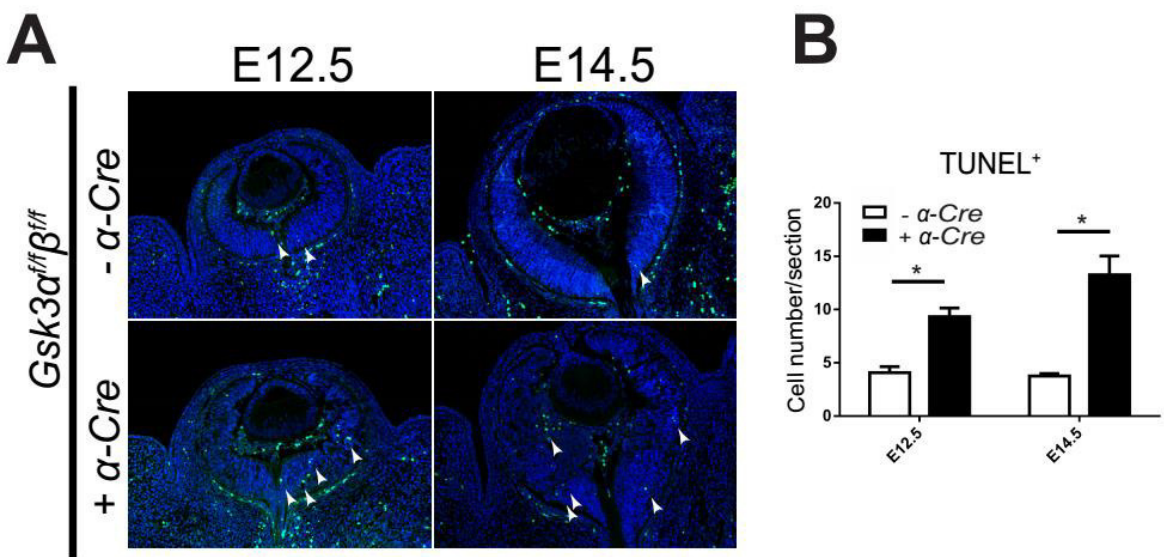


Figure 7. One allele of either *Gsk3 α* or *Gsk3 β* is sufficient for the development of a functional retina. (A) Immunoblot analysis of protein extracts from 2 months old animals with different combination of *Gsk3 α* and *Gsk3 β* floxed alleles (*Gsk3 α ^{f/f} β ^{+/+}*, *Gsk3 α ^{+/+} β ^{f/f}*, *Gsk3 α ^{f/+} β ^{f/f}* or *Gsk3 α ^{f/f} β ^{f/+}*) with or without *Cre* recombinase using anti-GSK3 antibody (recognizing both isoforms) reveals decreased expression of *Gsk3 α* or *Gsk3 β* (arrowheads). α -Tubulin is used as loading control. (B) IHC on 2 months retinal sections from control and *Gsk3 α ^{f/+} β ^{f/f}*; α -*Cre* retinas with or without *Cre* recombinase using anti-GSK3 β antibody showing ubiquitous *Gsk3* expression in all retinal layers, whereas its expression is lost in *Cre*-expressing retina. (C) Expression of only one *Gsk3* allele (*Gsk3 α*) is sufficient for proper photoreceptor development. IHC using anti-Rhodopsin (Rho) and anti-Cone arrestin (Arr3) antibodies to label rod and cone photoreceptors, respectively. (D) Expression of only one *Gsk3* allele (*Gsk3 α*) is sufficient for proper interneuron development. IHC using anti-Calretinin (Calr) and anti-Calbindin (Calb) antibodies to label horizontal and amacrine cells, respectively. (B-D) *onl*, outer nuclear layer; *inl*, inner nuclear layer; *gcl*, ganglion cell layer. Scale bar: 20 μ m. (E, F) Electroretinogram (ERG) recording in 2 months old *Gsk3 α ^{f/+} β ^{f/f}*; α -*Cre* animals and littermate controls. Photopic (cone) (E) and scotopic (rod) (F) response in *Gsk3 α ^{f/+} β ^{f/f}*; α -*Cre* animals are similar to controls. Mean \pm SEM intensity response curves of a- and b-wave responses averaged from 8 biological replicates of each genotype.

Figure 7.

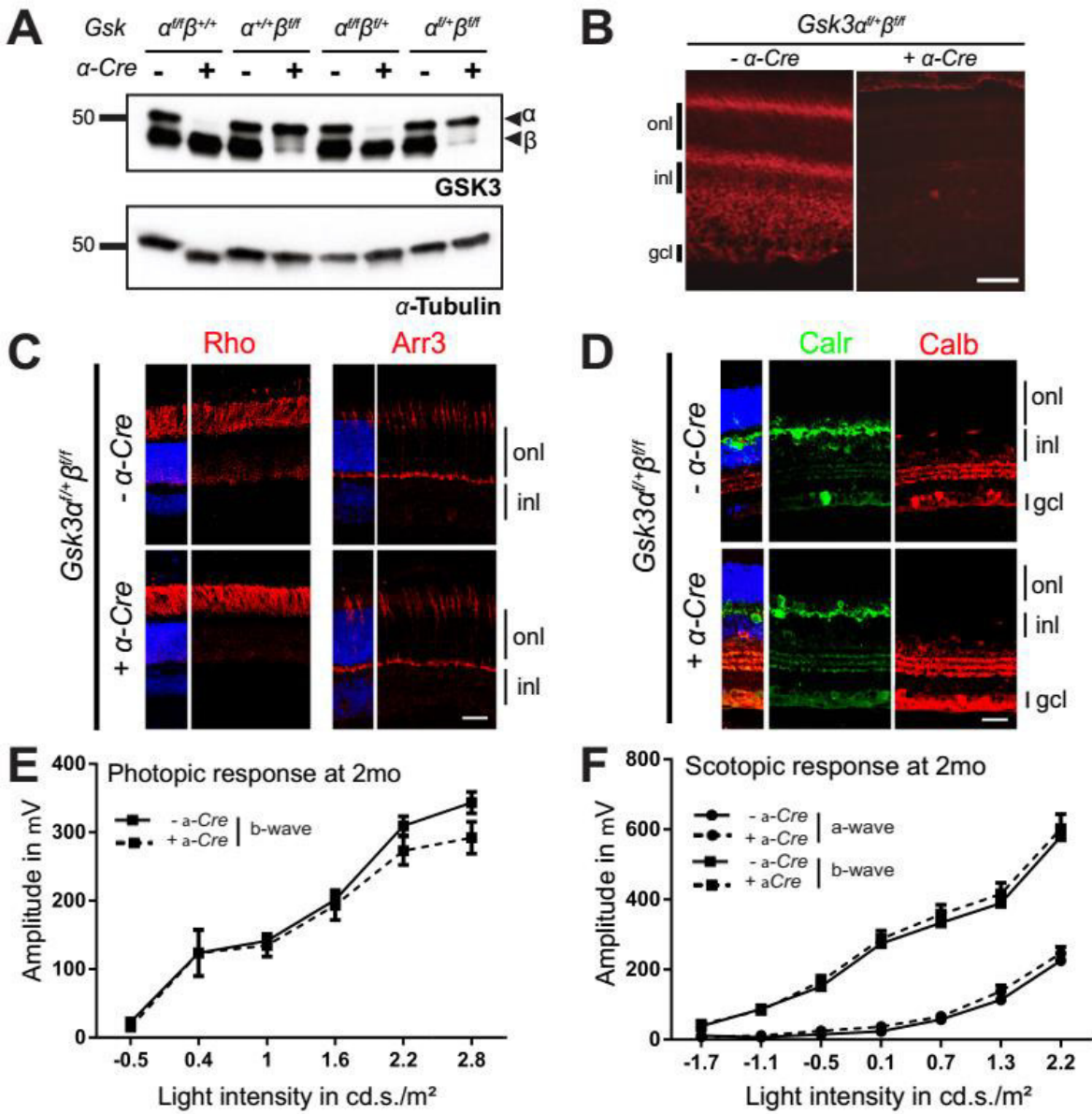


Figure 8. Gradual loss of *Gsk3α* and/or *Gsk3β* leads to an increased number of Brn3a-positive retinal ganglion cells displaced in the inner nuclear layer (INL). (A) Brn3a (red) and NF68 (green) IHC on 2 months old mouse retina reveal the presence of displaced retinal ganglion cells (dRGCs) in the INL of *Gsk3α^{f/+}β^{f/f}; α-Cre* compared to littermate controls. Top panel represents control retinas, middle panel a peripheral retinal area in *Gsk3α^{f/+}β^{f/f}; α-Cre* animals and bottom panel a more central area (white arrows). Scale bar: 20 μm. (B) Gradual loss of *Gsk3α* and *Gsk3β* alleles (*Gsk3α^{f/f}β^{+/+}*, *Gsk3α^{+/+}β^{f/f}*, *Gsk3α^{f/+}β^{f/f}* or *Gsk3α^{f/f}β^{f/+}*) leads to a gradual increase of Brn3a-positive RGC located to the INL, with the highest number observed in *Gsk3α^{f/+}β^{f/f}; α-Cre* animals. Left stacked histogram represents counting of the total number of Brn3a-positive cells per section located in the GCL (white bars) and in the INL (black bars) for different *Gsk3α* and/or *Gsk3β* floxed alleles combinations with or without *Cre* recombinase. Right histogram represents the percentage of the dRGC among the total number of Brn3a-positive cells per section for each combination. Mean ± SEM values are presented from 5-6 biological replicates, * indicates $P \leq 0.05$. (C) H&E staining of *Gsk3α^{f/+}β^{f/f}; α-Cre* retina (left panel) shows cells in the INL with a particular morphology showing thick axonal projection into the IPL with a large cell body, whereas such cells were absent in control retina. They may correspond to dRGC since their morphology is similar the one observed for dRGC labelled with Brn3a and NF68 antibodies (right panel). Scale bar: 20 μm. (D) dRGC send their axons into the optic nerve. Visualization of dRGCs after 3D reconstruction of 2-month old flat mount retina of control and *Gsk3α^{f/+}β^{f/f}; α-Cre* animals following retrograde labelling with Rhodamin-Dextran applied onto the optic nerve.

Figure 8.

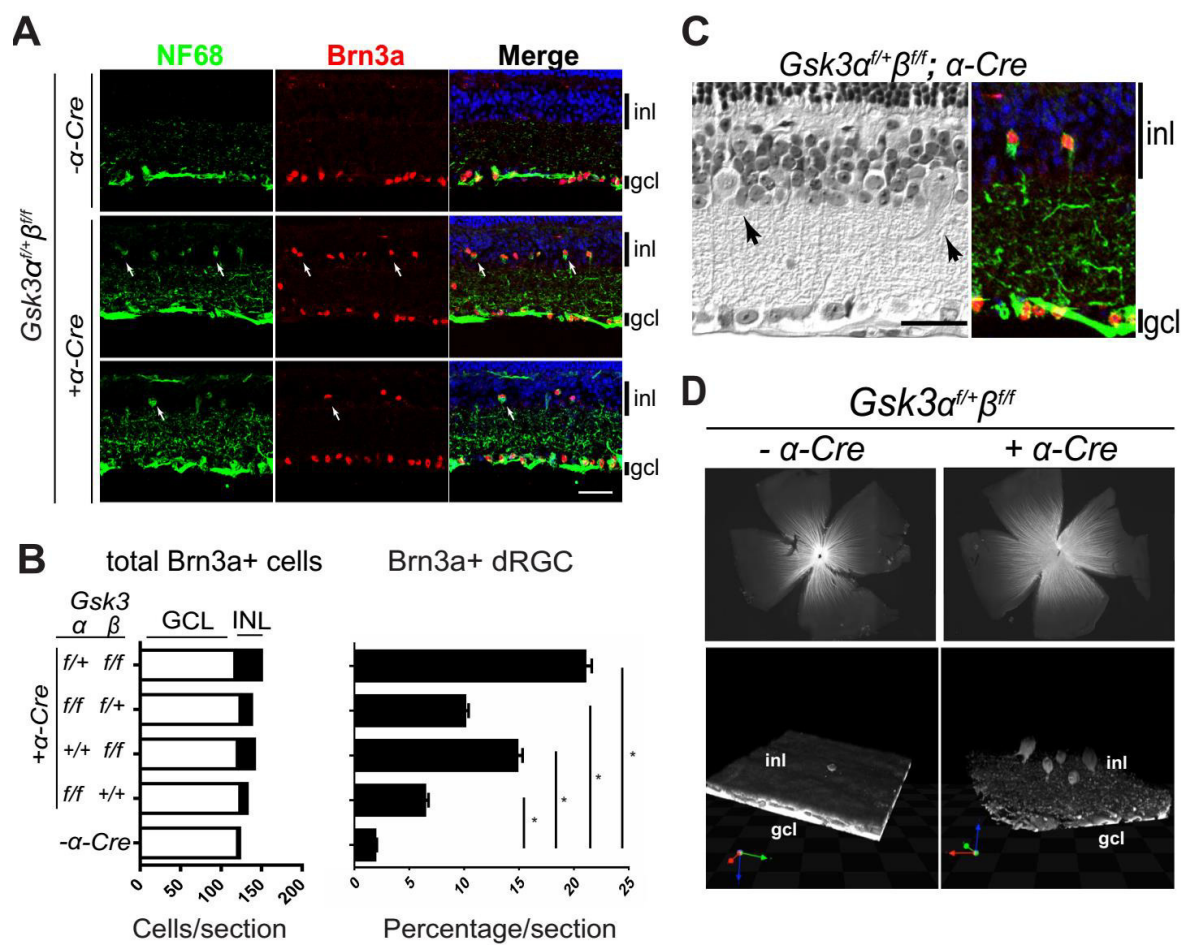


Figure 9. dRGCs are produced in the same differentiation wave as oRGC located in the GCL. (A) EdU- and Brn3a-positive cells were found both in the GCL and in the INL of 30-days old animals after a single injection of EdU at E12.5. (B) Percentage of EdU- and Brn3a-positive cells located either in the GCL or in the INL among total number of Brn3a-positive cells. Mean \pm SEM values are presented from 4 biological replicates, ns, not significant (C) Brn3a and NF68 immunostaining on P0 mouse retina revealed that a large number of dRGCs were already present in *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* but they were fewer in littermate controls (*white arrows*). (D) Left stacked histogram represents counting of the total number of Brn3a-positive cells per section located in the GCL (*white bars*) and in the INL (*black bars*) *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* retina. Right histogram represents the percentage of the dRGCs among the total number of Brn3a-positive cells per section. Mean \pm SEM values are presented from 4 biological replicates, * indicates $P \leq 0.05$. *inl*, inner nuclear layer; *gcl*, ganglion cell layer. *Scale bar: 20 μ m*

Figure 9.

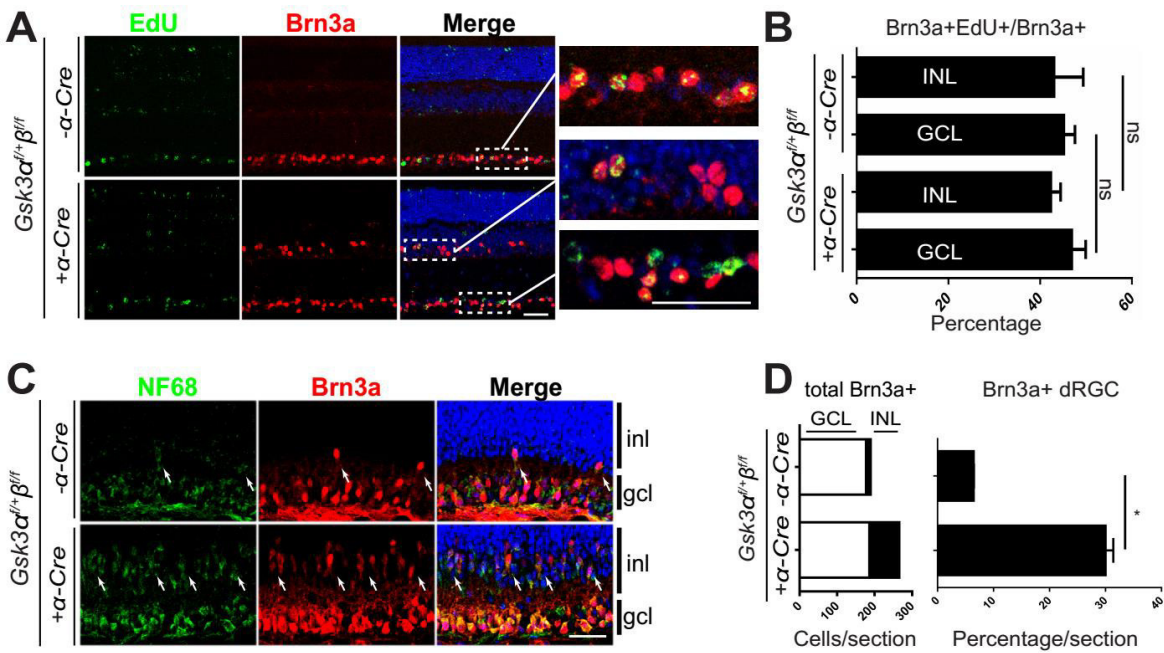
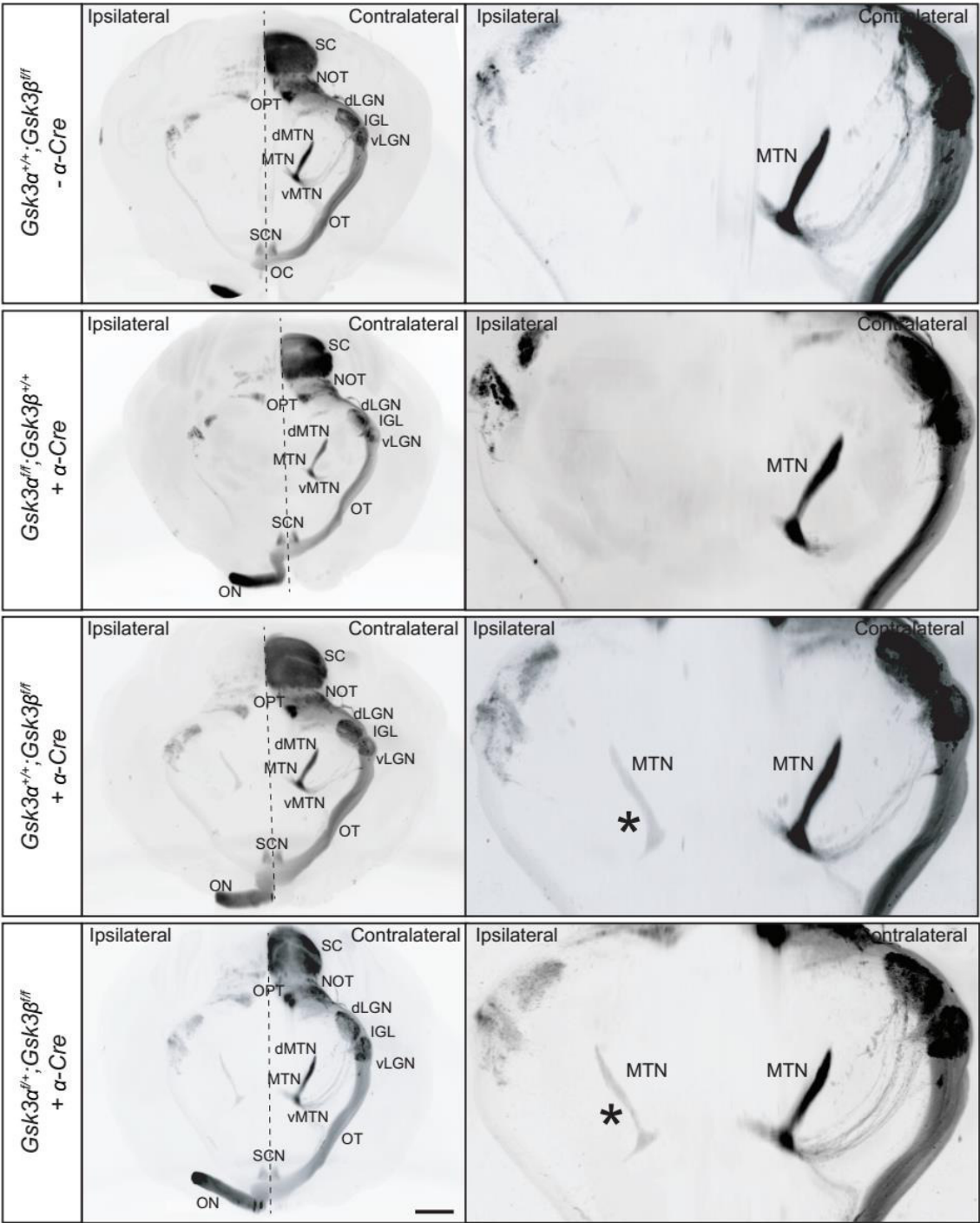


Figure 10. Lack of Gsk3 β results in RGC projections into the ipsilateral Medial Terminal Nucleus. All panels are light sheet fluorescence microscopy of solvent-cleared adult brain from control, *Gsk3 $\alpha^{f/f}\beta^{+/+}; \alpha-Cre$* , *Gsk3 $\alpha^{+/+}\beta^{f/f}; \alpha-Cre$* and *Gsk3 $\alpha^{f/+}\beta^{f/f}; \alpha-Cre$* animals after intravitreal injection of CTB coupled to either an Alexa-555 or -647. Ipsilateral projections of RGCs into the MTN was observed in the absence of *Gsk3 β* expression. SC, superior colliculus; NOT, nucleus of optic tract; dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus; IGL, intergeniculate leaflet; OPT, olivary pretectal nucleus; dMTN, dorsal medial terminal nucleus; MTN, medial terminal nucleus; vMTN, ventral medial terminal nucleus; OT, optic tract; SCN, suprachiasmatic nucleus; ON, optic nerve. Scale bar: 1mm; * indicates the ipsilateral MTN.

Figure 10.



Supplementary Table 1. List of primary and secondary antibodies used for immunohistochemistry (IF) and western blot (WB).

Primary antibodies

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
α -tubulin	mouse	SIGMA	T5168		1:200.000
GSK3 α / β	mouse	Thermo Fisher Scientific	44-610	1:250	1:1000
GSK3 β	mouse	BD	610201	1:250	
β -catenin	rabbit	Abcam	ab2365	1:200	
HES1	rabbit	Cell Signaling	11988S	1:200	
Brn3a	mouse	Santa Cruz	sc-8429	1:200	
GFP	goat	Abcam	ab290-50	1:500	
pH3	rabbit	EMD Millipore	06-570	1:300	
Calbindin D-28k	rabbit	Swant	300	1:100	
Calretinin	mouse	EMD Millipore	MAB1568	1:1.000	
Cone Arrestin	rabbit	EMD Millipore	AB15282	1:1.000	
Rhodopsin	Mouse	Abcam	MAB5316	1:2.000	

Secondary antibodies

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
Alexa Fluor 555 anti-mouse IgG2A	goat	Thermo Fisher Scientific	A21127	1:1000	
Alexa Fluor 555 anti-mouse IgG2B	goat	Thermo Fisher Scientific	A21147	1:1000	
Alexa Fluor 488 anti-rabbit	donkey	Thermo Fisher Scientific	A21206	1:1000	
Alexa Fluor 488 anti-mouse IgG1	goat	Thermo Fisher Scientific	A21240	1:1000	
Alexa Fluor 488 anti-rabbit	goat	Thermo Fisher Scientific	A21244	1:1000	
HRP anti-mouse IgG	goat	Sigma-Aldrich	A4416		1:5000

C.2 GSK3 function in retinal photoreceptors

As photoreceptors are the main retinal neurons, whose function is impaired in blinding diseases, we were specifically interested in studying the role of GSK3 in photoreceptor development, maturation and homeostasis. Our goal was to identify GSK3 targets critical for photoreceptors. Therefore, we opted for the strategy of conditional invalidation of both *Gsk3* in photoreceptor precursors. For this purpose, a mouse model carrying a specific deletion of both *Gsk3* α and *Gsk3* β in postmitotic photoreceptor precursors was generated. Using this mouse model, we studied the consequences of GSK3 loss in murine photoreceptors.

Associated publication (in preparation)

Braginskaja E, Gumerson J, Perron M, Swaroop A and Roger JE.

Glycogen Synthase Kinase 3 regulates NRL phosphorylation and stability establishing the fine tuning of gene regulatory networks required for rod photoreceptor maturation and survival.

Used animal models

To achieve conditional deletion of *Gsk3* α and *Gsk3* β specifically in postmitotic photoreceptor precursors, we used a mouse line carrying the *Crx*-promoter driving the *Cre* recombinase (*Crx-Cre*). This mouse line was kindly provided by Dr Tom Glaser (UC Davis, USA) (Prasov and Glaser, 2012b). *Crx-Cre* mice were mated with *Gsk3* α and *Gsk3* β floxed mice (*Gsk3* α ^{fl/fl} or *Gsk3* β ^{fl/fl}), which were generously provided by Dr Jim Woodgett (University of Toronto, Canada) (MacAulay et al., 2007, Patel et al., 2008) (Patel et al., 2008) to specifically delete both *Gsk3* α and *Gsk3* β in postmitotic photoreceptor precursors.

Phenotypic analysis

The mouse phenotypes were studied using classical laboratory methods such as immunohistochemistry, qPCR, immunoblotting but also electroretinography. Large scale transcriptome analyses were performed in collaboration with the team of Anand Swaroop at the NIH. Certain hypothesis concerning GSK3-mediated mechanisms of action were tested *in vitro*.

C.2.1 Main results

- Loss of both *Gsk3 α* or *Gsk3 β* impairs photoreceptor development and function, followed by their degeneration.
- Loss of *Gsk3* impairs NRL phosphorylation increasing its stability leading to its accumulation.
- NRL increase is not associated with an increase of its target genes.
- On the contrary, most of NRL target genes, co-regulated with CRX and implicated in photoreceptor development and homeostasis are downregulated upon *Gsk3* deletion in photoreceptors.

Altogether, our results suggest that GSK3 plays an essential role in regulating genes necessary for photoreceptor maturation and homeostasis, at least in part *via* the regulation of NRL stability. Such mechanism of posttranslational stability regulation may be deficient in retinitis pigmentosa patients carrying *NRL* mutations altering its phosphorylation.

C.2.2 Article n°2

Glycogen Synthase Kinase 3 regulates NRL phosphorylation and stability establishing the fine tuning of gene regulatory networks required for rod photoreceptor maturation and survival.

Elena Braginskaja, Jessica Gumerson, Muriel Perron*,
Anand Swaroop*, Jerome E Roger*

Glycogen Synthase Kinase 3 regulates NRL phosphorylation and stability establishing the fine tuning of gene regulatory networks required for rod photoreceptor maturation and survival

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Key words: Retinal degeneration; Photoreceptor development, Glycogen Synthase Kinase 3, NRL, CRX, Posttranslational Modifications

Running Title: GSK3 regulates NRL protein stability

ABSTRACT

Photoreceptors are highly differentiated and metabolically active neurons initiating the visual cascade, that require a tight control of gene expression by transcription factors for proper development, maturation and maintenance. As such, the regulation of these key genes can be done at the expression levels but also more rapidly by controlling their activity following posttranslational modifications (PTMs). Among the modifying proteins are the ubiquitously expressed and highly conserved Glycogen Synthase Kinases 3 (GSK3s), positioned at the crossroad of several signaling pathways and acting as molecular switches to regulate numerous cellular processes. To decipher their function *in vivo* during photoreceptor development and homeostasis, we deleted both *Gsk3 α* and *Gsk3 β* in postmitotic photoreceptor precursors. In this mouse model, photoreceptors response to light stimulus is largely reduced due to impaired maturation with progressive degeneration. At the molecular levels, GSK3-dependent phosphorylation regulates the stability of the rod specific transcription factor NRL by triggering its degradation by the proteasome. As such, lack of GSK3 activity led to high increase of its expression at the protein level, especially for certain phosphorylated isoforms. Strikingly, despite the high amount of NRL, whole transcriptome analysis revealed that only a subset of its target genes, related to vision pathway and co-regulated with CRX, were downregulated. In addition, our results shed more lights on disease mechanisms associated with autosomal dominant retinitis pigmentosa caused by NRL mutations altering its phosphorylation.

INTRODUCTION

The vertebrate retina is a neurosensory tissue organized in a laminar fashion, designed for converting light energy into visual information, initiated in particular by the presence of specialized neurons, the photoreceptors. Rod photoreceptors are highly sensitive neurons that can detect a single photon to mediate vision in low light conditions, while cone photoreceptors are responsible for color vision and acuity in brighter light conditions (1, 2). The gene regulatory networks underlying photoreceptor development has been extensively studied, with Orthodenticle Homeobox 2 (OTX2), RAR-related orphan nuclear receptor β (ROR β), Neural retina leucine zipper (NRL), Cone-rod homeobox (CRX), Nuclear receptor NR2E3 and the thyroid receptor TR β 2 being the key transcription factors regulating photoreceptor differentiation (3-5). Among them, NRL is a basic motif leucine zipper transcription factor of the v-maf musculoaponeurotic fibrosarcoma oncogene family protein (MAF)-subfamily (6) and is specifically expressed in rod photoreceptors and the pineal gland (7). NRL is necessary and sufficient for rod cell fate determination; its loss of function in mice leads to a cone-only retina (8), whereas ectopic expression of NRL in photoreceptor precursors directs the differentiation of a rod-only retina (9). NRL interacts with a variety of transcription factors including CRX and NR2E3 to synergistically activate the transcriptional program necessary for rod differentiation (10-13). In addition to the strict control of *Nrl* transcription, several lines of evidence strongly suggest that NRL transcriptional activity is temporally controlled by potential post-translational modifications (PTMs) during photoreceptor genesis. For instance, although NRL expression is detected as early as embryonic (E) day 12.5 during mouse retinal development, the rod visual pigment Rhodopsin, a direct downstream target, is mostly detectable after birth (7).

In addition, photoreceptor precursors express both NRL and cone patterning transcription factor TR β 2, during embryonic development (4, 14). Such results support the hypothesis that PTMs may offer a relevant mode of regulation of NRL transcriptional activity as well as for additional photoreceptor-expressed transcription factors, yet to be identified.

Coordinated and precisely controlled gene expression is essential for development and homeostasis and is governed primarily by *trans*-acting proteins functioning at *cis*-regulatory elements in target genes (15). Although control at the transcriptional level is central to gene regulatory networks, PTMs provide an additional level of complexity and order. Signaling molecules are key components in modifying the function of transcription factors, generally working through PTMs such as phosphorylation, sumoylation, ubiquitination and acetylation (16-18). These rapid and reversible covalent modifications allow efficient cellular adaptation to the changing extracellular microenvironment to influence the outcome in a positive or negative manner (16, 19, 20). Recent published work including ours demonstrated the importance of PTMs in regulating key photoreceptor-specific transcription factor functions. For instance, the expression of *Pias3*, an E3-SUMO ligase, is critical for proper cone differentiation (21, 22) and SUMOylation of NRL and NR2E3 is necessary to fully differentiate rod photoreceptors and activate their direct target genes (23, 24). In addition to SUMOylation, NRL has been shown to have multiple phosphorylated isoforms and human mutations altering such modifications have been reported to cause autosomal dominant retinitis pigmentosa (ADRP) (25-29). However, it remains to be clarified why NRL needs to be phosphorylated. PTMs in NRL are close to or within the minimum transactivation domain of the N-terminal region (30), as described for MAFA, another MAF subfamily protein (31).

MAFA also has multiple phosphorylated isoforms that are altered by mutation in codon 65, which corresponds to codon 50 in NRL (32). Phosphorylation of MAF enhances its transforming activity and is regulated by Glycogen Synthase Kinase 3 beta (GSK3 β) (33, 34).

The ubiquitously expressed and conserved serine/threonine GSK3 α and GSK3 β are the central kinases introducing posttranslational modifications. They were originally described as key regulators of glucose metabolism, directly inhibiting the enzyme Glycogen Synthase by phosphorylation (35). Two isoforms, GSK α and GSK3 β , encoded by two separate genes and sharing 95 % amino-acid sequence identity in the kinase domain (35), can compensate for each other, illustrating their functional redundancy (36). One of their major mode of regulation relies on controlling the protein stability of their numerous targets (37). They are implicated in development, energy homeostasis, survival and inflammation pathways (38). In the central nervous system, GSK3 kinases are important for neurogenesis, neuronal migration, neuronal polarization, microtubule dynamics, axon growth and guidance (35).

The critical role of PTMs, and especially those involving GSK3 in the context of neurodegenerative diseases has already been described in Alzheimer's or Parkinson's diseases (39) (40). Beneficial effects of GSK3 inhibitors have been shown for these diseases and for other neurodegenerative disorders (38, 41-43). In the adult retina, GSK3 kinases are expressed in all cell types, however very little is known about their function (44). Their role in controlling the functional timing of the proneural transcription factor NeuroD, has been reported in the *Xenopus* retina (45). In the context of *retinitis pigmentosa* (RP), the use of GSK3 inhibitors has beneficial effect on retinal degeneration (46).

We undertook this study to elucidate GSK3s function in photoreceptors and to identify their targets. Our results demonstrate a requirement of GSK3s for photoreceptor maturation and survival in the mouse retina. We showed that GSK3-dependent phosphorylation controls NRL stability and is required for the fine-tuning of a subset of genes co-regulated by both NRL and CRX and which are critical for photoreceptor development and homeostasis. Our study provides novel insights in both retinal diseases and developmental biology, with a system-wide understanding of complex gene regulation involving the modulation of transcription factor functions by PTMs in developing photoreceptors.

MATERIALS AND METHODS

Mice breeding, genotyping, animal facilities and ethic statements

All animal experiments have been carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EEC), European Union guidelines effective and with the Association for Research in Vision and Ophthalmology statement for the Use of animals in ophthalmic and visual Research. All animal care and experimentation were also conducted in accordance with guidelines, under the license APAFIS#1018-2016072611404304 by the Institutional animal care committee n°059 in France and by Animal Care and Use Committee at the National Institutes of Health (ASP#650). Both *Gsk3 α* and *Gsk3 β* floxed mice were kindly provided by Dr Jim Woodgett (Toronto, Canada) (47, 48). Mice carrying the *Crx*-promoter driving Cre recombinase (*Crx-Cre*) were kindly provided by Dr Tom Glaser (UC Davis, USA) (49). Conditional *Gsk3 α* and *Gsk3 β* knockout mice were generated using *Crx-Cre* (*Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre*) to achieve a specific deletion in postmitotic photoreceptor precursors. All mouse genotyping was performed as previously described (47-49). Animals were housed in groups of three to five and maintained in standard conditions (21°C, 12h/12h light/dark cycle, ad libitum access to dry food pellets and water) at the Gif-sur-Yvette animal care facilities in France, officially registered for experimental studies on rodents. Mice on C57Bl/6J background at different postembryonic ages rising from P6 to 3 months of either sex were used for experimental procedures. Animals were killed by CO₂ inhalation and cervical dislocation, and all efforts were made to minimize suffering.

Vectors and Plasmids

Human wild-type (WT) and mutant NRL cDNA were subcloned into pcDNA4c His/MaxC vector (Invitrogen) as previously described (28). FLAG-tev-GSK3 was subcloned into pDest-515 (N-terminal Flag tag) using Gateway® Recombination Cloning Technology (Life technologies).

RNA Extraction and Gene Expression Analysis by Real-Time PCR (qRT-PCR)

Total RNA was extracted from neural retina using RNeasy mini kit (Qiagen, Germantown, MD, USA) and treated with DNase I according to the manufacturer's instructions. RNA quantity and quality were assessed using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). For retro-transcription, 500 ng of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit according to manufacturer instructions (BioRad). Primers used for qRT-PCR have been described previously (50). For each qRT-PCR, 2 μ L of a ten-fold dilution of synthesized cDNA was used, and the reactions were performed in technical triplicates on a C1000 thermal cycler (CFX96 real-time system, BioRad) using SsoFast EvaGreen Supermix (BioRad) as previously described (51). qRT-PCR experiments were performed on three to four independent biological replicates. Differential expression was determined using the $\Delta\Delta$ Ct method (52) with the geometric average of *Hprt*, *Gak*, *Mrpl46* and *Srp72* as endogenous controls.

COS-7 cells maintenance and transfection

COS-7 cells (ATCC) were cultured at 37 °C, 5 % CO₂ and 80 % humidity in T75 flasks in DMEM supplemented with 10 % fetal calf serum (FCS), 100 units/mL penicillinG and 100 μ g/mL streptomycin.

The day before the transfection experiment 700.000 COS-7 cells were seeded in 2 mL medium per well in 6-well plates followed by an overnight incubation at 37 °C. Cells at 80 % confluence were transfected with plasmids at 500 ng/well each using Lipofectamine®2000 reagent (Thermo Scientific). If desired, cells were treated with 5 mM MG132, (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO) (Calbiochem) 2 h or with 0.5 µM Cycloheximide (Chx) (Sigma) for various periods of time before cell harvesting.

Protein extraction

Transfected COS cells or frozen retinas were lysed 48 h after transfection in lysis buffer (20 mM Na₂HPO₄, 250 mM NaCl, 30 mM NaPPi, 0.1% NP-40, 5 mM EDTA, 5 mM DTT) supplemented with protease inhibitor cocktail (P-2714, Sigma). After sonication and centrifugation, lysates concentration was determined using a Lowry protein assay kit (500011, DC Protein Assay, Bio-Rad). For phosphatase treatments, protein lysates were treated with 50 U of lambda phosphatase (Sigma) for 30 min at 37°C and processed thereafter for immunoblotting.

Immunoprecipitation and Immunoblotting

The supernatant was used for immunoprecipitation or immunoblot analysis. For immunoprecipitation, cell extracts were incubated with 1 µg of anti-Ub or anti-NRL antibody and immunoprecipitated was isolated on protein magnetic beads (Life technologies) according to the manufacturer instructions. 10 µg of cell protein extract and 20 µg retinal protein extracts were loaded per sample and separated on 12 % SDS-PAGE, then transferred onto nitrocellulose membranes. Immunoblotting was carried out as previously described (53). Primary and secondary antibodies are listed in Supplementary Table 1.

Proteins were visualized using enhanced chemiluminescence kit (Bio-Rad). α -tubulin was used as the loading control. Quantification was performed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Hematoxylin & Eosin (H&E) staining and Immunohistochemistry

Eyeballs were fixed in 4 % PFA for 60 min on ice after removal of the cornea and the lens. Methacrylate sections were used for H&E staining as previously described (54). For immunohistochemistry, fixed eye balls were incubated in an increasing concentration of sucrose (10 %, 20 % and 30 %) and embedded in OCT. Eyeballs were serially cut to 12 μ m sections using a cryostat. Immunohistochemistry was performed as previously described (24). Primary and secondary antibodies are listed in Supplementary table 1. Sections were counterstained with 1:1000 4',6-diamidino-2-phenylindole (DAPI) (1 mg/mL, 62248, Thermo Scientific). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays using in situ cell death detection kit according to the manufacturer's instructions (Promega). All images were acquired using a Zeiss LSM710 confocal microscope and Zen software (Zeiss).

Whole transcriptome sequencing (RNA-Seq) and data analysis

Whole transcriptome analysis (RNA-Seq) was performed using three independent biological replicates from P6, P10 and P14 *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$* retinas expressing or not *Crx-Cre*. RNA-seq libraries were constructed from 100 ng of total RNA using a modified TruSeq Stranded RNA Sample preparation kit protocol (54). Paired-end sequencing of 100 bases length was performed on a HiSeq 2500 system (Illumina). Pass-filtered reads were mapped using HiSAT2 (55) and aligned to Ensembl genome assembly GRCm38 (release 84).

This annotation includes cDNA, miRNA, long non-coding RNA, pseudogenes and gene predictions. Count table of the gene features was obtained using Feature Counts (56). For gene level analysis, EdgeR was used for normalization, differential expression analysis and to compute FPKM (fragments per kilobase of exon per million fragments mapped) values (57). An FPKM filtering cutoff of 1 in at least one of the 18 samples was applied. A False Discovery Rate (FDR) of less than or equal to 0.05 was considered significant and a cutoff of fold change of 2 was applied to identify differentially expressed genes. R packages and JMP Software (SAS) were used for data mining. GO annotation and pathways enrichment analysis were performed using Panther Classification System (<http://pantherdb.org/>).

Electroretinography

Electroretinogram (ERG) recordings were performed as described previously (21). Briefly, mice were dark-adapted overnight and prepared for the experiment under dim-red light. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and received topical proparacaine hydrochloride (0.5 %, Alcon) via eye drops. Pupils were dilated with tropicamide (1 %, Alcon) and phenylephrine (2.5 %, Alcon). Gold wire loop electrodes were lightly coated with GONAK hypromellose ophthalmic demulcent solution (2.5 %, Akorn) and placed directly on each cornea, and a reference electrode was placed in the mouth. ERG recordings were performed using an Espion E2 System (Diagnosys). Scotopic responses were elicited with a series of flashes of increasing light intensities from 0.001-10 cd.s/m². Photopic responses were elicited under rod-desensitizing background light with a series of flashes of increasing light intensities from 0.3-100 cd.s/m². Values of a- and b-wave were extracted and plotted for comparisons between groups.

RESULTS

Gsk3 α and Gsk3 β expression is required for photoreceptor homeostasis.

In order to investigate the role of GSK3 in photoreceptors, we took advantage of the mouse double floxed line *Gsk3 $\alpha^{f/f}\beta^{f/f}$* (47, 48) and a *Crx-Cre* driver lines (58). By crossing these lines, we generated *Gsk3 $\alpha^{f/f}\beta^{f/f}; Crx-Cre$* mice, that allow for *Gsk3* conditional knockout in photoreceptor precursors. The efficacy of the conditional deletion of both *Gsk3 α* and *Gsk3 β* was assessed by immunoblotting on whole retinal protein. At postnatal (P) day 6, *Gsk3 α* and *Gsk3 β* deletion did not significantly reduce the expression of the two kinases in *Crx-Cre* line compared to littermate controls, whereas at P10 and P14 expression of both *Gsk3 α* and *Gsk3 β* was severely reduced (Fig. 1A). The loss of both GSK3 kinases in the outer nuclear layer (ONL) was also confirmed by IHC on P14 retina using a pan antibody recognizing both *Gsk3 α* and *Gsk3 β* (Fig. 1B) (49). Thus, the *Gsk3 $\alpha^{f/f}\beta^{f/f}; Crx-Cre$* mouse line allows efficiently the deletion of both isoforms in photoreceptors after P6.

We next assessed retinal histology of *Gsk3 $\alpha^{f/f}\beta^{f/f}; Crx-Cre$* mice. Hematoxylin and eosin (H&E) staining on methacrylate sections from P10 to 3-months of age (3 mon) revealed proper lamination of the retina at P10 with no significant differences with control retina (littermates *Gsk3 $\alpha^{f/f}\beta^{f/f}$* without *Crx-Cre* recombinase) (Fig. 2A). From P28 onwards, retinal degeneration could be clearly observed on retinal sections. Measurement of the ONL thickness demonstrated a statistically significant loss of photoreceptors from P28 onward in the center and the periphery of *Gsk3 $\alpha^{f/f}\beta^{f/f}; Crx-Cre$* retinas compared to controls (Fig. 2A and 2B). At P18, photoreceptor degeneration was not yet not obvious. IHC using anti-GFAP indicated slight activation of Müller cells as early as P18, and a higher activation at P28 (Fig. S1).

Quantification and kinetic analysis of photoreceptor cell death, assessed by TUNEL assay, demonstrated a significant increase of apoptotic cell number in the ONL from P18 onwards in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* retinas compared to controls (Fig. 2C). Apoptotic cell death reached a peak between P21 and P28 in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* mice (Fig. 2C). Given the decreased thickness of the ONL and the increased cell death starting at P18, we assessed visual functions in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* animals by electroretinogram (ERG) recordings at P18, P21, P28, 2 and 3 months. At the onset of photoreceptor degeneration (P18) when the ONL is still preserved, scotopic a-wave corresponding to rod photoreceptor response to light stimulus, remained low in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* animals compared to controls despite increased light intensity stimulus (Fig. 3A). As a consequence of such low response of rod photoreceptors, scotopic b-wave (corresponding to the transmission of the visual signal through the inner retina) was also largely diminished. Overall, the scotopic ERG was constantly very low in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* compared to controls from P18 up to 3 months of age (Fig. 3B). As illustrated at 1 cd.s./m², an increase of b-wave amplitude still occurred in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* between P18 and P28 (from 100 to 250 μ V), although extremely limited compared to control animals (from 300 to 630 μ V) (Fig. 3B). Such results indicate that retinal maturation of the different retinal circuits still occurred in the absence of GSK3 but remained very limited. At 3 months, photoreceptors in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* animals showed some remaining a- and b-wave responses as still few rods were remaining in the ONL at this age (Fig. 3B and data not shown). After light adaptation, photopic ERG was recorded on the same animals. The cone b-wave response was significantly decreased in *Gsk3 α ^{f/f} β ^{f/f}; Crx-Cre* animals compared to littermate controls at all light intensities at P18 (Fig. 3C).

At 1 cd.s./m², photopic responses were also largely impaired from P18 to 3 months in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* animals compared to littermate controls (Fig. 3D). Nevertheless, and as observed for rods, cone response was also slightly increased from P18 to P21 as observed in control animals but decreased afterwards to become null at 3 months suggesting that cone photoreceptor maturation, although limited, still occurred in absence of GSK3 kinases (Fig. 3D). Altogether, scotopic and photopic ERG recordings demonstrate defects in both rod and cone photoreceptor function as early as P18. In addition, the kinetics of the decreased amplitude of the scotopic (from P28) and photopic b-wave (from P21) responses in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* animals suggests that cone photoreceptors may be more sensitive to GSK3 loss compared to rods.

Lack of GSK3 kinases impairs photoreceptor maturation.

Since visual function was largely impaired at P18 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* mutant mice prior the peak of apoptosis, we hypothesized that photoreceptor might not differentiate properly. Therefore, we performed a detailed phenotypic analysis on earlier stages, ranging from P6 to P18, using different photoreceptor-specific markers to detect potential developmental defects. IHC analysis of Rhodopsin expression revealed a delocalization of the protein in the ONL, as early as P6 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* compared to controls (Fig. 4). From P10 to P18, when photoreceptors mature, the lack of GSK3α and GSK3β impaired rod outer segment formation as inferred by the accumulation of Rhodopsin in the ONL compared to control retinas, where a vast majority of Rhodopsin protein was concentrated to the outer segments (Fig. 4). In cone photoreceptors, expression of cone Cone Arrestin was decreased at P14 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retina. At P18, Cone Arrestin staining revealed large defects in cone outer segment formation.

Indeed, cone photoreceptors in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* retina did not display a bright signal in the outer segment, as observed in control retina, but instead showed a more diffused signal delimitating a swollen segment, that may correspond to the inner segment (Fig. 4). S-opsin expression in the ventral part of the retina, where S-cones are abundant, was similar in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals to their littermate controls until P14, suggesting that S-cones developed normally up to this stage. However, at P18, S-Opsin expression was largely decreased and the remaining protein produced was mislocalized to the ONL (Fig. 4). Finally, expression of M-opsin was largely reduced in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls until P14, suggesting an impairment in M-cone development. However, M-opsin expression in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals seemed to reach an expression level close to controls at P18, despite the shortening of the outer segments as observed with Cone Arrestin (Fig. 4). Temporal expression analysis of these three cone markers suggests that different cone specific genes are differentially altered in the absence of GSK3 kinases but they commonly highlight defects in cone photoreceptor development. Altogether, our data demonstrate the critical role of GSK3s during photoreceptor maturation, especially in the formation of the outer segment of both rod and cone photoreceptors. Such impaired photoreceptor development observed in the absence of GSK3s explains the origin of the severe defects in visual responses observed as early as P18 by ERG recording (Fig. 3).

Lack of GSK3 kinases leads to a downregulation of genes related to retina development and function.

In order to get a global overview of transcriptional changes occurring in the absence of GSK3 in photoreceptors and to delve into the mechanisms underlying the impairment of photoreceptor function, we performed whole transcriptome analysis (RNA-Seq) of P6, P10 and P14 wild type and *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retina (Fig. 5).

Using a fold change (FC) cutoff of 1.5, a False Discovery Rate (FDR) ≤ 0.05 and an expression value ≥ 2 Fragment per kilobase of exon per million reads mapped (FPKM), we identified 154 differentially expressed genes (DEGs) at P6, 198 at P10 and 700 at P14. Volcano plot representation allowed the identification of several downregulated photoreceptor-expressed genes such as Cone Arrestin (*Arr3*), *Pde6h*, M-Opsin (*Opn1mw*), *Guca1b* or *Esrrb* (Fig 5A). During development, the number of DEGs largely increased with an increasing number of genes becoming downregulated. Among all DEGs, only 34 were downregulated at all stages, including important genes for retinal homeostasis and function, such as *Slc14a1*, *Esrrb*, *Pde6a* and uncharacterized transcripts such as *A930003A15Rik*, *Al847159* or *5930430L01Rik* that are mostly expressed in the eye according to EST databases (UNIGENE). Analysis using STRING highlighted a network of genes at all stages related to visual transduction, including several genes such as *Pde6a*, *Pde6c*, *Gnat1*, *Pde6h*, *Gucy2f*, *Guca1b* or *Gnat1* (data not shown). In addition, STRING analysis at P14 also identified a large network of genes coding for ribosomal proteins. Pathways analysis using PANTHER classification system for the DEG at each stage allowed the identification of biological processes, molecular function and cell compartments based on Gene Ontology that are statistically overrepresented (Fig. 5C).

For biological processes, several pathways related to retina development, visual perception, eye development and detection of light stimulus were found overrepresented at least in one stage. A large number of genes were also identified as belonging to cell compartment corresponding to photoreceptor inner and outer segments. A vast majority of the genes belonging to these different pathways were downregulated with a negative z-score. As found previously using STRING network analysis, a pathway corresponding to ribosomal subunit was found significantly overrepresented at P14 with a majority of these genes upregulated in the absence of GSK3 kinases.

Altogether these different analyses were strongly correlated with our phenotype and a large defect in photoreceptor maturation and impaired function. Therefore, we focused on groups of genes of the gene ontology (GO) related to vision (retina development in camera-type eye (GO:0060041), visual perception (GO:0007601), eye development (GO:0001654) and detection of light stimulus (GO:0009583)). As such, we isolated 56 DEGs belonging to at least one of this GO pathways out of 410 genes. After hierarchical clustering, we identified 42 genes showing increased expression during normal photoreceptor development but whose expression remained at low level in the absence of GSK3 (Fig. 5D). Among these downregulated genes, we identified few nuclear factors such as *Nrl* or *Essrb* and several disease-associated genes such as *Cep290*. However, other key transcription factors such as *Crx* or *Nr2e3* were unchanged in the absence of GSK3.

We next validated by qRT-PCR few DEGs identified following RNA-Seq analysis and appearing relevant to the observed phenotype according to their key function in photoreceptors (Fig. 6). In rods, *Rhodopsin* expression was about 2.5-fold decreased at P14 but unchanged at P6 and P10 in *Gsk3 $\alpha^{\text{f/f}}$ $\beta^{\text{f/f}}$; Crx-Cre* retina compared to controls.

This result was confirmed at protein levels (data not shown). In cones, *Cone Arrestin* expression was significantly reduced at all examined stages (P6, P10, and P14) in *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; Crx-Cre* animals compared to controls, preceding the protein decrease observed by IHC, that became obvious from P10-P14. *S-Opsin* was significantly decreased at P14, preceding the protein decrease observed by IHC from P18 onwards. *M-Opsin* was significantly decreased at P10 and P14 in *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; Crx-Cre* animals, which is consistent with the IHC results. Overall, our combined IHC, RNA-Seq and qPCR analyses demonstrate the importance of GSK3 for photoreceptor maturation.

Based on the downregulation of *Nrl* expression found by RNA-Seq analysis, we hypothesized that impaired photoreceptor maturation may be due in part to a decreased expression of NRL causing a downregulation of its target genes including additional photoreceptor specific transcription factors, such as CRX or NR2E3, that are known to function synergistically with NRL (4). Their expression was assessed by qRT-PCR and immunoblot from whole retinal extracts at stages P6, P10 and P14 in *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; Crx-Cre* animals and compared to littermate controls (Fig. 6B). CRX and NR2E3 expression did not change neither at the RNA nor at the protein level at all the three examined stages, confirming the RNA-Seq data. Strikingly, *Nrl* expression was significantly decreased at the RNA level at P10 and P14 but unexpectedly highly increased at the protein level (Fig. 5B). Moreover, we found a modification in the proportion of NRL isoforms expressed, and only three isoforms were upregulated compared to controls (Fig. 5B and Suppl. Fig. 2). Previous work identified the different NRL isoforms as phosphorylated (29), suggesting that lack of GSK3 has an impact on the phosphorylation profile of NRL and affects the ratio of the different isoforms.

GSK3-dependent phosphorylation regulates NRL protein stability.

Our results were puzzling: On one hand *Rhodopsin* expression that is directly regulated by NRL, NR2E3 and CRX, is suppressed in *Gsk3 α ^{ff/ff}; Crx-Cre* retinas, despite high amount of NRL protein, and on the other hand, other NRL target genes, such as *Nr2e3*, showed unchanged expression levels. We thus raised the hypothesis that changes in NRL phosphorylation status in absence of GSK3s could impair its transcriptional activity. We first investigated the molecular mechanisms leading to higher NRL protein amount in *Gsk3 α ^{ff/ff}; Crx-Cre* animals. *In silico* analysis of the human NRL protein revealed multiple potential phosphorylation sites (<http://www.cbs.dtu.dk/services/NetPhos/>) - Serine or Threonine residues that are evolutionarily highly conserved in vertebrates and in other MAF-subfamily members (Fig. 7A). As described for MAFA (33) (34), NRL protein sequence includes putative GSK3 sequential phosphorylation sites at residues 46, 42 and 38, after a predicted priming phosphorylation on Serine 50 (S50). In contrast to other MAF-subfamily members, the Serine at residue 34 is absent. Therefore, NRL would likely have at least four phosphorylated isoforms if no other Serine/Threonine/Tyrosine residues are modified (Fig. 7A). To determine if mutations on putative phosphorylation sites disrupt sequential NRL phosphorylation, we transfected putative NRL phosphorylation mutants (NRL-S50A, NRL-S46A, NRL-T42A or NRL-S38A) or WT-NRL. As reported previously, we can distinguish multiple phosphorylated isoforms by immunoblot (four bands depending on the exposure time but only one with NRL-S50A mutant) (28, 29) (Fig. 7B). In concordance with the predicted number of isoforms produced for each mutant (see Fig. 7A), NRL-S46A presents only two isoforms, including one non-phosphorylated form, as detected for NRL-S50A mutant (Fig. 7B). Mutation at Threonine 42 (NRL-T42A) resulted in an additional isoform compared to NRL-S46A.

We predicted that the transfection of WT-NRL could produce five different isoforms, but we observed a maximum of four under our experimental conditions. The highest band on the immunoblot was detectable only after a very long-time exposure. Phosphatase treatment on protein extracts from transfected cells with NRL-WT, led to the detection to only one isoform, confirming that the multiple NRL isoforms are indeed phosphorylated (Fig. 7C). Treatment of the transfected cells with a GSK3 inhibitor (2',3'-bisphosphoryl-6-bromoindirubin-3'-oxime (BIO) reduced to one the number of phosphorylated isoforms of NRL-WT (Fig. 7C), suggesting that NRL phosphorylation is GSK3-dependent. As expected, NRL-S50A was insensitive to BIO inhibitor or phosphatase treatment (Fig. 7C). As GSK3 β can sequentially phosphorylate target proteins (14), we co-transfected WT-NRL, NRL-S38A, NRL-S42A, NRL-S46A, NRL-T50A with GSK3 β expression construct.

A gradual increase in the ratio of higher isoforms was observed in all except NRL-S50A mutant (Fig. 7D), suggesting that GSK3 sequentially phosphorylates NRL from codon 46 to codon 38. We then evaluated the effect of GSK3 β on NRL-S50T and NRL-P51S, two NRL mutants associated with human ADPR (Fig. 7E) (26) (59) (60). Co-expression of NRL-S50T with GSK3 β produced one additional isoform with higher molecular weight in contrast to NRL-S50A and NRL-P51S, though at a very low expression level (Fig. 2E), suggesting that patients carrying NRL-S50T mutations may still have potentially some remaining degree of NRL regulation by PTMs. Altogether, these results demonstrate GSK3-dependent phosphorylation of NRL.

We next sought to investigate the consequence of such PTMs on NRL. Based on our *in vivo* results revealing NRL protein level increase in absence of GSK3, we hypothesized that GSK3-dependent phosphorylation regulates NRL stability through degradation by the Ubiquitin Proteasome System (UPS), as described previously for MAFA (33) (34). We therefore transfected WT-NRL with or without GSK3 β and 48 hours (h) later, cells were treated with Chx to inhibit protein synthesis during the indicated periods of time (0 min, 30 min, 1 h, 1 h 30 min, 3 h) (Fig. 7F). After 30 min treatment with Chx, WT-NRL protein was almost undetectable in presence of GSK3 β in contrast to WT-NRL without GSK3, suggesting a decreased stability of WT-NRL in the presence of GSK3 β . We then compared the degradation rate of WT-NRL and of the two human ADRP-associated mutants in the presence of GSK3 β , following Chx treatment (Fig. 7G, H). The half-life (50 % of the initial protein quantity) of WT-NRL + GSK3 β was about 30 min. In contrast, the NRL-S50T and NRL-P51S mutants were more stable even in the presence of GSK3 β , with a half-life of 75 min for the NRL-S50T (that exhibits a slight phosphorylation) and around 100 min for the NRL-P51S mutant (Fig. 7G and 6H).

We then tested the hypothesis that multiple phosphorylation modulated by GSK3 β target the NRL protein to the UPS, as it was demonstrated for β -catenin in the Wnt signal transduction pathway (61) (62). We therefore co-transfected WT-NRL or NRL-S50A with or without GSK3 β in COS-7 cells, which were then harvested 48 h later, following a two-hour treatment with MG132, an inhibitor of the proteasome (63) (Fig. 7I). The inhibition of the UPS resulted in a large increase in the amount of WT-NRL protein when co-transfected with GSK3 β , however, the amount of NRL-S50A protein remained unaltered.

Altogether, these experiments suggest that NRL phosphorylation by GSK3 β controls the stability of NRL protein by targeting it to the UPS. The observed increased level of NRL protein in absence of GSK3 results from an increased stability due to impaired phosphorylation. This mechanism may as well be strongly reduced in retinopathy-associated NRL mutants.

Increased amount of NRL and alteration of its phosphorylated isoforms impair the expression of a subset of rod photoreceptor-expressed genes co-regulated by CRX.

We next sought to explore the mechanism underlying the decreased expression of a subset of NRL target genes in GSK3 mutant mice, despite a higher stability of the protein. We combined our RNA-Seq data with published ChIP-Seq data for NRL and CRX in order to better delineate the subset of genes deregulated and targets of NRL or CRX or both. Between 30 % and 45 % of the DEGs had at least one binding site for either CRX or NRL or both (Fig. 7A). Among them, an increasing proportion of these DEG from P6 to P14 had both NRL and CRX binding sites (from 54.9 % at P6 to 64 % at P14) with a concomitant decreased proportion of DEGs with only NRL binding sites in *Gsk3 $\alpha^{fl/fl}\beta^{fl/fl}$; Crx-Cre* retinas (Fig 7B). In contrast, only 38.8 % of genes showed NRL and CRX binding sites in WT adult retina, suggesting that lack of GSK3 altered mainly the expression of genes regulated by both transcription factors.

The same analysis performed on the 56 genes related to vision and clustered previously (Fig. 5 D) identified 30 deregulated genes with binding sites for either NRL or CRX or both. Among them, less than 70 % have binding sites for both NRL and CRX at P6, as found in WT retina. Strikingly, at P10 and P14 more than 90 % of the DEGs are regulated by both CRX and NRL with only two genes upregulated across all stages, *Gnat1* and *Fscn2* (Fig. 8C). Hierarchical clustering on NRL targets genes belonging to vision-related pathways revealed 28 genes with both NRL and CRX target genes and only two (*Lrit3* and *Pde6c*) with only NRL binding sites (Fig. 8D). Such results strongly suggest that alteration of NRL phosphorylated isoforms and increased amount of NRL preferentially alters a subset of genes regulated by both transcription factors, NRL and CRX.

DISCUSSION

PTMs exert a key control on transcriptional gene regulation. Multi-site phosphorylation is a common, sophisticated and versatile mechanism for regulating protein function and activity, particularly in case of transcription factors (18). Phosphorylation can modify transcription factor stability, nuclear localization, protein-protein interactions, DNA-binding, transcriptional activity and transactivation-induced inactivation (64). In regards to the central function of GSK3 kinases in the CNS, we focused on their unknown function specifically in photoreceptors. We found that the absence of GSK3s leads to impaired photoreceptor maturation and to their subsequent degeneration. Our data strongly suggest that increased amount of NRL protein and alteration of its phosphorylation may contribute to the phenotype. Our *in vitro* data demonstrates a major role of GSK3-dependent multi-site phosphorylation in controlling the amount of NRL protein by promoting its degradation by the proteasome. Such findings provide evidence for the mechanisms leading to the loss of NRL protein regulation *in vivo*. Therefore, we propose a model where the availability of NRL protein (stability) and strict control of the amount of its different phosphorylated isoforms would be critical for tightly initiating and maintaining gene regulatory networks and promoter occupancy during rod development and homeostasis. Our studies therefore provide the framework for integrating signaling pathways in the regulation of NRL activity via PTMs.

GSK3 kinases are involved in photoreceptor development and homeostasis.

In this study, we found that the absence of GSK3 leads to both developmental defects and subsequent photoreceptor degeneration. Whether these two phenotypes are independent or associated is an open question.

Indeed, the arrest of photoreceptor differentiation described in *Crx^{Rip/+}* dominant mouse model of human congenital blindness does not lead to massive subsequent degeneration indicating that photoreceptor degeneration occurring in the absence of GSK3 kinases could be independent from their impaired differentiation (54). In contrast, in some syndromic diseases affecting the retina, such as Bardet-Biedl (BBS) or Usher syndrome, photoreceptor cell death is a consequence of developmental defects, *i.e.* disorganization and shortening of forming photoreceptor OS (65) (66). Therefore, further investigations will be required to focus specifically on the role of GSK3 in photoreceptor maintenance in a context where photoreceptor develop properly. Surprisingly, complete loss of GSK3 leads to photoreceptor degeneration, whereas the presence of only one allele not invalidated (*Gsk3 α ^{f/+} β ^{f/f}; Crx-Cre* or *Gsk3 α ^{f/f} β ^{f/+}; Crx-Cre*) completely rescues the phenotype with normal ERG recording and histology (data not shown). Interestingly, the use of GSK3 inhibitors showed beneficial effect on retinal degeneration in the context of retinitis pigmentosa, as described in several model of neurodegeneration in the CNS (46, 67). GSK3 dual function in promoting either cell death or survival, depending on its kinase activity and the cellular context, has been well established (46, 68). Therefore, further experiments using different models of injury, such as MNU injection or light exposure, need to be performed to assess the resistance of the photoreceptors in retina expressing only one copy of either *Gsk3 α* or *Gsk3 β* . In addition, to determine the effects of the complete loss of GSK3 functions in adult photoreceptors, subretinal injection of an AAV vector expressing *Cre* recombinase could be performed. Altogether, the obtained results could pave the way to new therapeutic approach for retinal dystrophies.

Disease-causing-mutations increase NRL transcriptional activity through increased stability.

NRL and MAFA belong to the large Maf sub-family of bZIP transcription factors with a highly conserved sequence around the minimum transactivation domain, which contains the amino acid residues phosphorylated by GSK3 β . In myeloma cells, GSK3 β -dependent phosphorylation increases MAFA transforming activity and enhances its degradation, while MAFA phosphorylation mutant S65A (corresponding to S50A in NRL) shows lower transcriptional activity on target genes (34, 69). However, in pancreatic β -cells, MAFA is degraded under low glucose concentration, while the phosphorylation mutant of MAFA has a higher transcriptional activity on the insulin promoter due to an increase in MAFA stability (33). NRL phosphorylation mutants exhibit a similar increase in stability and transcriptional activity, but more importantly, our study highlights the complexities associated with PTMs of transcription factors. While phosphorylation induces the degradation of MAFA and NRL, the lack of phosphorylation seems to have different consequences depending on the cell type or the cellular context. NRL mutations at codons 50 and 51 alter its phosphorylation and are associated with retinal degeneration in humans (25-28, 60). The higher transcriptional activity observed *in vitro* for these two mutants on the *Rhodopsin* promoter suggests that a tight regulation of NRL direct targets is crucial for maintaining rod function. NRL phosphorylation mutants (S50 or P51) are still able to bind to *Rhodopsin* promoter and it was proposed that NRL phosphorylation might regulate its interaction with other nuclear factors affecting the composition of the transcriptional complexes (28, 30). We propose that increased *Rhodopsin* promoter activation may also originate from NRL-S50T and NRL-P51S longer half-life allowing the presence of more NRL in the cells and therefore more transcription of the target genes.

Our *in vitro* results showed that conversion of Serine 50 to Threonine largely prevents NRL phosphorylation even in the presence of GSK3 β whereas the change of codon 51 from Proline to Serine completely block it, suggesting the critical role of P51 in maintaining NRL tertiary structure and importance of the consensus sequence in determining PTM-mediated regulation. The longer half-life of NRL-P51S mutant compared to NRL-S50T can be explained by the fact that NRL-S50T can still be phosphorylated albeit at a reduced level. Our data thus clarify the genotype-phenotype correlations and explain a more severe clinical manifestation observed in the NRL-P51S patients compared to those carrying the S50T mutation (27, 59). Altogether, our results demonstrate that GSK3-dependent phosphorylation strictly controls the amount of NRL in rods. The loss of such level of regulation in the case of NRL ADRP-associated mutations increases its amount at the protein level altering the expression of its target genes causing photoreceptor cell death.

Different NRL-phosphorylated isoforms regulate different subsets of target genes.

We were unable to detect *in vivo* which amino acids were phosphorylated in retinal extracts from controls and *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* due to the impossibility to properly digest NRL protein and therefore produce peptides suitable for mass spectrometry analysis. Despite the lack of such information, our *in vitro* data confirmed the predicted GSK3 phosphorylation sites sequentially modified from codon 46 to codon 38 similarly to MAFA (33, 34). In addition, our *in vivo* data obtained in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* retina clearly showed higher amount of NRL protein, which can be correlated to our *in vitro* data showing enhanced protein stability in absence of GSK3-dependent phosphorylation.

Surprisingly, NRL protein still shows some degree of phosphorylation *in vivo* in the absence of GSK3 kinases although the increase mostly concerns isoforms with lower phosphorylation based on their lower molecular weight.

In *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retina, the action of the priming kinase could still occur but subsequent GSK3-dependent phosphorylation of S46, T42 and S38 amino acids is impaired. We cannot exclude that additional kinases can also phosphorylate NRL, as showed *in vitro* with MAPKs (70). These kinases can also be one of the priming kinase initiating GSK3-dependent phosphorylation as described for MAFA (34).

In contrast to NRL-S50A mutants with increased transcriptional activity on *Rhodopsin* promoter *in vitro*, altered NRL phosphorylation *in vivo* led to the opposite effects with reduced expression of a subset of rod expressed genes, including *Rhodopsin* whereas other genes, such as *Nr2e3*, were normally expressed. Altogether, these results strongly support that distinct NRL isoforms differentially control target gene expression clearly demonstrating that PTMs add a higher degree of complexity beyond the regulation of gene expression at the transcriptional level. In the case of NRL and since no splicing events have been reported so far, we can predict that from a unique transcript, PTMs lead to the production of more than 5 phosphorylated isoforms, which can be independently combined to sumoylation (24). In this context, our work deciphered only a small part of this complex regulation and further study will be required to better understand the role of each NRL phosphorylated isoforms.

Lack of GSK3-dependent phosphorylation of NRL deregulate a subset of photoreceptor expressed genes co-regulated with CRX.

Phosphorylation can quantitatively modulate the promoter occupancy in a rheostat-like manner, as demonstrated for the bHLH transcription factor Neurogenin 2 (Ngn2) (71). Highly phosphorylated Ngn2 is targeted to the proteasome, leading to reduce promoter occupancy in dividing progenitors, whereas unphosphorylated Ngn2 binds stably to target gene promoters, such as NeuroD, initiating neuronal differentiation. The concept of regulation of homeostasis by protein degradation is also gaining strength (72).

Mutations in KLHL7, a BTB-Kelch protein implicated in the UPS, are associated with retinitis pigmentosa (73, 74). A deficiency in TRIM2 ubiquitin ligase activity leads to protein aggregation and neurodegeneration, notably in the retina, cerebellum, cerebral cortex and spinal cord (75). We can thus hypothesize that signaling pathways modulate NRL stability, thereby modifying promoter target gene occupancy by NRL-containing transcriptional complexes, to fine tune photoreceptor gene regulatory networks. Since CRX, NRL and NR2E3 activate synergistically their target gene expression, we can also speculate that altered NRL phosphorylation and/or protein accumulation would lead to accumulation of NRL homodimers on its target genes, preventing the formation of a highly active transcriptional complex comprising CRX among other transcription factors. Our results clearly support such mechanism involving a modification in the composition of NRL transcriptional complexes reducing the expression of a subset of their target genes co-regulated with CRX (10, 13).

In conclusion, our work suggests that GSK3-dependent phosphorylation of NRL produces distinct NRL isoforms, regulates NRL protein stability and controls target gene promoter occupancy. In this context, the identification of inhibitors or activators of signaling pathways leading to a strict control of NRL post-translational modification could therefore be exploited for small molecule therapeutics to treat retinal diseases.

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FIGURE LEGENDS

Figure 1. Conditional deletion of *Gsk3 α* and *Gsk3 β* in photoreceptors using the *Crx-Cre* line. (A) Immunoblot on whole retinal protein extracts showing GSK3 α and GSK3 β expression in *Crx-Cre* line at P6, P10 and P14. Reduced expression of GSK3 α and GSK3 β was detectable starting at P10. (B) Immunohistochemistry using an antibody that detects both GSK3 α and GSK3 β at P14 revealed reduced expression of both kinases in photoreceptors using the *Crx-Cre* line. onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 20 μ m

Figure 1.

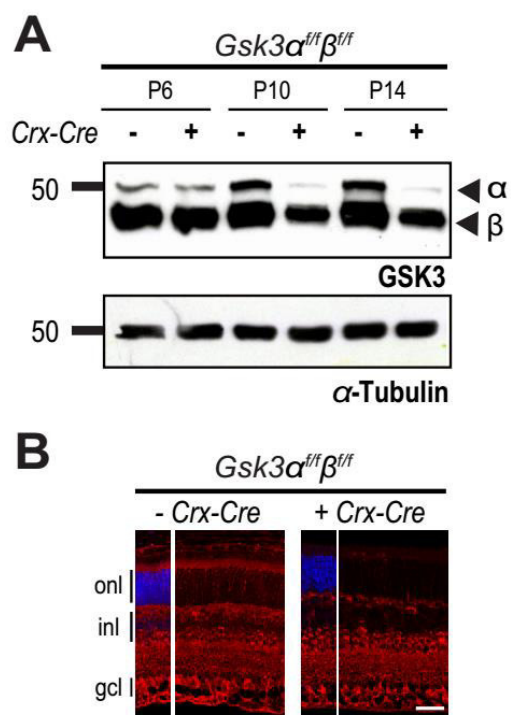


Figure 2. Lack of *Gsk3 α* and *Gsk3 β* expression in photoreceptors leads to photoreceptor degeneration. (A) The overall retinal histology was assessed by hematoxylin and eosin (H&E) staining in *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; Crx-Cre* animals and compared to littermate controls at P10, P18, P28 and 3 months. At P10, no histological changes were detectable. Outer nuclear layer (ONL) started to degenerate at P18, along with a shortening of outer and inner segments. At 3 months, only two to three rows of photoreceptors remained. rpe, retinal pigment epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer, mon, month. Scale bar: 20 μ m. (B) Measurement of the ONL thickness in central retina and peripheral retina. ONL is significantly thinner at P28, both in the central and in the peripheral retina. Mean \pm SEM values are presented from 4 biological replicates, * indicates p-value < 0.05, ** p-value < 0.01 and *** p-value <0.001. (C) Apoptotic cells were quantified by TUNEL assay. Photoreceptor cell death significantly increased from P18 onwards in *Gsk3 $\alpha^{f/f}$ $\beta^{f/f}$; Crx-Cre* animals compared to littermate controls, with a peak of cell death between P21 and P28. Mean \pm SEM values are presented from 3 biological replicates, * indicates p-value <0.05.

Figure 2.

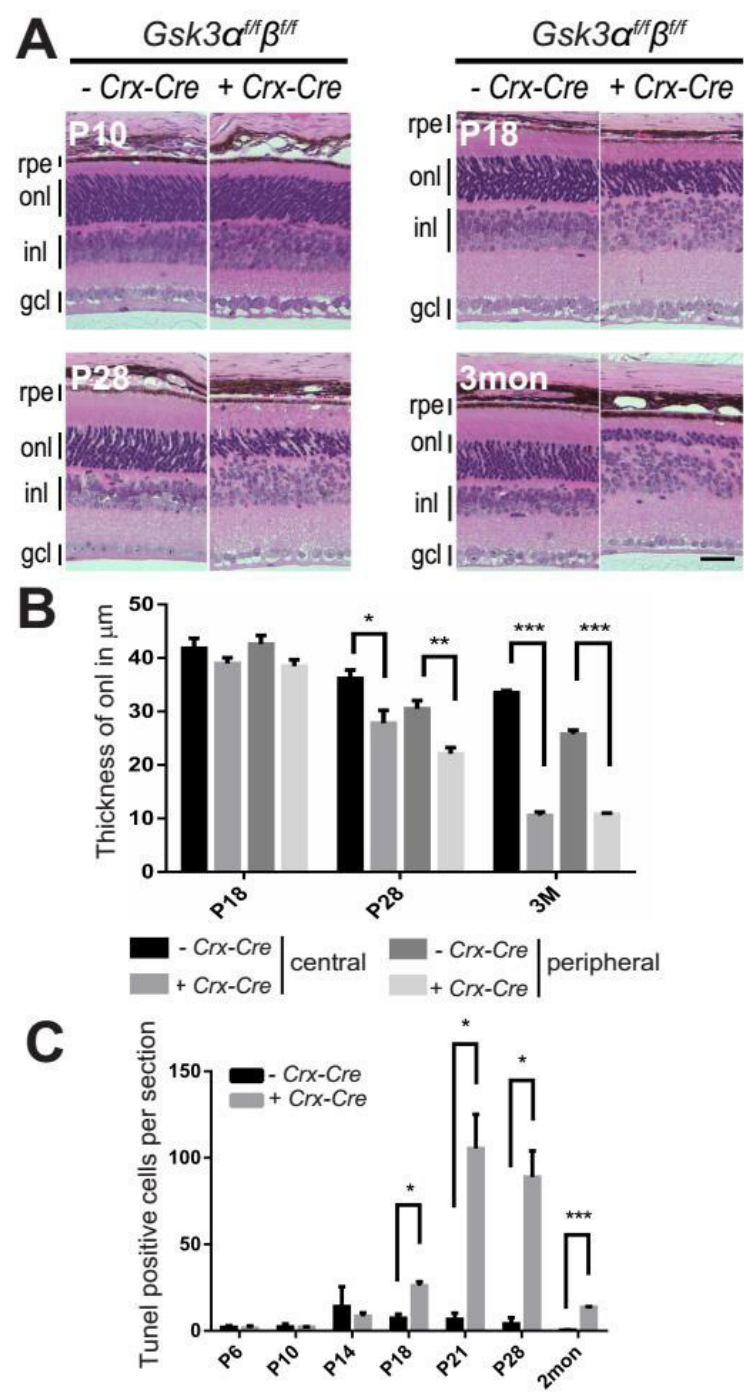


Figure 3. Lack of GSK3 in photoreceptors severely impairs rod and cone retinal function. (A, B) Dark-adapted electroretinogram (scotopic ERG) recordings in P18, P21, P28, 2 and 3-month-old *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals and littermate controls. Mean \pm SEM intensity response curves of a- and b-wave responses were calculated from 4-8 biological replicates of each genotype. (A) At P18, the a- and b-wave scotopic responses were significantly decreased in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls. (B) At 1 cd.s./m², scotopic response was largely decreased from P18 to 3 months in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* compared to controls. At 3 mon, photoreceptors in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals still showed some remaining function. (C, D) Mice were light-adapted to record photopic ERG. Mean \pm SEM intensity response curves of b-wave are calculated from 4-8 biological replicates of each genotype. (C) At P18, the cone b-wave responses were significantly decreased in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls at all light intensities. (D) At 1 cd.s./m², photopic responses were largely impaired from P18 to 3 mon in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls. Cone response was slightly increased from P18 to P21 as observed in controls before decreasing thereafter to fall to zero at 3 months (3 mon).

Figure 3.

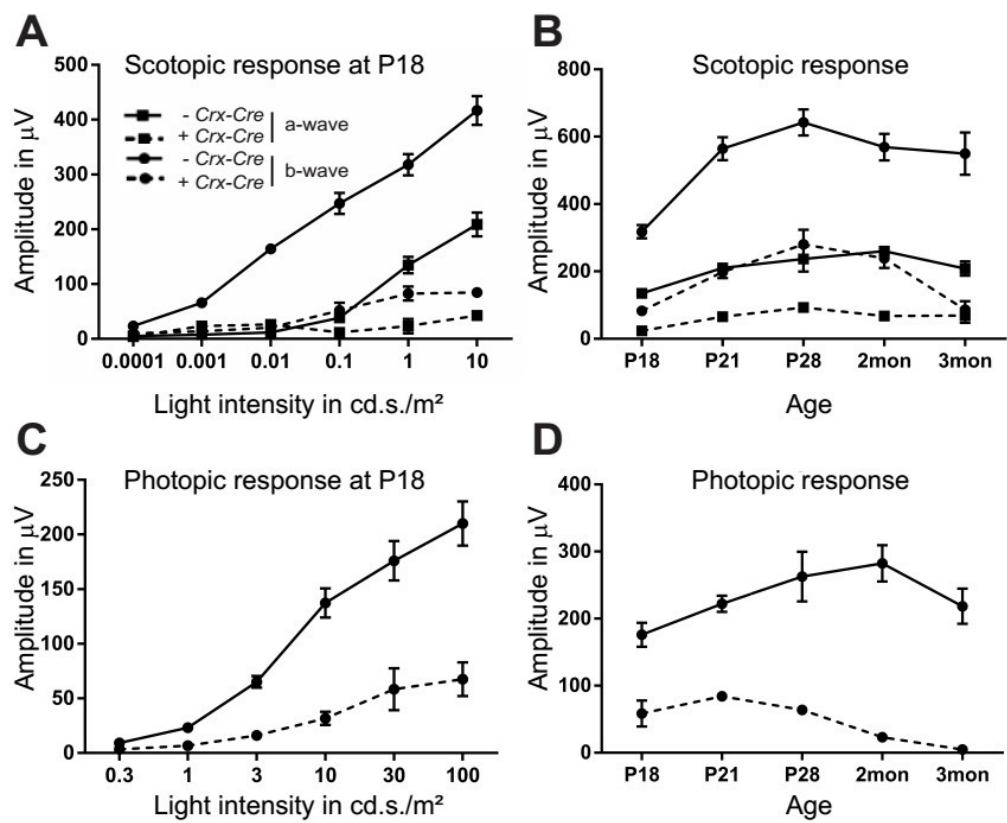


Figure 4. Lack of GSK3 in photoreceptors impairs rod and cone maturation.

Immunostaining of controls and *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* mouse retina sections at P6, P10, P14 and P18. Primary antibodies were used to detect rod photoreceptors (Rhodopsin), cone photoreceptors (Cone Arrestin), S-cones (S-Opsin) and M-cones (M-Opsin).

(A) Rhodopsin mislocalization to the ONL suggests impaired outer segment formation in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls from P6 onwards.

(B) Expression of Cone Arrestin was decreased by P14, at P18 cone segments underwent a visible thickening in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls.

(C) Expression of S-opsin in the ventral part of the retina, where S-cones are abundant, was normal in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls until P14 but decreased by P18 in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls.

(D) Expression of M-opsin in the dorsal part of the retina, where M-cones are abundant, was delayed in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls until P14.

onl, outer nuclear layer; inl, inner nuclear layer. Scale bar: 20 μ m

Figure 4.

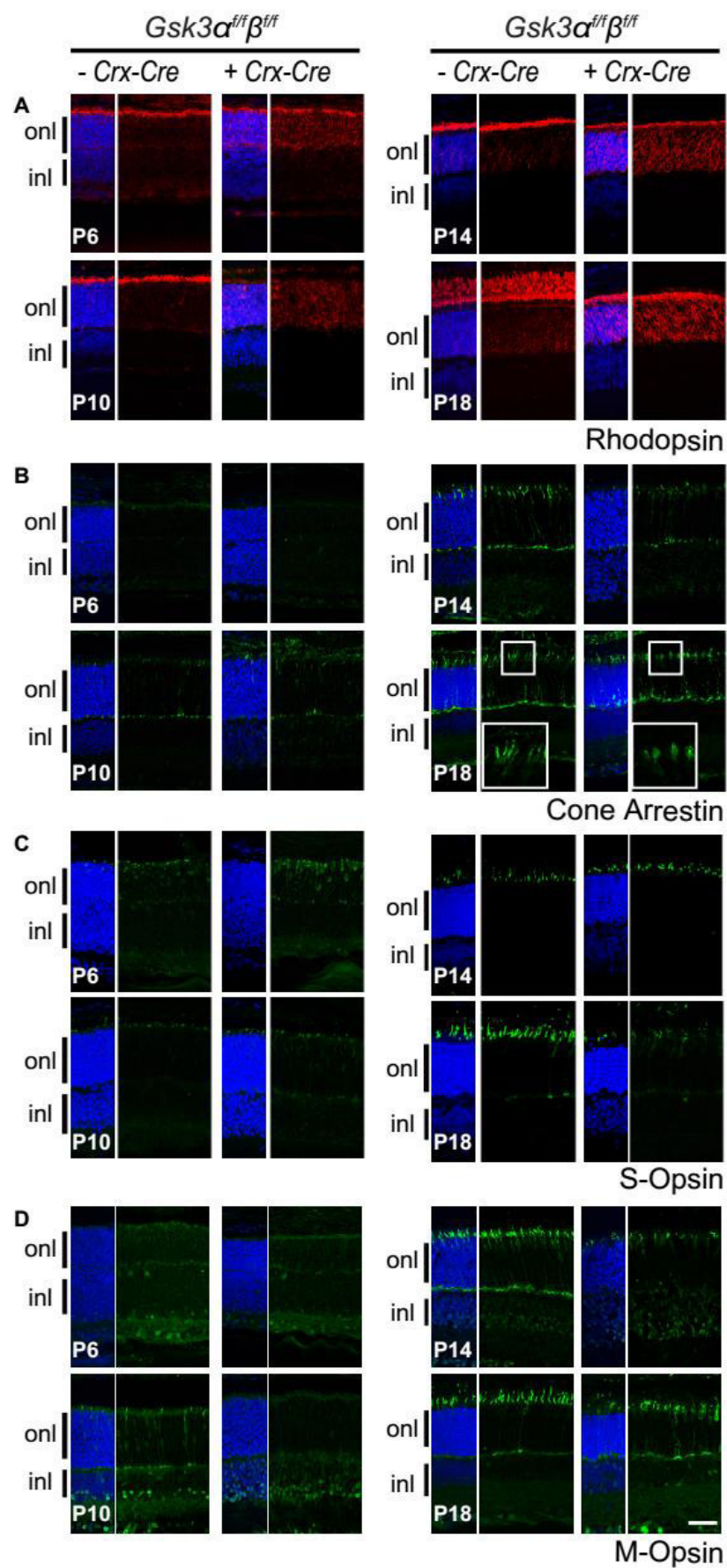


Figure 5. RNA-Seq analysis of *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$; Crx-Cre* retina revealed alteration of pathways related to retinal development and visual transduction.

(A) Volcano plots of differentially expressed genes in *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$; Crx-Cre* retina at P6, P10 and P14. Difference in RNA expression (FC) between *Gsk3 $\alpha^{fl/fl}$ $\beta^{fl/fl}$; Crx-Cre* and control retinas genes is plotted on the x-axis (log2 scale), and False Discovery Rate (FDR) adjusted significance is plotted on the y-axis (log2 scale). Genes up- or down-regulated by a factor ≥ 1.5 with $FDR \leq 0.05$ are indicated in *red*. Vertical dashed lines represent $FC=1.5$. and FDR of 0.05. (B) Venn diagram representing number of differentially expressed genes (DEG) that are unique or common between P6, P10 and P14 stages. Red and blue arrows represent the number of up-and down-regulated genes, respectively. (C) Bar plots representation of the top over-represented GO pathways amongst DEGs at P6, P10 and P14 for biological process (BP) and Cellular Component (CC). Pathways were identified by GO enrichment analysis using PANTHER. Z-score values indicate whether an entire biological process contains more up- (*red*) or down- (*blue*) regulated genes within it. (D) Hierarchical clustering analysis at P6, P10 and P14 of 56 DEG belonging to GO pathways related to retina and vision: detection of light stimulus (GO:0009583), eye development (GO:0001654), visual perception (GO:0007601) and retina development in camera-type eye (GO:0060041). The z-score heatmap was derived from the average of the triplicates collected at each time point. *blue*: low expression; *red*: high expression.

Figure 5.

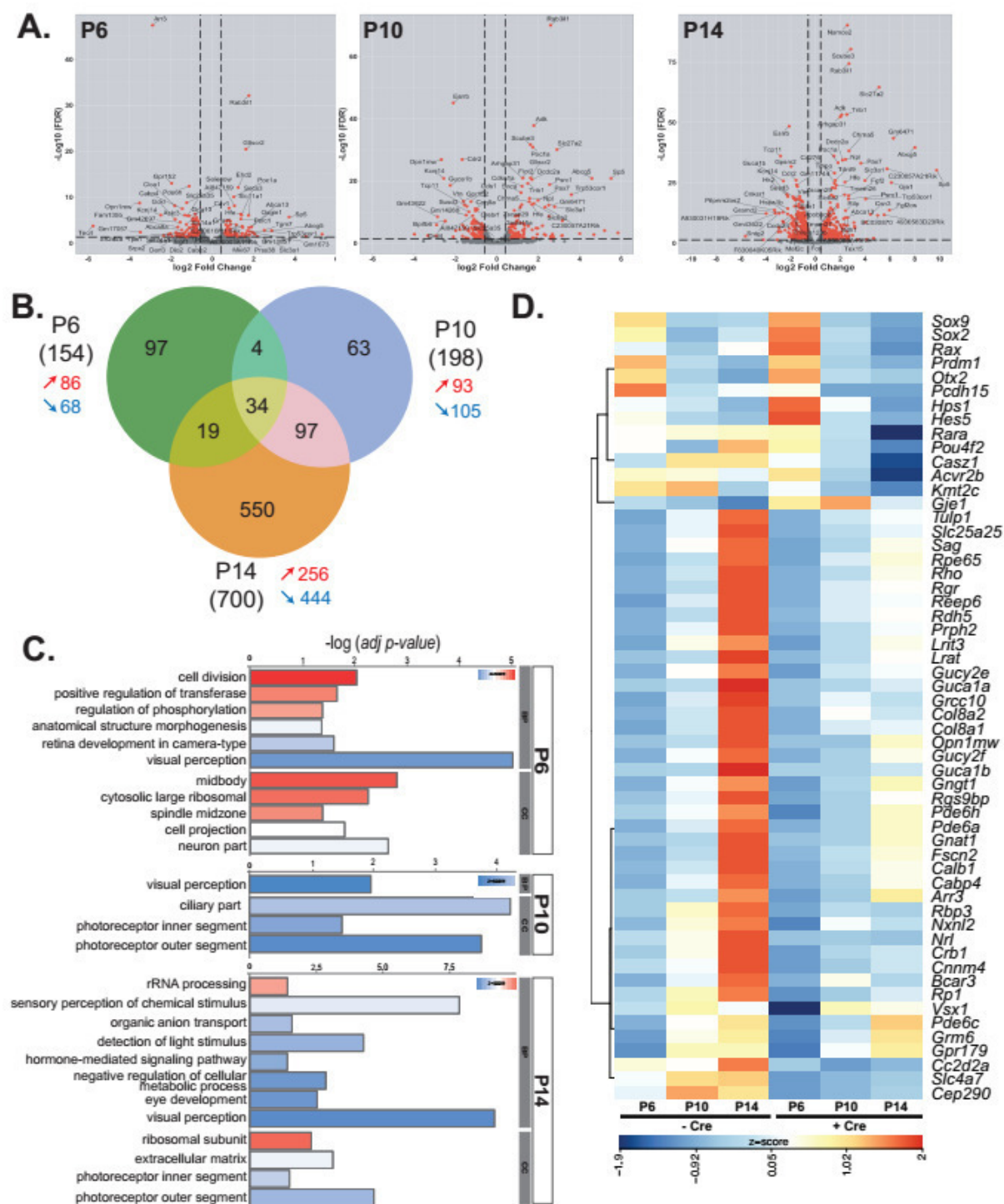


Figure 6. Lack of GSK3 in photoreceptors leads to selective gene expression alterations.

(A) qPCR was performed on whole retinal extracts at stages P6, P10 and P14 in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls. *Rhodopsin* expression was significantly decreased at P14. *Cone Arrestin* expression was significantly reduced at all examined stages P6, P10, P14 in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals. *S-* and *M-opsin* displayed a significant decrease at P14 in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls. Mean \pm SEM values are presented from 3 to 4 biological replicates, * indicates p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001. (B) Relative expression level of the main photoreceptor specific transcription factors CRX, NR2E3 and NRL was assessed by qPCR (*left panels*) and immunoblot (*right panels*) from whole retinal extracts at stages P6, P10 and P14 in *Gsk3 $\alpha^{f/f}\beta^{f/f}$; Crx-Cre* animals compared to littermate controls. Photoreceptor specific transcription factors *Crx* and *Nr2e3* did not show any significant expression alterations at both RNA and protein levels. *Nrl* expression was significantly decreased at RNA level at P10 and P14 but the NRL protein amount was highly increased.

Figure 6.

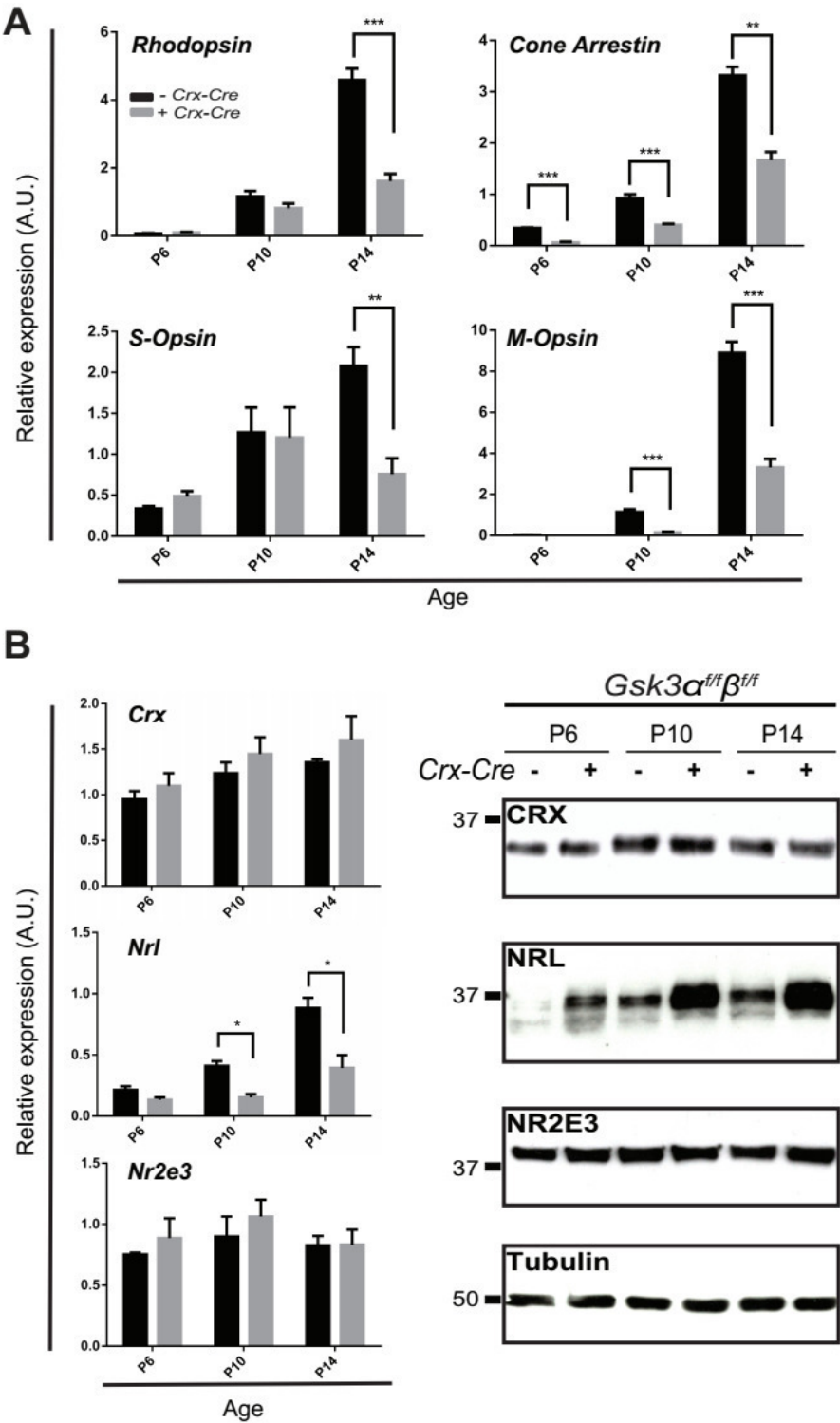


Figure 7. GSK3-dependent phosphorylation of NRL regulates its protein stability.

(A) Alignments of NRL protein across different species (adapted from Roger et al. 2010). Identical amino acids are shown in *red*, highly conserved amino acids in *blue*, less conserved in *green*, and not conserved in *black*. Black arrowheads indicate amino acids in NRL predicted to be phosphorylated by GSK3 and arrow indicates putative priming kinase site. MTD, minimal transactivation domain; Hinge, hinge domain; EHD, extended homology domain; BM, basic motif; Leu Zipper, leucine zipper. *Hsap*, *Homo sapiens* (human); *Ptro*, *Pan troglodyte* (chimpanzee); *Mmus*, *Mus musculus* (mouse); *Rrat*, *Rattus norvegicus* (rat); *Cfam*, *Canis familiaris* (dog); *Btau*, *Bos taurus* (cow); *Ecab*, *Equus caballus* (horse); *Xlae*, *Xenopus laevis* (Xenopus); *Brer*, *Brachydanio rerio* (zebrafish). (B) COS-7 cells were transfected with WT-NRL or phosphorylation mutants (NRL-S50A, NRL-S46A, NRL-T42A or NRL-S38A). Immunoblotting of the cell lysates using anti-Xpress to detect NRL revealed a sequential phosphorylation of NRL. Tubulin was used as loading control. (C) COS-7 cells were transfected with WT-NRL or NRL-S50A phosphorylation mutant constructs and treated 48 h after with GSK3 inhibitor (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO) for 2 h. Protein lysates were treated or not with phosphatase to demonstrate NRL phosphorylation. NRL phosphorylation was abolished using GSK3 inhibitor BIO, whereas BIO had no effect on the phosphorylation mutant S50A. Tubulin was used as loading control. (D) COS-7 were transfected with WT-NRL or phosphorylation mutants (NRL-S50A, NRL-S46A, NRL-T42A or NRL-S38A) and co-transfected with GSK3 β -GFP construct. Immunoblotting of the cell lysates probed with anti-Xpress revealed a sequential phosphorylation of NRL. Tubulin was used as loading control.

Figure 7.

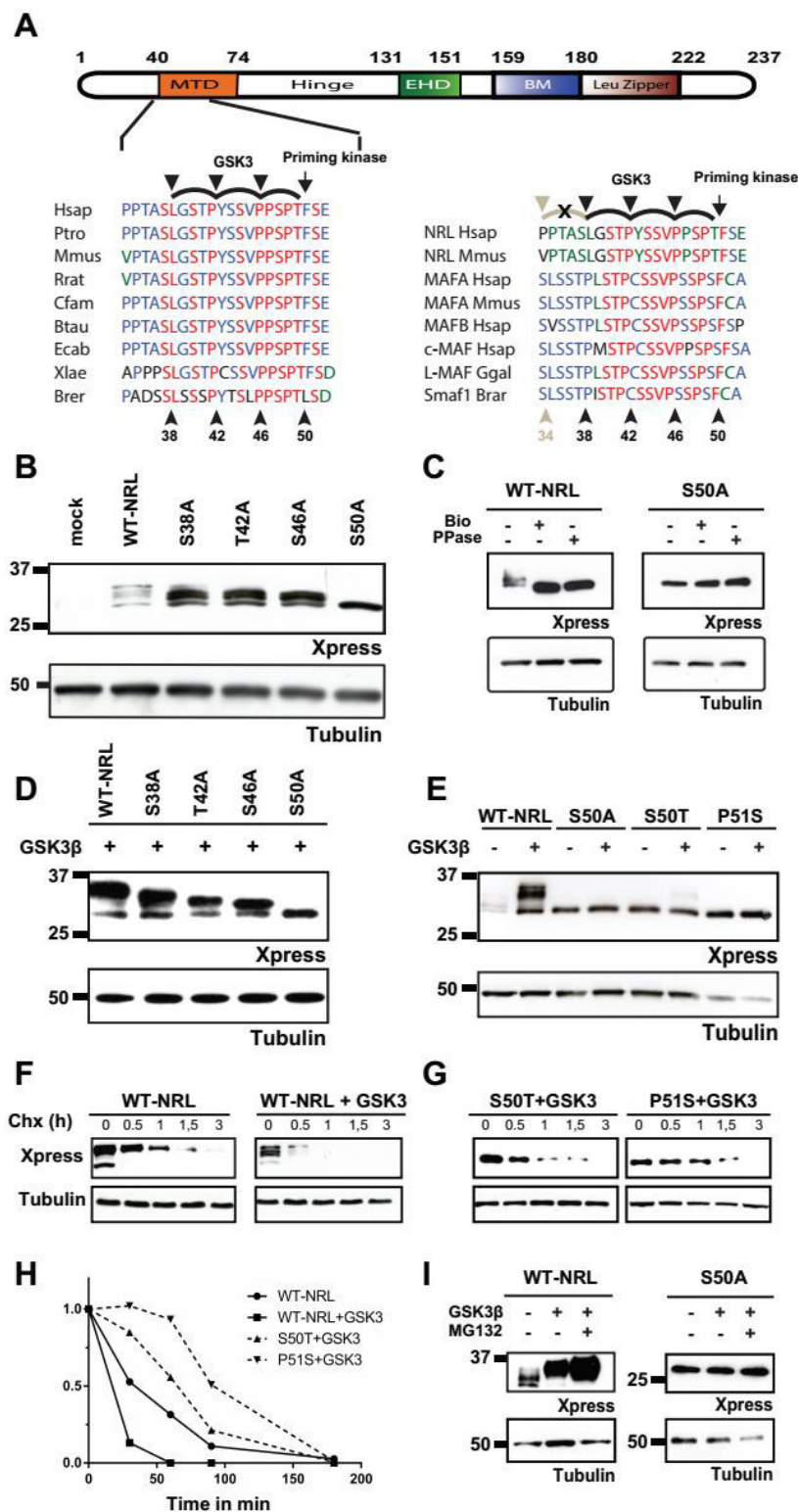
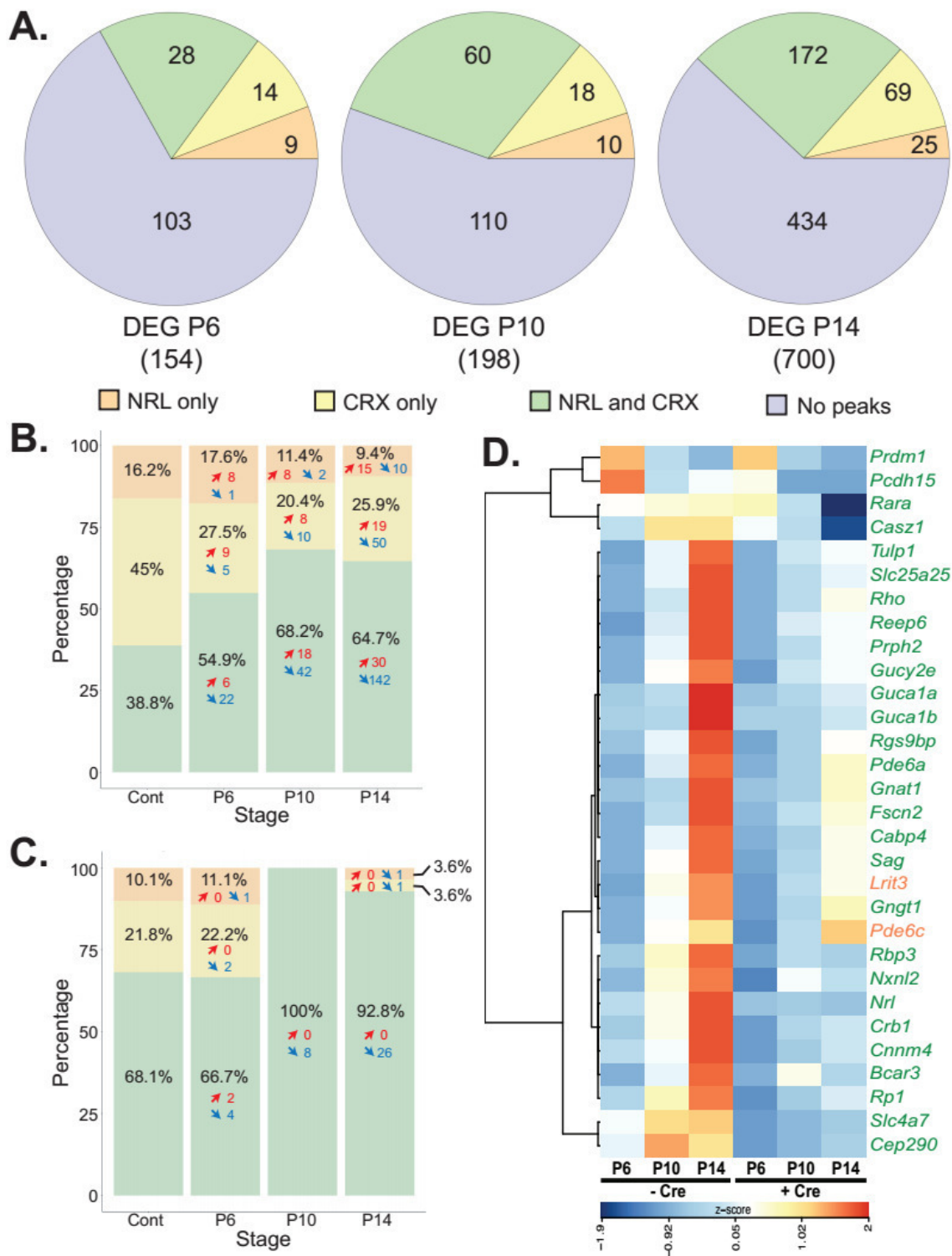


Figure 7 (continued). (E) COS-7 cells were co-transfected with constructs encoding GSK3 β -GFP and either WT-NRL, phosphorylation mutants (NRL-S50A) or mutants associated with Autosomal recessive retinitis pigmentosa (ADRP) (i.e. NRL-S50T, NRL-P51S). Protein extracts were immunoblotted with anti-Xpress. Tubulin was used as loading control. GSK3 phosphorylates WT-NRL, but is unable to phosphorylate NRL-P51S. A low level of phosphorylated isoforms can be detected for NRL-S50T. (F) COS-7 cells were transfected with WT-NRL, co-transfected or not with GSK3-GFP and treated 48 h later with the protein synthesis inhibitor, cycloheximide (Chx) for indicated periods of time. NRL stability is decreased in presence of GSK3 β compared to ADRP associated mutants. Tubulin was used as loading control. (G) COS-7 cells were transfected with constructs encoding WT-NRL or ADRP NRL mutants (NRL-S50T, NRL-P51S), co-transfected with GSK3 β -GFP, and treated 48 h later with Chx for indicated periods of time. NRL stability is decreased compared to ADRP associated mutants. Tubulin was used as loading control. (H) Quantification of immunoblots showed in E and F. (I) COS-7 cells were transfected with WT-NRL or NRL-S50A mutant, co-transfected or not with GSK3 β . Cells were treated 48 h after transfection with the proteasome inhibitor MG132 for 2 h. GSK3-mediated phosphorylation of NRL leads to its degradation via the proteasome. NRL stability is increased when the proteasome is inhibited, whereas the stability of the S50A mutant remains not affected.

Figure 8. GSK3-dependent phosphorylation of NRL regulates a subset of CRX and NRL target genes involved in photoreceptor development and function.

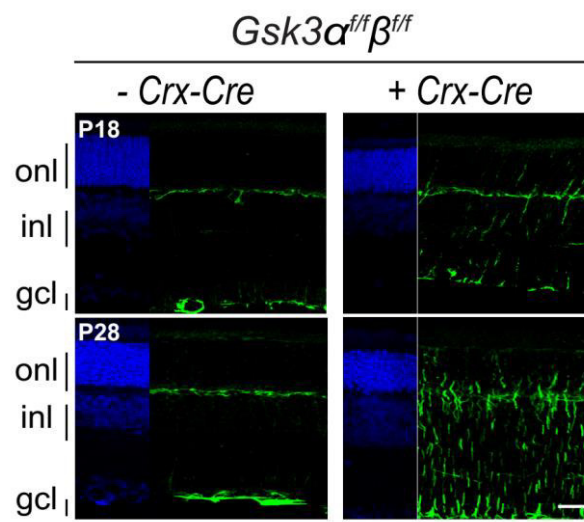
(A) The proportion of DEG regulated by NRL and/or CRX in mice lacking *Gsk3α* and *Gsk3β* increased during photoreceptor development. Pie chart representing the number of DEG with or without NRL and/or CRX binding sites at P6, P10 and P14 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retinas compared to controls. (B) Among NRL and CRX target genes, lack of *Gsk3α* and *Gsk3β* in photoreceptors alters primarily genes that are downregulated and with binding sites for both transcription factors. Stacked bar charts representing the percentage of DEG with only NRL or CRX binding sites or with both at P6, P10 and P14 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retina compared to control. The number of genes up- and down-regulated are indicated in *red* and *blue*, respectively. (C) A vast majority of DEG in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retina belonging to GO pathways related to retina and vision are downregulated with both NRL and CRX binding sites. Stacked bar charts representing the percentage of DEG related to retina and vision with only NRL or CRX binding sites or with both at P6, P10 and P14 in *Gsk3α^{fl/fl}β^{fl/fl}; Crx-Cre* retina compared to control. The number of up- and down-regulated genes is indicated in *red* and *blue*, respectively. (D) Hierarchical clustering analysis at P6, P10 and P14 of 30 DEG with NRL binding sites belonging to vision-related GO pathways: detection of light stimulus (GO:0009583), eye development (GO:0001654), visual perception (GO:0007601) and retina development in camera-type eye (GO:0060041) with NRL binding sites. The z-score heatmap was derived from the average of the triplicates collected at each time point. *green* indicates 28 genes with both NRL and CRX binding sites; *orange* indicates 2 genes with only NRL binding sites.

Figure 8.



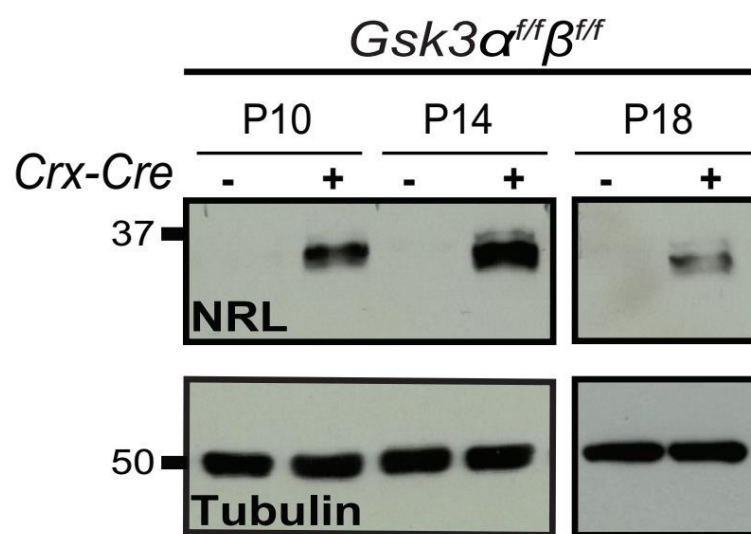
Supplementary Figure 1. Reactive gliosis of Müller cells during retinal degeneration of *Gsk3 α ^{ff/ff}; Crx-Cre* mice. Immunostaining with anti-GFAP antibody (*green*) on retinal sections of *Gsk3 α ^{ff/ff}; Crx-Cre* and control animals at P18 and P28. Nuclei are DAPI counterstained (*blue*). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 20 μ m

Supplementary Figure 1.



Supplementary Figure 2. Lower exposure of NRL immunoblot partly presented in Figure 6B. NRL expression level was assessed by immunoblot from whole retinal extracts at stages P10, P14 and P18 in *Gsk3 α ^{fl/fl} β ^{fl/fl}; Crx-Cre* animals compared to littermate controls. Only 2 or 3 phosphorylation isoforms of the NRL protein are increased in *Gsk3 α ^{fl/fl} β ^{fl/fl}; Crx-Cre* animals. P18 was included for a better isoform distinction and reveals upregulation of three NRL isoforms compared to controls even after the onset of degeneration. Tubulin was used as loading control.

Supplementary Figure 2.



Supplementary Table 1. List of primary and secondary antibodies used for immunohistochemistry(IF) and western blot (WB).

Primary antibodies

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
GSK3 α/β	mouse	Thermo Fisher Scientific	44-610	1:1000	1:1000
Xpress	mouse	Thermo Fisher Scientific	R910-25		1:2.000
NRL	rabbit	EMD Millipore	ABN1712		1:1.000
α -tubulin	mouse	SIGMA	T5168		1:200.000
Cone Arrestin	rabbit	EMD Millipore	AB15282	1:1.000	
M-Opsin	rabbit	EMD Millipore	AB5405	1:1.000	
S-Opsin	rabbit	EMD Millipore	AB5407	1:500	
Rhodopsin	Mouse	Abcam	MAB5316	1:2.000	

Secondary antibodies

ANTIGEN	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
Alexa Fluor 555 anti-mouse IgG2A	goat	Thermo Fisher Scientific	A21127	1:1000	
Alexa Fluor 488 anti-rabbit	donkey	Thermo Fisher Scientific	A21206	1:2.000	
Alexa Fluor 647 anti-rabbit	goat	Thermo Fisher Scientific	A-20991	1:2.000	
HRP anti-mouse IgG	goat	Sigma-Aldrich	A4416		1:5.000
HRP anti-rabbit IgG	donkey	GE Health	NA934V		1:5.000

D Discussion and perspectives

D.1 GSK3 in the context of retinal development

D.1.1 Short-term perspectives

To decipher GSK3 function in the retina, my Ph.D. work focused on two aspects of GSK3 function in the retina. On the one hand, we described the general role of GSK3s during early development of retinal progenitors and on the other hand their function in photoreceptors, the main cells affected in many retinal dystrophies. Taken together, our results highlight for the first time the central role of GSK3s in both retinal progenitor maintenance and in photoreceptor maturation and survival.

Complete loss of GSK3 in retinal progenitors resulted in **severe morphological defects**. As described in the brain, we found impaired neural differentiation (Kim et al., 2009), but in addition neural proliferation was also affected upon GSK3 loss in the retina resulting in microphthalmia. This suggests that retinal progenitors are more sensitive to the lack of GSK3 activity than brain progenitors. Interestingly, the effects of *Gsk3* loss-of-function in the retina are very similar those found in β -catenin gain of function. In both cases, both proliferation and neural differentiation are impaired (Fu et al., 2006) (Ouchi et al., 2011).

Noteworthy, the expression of **only one allele** of *Gsk3 α* or *Gsk3 β* was sufficient to **completely rescue retinal development, maturation and function**.

Further analysis of mouse retinas with only one allele of *Gsk3* in retinal progenitors, and in particular in *Gsk3 α ^{f/+} β ^{f/f}; α -Cre* retina, revealed a strikingly **high number (25 %) of displaced RGCs (dRGCs)** located in the INL. These cells, produced at the same time as other RGC, are usually present in low numbers and account for nearly 2 % of total RGCs in wildtype retina (Nadal-Nicolas et al., 2014). Consequently, they remain poorly described and our model could therefore offer a great opportunity to investigate their functions and to unravel the limiting factors and signaling pathways involved in their production.

It would be interesting to assess whether the overproduced dRGCs alter the visual function in *Gsk3 α ^{f/+} β ^{f/f}; α -Cre* mice. So far, ERG recordings did not reveal any differences with control animals. However, we could specifically measure RGCs function by taking advantage of methods known to better assess RGCs functions such as photopic negative response (PhNR), pattern reversal ERG (PERG) (Porciatti, 2015) (Wilsey and Fortune, 2016), or multifocal ERG (mfERG) (Wilsey et al., 2017). In addition, multielectrode arrays could help analyzing simultaneous electrophysiological responses of RGCs (Sekirnjak et al., 2006).

This method could unveil differences between dorsal and ventral RGCs responses (Hilgen et al., 2017). Indeed, dRGC number is much higher in the ventral region in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice than in the dorsal region (data not shown). However, such methods do not guarantee the ability to record specifically dRGCs.

A first series of anterograde labelling that aimed to trace RGC projections into the visual brain area, indicated altered RGCs projections in retinal progenitors following a loss of *Gsk3 β* . More precisely, a clear fluorescent signal was detected in the medial terminal nucleus (MTN) on the ipsilateral side whereas it was barely detectable in control animals. Our hypothesis is that the signal observed in the ipsilateral MTN of *Gsk3 α ^{f/+} β ^{ff}; α -Cre* retina mostly originates from dRGCs as the number of oRGCs remains unchanged. To test whether the altered projections into the ipsilateral MTN arise from dRGCs, a retrograde tracer, such as indocyanine green (ICG) could be stereotactically injected into the MTN. However, since dRGCs may also project to other brain areas, additional injections could be performed into the superior colliculus (SC), an area that collects projections from both oRGCs and dRGCs (Salinas-Navarro et al., 2009a) (Salinas-Navarro et al., 2009b) (Salinas-Navarro et al., 2009c) (Nadal-Nicolas et al., 2014). This kind of retrograde labelling is efficient and has been successfully applied to label rare populations of RGCs (Krause et al., 2014). To complete this analysis and to specifically identify nuclei where RGC projections are increased, the volume of the different fluorescent areas will be precisely measured. From a functional point of view, neurons in the MTN are known to code predominantly for vertical stimulus movements and to mediate vertical optokinetic responses (Simpson et al., 1988) (Giolli et al., 2006). Therefore, we could investigate whether optokinetic responses are modified in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* retinas compared to controls.

So far at the molecular level, we could not characterize these dRGCs beyond their immunoreactivity for Brn3a. The use of several markers described to specifically label ipsilateral RGCs, including cyclin D2 (Marcucci et al., 2016) and Zic2 (Herrera et al., 2003) (Garcia-Frigola et al., 2008) could shed more light on the molecular signature of these cells. Other markers, such as Sema6A and PlexinA2/A4 could also be used since they label RGCs projecting to the MTN (Sun et al., 2015). A global transcriptomic approach could also reveal critical factors involved in dRGC production and projections. Such analyses could succeed since only these cells are highly increased in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* retina. However, we cannot exclude that transcripts expressed by the RGCs will be poorly sequenced since these cells represent a low proportion of retinal cells. Their expression could be “diluted” among other cell type transcripts such as photoreceptors.

To circumvent this problem, several alternative methods could be used such as immune-panning techniques, allowing for the enrichment of RGCs using an anti-Thy1 antibody (Butowt et al., 2000) (Yin and Benowitz, 2018). The use of a reporter line expressing fluorescent proteins specifically in RGCs could also allow for the purification of these cells by flow-sorting. For instance, knock-in *Hox10-GFP* or *Drd4-GFP* (Yonehara et al., 2016) (Huberman et al., 2009) that express *GFP* in all RGCs could be crossed to *Gsk3 $\alpha^{f/f}; \alpha$ -Cre* mice. Overall, these approaches in combination with the use of our genetic model of partial *Gsk3* deletions, and particularly the *Gsk3 $\alpha^{f/f}; \alpha$ -Cre* mouse model, could pave the way for a better **characterization of dRGCs** and **determination of their function in wildtype *in vivo***.

In addition, it would be interesting to specifically assess the **mechanistic role of GSK3 in dRGC genesis**. As demonstrated in the literature, GSK3 regulates the activity of proneural transcription factors such as NeuroD (Moore et al., 2002) or Neurog2 (Li et al., 2012) that both have an impact on cell-types specification in the retina. Therefore, it would be interesting to identify specific GSK3-dependent factors that could control RGC or dRGC genesis and that could be deregulated upon partial GSK3 loss.

Regarding the role of **GSK3 during photoreceptor development and homeostasis, loss of both GSK3** profoundly impairs photoreceptor maturation leading to their **degeneration**. At the molecular level, we identified a GSK3-dependent regulation of the rod-specific transcription factor NRL. Indeed, lack of GSK3 leads to an increased amount of NRL protein with an alteration in the number of phosphorylated isoforms. Only two or three NRL phosphorylated isoforms were upregulated in *Gsk3 $\alpha^{f/f}; Crx-Cre$* retinas compared to controls thus revealing an altered NRL phosphorylation pattern in the absence of GSK3. This result remains to be confirmed by using a specific Phos-tag™ gel designed to improve separation of phosphorylated proteins.

Our results suggest that NRL stability control could be absent in disease conditions, in *Retinitis Pigmentosa* patients carrying *NRL* mutations at the priming kinase site or adjacent to the priming kinase site of GSK3 (*NRL-S50T* and *NRL-P51S*) (Bessant et al., 1999) (Martinez-Gimeno et al., 2001) (DeAngelis et al., 2002) (Kanda et al., 2007) (Swain et al., 2007). However, our data are inconsistent with *in vitro* findings using *NRL-S50T* and *NRL-P51S* that displayed an increased transcriptional activity of NRL target genes, such as *Rho* (Swain et al., 2007), whereas NRL target genes were downregulated or unchanged in our model. This “discrepancy” could be due to the differential isoforms present in *Gsk3 $\alpha^{f/f}; Crx-Cre$* retinas compared to *NRL-S50T* and *NRL-P51S* mutants that lack phosphorylation.

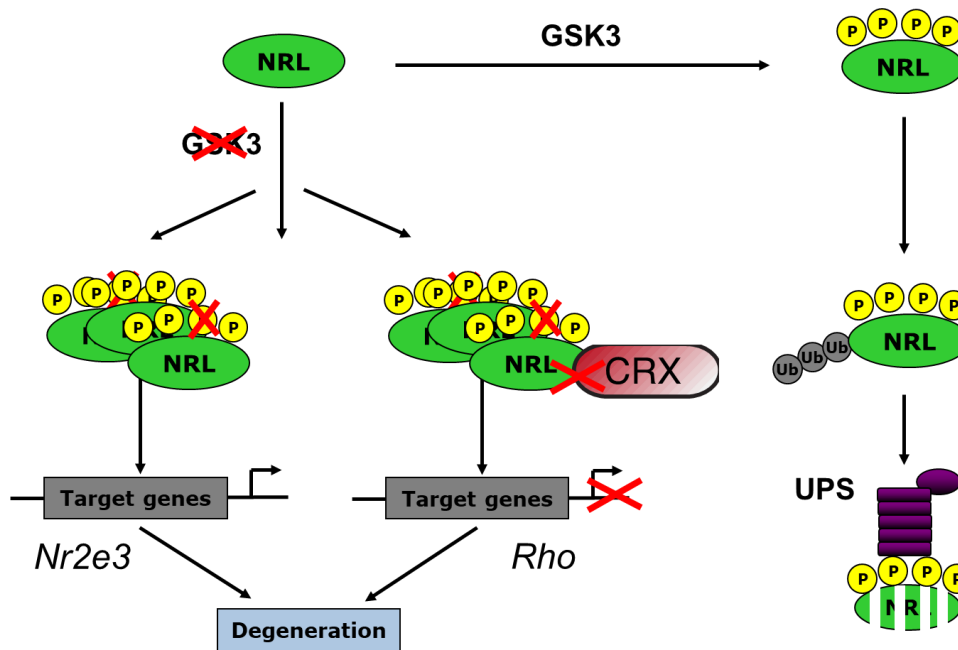


Figure 34. Summary of GSK3-dependent NRL regulation and the (potential) outcomes.

GSK3 phosphorylates NRL on 4 consecutive amino acids triggering its poly-ubiquitylation and proteasomal degradation (**right**). Lack of GSK3 leads to an altered NRL phosphorylation and accumulation. However, despite NRL accumulation, NRL targets are either unchanged, such as *Nr2e3* (**left**) or downregulated, such as *rhodopsin* (*Rho*) (**middle**), resulting in impaired photoreceptor maturation followed by their degeneration. To regulate the majority of its downregulated targets, NRL acts in synergy with CRX (**middle**).

Indeed, phosphorylated NRL isoforms remain in *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retinas, which are likely phosphorylated by other kinases (Figure 6B of the manuscript). These stabilized isoforms are likely responsible for the decreased expression of NRL target genes.

Importantly, our meta-analysis of RNA-Seq data in combination with available NRL and CRX ChIP-Seq data (Hao et al., 2012) (Corbo et al., 2010) demonstrated that most of the downregulated genes related to photoreceptor function have both NRL and CRX binding sites. The next step would be to understand why despite such a **huge accumulation of NRL at the protein level**, most NRL target genes that are co-regulated by CRX and critical for photoreceptor development and function, are **downregulated** in *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retinas compared to controls (Figure 34). Several hypotheses can be advanced:

- **Altered NRL phosphorylation in the absence of GSK3 may result in an impaired NRL binding to regulatory elements on target genes.** In addition, NRL accumulation may prevent CRX binding to these regulatory elements through stereo-inhibition. To address the question of whether NRL binding to target genes is affected in *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retinas, Chromatin-Immunoprecipitation (ChIP) experiments were initiated. In combination with RNA-Seq, we could specifically determine that impaired transcription factor binding to DNA is responsible for the downregulation of the genes of interest (such as phototransduction genes, involving *Rho*). This study is still in progress.
- **Altered NRL phosphorylation and/or accumulation can impair its interaction with CRX in *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retinas compared to controls.** For many downregulated NRL target genes, NRL acts in synergy with CRX. An impaired NRL-CRX interaction may explain their downregulation. The required Co-Immunoprecipitation (CoIP) experiments to test NRL-CRX interaction are challenging since only rabbit polyclonal are available both proteins. To circumvent this issue, NRL IP could be performed prior to the MS/MS analysis of pulled down complexes. It remains to be clarified whether NRL mutations at critical phosphorylation sites hinder its interaction with CRX.
- Finally, even if NRL binding or its interaction to CRX are unaffected in *Gsk3 $\alpha^{fl/fl}$; Crx-Cre* retinas, it remains feasible that the **NRL-CRX complex displays a transcriptionally activity more or less potent that could** account for the downregulation of target genes. Indeed, NRL transcriptional activity is largely increased when it acts in synergy with CRX (Mitton et al., 2000b).

We could perform an *in vitro* luciferase-reporter assay to test for the transcriptional activity of the complex formed by NRL-WT *versus* NRL phosphorylation mutants with CRX using *Rho* and *Nr2e3* promoters, respectively corresponding to downregulated and unchanged NRL target genes in *Gsk3 α ^{ff} β ^{ff}*; *Crx-Cre* retinas. We could identify using the *Rho* promoter if GSK3-phosphorylation NRL mutants (S38A, T42A and S46A) are less active and if S50 is more active (as previously shown) compared to NRL-WT.

Hence, these approaches could help explaining why despite NRL accumulation, the majority of its target genes are downregulated. They should also shed more light on the mechanisms underlying *NRL* mutations in *Retinitis Pigmentosa* patients.

Finally, we need to functionally **demonstrate that the observed phenotype in *Gsk3 α ^{ff} β ^{ff}*; *Crx-Cre* retinas is indeed due to NRL deregulation**. We cannot exclude that the phenotype observed is independent of GSK3-mediated phosphorylation of NRL. GSK3 could also act on other factors leading to downregulation of a subset of rod expressed genes. To test this hypothesis, we could perform luciferase assays upon pharmacological treatment of the cells with BIO, an inhibitor of GSK3, using the NRL-WT in the presence or absence of CRX and a reporter construct (including *Rho* promoter) . If the downregulation of *Rho* expression originates from GSK3-dependent NRL phosphorylation, *Rho* promoter activation should be decreased in presence of the inhibitor. This kind of approach was performed using MAPK inhibitors. In these conditions, the activation of the *Rho* promoter was decreased with a concomitant reduction of NRL phosphorylation (Swain et al., 2007). As described for MAFA, a member of the same family as NRL, MAPK could be the priming kinase triggering subsequent NRL phosphorylation by GSK3 (Herath et al., 2014). Such *in vitro* experiments would provide further evidence that altered NRL phosphorylation due to lack of GSK3 activity leads to downregulation of a subset of rod genes.

The same issue could be addressed by *in vivo* electroporation of different NRL phosphorylation mutants (S38A, T42, S46 and S50A) in *Nrl^{-/-}* mice that lack the expression of rod-specific genes including *Rho*. We could assess whether rod development occurs and whether developing rods display outer segments and degenerate. According to our expectation, electroporation of the S38A, T42A, and S46A isoforms should mimic the phenotype observed in *Gsk3 α ^{ff} β ^{ff}*; *Crx-Cre* mice with a decreased *Rho* expression, whereas electroporation of S50A should mimic the phenotype observed in *Retinitis Pigmentosa* patients with increased *Rho* expression. Thus, such an experiment could explain the **different outcome** (increased or decreased target gene expression) **upon different NRL isoform stabilization**.

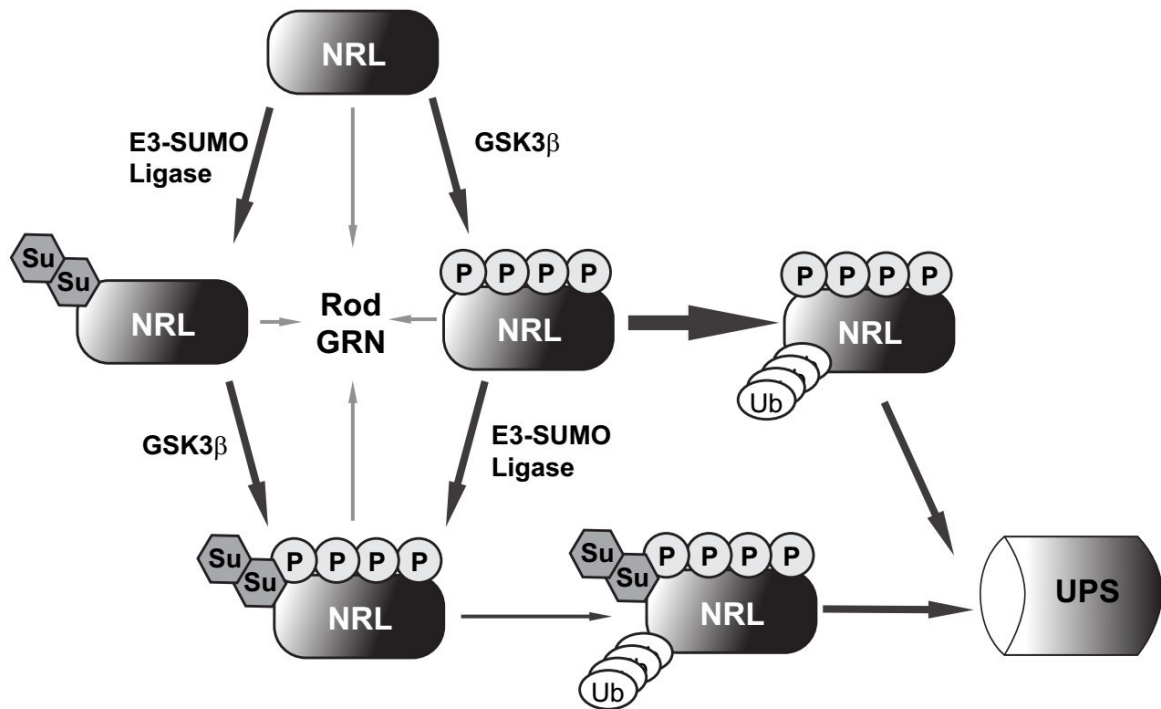


Figure 35. Model of NRL regulation by posttranslational modifications.

NRL can be phosphorylated or sumoylated by GSK3 β or an E3-SUMO ligase, respectively. NRL isoforms participates in the establishment of rod photoreceptor gene regulatory network (GRN) (*grey arrows*). Complete phosphorylation of NRL on S50, S46, T42 and S38 leads to polyubiquitination and its degradation by the ubiquitin-proteasome system (UPS). The stability of NRL is increased by sumoylation (*thin arrow*) compared to the non-sumoylated forms (*thick arrow*). P: phosphorylation; Ub: ubiquitin; Su: sumoylation.

We demonstrated the effects of NRL phosphorylation. But what about the **impact of other NRL PTM** described in the literature? As presented in the Introduction, NRL sumoylation promotes target gene expression to settle down rod differentiation (Roger et al., 2010). In addition, our preliminary data revealed decreased NRL stability when sumoylation is blocked (NRL-K20/K2) (data not shown). We thus suppose a fine-tuning of the NRL amount during development by controlling the balance between phosphorylation and sumoylation (Figure 35).

Additional PTMs could also occur to fine-tune the transcriptional activity of NRL, bringing even more complexity to the proteome. This kind of regulation has been demonstrated for p53, which can be modified by over 50 individual posttranslational modifications and has a wide range of downstream targets in the retina exerting a role in development and disease (Meek and Anderson, 2009). The identification of inhibitors or activators of signaling pathways and modulation of NRL post-translational modifications could be exploited for small molecule therapeutics to treat retinal diseases (Marchena et al., 2017).

D.1.2 Long-term perspectives

➤ Identification of additional GSK3 targets

It is unlikely that NRL accumulation resulting in deregulation of its target genes is the only explanation for the complex phenotype observed in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* mice. We cannot exclude additional NRL-independent mechanisms that contribute to the lack of photoreceptor maturation and degeneration in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* mice. This is even more relevant since cone maturation is also altered despite that NRL is normally not expressed in cone photoreceptors. As such, RNA-Seq analysis revealed a large number of differentially expressed genes in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* retina with no NRL binding sites but that were crucial for photoreceptor development and homeostasis. Thus, our long-term goal is to **identify and validate further GSK3 targets** constituting the complex gene regulatory network critical for photoreceptors development and maintenance. In this context, it will be of particular interest to identify cone-specific transcription factors that are regulated by GSK3 in a similar way as NRL to better explain cone developmental defects and degeneration in absence of GSK3.

In the search for additional GSK3 targets, we performed a preliminary mass spectrometry analysis on *Gsk3 α^{ff} β^{ff} ; Crx-Cre* retinas at P14, shortly before the onset of photoreceptor degeneration. To select the most relevant putative GSK3 targets, we kept only proteins that were upregulated in the absence of GSK3 but decreased or unchanged at the transcript level consistent with our findings showing NRL protein accumulation in the absence of GSK3 (Figure 36).

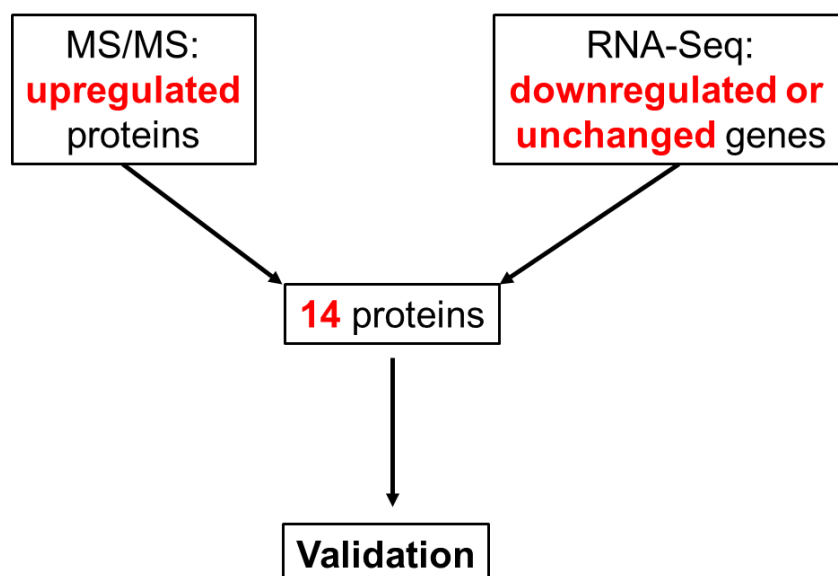


Figure 36. Workflow for GSK3 target identification.

Preliminary analysis of the large-scale proteomic (MS/MS) and transcriptomic (RNA-Seq) data to identify putative GSK3 targets. Validation of selected GSK3 targets will be performed using the same approaches as for NRL.

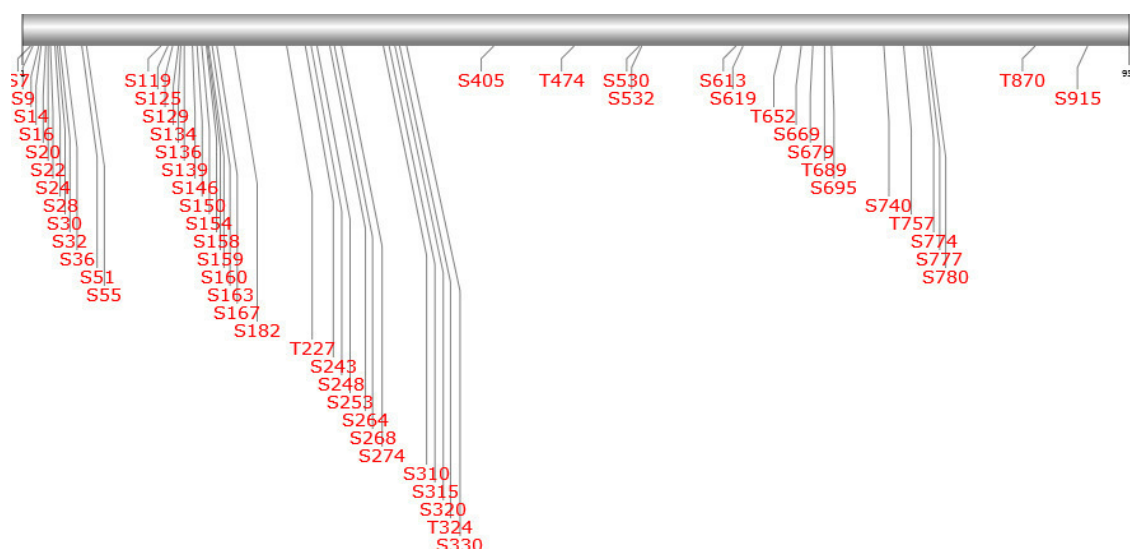


Figure 37. In silico identification of GSK3 phosphorylation sites on TRAP150.

Based on in silico analyses, Thyroid hormone receptor-associated protein 3 (TRAP150), which is among the 14 putative GSK3 targets (Figure 36) exhibits numerous potential GSK3 phosphorylation sites as well as phosphorylation motifs every four amino acids, e.g. S16-S20-S24-S28-S32-S36; S146-S150-S154-S158.

Among the 14 candidates, one putative candidate unchanged at the RNA level but highly upregulated at the protein level in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* retinas is the Thyroid hormone receptor-associated protein 3 (Trap150).

Based on *in silico* analyses, we found Trap150 to be potentially sequentially phosphorylated by GSK3. Indeed, it exhibits several GSK3 phosphorylation sites, including specific GSK3 phosphorylation motifs every four amino acids (Figure 37). Notwithstanding the role of this factor in photoreceptors, its regulation by GSK3 as well as its possible link with the Thyroid hormone receptor important in regulating the cone S-M- cone gradient in the retina, remains to be determined.

➤ **GSK3 and glucose metabolism**

As presented in the introduction, **GSK3 has a central role in glucose metabolism**. The enzyme glycogen synthase (GS) regulates blood glucose levels. GS is the key enzyme in gluconeogenesis; it converts glucose into glycogen, while GSK3 phosphorylates GS and suppresses its action (Figure 38). In this context, we can speculate that the absence of GSK3 would lead to an hyperactivation of GS, reducing the levels of free glucose. Such imbalance in the available glucose can be deleterious for photoreceptors since they are highly metabolically active cells. Indeed, to meet their large energy demands, photoreceptors mainly rely on glucose. Starvation affects photoreceptor, and mainly cone photoreceptor survival (Punzo et al., 2009). Hence, the lack of glucose in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* retinas could explain increased cone degeneration. This hypothesis needs to be tested in *Gsk3 α^{ff} β^{ff} ; Crx-Cre* retinas in comparison to controls.

Similar to cancer cells, photoreceptors metabolize glucose by aerobic glycolysis (Leveillard, 2015) (Leveillard and Sahel, 2017), which is an inefficient way to produce ATP. In fact, glucose is metabolized to lactate despite the presence of oxygen, producing only 2 ATP per mole of glucose (in contrast to 32 ATP per mole of glucose produced by oxidative phosphorylation). Whether GSK3 can affect this metabolism remains to be clarified. Using combined RNA-Seq and MS/MS data, we identified several altered pathways related to glucose metabolism with more than 200 deregulated genes and proteins. Interestingly, among downregulated NRL targets, we identified **Hexokinase 2 (HK2)**, the key enzyme catalyzing the first step of glycolysis. In photoreceptors, HK2 is expressed in the outer retina and is a marker for **aerobic glycolysis** (Reidel et al., 2011) (Ait-Ali et al., 2015). Hence, NRL deregulation could inhibit the key enzyme for aerobic glycolysis to reduce inefficient glucose utilization.

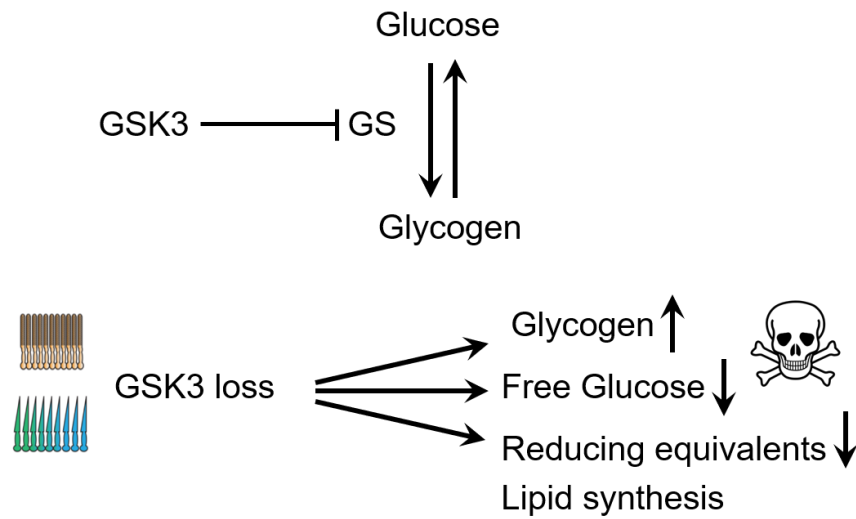


Figure 38. Proposed model of GSK3 lack leading to the lack of glucose.

Glycogen synthase (GS), the key enzyme in gluconeogenesis, converts glucose into glycogen, while GSK3 phosphorylates GS and suppresses its action. Therefore, GSK3 loss might increase glycogen, decrease free glucose levels, and decrease reducing equivalents produced in the pentose phosphate pathway as well as lipid synthesis.

In line with that, enzymes catalyzing the key steps of the citrate cycle, such as pyruvate dehydrogenase and isocitrate dehydrogenase were found to be upregulated in *Gsk3 α ^{fl/fl}; Crx-Cre* retinas. Therefore, we advance the following hypothesis: to compensate for the lack of glucose and energy, **photoreceptors could switch from their usually inefficient aerobic glycolysis to oxidative phosphorylation**, probably resulting in an overproduction of ATP. To support this hypothesis, we first need to measure ATP levels in *Gsk3 α ^{fl/fl}; Crx-Cre* photoreceptors in comparison to controls. Importantly, increased ATP levels have been reported to accelerate photoreceptor cell death (Notomi et al., 2013). In addition, an “ATP overflow” has been reported to decrease protein biosynthesis (Pontes et al., 2015). Therefore, photoreceptors could prefer the inefficient glucose utilization to sustain the high amount of protein required for the OS visual transduction and OS renewal.

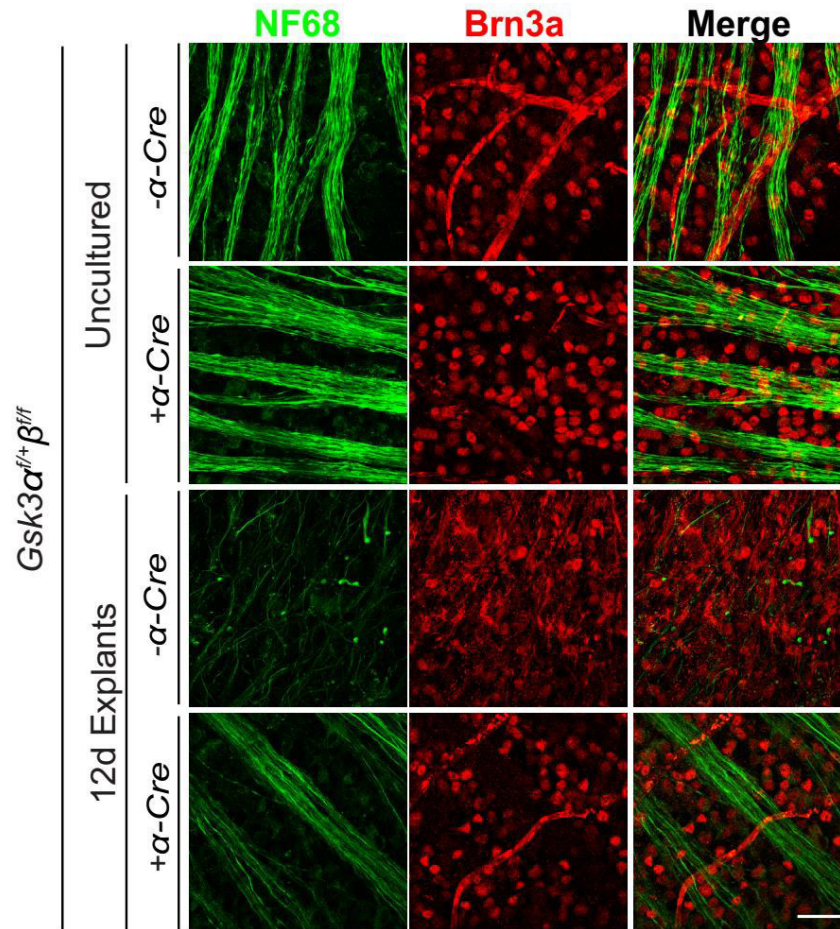


Figure 39. Complete loss of GSK3 β has a neuroprotective effect on RGCs in retinal explant culture.

Retinal explants from *Gsk3 $\alpha^{f/+}$ $\beta^{fl/fl}$* retinas, positive or negative for α -Cre recombinase expression, were cultured for 12 days to induce RGC cell death (**lower panels**). Uncultured retinas (**upper panels**) served as controls. Flat mount retinas were immunolabeled with anti-Brn3a to visualize RGCs and anti-neurofilament 68 (NF) to mark axons. RGCs were very well preserved upon partial GSK3 loss in *Gsk3 $\alpha^{f/+}$ $\beta^{fl/fl}$; α -Cre* retinas, with a limited loss of cells and a very good preservation of the axon bundles compared to the control situation. Scale bar: 20 μ m.

D.2 GSK3 in the context of stress and disease

The subsequent step is to transfer the acquired knowledge, “to build the translational bridge” of GSK3 in retinal development to adult retina and especially to pathological, diseased conditions. Indeed, the role of GSK3 in disease and stress conditions remains to be clarified. As depicted, the expression of **only one allele** of *Gsk3 α* or *Gsk3 β* is sufficient to entirely rescue microphthalmia *Gsk3 $\alpha^{ff}\beta^{ff}; \alpha$ -Cre* mice. It also completely **rescues photoreceptor degeneration** and restores normal photoreceptor development, maturation, and function. Therefore, it seems that complete GSK3 loss is rather detrimental, whereas partial loss has no obvious impact on retinal function and maintenance. Interestingly, a recent study showed that GSK3 inhibition using small molecules had a protective effect in *Retinitis Pigmentosa* and in glaucoma models (Marchena et al., 2017). Such results led us to speculate that **partial loss of *Gsk3 α* or *Gsk3 β* in mice may have protective effects under stress conditions**. To test this hypothesis, we used different stress conditions to assess RGC and photoreceptor survival, the two main cells affected in retinal dystrophies. To test RGC survival in a context of partial loss of GSK3 activity and under stress conditions, we used an adult retinal explant model, in which RGC cell death is known to be triggered rapidly following the optic nerve resection. Retinal explants of *Gsk3 $\alpha^{f/+}\beta^{ff}; \alpha$ -Cre* or control retinas were kept in culture for 12 days. As expected, we observed a massive RGC loss in control explants after 12 days of culture with an almost complete loss of RGC axons (labelled with anti-NF68). Stuningly, in the same conditions, RGCs were very well preserved in *Gsk3 $\alpha^{f/+}\beta^{ff}; \alpha$ -Cre* retinas, with a limited loss of cells and a very good preservation of the axon bundles (Figure 39). Importantly and in consistence with the published data, in which small molecule inhibitors were used (Marchena et al., 2017), our preliminary data provide the first genetic evidence that GSK3 partial inhibition is able to significantly prevent RGC cell death in adult retina. It is striking that a single allele of *Gsk3 α* not only completely rescues the microphthalmia but has additionally such neuroprotective effect on RGCs upon stress exposure. To conclude, it seems that the **degree of *Gsk3* inhibition is decisive**. The question arises about the mechanisms underlying *Gsk3 α* and *Gsk3 β* implication in neuroprotection. It will be interesting to investigate if such protective effects can occur in additional models. For example, N-methyl-D-aspartate (NMDA) can be applied to activate the NMDA receptors and induce excitotoxicity to promote cell death in the retina. Such excessive NMDA receptor activity has been linked to glaucoma (Lam et al., 1999) (Manabe and Lipton, 2003). Alternatively, an optic nerve crush can be performed on these mice to test the possible neuroprotective effect of a partial GSK3 loss. Altogether, these approaches might lead to the **identification of neuroprotective effects regulated by GSK3** and are relevant for **diseases affecting RGC survival** such as glaucoma.

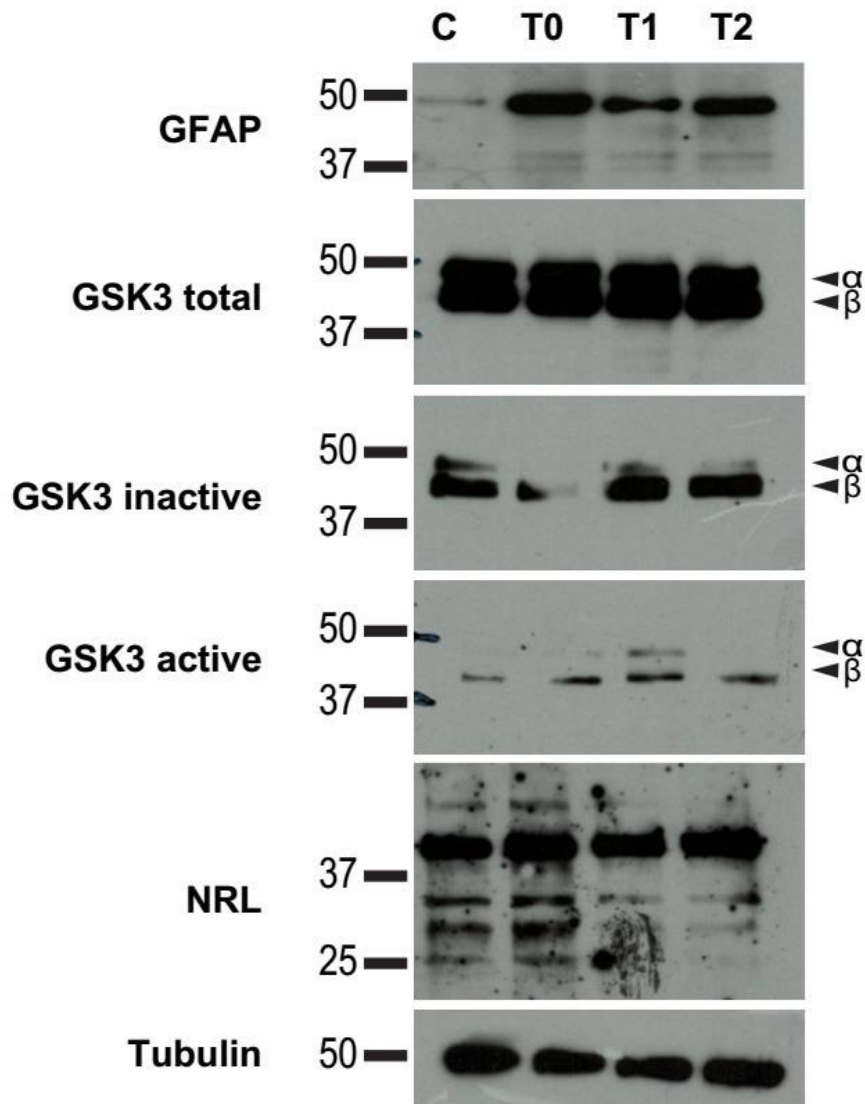


Figure 40. GSK3-mediated NRL regulation in response to stress.

In this preliminary experiment, wildtype mice were dark-adapted overnight. They were exposed to light at an intensity of 12.000 Lux for one hour and put back in the dark. Non-exposed animals were used as controls. Retinal proteins were extracted. GFAP, total GSK3 as well as active and inactive form of GSK3 and NRL were detected by immunoblot. **C0**: non-exposed control; **T0**: right after light exposure; **T1**: one hour after light exposure; **T2**: two hours after light exposure. Tubulin was used as loading control.

Concerning photoreceptors, specific models are available to assess their neuroprotection upon partial GSK3 loss in a neurodegenerative context. For example, we could use a pharmacological model to trigger photoreceptor degeneration in *Gsk3α^{f/+}β^{ff}*; *Crx-Cre* mice compared to controls. The pharmacological agent N-methyl-N-nitrosourea (MNU), which induces double-strand DNA breaks, could be intraperitoneally injected in *Gsk3α^{f/+}β^{ff}*; *Crx-Cre* and control mice. MNU induces rapid apoptotic photoreceptor cell death so that photoreceptors are completely lost 7 days after injection (Yuge et al., 1996). It will be interesting to assess whether partial GSK3 loss could rescue photoreceptor cell death in *Gsk3α^{f/+}β^{ff}*; *Crx-Cre* mice compared to controls.

Bright light exposure is an additional model of **induced stress leading, under certain conditions, to photoreceptor cell death**. In this model, mice are dark-adapted overnight and exposed for various time windows to a bright light before going back to the dark. The light will induce a strong retinal stress that may trigger subsequent photoreceptor degeneration depending on the mouse strain, the time of exposure, and the light intensity. We hypothesized that GSK3 may play a protective role under stress condition and that its activation may trigger rapid degradation of factors to mediate neuroprotection. Such factors have to be identified. Therefore, mice lacking partially GSK3 activity, such as *Gsk3α^{f/+}β^{ff}*; *Crx-Cre* or *Gsk3α^{ff}β^{f/+}*; *Crx-Cre* line, should be more sensitive to light exposure than control C57Bl6/J. A preliminary experiment has already been initiated to setup the experimental procedure. A one-hour 12.000 Lux exposure was tested. We harvested retinas after dark-adaptation, at t=0 when light was turned on, and then after 1 and 2 hours (Figure 40). We observed a rapid induction of the glial fibrillary acidic protein (GFAP) expression as soon as the bright light is on, reflecting the activation of Müller cells in response to bright light exposure. We then assessed GSK3 activation and inactivation and found an increased activation of GSK3α from t=0 to one hour after the beginning of the exposure with a concomitant decrease of the inactive form of GSK3α. In our conditions, we could not clearly rule out whether GSK3β becomes activated in such light-induced stress conditions. It seems to slightly increase right after the beginning of the light exposure. Total GSK3 was quantified for normalization. Such data support the advanced hypothesis that GSK3 becomes activated upon bright light exposure. We next will assess if photoreceptors are more sensitive upon partial loss of GSK3α or GSK3β. However, photoreceptors should be more prone to cell death in *Gsk3α^{ff}β^{f/+}*; *Crx-Cre* line since only GSK3α was activated. Such specific activation strongly suggests that this isoform plays a critical role in photoreceptor response to stress. This is in line with the large decreased expression in *Gsk3α* in *Gsk3α^{ff}β^{ff}*; *Crx-Cre* line (Figure 1A of the manuscript).

Finally, based on our findings of GSK3-mediated regulation of NRL stability, we hypothesized that rapid downregulation of NRL protein may trigger a quick response to stress and may potentially contribute to a neuroprotective response. Indeed, NRL protein amount was largely decreased after one hour when GSK3 α activation was at the maximum and maintained decreased for 2 hours following the beginning of the illumination. Therefore, GSK3-mediated NRL degradation may play a role in rod photoreceptor response to stress. We speculate that such stress-regulating mechanism could be absent in *Retinitis Pigmentosa* patients carrying *NRL* mutations (*NRL-S50T*, *NRL-P51S*) in which phosphorylation by GSK3 is impaired.

To conclude, the above presented findings underline **GSK3 implication in the retinal stress response for both RGC and photoreceptors**. Importantly, while a clear neuroprotective effect compared to wildtype has been established upon partial *Gsk3* deletion for RGCs, the situation upon partial *Gsk3* deletion compared to wildtype needs to be clarified in photoreceptors. Further experiments will be required to decipher the underlying molecular mechanisms, as well as GSK3 targets in this context. These findings may pave the way for new therapeutic approaches targeting glaucoma and photoreceptor degenerative diseases.

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Titre : Rôle de la Glycogène Synthase Kinase 3 dans le Développement et l'Homéostasie de la Rétine

Mots clés : Rétine, Glycogène Synthase Kinase 3, Développement, Neurodégénérescence, Photorécepteurs, Cellules ganglionnaires

Résumé : Les modifications post-traductionnelles (MPTs) permettent un haut degré de régulation de l'expression des gènes en générant une diversité fonctionnelle au niveau du protéome. Dans le système nerveux, les MPTs régulent entre autres des facteurs de transcription permettant une adaptation rapide à un microenvironnement dynamique. Dans ce contexte, je me suis concentrée sur l'étude des Glycogène Synthase Kinases 3 (GSK3s). Elles sont au centre de la régulation de nombreuses voies de signalisation et contrôlent la stabilité de multiples cibles par phosphorylation. Au cours du développement du cerveau, les kinases GSK3 contrôlent la balance entre la prolifération et la différenciation cellulaire. De nombreuses études ont montré que la dérégulation de l'activité des kinases GSK3 est impliquée dans le développement des maladies neurodégénératives du cerveau. En revanche, leur rôle dans les maladies neurodégénératives de la rétine ou au cours du développement rétinien reste une question ouverte. L'objectif de ma thèse était d'étudier le rôle de ces kinases au cours du développement et de l'homéostasie rétinienne. J'ai montré que l'absence totale de *Gsk3 α* et de *Gsk3 β* très tôt au cours du développement rétinien entraîne une microphthalmie chez l'adulte due à des défauts sévères de prolifération et de différenciation conduisant à la mort des progéniteurs. Ces données suggèrent que les kinases GSK3s sont essentielles au maintien des progéniteurs rétiens. Les deux kinases jouent des rôles redondants puisque l'expression d'un seul allèle *Gsk3* est suffisante pour prévenir le phénotype de microphthalmie. Cependant, une analyse phénotypique approfondie dans ce contexte génétique (un seul allèle *Gsk3*) a révélé une forte augmentation du nombre de cellules ganglionnaires déplacées (dRGCs) dans la couche nucléaire interne, qui apparaissent normalement en très faible nombre dans une rétine sauvage. Le traçage des axones de toutes les cellules ganglionnaires par marquage antérograde a

mis en évidence des projections dans un noyau ipsilatéral qui n'est pas visible chez les animaux sauvages. Dans l'ensemble, ces données suggèrent que les kinases GSK3s sont impliquées dans la genèse des dRGCs, dont les projections au cerveau sont particulières et dont la fonction est méconnue. Le modèle génétique que j'ai développé offre par conséquent un modèle de choix pour étudier l'ontogenèse et la fonction de ces cellules. En dehors du rôle de GSK3 au cours du développement précoce de la rétine, mes travaux de thèse se sont également concentrés sur leur rôle de GSK3 dans les photorécepteurs, dont les défauts de développement conduisent très souvent à leur dégénérescence et donc à la cécité. Pour cela, j'ai utilisé des souris invalidées de manière conditionnelle pour *Gsk3 α* et *Gsk3 β* spécifiquement dans les précurseurs des photorécepteurs. L'absence de GSK3 conduit à une altération de leur maturation et de leur fonction, suivie de leur dégénérescence. Des analyses combinées de transcriptomique et d'approches *in vitro* et *in vivo* m'ont permis de montrer que l'absence de GSK3 dans les photorécepteurs conduit à des défauts de phosphorylation de NRL (facteur de transcription nécessaire au développement des photorécepteurs de type bâtonnet), associée à une augmentation de sa stabilité. J'ai également montré que l'absence de GSK3 dans les photorécepteurs conduit à la diminution d'expression d'un sous-ensemble de gènes cibles de NRL, co-régulés par CRX, et impliqués dans le développement et l'homéostasie des photorécepteurs. Mes travaux suggèrent donc que GSK3, *via* la régulation de la stabilité de NRL, joue un rôle essentiel dans le contrôle de l'expression de gènes nécessaires à la maturation et l'homéostasie des photorécepteurs. Un tel mécanisme de régulation pourrait être déficient chez les patients atteints de rétinites pigmentaires et porteurs de mutations dans *NRL* connues pour bloquer sa phosphorylation.

Title : Role of the Glycogen Synthase Kinase 3 for the Retinal Development and Homeostasis

Keywords : Retina, Development, Neurodegeneration, Glycogen Synthase Kinase 3, Photoreceptors, Ganglion cells

Abstract: Posttranslational modifications (PTMs) allow a higher degree of regulation for the control of gene expression by generating functional diversity at the proteome level. In the central nervous system, PTMs regulate stability or activity of transcription factors allowing a rapid response to a dynamic cellular microenvironment. In this context, I focused on the ubiquitously expressed and highly conserved Glycogen Synthase Kinases 3 (GSK3s). They are at the crossroad of multifunctional signaling pathways and control the stability of various targets by phosphorylation. During mammalian brain development, GSK3 kinases control the balance between proliferation and differentiation. Deregulation of GSK3 kinases activity has also a key role in neurodegenerative diseases. Drugs targeting GSK3s hold a lot of promises to treat such diseases. Whether these kinases are also important during retinal neurodegenerative diseases or during retinal development remains an open question. In this context, the objective of my Ph.D. was to investigate the role of these kinases during retinal development and homeostasis. I found that the complete lack of *Gsk3 α* and *Gsk3 β* lead to microphthalmia in adults due to severe defects of both proliferation and neuronal differentiation leading to cell death. These data suggest an essential role for GSK3s to maintain retinal progenitors. Interestingly, the expression of only one *Gsk3* allele was enough to rescue the phenotype demonstrating the functional redundancy of the two kinases. However, further analysis revealed a large number of displaced retinal ganglion cells (dRGCs) in the inner nuclear layer, which normally appear at very low number in a wildtype retina. Strikingly, anterograde tracing

of all retinal ganglion cells revealed altered axonal projections into an ipsilateral nucleus. Altogether, our data suggest an essential role of GSK3s in dRGC genesis, whose projections to the brain are peculiar and whose function remains poorly known. Importantly, the genetic model that I developed offers a powerful system to further study the ontogenesis and the function of dRGCs. The focus of my Ph.D. work was on the role of GSK3 specifically for photoreceptors, the main cells for vision, whose developmental defects are associated with retinal degenerative diseases. Lack of both GSK3 kinases in photoreceptor precursors resulted in an impaired photoreceptor maturation and function followed by their degeneration. Using combined transcriptomic as well as *in vivo* and *in vitro* approaches, I demonstrated that the absence of GSK3 in photoreceptors was associated with an impaired phosphorylation of NRL (transcription factor required for rod photoreceptor development) resulting in its increased stability and accumulation. Despite such NRL accumulation, most of NRL target genes, co-regulated with CRX and known for their role during photoreceptor development and homeostasis, were downregulated. This suggests that GSK3 plays an essential role in regulating genes necessary for photoreceptor maturation and homeostasis, *via* regulation of NRL stability. Such mechanism of posttranslational stability regulation may be deficient in *Retinitis Pigmentosa* patients carrying *NRL* mutations altering its phosphorylation. Our studies therefore provide the framework for integrating signaling pathways in the regulation of NRL activity via PTMs and pave the way for developing therapies in patients with NRL mutations.