Role of Otx2 in mature retinal photoreceptors

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Rôle d'Otx2 dans les photorécepteurs de la rétine mature

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Titre de la thèse: Rôle d'Otx2 dans les photorécepteurs de la rétine mature

Mots-clés: rétine, photorécepteurs, Otx2, adaptation à la lumière, transfert d'homéoprotéines

Résumé

Si le rôle du facteur de transcription Otx2 dans le développement de la rétine est bien compris, sa fonction dans la rétine adulte reste floue. L'expression d'*Otx2* est maintenue dans l'épithélium pigmenté rétinien (RPE), les photorécepteurs (PR) et les cellules bipolaires durant toute la vie. Des travaux antérieurs ont montré que l'ablation du gène *Otx2* dans la rétine adulte conduit à une dégénérescence des PR, suggérant qu'il a un rôle dans leur maintenance. Il a ensuite été montré que l'expression d'*Otx2* restreinte dans le RPE est à la fois nécessaire et suffisante pour la survie des photorécepteurs, ce qui indique une fonction neuroprotectrice non autonome dans ces cellules. Ces résultats n'expliquent pas la fonction de la protéine Otx2 endogène dans les PR. Afin de l'élucider, nous avons utilisé la lignée de souris *Crx-CreER^{T2}* inductible pour procéder à la délétion du gène *Otx2* spécifiquement dans les PR. L'histologie a confirmé que l'expression d'*Otx2* endogène n'est pas nécessaire à la survie des PR ni au maintien de leur identité cellulaire chez l'adulte. Cependant, nous avons constaté qu'Otx2 est impliqué dans l'adaptation visuelle en régulant le mouvement de l'arrestine induit par la lumière, dans les photorécepteurs. L'arrestine est transportée entre les segments externes du PR, où il s'accumule à la lumière, et les segments internes et le soma, où il s'accumule dans l'obscurité, pour moduler la sensibilité du PR, en réponse à différentes intensités lumineuses. Ce trafic est compromis après délétion d'Otx2 dans les PR : l'arrestine reste bloquée dans les segments internes et dans le soma, même après une exposition prolongée à la lumière. Des tests comportementaux ont montré que les souris mutantes sont fortement photophobes. Les mécanismes détaillés restent à élucider.

Pour déchiffrer le réseau de gènes contrôlé par Otx2 dans les PR, nous avons effectué des analyses temporelles par RNA-seq au décours de l'inactivation d'*Otx2* dans les PR. Aucun gène de la cascade de phototransduction ne semble dérégulé. Par contre, l'expression de certains gènes de la matrice extracellulaire et, de manière surprenante, d'un groupe de gènes spécifiquement impliqués dans la mélanogénèse dans le RPE, et déjà connus pour être des cibles directes d'Otx2, est diminuée. Un examen attentif des souris porteuses d'une délétion d'Otx2 spécifique dans les PR a confirmé que le niveau de protéine Otx2 dans les noyaux du RPE est réduit, tandis que la protéine Otx2 peut maintenant être détectée dans les segments externes de PR. Au vu de ces données et de l'effet neuro-protecteur d'Otx2 joué par le RPE, nous avons émis l'hypothèse d'un transfert direct de la protéine Otx2 du RPE aux PR. Pour prouver cette hypothèse, des vecteurs viraux dirigeant l'expression d'une forme d'Otx2 étiquetée, spécifiquement dans les cellules du RPE, ont été générés et utilisés pour l'injection sous-rétinienne chez la souris. Après transduction du RPE, nous avons recherché si la forme d'Otx2 étiquetée pouvait être détectée dans les PR. Nous avons constaté que dans la rétine contrôle, un transfert constitutif d'Otx2 est actif à faible débit. Après l'induction du KO dans les PR, le taux de ce transfert est augmenté, agissant probablement comme une réponse neuro-protectrice. Une telle augmentation du transfert explique bien la réduction du taux de protéine Otx2 dans le RPE et la diminution d'expression des gènes de la mélanogenèse. La protéine Otx2 transférée semble être transportée des segments externe et interne des PR vers les

synapses situées dans la couche plexiforme externe, ce qui suggère que sa fonction neuro-protectrice met en jeu des mécanismes différents de sa fonction génomique classique de facteur de transcription.

En conclusion, cette étude a révélé une nouvelle fonction de la protéine Otx2 endogène dans l'adaptation

des PR à la lumière et a démontré l'existence d'un transfert de protéine Otx2 exogène, du RPE vers les PR, avec un rôle neuro-protecteur présumé.

Thesis title: Role of Otx2 in mature retinal photoreceptors

Keywords: retina, photoreceptors, Otx2, light adaptation, homeoprotein transfer

Abstract

Although the role of Otx2 transcription factor in retinal development is well understood, its function in the adult retina remains unclear. *Otx2* expression is maintained in Retinal Pigmented Epithelium (RPE), photoreceptors (PRs) and bipolar cells throughout life. Previous works from our lab showed that *Otx2* ablation in the adult retina leads to photoreceptor degeneration, suggesting a role in their maintenance. It was then showed that RPE-restricted *Otx2* expression was both necessary and sufficient for photoreceptor maintenance, indicating a non-cell-autonomous neuroprotective function. This left unexplained the function of endogenous Otx2 in PRs. In order to elucidate it, we performed PR-specific *Otx2* knockout, using the inducible PR-specific *Crx-CreER*^{T2} mouse line. Histology confirmed that endogenous Otx2 is not required for PR survival or maintenance of their cell identity in the adult. However, we found that Otx2 is implicated in visual adaptation, by regulating the light induced movement of arrestin in photoreceptors. Arrestin shuttles between PR outer segments, where it accumulates in the light, and inner segments and soma, where it accumulates in the dark, to modulate PR sensitivity, in response to different light intensities. Such trafficking is compromised in PR-specific *Otx2* KO mice, where arrestin remains blocked in inner segments and soma, even after prolonged light exposure. Behavioural tests showed that PR-specific *Otx2* KO mice are strongly photophobic. The detailed mechanisms remain to be elucidated.

To decipher the genetic network controlled by Otx2 in PRs, we carried out time series RNA-seq analyses following PR-specific *Otx2* knockout. No photo-transduction gene was found to be deregulated. Instead of that, we found down-regulation of extracellular matrix genes and, surprisingly, of a group of RPE-specific genes involved in melanogenesis, genes that were already known to be direct targets of Otx2. Careful examination of PR-specific *Otx2* KO mice confirmed that Otx2 protein level in the RPE nuclei was reduced while some Otx2protein could now be detected in PR outer segments. According to such data and to the neuroprotective effect of RPE-restricted Otx2, we hypothesized a direct Otx2 protein transfer from RPE to PRs. To prove this hypothesis, viral vectors driving tagged-Otx2 expression specifically in RPE cells were generated and used for sub-retinal injection in mice. After RPE transduction, tagged-Otx2 could be detected in the PRs. We found that in wild type retina, constitutive Otx2 transfer is active at low rate. After KO induction in the PRs, the transfer rate is increased, probably acting as a neuroprotective response. Such increment in the transfer explains well the reduction of Otx2 protein level in the RPE and the subsequent downregulation of melanogenesis genes. Transferred Otx2 appears to travel across PR cells from the inner and outer segments to the synapse in the outer plexiform layer, suggesting its

neuroprotective function involves mechanisms that are different from its classical genomic function of transcription factor.

In conclusion, this study disclosed a new function of endogenous Otx2 in PR light adaptation and demonstrated the existence of a transfer of Otx2 from RPE to PR cells, with a putative neuroprotective role.

Al Mio Amato Nonno Angelo

To My Lovely Angel Grandpa

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I thank Nuria, fantastic post-doc, who has always invited me (without too much success, I'm sorry for her) to try to avoid long circles of sentences for speaking about science and to go straight to the point. (her words "Pasquale GO TO THE POINT" are still in my mind ☺).

I thank Salsabiel, Malala, Vishnu and Benedicte and all the "old" PhD students of the seventh floor of the natural sciences department for sharing their time with me. In particular I will miss the sarcasm of Salsabiel and the long discussions with Malala about how to use cow poop ☺.

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List of abbreviations

AAV	Adenoassiociated virus
ACs	Amacrine cells
Arr1	Arrestin 1 (visual arrestin)
Arr4	Arrestin 4
BPs	Bipolar cells
Brn3A	Brain 3A
Brn3B	Brain 3B
cGMP	Cyclic guanosine monophosphate
ChABC	chondroitinase ABC
Chx10	Ceh-10 Homeodomain-Containing Homolog
Cox7c	Cytochrome c oxidase subunit 7C, mitochondrial
CP	Critical period
Crx	Cone-rod homeobox
DAPI	4′,6-diamidino-2-phenylindole
Dct	Dopachrome tautomerase
E10 (and similar)	Embryonic day 10
ECM	Extracellular matrix
EFTFs	Eye-field transcription factors
En1, En2	Engrailed 1, 2
ERG	Electroretinogram
Fgf	Fibroblast growth factor
Gadd45b	Growth arrest and DNA-damage-inducible, beta,
GAGs	Glycosaminoglycans
gc	Gene Copies
GC1	Guanylate cyclase 1
GC2	Guanylate cyclase 2
GCAP	Guanylate cyclase activating protein
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Gldc	Glycine decarboxylase/ glycine dehydrogenase
Gpr143	G-protein coupled receptor 143
GRK	G protein-coupled receptor kinases
Gα	Alpha subunit of transducin
HCs	Horizontal cells
INL	Inner nuclear layer
IPL	Inner plexiform layer
IPM	Interphotoreceptor matrix
IRBP	inter-photoreceptor retinoid binding protein
IRES	Internal ribosome entry site
IS	Inner segment
KO	Knock Out

Lhx2	LIM homeobox 2
Loxl1	Lysyl oxidase homolog 1
LRAT	Lecithin retinol acyltransferase
Maob	Monoamine oxidase B
mDAs	mibrain dopaminergic neurons
MGCs	Muller glial cells
Mitf	Microphtalmia associated factor / Melanocyte Inducing Transcription Factor)
Mlana	Protein melan-A
Mlph	Melanophilin
NFL	Nerve fibre layers
Nrl	Neural retina-specific leucine zipper
Oca2	oculocutaneous albinism II
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
Otd	orthodenticle
Otx1	Orthodenticle Homeobox 1
Otx2	Orthodenticle homeobox 2
P10 (and similar)	Post-natal day 10
Pax6	Paired-box gene 6
PDE	Phosphodiesterase
PEDF	Pigment epithelium derived factor
PGs	Proteoglycans
Pitx	Paired-like homeodomain transcription factor/pituitary homeobox
PLC	Phospholipase C
Pmel	Premelanosome protein
PNN	Perineuronal net
PP2A	Protein Phosphatase 2A
PRs	Photoreceptors
PV-cells	Parvalbumin cells
*R**	Bleached rhodopsin
Rax	Retina And Anterior Neural Fold Homeobox
RGCL	Retinal ganglion cell layer
RGCs	Retinal ganglion cells
RGS9	Regulator of G-protein signalling 9
RK	Rhodopsin Kinase
RPCs	Retinal precursor cells
RPE	Retinal pigmented epithelium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
Rx	Retinal homeobox
Shh	Sonic Hedgehog Homolog
Six3	Sineoculis homeobox homolog 3
Six6	Sineoculis homeobox homolog 6

Slc45a2	Solut carrier member family 45
SPACRCAN	Syaloproteoglycan associated with cones and rods
Suclg2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial
TGFβ	Transforming growth factor beta
Tyr	Tyrosinase
Tyrp-1	Tyrosinase related protein 1
Vsx2	Visual System Homeobox 2

List of figures

Résumé détaillé

Rôle d'Otx2 dans les photorécepteurs de la rétine mature

Si le rôle du facteur de transcription Otx2 dans le développement de la rétine est bien compris, sa fonction dans la rétine adulte reste floue. L'expression du gène *Otx2* est maintenue dans l'épithélium pigmenté rétinien (RPE), les photorécepteurs (PR) et les cellules bipolaires durant toute la vie. Des travaux antérieurs de notre laboratoire ont montré que l'ablation du gène *Otx2* dans la rétine adulte conduit à une dégénérescence des photorécepteurs, suggérant qu'il joue un rôle dans leur maintenance. Il a ensuite été montré que l'expression d'*Otx2* restreinte dans le RPE est à la fois nécessaire et suffisante pour la survie des photorécepteurs, ce qui indique une fonction neuro-protectrice non autonome dans ces cellules. Ces résultats n'expliquent pas la fonction de la protéine Otx2 endogène dans les PR. Afin de l'élucider, nous avons utilisé la lignée de souris *Crx-CreER^{T2}* inductible pour procéder à la délétion du gène *Otx2* spécifiquement dans les PR. L'histologie a confirmé que l'expression d'*Otx2* endogène n'est pas nécessaire à la survie des PR ni au maintien de leur identité cellulaire chez l'adulte. Cependant, nous avons constaté qu'Otx2 est impliqué dans l'adaptation visuelle en régulant le mouvement de l'arrestine induit par la lumière, dans les photorécepteurs. L'arrestine est transportée entre les segments externes du PR, où elle s'accumule à la lumière, et les segments internes et le soma, où elle s'accumule dans l'obscurité, pour moduler la sensibilité du PR, en réponse à différentes intensités lumineuses. Ce trafic est compromis chez les souris après délétion d'Otx2 dans les PR: l'arrestine reste bloquée dans les segments internes et dans le soma, même après une exposition prolongée à la lumière. Des tests comportementaux ont montré que les souris mutantes sont fortement photophobes. Les mécanismes détaillés restent à élucider.

Pour déchiffrer le réseau de gènes contrôlé par Otx2 dans les PR, nous avons effectué des analyses temporelles par RNA-seq au décours de l'inactivation d'*Otx2* dans les PR. Aucun gène de la cascade de photo transduction ne semble dérégulé. Par contre, l'expression de certains gènes de la matrice extracellulaire et, de manière surprenante, d'un groupe de gènes spécifiquement impliqués dans la mélanogénèse dans le RPE, et déjà connus pour être des cibles directes d'Otx2, est diminuée. Un examen attentif des souris porteuses d'une délétion d'Otx2 spécifique dans les PR a confirmé que le niveau de protéine Otx2 dans les noyaux du RPE est réduit, tandis que la protéine Otx2 peut maintenant être détectée dans

les segments externes de PR. Au vu de ces données et de l'effet neuro-protecteur d'Otx2 joué par le RPE, nous avons émis l'hypothèse d'un transfert direct de la protéine Otx2 du RPE aux PR. Pour prouver cette hypothèse, des vecteurs viraux dirigeant l'expression d'une forme d'Otx2 étiquetée, spécifiquement dans les cellules du RPE, ont été générés et utilisés pour l'injection sous-rétinienne chez la souris. Après transduction du RPE, nous avons recherché si la forme d'Otx2 étiquetée pouvait être détectée dans les PR. Nous avons constaté que dans la rétine contrôle, un transfert constitutif d'Otx2 est actif à faible débit. Après l'induction du KO dans les PR, le taux de ce transfert est augmenté, agissant probablement comme une réponse neuro-protectrice. Une telle augmentation du transfert explique bien la réduction du taux de protéine Otx2 dans le RPE et la diminution d'expression des gènes de la mélanogénèse. La protéine Otx2 transférée semble être transportée des segments externe et interne des PR vers les synapses situées dans la couche plexiforme externe, ce qui suggère que sa fonction neuro-protectrice met en jeu des mécanismes différents de sa fonction génomique classique de facteur de transcription.

En conclusion, cette étude a révélé une nouvelle fonction de la protéine Otx2 endogène dans l'adaptation des PR à la lumière et a démontré l'existence d'un transfert de protéine Otx2 exogène, du RPE vers les PR, avec un rôle neuro-protecteur présumé.

Detailed summary

Role of *Otx2* in the photoreceptors of the mature retina

Although the role of Otx2 transcription factor in retinal development is well understood, its function in the adult retina remains unclear. *Otx2* expression is maintained in Retinal Pigmented Epithelium (RPE) cells, photoreceptors (PRs) and bipolar cells throughout life. Previous works from our lab showed that *Otx2* ablation in the adult retina leads to photoreceptor degeneration, suggesting a role in their maintenance. It was then shown that RPE-restricted *Otx2* expression was both necessary and sufficient for photoreceptor maintenance, indicating a non-cell-autonomous neuroprotective function. This left unexplained the function of endogenous Otx2 in PRs. In order to elucidate it, we performed PR-specific *Otx2* knockout, using the inducible PR-specific *Crx-CreER*^{T2} mouse line. Histology confirmed that endogenous Otx2 is not required for PR survival or maintenance of their cell identity in the adult. However, we found that Otx2 is implicated in visual adaptation, by regulating the light-induced movement of arrestin in photoreceptors. Arrestin shuttles between PR outer segments, where it accumulates in the light, and inner segments and soma, where it accumulates in the dark, to modulate PR sensitivity, in response to different light intensities. Such trafficking is compromised in PR-specific *Otx2* KO mice, where arrestin remains blocked in inner segments and soma, even after prolonged light exposure. Behavioural tests showed that PR-specific *Otx2* KO mice are strongly photophobic. The detailed mechanisms remain to be elucidated.

To decipher the genetic network controlled by Otx2 in PRs, we carried out time-series RNA-seq analyses following PR-specific *Otx2* knockout. No phototransduction gene was found to be deregulated. Instead of that, we found down-regulation of extracellular matrix genes and, surprisingly, of a group of RPE-specific genes involved in melanogenesis, that were already known to be direct targets of Otx2. Careful examination of PR-specific *Otx2* KO mice confirmed that Otx2 protein level in the RPE nuclei was reduced while some Otx2 protein could now be detected in PR outer segments. According to such data and to the neuroprotective effect of RPE-restricted Otx2, we hypothesized a direct transfer of Otx2 protein from RPE to PR cells. To prove this hypothesis, viral vectors driving tagged-Otx2 expression specifically in RPE cells were generated and used for sub-retinal injection in mice. After RPE transduction, tagged-Otx2 could be detected in the PRs. We found that in wild

type retina, constitutive Otx2 transfer is active at low rate. After KO induction in the PRs, the transfer rate is increased, probably acting as a neuroprotective response. Such increment in the transfer explains well the reduction of Otx2 protein level in the RPE and the subsequent downregulation of melanogenesis genes. Transferred Otx2 appears to travel across PR cells from the inner and outer segments to the synapse in the outer plexiform layer, suggesting its neuroprotective function involves mechanisms that are different from its classical genomic function of transcription factor.

In conclusion, this study disclosed a new function of endogenous Otx2 in PR light adaptation and demonstrated the existence of a transfer of Otx2 from RPE to PR cells, with a putative neuroprotective role.

INTRODUCTION

I Visual perception

Vision is the most important of the five senses for humans. Every day we perceive many visual impressions from the environment. We can see thousands of colours; we can distinguish structures and patterns. Visual perception is the ability to interpret light reflected by objects, converting it in signals, that the brain can integrate and elaborate.

1.1 General anatomy of the eye

The eye is the organ where the phenomenon of vision is initiated. In higher organisms, eyes are very complex optical systems, able to detect light from the surrounding environment, regulate its intensities, focus it to form an image and convert it in electrical signals, that the brain can process and elaborate. These functions are achieved thanks to specialized regions of the eye. The general anatomy of the eye is well conserved in higher organisms (**Figure 1**).

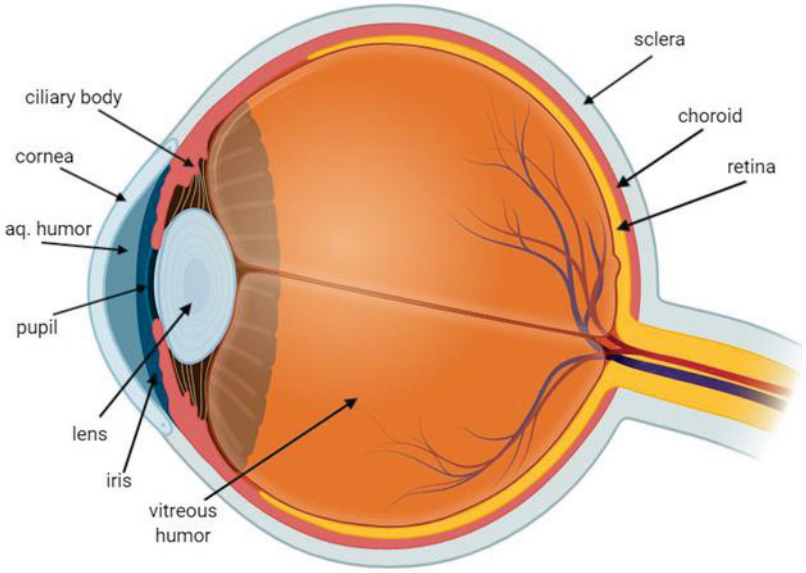


Figure 1. General anatomy of the human eye. The human eye is organized in three layers: the outermost, in white, is composed by the cornea and sclera; the middle layer, in red, consists of the ciliary body, the iris and the choroid; the innermost layer, in yellow, is represented by the retina. Created with BioRender.

The main compartments of the eye are:

The cornea: the only transparent tissue of the human body; it is placed at the front of the eye, where it covers the pupil (the opening at the centre of the eye), the iris (the coloured part of the eye) and the anterior chamber (the fluid-filled inside the eye, in front of the lens). Its main function is to refract or bend the light entering the eye to focus it on the retina.

The sclera: the "white" of the human eye; it is the outermost layer of the eye and it is continuous with the cornea. Together they form the fibrous tunic. Its main function is to protect the eyeball.

The uvea: the vascular middle layer of the eye; it is involved in nutrients and oxygen exchange of the eye and it is composed of three different structures: iris and ciliary body in the anterior part, choroid in the posterior part. The iris is the coloured part of the eye and it acts as a diaphragm, regulating the amount of light reaching the retina. Indeed, it controls the size and diameter of the pupil, the central opening of the iris. The ciliary body is composed by the ciliary muscle, that controls the shape of the lens, and by the ciliary epithelium, that produces the aqueous humor (from the pigmented part of the epithelium) and the vitreous humor (from the non-pigmented part of the epithelium). The choroid, instead, is the vascular component of the uvea that supplies the retina with oxygen and nutrients. It contains blood vessels and connective tissue. Connected to the ciliary body and bathed in the vitreous and aqueous humor there is the lens. It is a biconvex protein structure, that, along with the cornea, helps to refract light to be focused on the retina, changing shape.

The retina: the innermost layer of the eye is divided in a non-neural region, the retinal pigmented epithelium (RPE) and a neural region, which consists of different interconnected neuronal populations. Because this thesis is focused on photoreceptor cells, the most important population of the retina, a more detailed description of its structure and function is following in the next paragraphs.

1.2 Laminar organization of the retina

The retina is a multilayer structure positioned in the innermost part of the eye and encompasses the RPE and the neural retina. It is characterized by a conserved laminar organization (Figure 2).

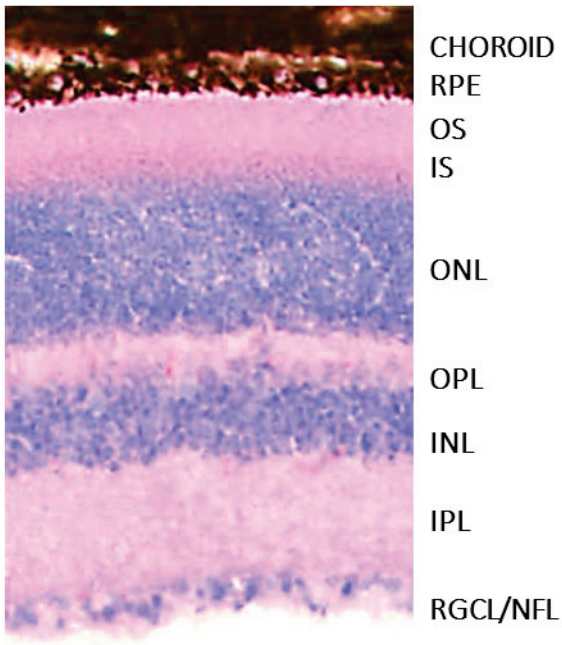


Figure 2. Histological organization of the retina. Eosin/haematoxylin staining of an adult mouse retina vertical section. The retina has a laminar organization. The RPE is positioned between the choroid and the photoreceptor layer. The OS, the photoreceptor compartment associated to phototransduction, and the IS, the one important for ATP synthesis, are above the ONL, the layer including photoreceptor cell bodies. The OPL includes synaptic connection between photoreceptors and cells from the next layer, the INL. The INL contains the cell bodies of horizontal, bipolar, amacrine, Muller and displaced retinal ganglion cells. The IPL is formed by synaptic connections between bipolar cells and amacrine cells of the INL and retinal ganglion cells, whose cellular bodies are located in the RGCL. Axons of retinal ganglion cells, in the NFL, surrounded by astrocytes, are forming the fibres of the optic nerve. See text for abbreviations.

The RPE layer is positioned between the neural retina and the choroid, and it supports neural retinal cells, in particular, photoreceptors, by protecting them from light-induced oxidative stress and by regulating exchanges of metabolites and ions between photoreceptors and choroid. Moreover, it contributes to the phototransduction, by recycling some important components of the cascade (Strauss 2005).

The Outer Segment/Inner segment (OS/IS) layer includes the outer segment and the inner segment of photoreceptor cells. While outer segments are compartments specialized for the phototransduction, the main functions of inner segments are to host protein translation machinery and a high number of mitochondria involved in ATP production.

The Outer Nuclear Layer (ONL) includes the cell bodies of the photoreceptors, the cells responsible to convert the perceived light into electrical stimuli, that will be conveyed to the brain.

The Outer Plexiform Layer (OPL) is a dense synaptic region, incorporating connections between axon terminals of the photoreceptors and dendrites of the bipolar and horizontal cells.

The Inner Nuclear Layer (INL) includes the cell bodies of different neuronal cells (bipolar cells, amacrine cells, horizontal cells, displaced retinal ganglion cells) and non-neuronal populations (Muller Glial Cells).

The Inner Plexiform Layer (IPL) consists of a dense synaptic region, incorporating connections between bipolar or amacrine axon terminals and retinal ganglion cells dendrites. This layer is thicker than the OPL.

The Retinal Ganglion Cells Layer (RGCL or GCL) includes the cell bodies of retinal ganglion cells and displaced amacrine cells.

The Nerve fibre layer (NFL) consists of the retinal ganglion cells axonal projections, which form the optic nerve. These axonal projections are surrounded by astrocytes, that are forming a bundle through which axons run.

1.3 Cellular organization of the retina

Retina is predominantly represented by different types of neurons. In particular, the mammalian retina consists of over 60 different neuronal cell types, a number that continues to be redefined (Richard H. Masland 2012). On top of that, some non-neural cell types are also very important for the correct functionality of retina, supporting the neuronal population in different ways. Here, a short description of retinal cells will be given, focusing more in the next paragraphs on RPE and, moreover, on photoreceptors, exploring also the very strict relation, that makes these two cell populations an almost unique functional element.

Moving from the RPE layer to the RGCL, 7 principal classes of retinal cells can be defined (Figure 3):

RPE cells: this non-neural population is organized in a monolayer of pigmented cells that take care of photoreceptors, supplying nutrients, removing waste end-products and actively participating in the visual cycle.

Photoreceptor cells (PRs): these cells are responsible for light perception and transduction. Two PRs populations are defined: rods and cones. Rods provide vision in dim-light conditions

as in the night (scotopic vision), while cones are important in bright light conditions and in colour discrimination (photopic vision). In the dark, photoreceptors are depolarized, releasing glutamate. In response to the light, these cells become hyperpolarized with less glutamate released.

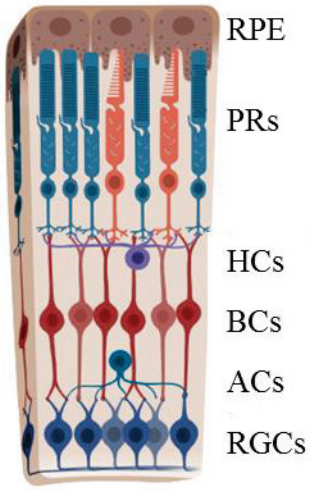


Figure 3. Schematic structure of the retina. The diagram presents the principal cell populations of the retina and their interconnections. RPE: retinal pigmented epithelium; PRs: photoreceptors (rods in blue, cones in orange); BCs: bipolar cells; ACs: amacrine cells; RGCs: retinal ganglion cells. Created with BioRender.

Bipolar cells (BCs): these cells are interneurons that receive input stimuli from axonal terminals of the photoreceptors, rods and cones. Bipolar cells receiving signals from cones are called cone-bipolar cells, while the ones receiving inputs from rods are called rod-bipolar cells. Over 15 different classes of bipolar cells have been described (Euler et al. 2014). According to the response elicited when the glutamate released by photoreceptor terminals binds their receptors at the dendritic level, ON- bipolar cells and OFF-bipolar cells can be distinguished. Glutamate released in the dark by photoreceptors inhibits ON-bipolar cells and activates OFF-bipolar cells. With the light onset and less glutamate released by PRs, ON-bipolar cells become active (depolarized), while OFF-bipolar cells are inhibited (hyperpolarized). These properties arise by the two different glutamatergic receptors present on the dendrites of these two classes of bipolar cells. Indeed, OFF-bipolar cells are characterized by an ionotropic glutamate receptor, whereas ON-bipolar cells express a metabotropic glutamate receptor. Only one group of rod bipolar cells has been described in

the mammalian retina, that are all ON type. Cone-bipolar cells, on the other side, can belong to ON or OFF type. Photoreceptors are the main input on bipolar cells, but this input signal can be modulated by horizontal cells. The main output cells are the retinal ganglion cells, but both ON- and OFF- signals can be modulated by amacrine cells at the IPL level, before reaching them (Diamond 2017). As for photoreceptors, their neurotransmitter is glutamate.

Horizontal cells (HCs): according to the species there are 1 or 2 subclasses of described horizontal cells (Richard H. Masland 2012). Their two main functions are the lateral inhibition of photoreceptors axonal terminals and feedforward lateral inhibition of bipolar dendritic terminals. They operate all in the OPL. Even if they are GABAergic neurons, there is a debate on the real released messenger, which is implicated in the lateral feedback and feedforward inhibition (Kramer & Davenport 2015; Thoreson & Mangel 2012; Kamermans & Spekreijse 1999). The general effect of HCs on the vision is to modulate the contrast in bright and dim light conditions.

Amacrine cells (ACs): they constitute the more diversified class of retinal cells. At least 30 different subclasses have been identified, but the real number could still be underestimated. (Bloomfield 2009; Richard H Masland 2012) (Pérez de Sevilla Müller et al. 2019). As horizontal cells in the OPL, amacrine cells are responsible for lateral inhibition in the IPL. They can integrate and modulate the input signal from bipolar cells, providing an inhibitory signal to the dendrites of the retinal ganglion cells or of other amacrine cells. Moreover, they can give a negative feedback also retro-acting on the axonal terminals of the same input bipolar cells. The most known classes are the glycinergic and the GABAergic ones, but dopaminergic as well as cholinergic populations have been identified. Some of these amacrine cells are displaced in the RGCL with a not yet clear function (Pérez De Sevilla Müller et al. 2007).

Retinal ganglion cells (RGCs): these are the only cells conveying to the brain the elaborated and integrated signal received by photoreceptors. Indeed, their axons escape from the orbit eye through an opening in the sclera and, then, they reach their final target regions. To date, according to their position, shape, sub-laminar arborization and activity, more than 25 classes of RGCs have been identified in the mouse retina, a number that seems destined to increase (Sanes & Masland 2015). Their projections at the level of the optic chiasm can be ipsilateral or contralateral. In human retina, 45% of RGCs are ipsilateral, ensuring an efficient binocular vision. In contrast, mice have a very limited binocular vision with only 3% of RGCs

projecting ipsilaterally. Melanopsin-containing RGCs have been also described (Schmidt et al. 2011). These cells are intrinsically photosensitive and have a function in the establishment of the circadian rhythm and in the mood (Lazzerini Ospri et al. 2017).

Glial Cells: three different classes of glial cells have been identified in the retina. These are Muller glial cells (MGCs), astrocytes and microglial cells. MGCs have a range of functions that are vital for the neuronal populations of the retina. Indeed, they supply neural cells with end-products, derived from their anaerobic metabolism to accelerate their aerobic metabolism. Moreover, they mop up some waste products and eliminate the excess of neurotransmitters such as glutamate, by taking it up and degrading it. Interestingly, some vertebrate like zebrafish, but not mammals, can de-differentiate MGCs upon neuronal cell damage, reconverting these cells into the ones that have been lost (Nagashima et al. 2013; Sifuentes et al. 2016). For that reason, several studies have been conducted to investigate the potential to re-activate a pluripotent state also in mammalian MGCs, converting them into photoreceptors or other neural cell types of the retina, that could be transplanted in human patients affected by retinal diseases (Xiong et al. 2019; Sanges et al. 2016). Microglial cells act as macrophages in the retina and can interact with the other glial cell types and neuronal population of the retina, by secreting growth factors. Astrocytes are covering the fibres of the optic nerve, consisting of the axons of retinal ganglion cells. Together with this structural function, they can also participate to the retinal physiology by secreting neurotrophic factors that are important for the neuronal populations of the retina.

1.4 Photoreceptor structure and distribution

Photoreceptors are the cells responsible to convert light into electrical stimuli, that are transmitted to bipolar cells and, then, to retinal ganglion cells (vertical transmission), after a sophisticated signal elaboration and integration regulated by horizontal and amacrine cells in the horizontal axis (horizontal transmission). Two different types of photoreceptors exist: rods and cones (Figure 4). Even if the general features are conserved, rods and cones acquired, during the course of evolution, well-specialized characteristics, that adapted each cell type to specific functions. The general structure of mammalian photoreceptors can be summarized in Figure 4.

Outer Segment (OS): this is a specialized compartment, containing most of the molecular phototransduction machinery. It consists of folded membranes, called discs, where opsins and other members of the phototransduction machinery are integrated. No organelles are present here (Baker & Kerov 2013).

Inner Segment (IS): this compartment hosts the organelles involved in protein synthesis and post-translational modification (Golgi, Endoplasmic Reticulum, ribosomes, etc.) and a lot of mitochondria, for a sustained ATP production (Baker & Kerov 2013).

Primary connecting cilium: this structure is located between the OS and the IS. The main function of this compartment is the trafficking of proteins addressed from the innermost compartments of the photoreceptors, the soma and the IS, to the OS, the outermost one and vice versa. The primary cilium hosts cytoskeleton elements and motor proteins. The most transported proteins are involved in phototransduction. These proteins need to shuttle between OS and IS, for physiological reasons (for instance visual adaptation), making the primary cilium a critical compartment for the correct functionality of photoreceptors (Khanna 2015; Schmitt & Wolfrum 2001).

Soma: this compartment mainly hosts the nucleus and a very thin cytoplasm surrounding it.

Axon terminals and synaptic structures: axons of the photoreceptors are unmyelinated and, thus, transparent, allowing light to pass through them and reach the OS. The synaptic terminals of photoreceptors are organized as ribbon synapses. A ribbon synapse contains an electron-dense structure, the synaptic ribbon, where vesicles containing neurotransmitters are tightly packed, ensuring a sustained release of these molecules. The synapse structure is quite different between rods and cones. The ribbon synapse of rods is called spherule, while the one of the cones is called pedicle. Rods spherules are generally smaller than pedicles and, consequently, they incorporate less synaptic ribbons (for instance only one in the mouse), while cone pedicles contain a higher number of synaptic ribbons. However, spherules are more electron-dense than pedicle and, despite their number of synaptic ribbons is smaller, these are longer in spherules than in pedicles, ensuring a good number of vesicles (Carter-Dawson & Lavail 1979; Tarboush et al. 2012).

Rods are well adapted to detect light in very dim conditions. Indeed, these cells are ultrasensitive, being able to detect single photons. Cones, on the other side, need a larger number of photons to translate them into a signal and they are mainly used in bright light conditions. Moreover, cones are adapted to distinguish colours.

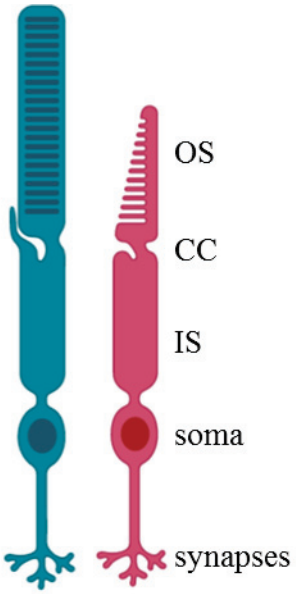


Figure 4. General structure of photoreceptors: cones (red) and rods (green) share the general organization. The outer segments (OS) is adapted for phototransduction. Inner segments (IS) are enriched in mitochondria for ATP synthesis and organelles important in protein translation and modification. The connecting cilium (CC), between them, is important for protein trafficking. The soma oma hosts the nucleus and thin cytoplasm. At the synaptic level ribbon synapses release glutamate. Created with BioRender.

The number of rods and cones and their ratio varies across the different species. A general rule is that nocturnal animals have more rods than diurnal animals, and then, a higher ratio between rods and cones (Figure 5. Kaskan et al. 2005). For instance, the human retina consists of about 120 million rods and 6 million cones (rod to cone ratio 20:1), while nocturnal rodents retina has around 6 million rods and 200 thousand cones (rod to cone ratio 30:1) (Ortiń-Martínez et al. 2014; Jeon et al. 1998).

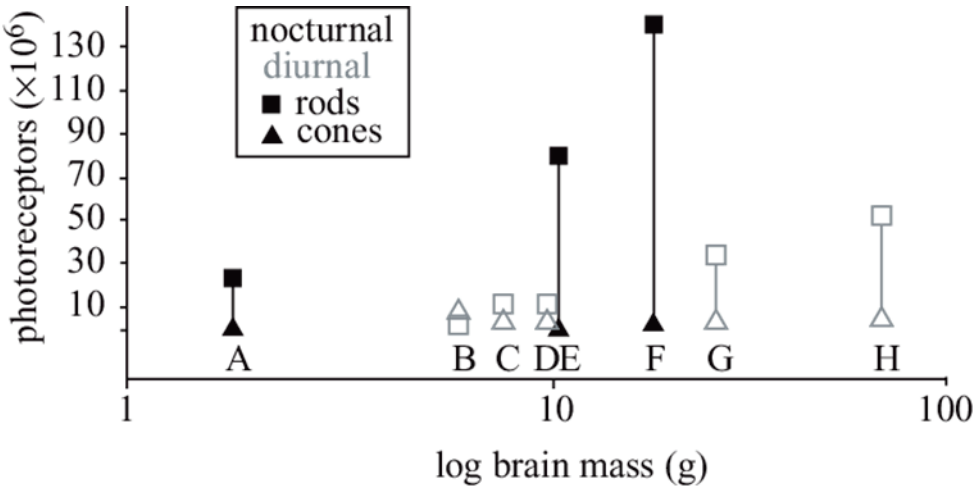


Figure 5. Rod and cone composition of the retina in nocturnal and diurnal species. Comparisons of total rod and cone numbers in different nocturnal and diurnal species. From left to right, species are A, Rattus rattus (black rat), B, Spermophilus beecheyi (squirrel), C, Callithrix jacchus (marmoset), D, Saguinus m. niger (black tamarin), E, Galago garnetti (galago) , F, Aotus sp., G, Saimiri ustius (saimiris), and H, Cebus paella (tufted capuchin). The common rat (A) has eight times more rods than the much larger diurnal ground squirrel (B). The nocturnal owl monkey (F) has four times more rods than the diurnal squirrel monkey (G). The nocturnal galago (E) has about ten times more rods than the diurnal tamarin (D). Adapted from Kaskan et al., 2005.

In Primates, this ratio dramatically changes in the specialized region called *fovea*, (a sub-region of the macula lutea), where cone density dramatically increases (Figure 6). Human fovea, in fact, has an almost inverted ratio of rods versus cones. For that reason, the fovea is a well-adapted eye structure important for visual acuity. Only humans and monkeys have this kind of structure. Rodents do not have rich cone areas like the *fovea* (Volland et al. 2015).

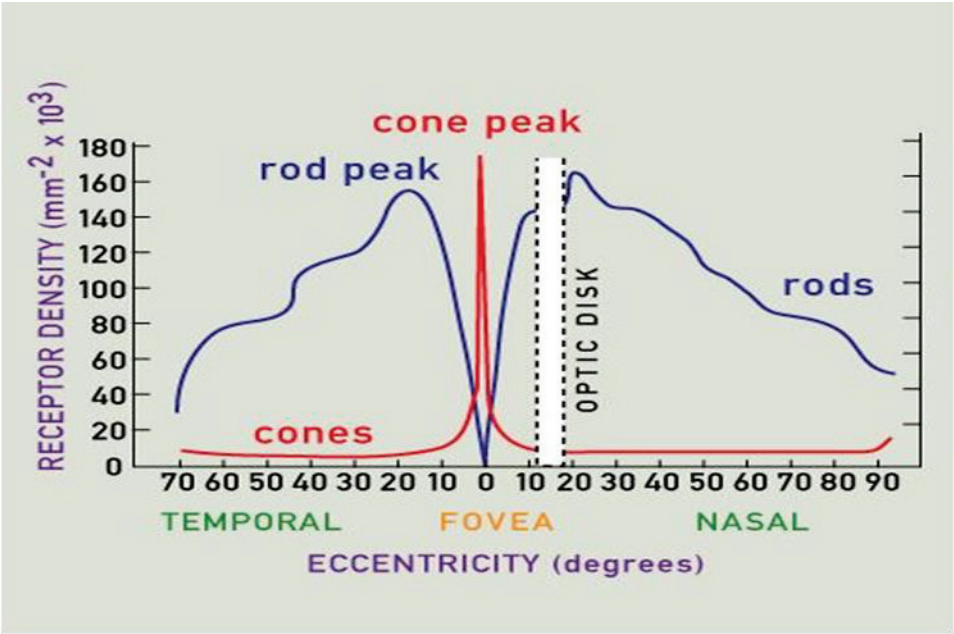


Figure 6. Distribution of rods and cones in the human retina. The graph illustrates that cones (red) are present at a low density throughout the retina, with a sharp peak in the centre of the fovea. Conversely, rods (blue) are present at high density throughout most of the retina. Adapted from http://www.webexhibits.org/causesofcolor/1H.html

1.5 Visual pigments

A critical feature that distinguishes rods from cones is their visual pigment. Opsins (Terakita 2005) are a family of photosensitive G-protein coupled receptors, that consist of a protein region and a non-protein region, a chromophore. Rhodopsin is the unique opsin of the rods and has an absorption peak at 495 nm, whereas in humans there are 3 different cone opsins (2 in mice). According to the different wavelengths they respond to, cone opsins are referred to as L-opsin (Long-wavelength, absorption peak at 560 nm), M-opsin (Medium wavelength, absorption peak at 530 nm) and S-opsin (Short-wavelength, absorption peak at 420 nm). Only M- and S-opsins are present in mice.

In general, it is considered that individual cones can have only one type of opsin. Recently, at least in mice, this rule has been put in discussion. Indeed, while single M-opsin cones are enriched in the dorsal region of the retina, the lateral and ventral regions are enriched in double-positive M- and S- cones (Applebury et al. 2000).

The chromophore present in rhodopsin and cone opsins is the 11-cis retinal, a product derived from vitamin A. Thus, what makes rhodopsin and photopsins (cone opsins) able to

respond to different wavelengths is the presence of specific amino acids in each protein sequence (Nathans et al. 1986).

1.6 Phototransduction

Light conversion into electrical signals starts in photoreceptors by a cascade known as phototransduction (Figure 7) (Leskov et al. 2000). This process has been well characterized for rods, while for cones there are some remaining ununderstood points. One photon is sufficient to activate rhodopsin, converting the 11-cis retinal into all-trans retinal (Baylor et al. 1979; Lamb & Pugh 2006; Sampath & Rieke 2004). Activated rhodopsin becomes colourless (bleached), undergoing different quick conformational changes. The 11-cis retinal is then, an antagonist for the reactions, preventing opsins to operate in the absence of light. All-trans retinal works, instead, as an agonist. Active rhodopsin (known as metarhodopsin, referred to as R*) induces the activation of transducin (Palczewski 2006). Transducin is a heterotrimeric G protein, with three subunits, Gα, Gβ, and Gγ. In its inactive form, transducin binds to GDP. R* promotes the replacement of GDP by GTP. The α subunit of the transducin, referred to as G*, then, is uncoupled and, diffuses along the membrane, where it meets and binds to the phosphodiesterase PDE. This enzyme will degrade cGMP, reducing its cellular level. The effect of cGMP reduction is the closure of cGMP-gated channels. In rods, 85% of these channels allow, in the dark, the entrance of Na^+ ions, while the remaining 15% allow the entrance of Ca^{2+} ions. Na^+ accumulation generates a depolarization current, known as the dark current, that allows the release of glutamate. In light conditions, the decrease of Na^+ level reduces the dark current in the photoreceptors, inducing a hyperpolarization of the photoreceptors. As a consequence, less glutamate is released under light conditions (Fu & Yau 2007). Phototransduction has several steps of amplification. For instance, a single R* can activate numerous transducins. In other words, one photon can indirectly activate several transducins (around 1:16 in mice, 1:60 in frogs, Arshavsky & Burns 2014). One transducin can activate only one PDE, but one PDE can hydrolyse many cGMP molecules, amplifying again the original signal.

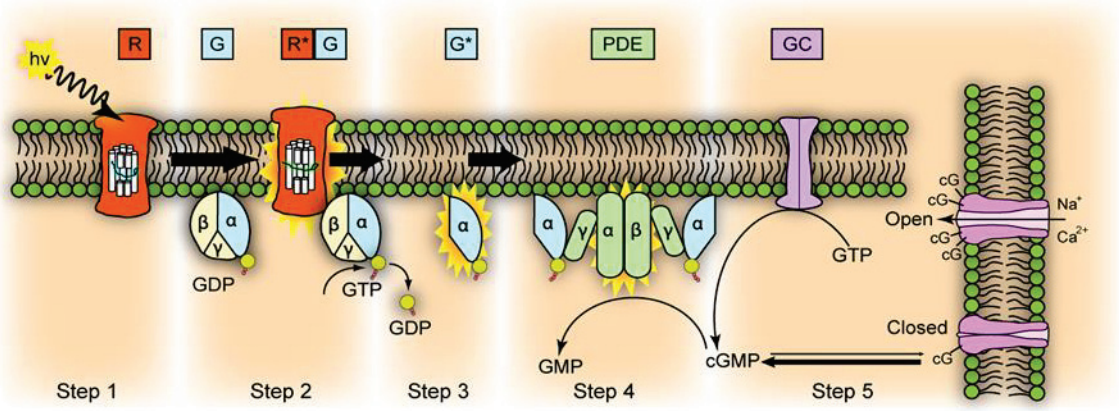


Figure 7. Molecular mechanism of phototransduction. Light conversion starts with photoconversion of the rhodopsin (R → R*). R* can activate transducin (G →) G*). G-alpha subunit diffuses along the membrane, meeting and activating phosphodiesterase (PDE). PDE converts cyclic GMP (cGMP) into GMP. Upon decreasing cGMP level in the cells, cGMP gated channels (GC) close, reducing the import of Na+ and Ca2+. As consequence cells hyperpolarize and reduce the release of glutamate. From Leskov et al. 2000.

Thus, the very high sensitivity of the photoreceptors (in particular of the rods) and the very strong amplification steps of phototransduction allow the retina to respond to very low intensities. However, these two features can have an adverse effect. Rods can be hyperactivated and, then, phototransduction saturated, at moderate intensities. Thus, the brain would not be able to discriminate higher intensities, if the cascade is already saturated. To avoid this saturation and to be able to react to different light conditions, different mechanisms of light and dark adaptation have been evolved. Part of the visual adaptation process is based on the same mechanisms used by photoreceptors to inhibit phototransduction in the dark. As mentioned, closure of the cGMP-gated channels has an effect on ion concentration in the cell, reducing Na^+ and Ca^{2+} levels. While sodium reduction has as consequence the hyperpolarization of the photoreceptors, reduction of calcium has also an important effect on several proteins involved in the block of phototransduction. To have a complete block of phototransduction, R* has to be inactivated and the cGMP level has to be restored to re-induce the dark current. R* is inhibited in 2 steps. The first step is performed by the GRK (G protein-coupled receptor kinase), also referred to as rhodopsin kinase. In the dark, this protein is bound to recoverin, a calcium-sensitive protein. Decrease of Ca^{2+} after closure of the cGMP-gated channels promotes uncoupling between rhodopsin kinase and recoverin. GSK can, then, phosphorylate R* at different residues, reducing its activity (Mendez et al. 2000). Moreover, rhodopsin phosphorylation increases its affinity for

a third protein, called arrestin. Arrestin works as an antagonist of transducin, competing with it to bind rhodopsin. These two steps are fundamental to block the activity of R* and prevent further activation of transducins.

Another effect of calcium level reduction is the hyperactivation of guanylate cyclases (GC1 and GC2), the enzymes that produce cGMP (Burns 2010). In the dark, GC activity is sufficient to have a normal amount of cGMP to keep the channels open. GCAP, a regulator of the GCs, is a Ca^{2+}-modulated protein too. In the dark, when calcium concentration is sufficient, GCAP binds Ca^{2+} ions and is high affine to GC, reducing its activity. In the light, when PDE starts to degrade cGMP, calcium level quickly decreases. The reduction of calcium promotes the dissociation of GCAP and GC, increasing GC activity. cGMP level is then restored; channels are, then, re-opened and the calcium level is therefore restored to the "dark level". Thus, rhodopsin kinase will be also inhibited. R* will be finally dephosphorylated by phosphatase PP2A (Sommer et al. 2005; Vishnivetskiy et al. 2007).

The final steps, to restore the "dark" conditions and make the photoreceptors ready to respond to new stimuli are:

decay of R*: as described, metarhodopsin is phosphorylated by GRK and, then bound by arrestin. The agonist of the phototransduction (all-trans retinal) is still present at this stage. Then, the role of arrestin and GRK is to block this agonist effect. When all-trans retinal is spontaneously released, metarhodopsin becomes apo-opsin, an inactive form; at this point, arrestin and phosphorylation are no longer required. Indeed, the release of all-trans retinal facilitates the dissociation of arrestin from apo-opsin (Sommer et al. 2005; Vishnivetskiy et al. 2007) and its following dephosphorylation mediated at least in part by the enzyme PP2A.

recycling of all-trans retinal into 11-cis retinal: once released, all-trans retinal accumulates in the photoreceptor cytoplasm, where it is reduced to all-trans retinol by the all-trans retinol dehydrogenase. All-trans retinol is released outside the cells in the interphotoreceptor matrix where it binds to IRBP (inter-photoreceptor retinoid binding protein), that will allow its uptake by the neighbouring RPE cells. There, all-trans retinol is converted into all-trans retinyl ester by LRAT (Lecithin retinol acyltransferase) and, then, all-trans retinyl ester is converted into 11-cis retinol by the isomerohydrolase encoded by the *RPE65* gene. Finally, 11-cis retinol is converted into 11-cis retinal by the 11-cis retinol dehydrogenase. 11-cis retinal is released by the RPE and, through IRBP transport, is delivered to photoreceptors to start a new visual cycle (Jones et al. 1989; Okajima et al. 1989; Stecher et al. 1999).

While the RPE has the main role in rod chromophore recycling , for cones, the reconversion of all-trans retinal into 11-cis retinal is performed by Muller glial cells (Mata et al. 2002; Muniz et al. 2007).

1.7 Visual adaptation

As mentioned above, rods can respond to a single photon. The reaction is amplified in the following steps, promoting photoreceptor hyperpolarization and subsequent activation of the bipolar and ganglion cells. Animals, whose eyes are subjected to different light intensities that can change dramatically during the day or when moving from bright area to dark area, have evolved mechanisms that allow them to adapt to intensities covering 10 orders of magnitude (Govardovskii et al. 2000). When eyes face bright light, because of the high sensitivity and amplification steps, phototransduction can be quickly saturated. To avoid that and to adapt the response to different light intensities, there are several mechanisms grouped as **light adaptation** mechanisms. On the opposite side, in very dim light or dark conditions, eyes need to keep high sensitivity to respond to few photons. At the transition, when eyes have been exposed to bright light for a long time, rhodopsin is bleached and partially phosphorylated, reducing its sensitivity. Moving into the dark, photoreceptors need to recover high sensitivity and reactivate the amplification steps. To do that, mechanisms referred to as **dark adaptation** mechanisms have been evolved.

Light adaptation can be described as the mechanisms involved to reduce and adapt photoreceptors sensitivity and block amplification steps of the phototransduction, while dark adaptation is the process by which photoreceptors improve or recover high sensitivity and reactivate amplification steps in phototransduction cascade (Reeves 2009; Reidel et al. 2008). While light adaptation takes a few seconds, dark adaptation can take up to 10 minutes and, in extreme conditions, up to 30 minutes. This period is depending on the light intensity and the exposure time before going into dark conditions. Higher intensities induce longer dark adaptation times. (Hecht et al. 1937; Haig 1941; Lamb & Pugh 2006; Stokkermans et al. 2016). Indeed, higher intensities and longer time of exposure bleach a higher number of opsins. In the dark, then, the visual cycle to regenerate functional opsin is taking time because it requires several enzymatic reactions, that take place into 2 different cell-types (RPE and photoreceptors). On the other side, light exposure will comport almost

immediate closure of cGMP-gated channels, with a quick reduction of Ca^{2+} in the photoreceptors. As mentioned above, a decrease of calcium concentration will imply a dramatic, but quick, change in the biochemistry of rods and cones, that will promote, as one of the cited consequences, the block of bleached opsins and then the block of one of the amplification steps of phototransduction.

Dark adaptation mechanisms: a first mechanism, used by the eye to collect as many photons as possible in the dark involves the dilatation of the pupil, the opening in the centre of the iris.

At the molecular level, photoreceptors pre-exposed to light will have their rhodopsin partially bleached and phosphorylated. Restoring the 11-cis retinal is one of the most important mechanisms underlying dark adaptation (Lamb & Pugh 2006). It emphasizes the strong relationship between photoreceptors and RPE, where all-trans-retinol oxidation and isomerization regenerate 11-cis retinal (Lamb & Pugh 2004). Moreover, once all-trans retinal is released, arrestin uncoupling and dephosphorylation of R* are critical steps to restore an active rhodopsin ready to respond to new signals.

Light adaptation mechanisms: pupil constriction is a spontaneous reflex, triggered by bright light, that limits illumination of the neural retina. However, the stronger the light, the higher the number of rhodopsin molecules activated. Rhodopsin bleaching is a first molecular mechanism for light adaptation: indeed, once bleached, rhodopsin becomes unable to respond quickly to new stimuli. However, a single rhodopsin can activate several transducins, and each phosphodiesterase, activated by one transducin, can degrade several cGMP molecules. To avoid saturation, modulation of amplification processes in response to different light intensities are at work. The mechanisms involved in phototransduction termination, are also used to adapt the response to the light (DeGrip et al. 2000) (Pugh et al. 1999). Rhodopsin phosphorylation reduces the response to the light, by reducing the number of transducins activated by R*. In addition, the recruitment of arrestin completely quenches rhodopsin, achieving light adaptation (Berry et al. 2016).

1.8 Protein trafficking and redistribution in light and dark conditions as additive mechanisms for visual adaptation

Several proteins involved in phototransduction change their localization among photoreceptor compartments in response to light conditions. This phenomenon is well described in Vertebrates as in Invertebrates and has been reported by some authors as participating to the mechanism of visual adaptation (Fain et al. 2001; Elsaesser et al. 2010; Orisme et al. 2010). Recoverin, the protein that inhibits rhodopsin kinase activity, accumulates in the outer segments of dark pre-adapted mice. When mice are exposed to light, recoverin is transferred toward the soma of photoreceptors, accumulating in the inner segments (Strissel et al. 2005). In this way, more rhodopsin kinase becomes available to phosphorylate rhodopsin and modulate the response to the light.

Another case of protein redistribution concerns the protein RGS9, which converts the GTP associated with the alpha subunit of the transducin into GDP, thus reducing the numbers of PDE that will be activated downstream. In dark-adapted mice, most of RGS9 is distributed in the inner segments and soma. After light onset, RGS9 relocalizes to the outer segments where it regulates the amount of active PDE and, then, the response to light.

Light-induced relocalization also concerns arrestin and the alpha subunit of transducin (Slepak & Hurley 2008). In the dark, arrestin 1, the major form of arrestin in the retina, is distributed in the IS and soma of photoreceptors, while the alpha subunit of transducin is localized in the OS, associated to the beta and gamma subunits of transducin. Upon light exposure, arrestin 1 is readdressed to the OS, where it blocks activated rhodopsin R*, preventing its interaction with transducin while transducin alpha subunit, separated from transducin beta and gamma subunits partially relocalizes to the soma and IS, avoiding hyperstimulation of the PDE (Figure 8).

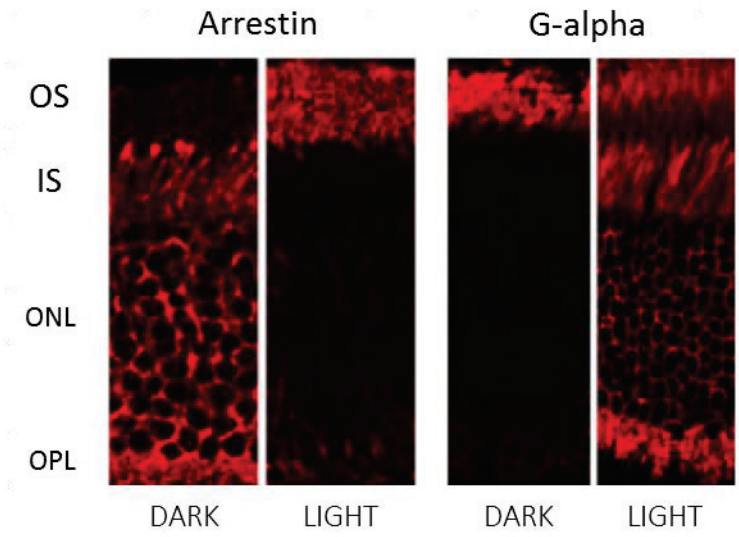


Figure 8. Light-dependent redistribution of arrestin and transducin G-alpha subunit. Immuno-staining of arrestin and transducin alpha subunit (G-alpha) in vertical sections of adult dark- or light-adapted mice. On the left, arrestin is localized in the IS, ONL (soma) and OPL in dark-adapted mice. Light induces the complete re-localization of arrestin to the OS. On the right, G-alpha subunit is localized in the OS in dark adapted mice. Light exposure induces G-alpha subunit re-localization to the IS, ONL (soma) and OPL. (adapted from Slepak & Hurley, 2008).

The main factors controlling the magnitude of protein redistribution appear to be the intensity and the duration of exposure to the light: increasing light intensities speeds up the redistribution, while increasing time exposure to a fixed intensity increases the amount of redistributed proteins (Broekhuyse et al. 1985; Sokolov et al. 2002; Whelan & McGinnis 1988; Elsaesser et al. 2010). The redistribution process is orchestrated by rhodopsin light-activation and requires functional rhodopsin (Strissel et al. 2006). Indeed, $RPE65^{-/-}$ mice, which are deficient in the enzyme that catalyses the conversion of the all-trans-retinyl ester into 11-cis-retinol, preventing rhodopsin activity, do not undergo arrestin 1 relocalization under light conditions (Mendez et al. 2003). Moreover, a light threshold is required to initiate redistribution. For alpha subunit (Sokolov et al. 2002; Lobanova et al. 2007), indeed, dark pre-adapted mice were exposed for 1 hour at different light intensities (from 10 scotopic cd/m^2 to 10^4 cd/m^2) and retinal sections were immuno-stained to detect the localization of this protein subunit. A minimal translocation from the OS to the IS was observed at 20 scotopic cd/m^2. At 40 scotopic cd/$m^{2,}$ half of the transducin subunit was translocated after 1 hour. At almost 200 cd/m^2 almost 100% of alpha subunit was transferred. This saturating intensity was then fixed and mice were exposed to it for

different durations. The longer the exposure time was, the higher was the amount of protein transferred from OS to IS (Sokolov et al. 2002; Lobanova et al. 2007). In a similar way, this light-threshold and exposure duration dependence have been confirmed also for arrestin 1 translocation from IS to OS (Strissel et al. 2006; Elsaesser et al. 2010). Although transducin and arrestin relocalization require functional rhodopsin, the process does not seem to rely on phosphorylation of this protein. Indeed, using two genetic models of mice lacking rhodopsin phosphorylation Mendez et al. concluded that rhodopsin phosphorylation and protein translocation are two independent, unrelated, processes (Mendez et al. 2003).

The mechanisms of protein transport between photoreceptor compartments have been investigated. An initial model, based on *in vitro* measures of affinity for rhodopsin forms and on theoretical calculations, suggested that spontaneous diffusion of arrestin 1 and transducin across photoreceptors was sufficient to account for their relocalization (Peet et al. 2004; Nair et al. 2005). In this model, microtubules are suggested to be only "sinks" to anchor proteins before their passive diffusion. Moreover, this model claimed that the translocation of these proteins was energy independent. These conclusions were reached by soaking mice eyecups in a glucose-free medium containing KCN, an inhibitor of the respiratory chain.

More recent data have suggested that the movement of these proteins depends on direct transport mediated by elements of the cytoskeleton, which opposes to the model of passive diffusion. Different studies in mice and Xenopus, using depolymerizing drugs, have shown that microtubule and microfilaments are, indeed, required for proteins movement between OS and IS or vice versa (Reidel et al. 2008; Peterson et al. 2005). Whether motor proteins are required, and which ones is still a matter of debate. For instance, the role of KIF3A, a kinesin motor protein, is unclear. The absence of this protein in mouse rods affects the transport of arrestin 1, but not the transport of transducin alpha subunit (Marszalek et al. 2000). This suggests that at least for arrestin 1, a motor protein is required. What is surprising is the mis-localization reported for rhodopsin, which accumulates in the inner segments of *KIF3A* KO mice photoreceptors. Because rhodopsin activity is required for correct arrestin 1 and transducin alpha subunit re-localization, the arrestin 1 mis localization observed in *KIF3A* KO mice thus could be an indirect consequence of rhodopsin mis-distribution. The actin cytoskeleton might also be involved. For instance, Reidel et al., 2008, showed that depletion of non-muscle myosins does not affect arrestin 1 trafficking in mice,

while in Drosophila, depletion of the same class of proteins affects the transport of arrestin (Lee & Montell 2004; Strissel & Arshavsky 2004). Whether this incongruence is based on the difference in the structure of Vertebrate and fly photoreceptors is still an unresolved question.

A definitive proof against passive diffusion, energy independent model has finally been given in a study using a similar system with mice eyecups, cultured in medium containing KCN (Orisme et al. 2010). Here, by contrast to Nair et al., the authors demonstrated that ATP is required for the correct re-localization of arrestin 1. The discrepancy between the two models has been explained by looking at the level of ATP depletion. Indeed, in the experiment of Nair et al. (2005) ATP depletion was not complete (only 100-fold reduction), while in Orisme et al. the depletion reached 3 orders of magnitude. The request for ATP better fits with the direct role of the cytoskeleton.

Moreover, Orisme et al. showed the importance of the PLC (phospholipase C) signalling in this mechanism. Blocking this cascade with PLC inhibitors inhibited arrestin 1 translocation. This paper also raised the hypothesis that PLC cascade could start from rhodopsin with the implication of a downstream G-protein, different from the transducin used for the phototransduction. In parallel, a study on Drosophila revealed the importance of the small GTPase protein Rac2 for arrestin 1 relocalization (Elsaesser et al. 2010). Activity of this protein, again, appears to be depending on rhodopsin light activation. According to this study, light-activated rhodopsin could simultaneously operate through an alternative cascade to the one used for phototransduction, which would, indirectly or directly, regulate arrestin 1 localization. Since it has been reported that Rac2 directly activates PLC (Illenberger et al. 2003), it is possible that Rac2 and PLC participate in the same rhodopsin alternative pathway leading to arrestin 1 relocalization. Further investigations are still needed to clarify this aspect. However, the necessity of a GTPase Rac2 in Drosophila and the requirement of PLC cascade for protein translocation, is also reinforcing the idea that ATP could be required.

The significance of the mechanism of protein relocalization is still a matter of debate. Many authors consider arrestin 1 and transducin alpha subunit redistribution as a light adaptation system. A common criticism of this hypothesis is that to be transported, arrestin 1 needs higher light intensities than the ones required for rhodopsin activation. In other words, rhodopsin bleaching can happen before arrestin 1 trafficking starts, confirming that rhodopsin activation is not the signal that initiates arrestin 1 relocalization. The situation is

quite different in bright light, where arrestin 1 binding inhibits rhodopsin-transducin interaction and limits the amplification step of photo response. Therefore, while rhodopsin can be bleached without interacting with arrestin 1 at lower intensities, where rods need to be fully activated to achieve maximal sensitivity, at higher intensities, arrestin 1 acts to prevent over-saturation of the phototransduction cascade in a light-dependent manner, by blocking a number of rhodopsin molecules proportional to light intensity and time exposure. This way of action fits with a mechanism of adaptation to bright light.

In Drosophila, a direct correlation between arrestin 1 deprivation and light adaptation defect has been shown, supporting the view of arrestin relocalization as a light adaptation mechanism (Lee et al. 2003).

By contrast, *arrestin 1* KO mice, that undergo photoreceptor degeneration after a long period of exposure to medial light intensities or after seven days at high intensities, rather suggest a neuroprotective role of arrestin 1. Although a light adaptation defect was observed in ERG tests of *Arrestin* 1 KO mice, Xu et al., 1997 initially concluded that this defect was arrestin 1 independent. Recent studies on the cones opened a new interesting perspective. Cones express two different arrestins, arrestin 1, that is also expressed by rods, and the cone-specific arrestin 4. *Arrestin 4* KO mice show cone degeneration together with defects in contrast sensitivity and visual acuity, but do not display light adaptation defects, supporting the idea that, by contrast to arrestin 1, arrestin 4 does not play a role in visual adaptation (Deming, Pak, Brown, et al. 2015). Yet, *arr1*$^{-/-}$ mutant mice show cone dystrophy and, surprisingly, a defect in light adaptation, revealed by ERG in photopic cone conditions. This phenotype has been recapitulated in *arr 1*$^{-/-}$;arr 4$^{-/-}$ double KO. These results have been further confirmed by analysing arrestin mutations on an *Nrl* (*Neural retina leucine zipper*) null background. *Nrl*$^{-/-}$ mice do not form rods and develop a full-cone retina and thus represent an interesting model to study the function of arrestins independently of rods (Mears et al. 2001).

While neither single *Nrl*$^{-/-}$ *or arr4*$^{-/-}$ KO mice nor *Nrl*$^{-/-}$*;arr4*$^{-/-}$ double KO mice show light adaptation defect, ablation of arrestin 1 in concomitance with *Nrl* KO (Nrl$^{-/-}$;arr 1$^{-/-}$) or *Nrl* + *arrestin 4* KO (Nrl$^{-/-}$;arr 4$^{-/-}$;arr 1$^{-/-}$) results in light adaptation defects revealed by ERG in photopic conditions (Deming, Pak, Shin, et al. 2015).

All together these observations suggest that arrestin 1 is implicated in light adaptation of cones, and plays a different role in rods, where it exerts a neuroprotective action upon long

exposure to bright light. The mechanisms of this neuroprotective action in rods is not known.

1.9 RPE and photoreceptors: a strong structural and functional relationship

Photoreceptor survival and maintenance depend on the RPE. The RPE is a specialized monolayer of pigmented cells, with its basal side facing the choroid and its apical side facing photoreceptors. Thanks to its tight junctions, it is one of the main components of the blood retina barrier, preventing the contact of pathogens with the neural retina. Besides this insulating role, It exerts many diversified and essential functions (Figure 9) (Strauss 2005; Sparrrow et al. 2010).

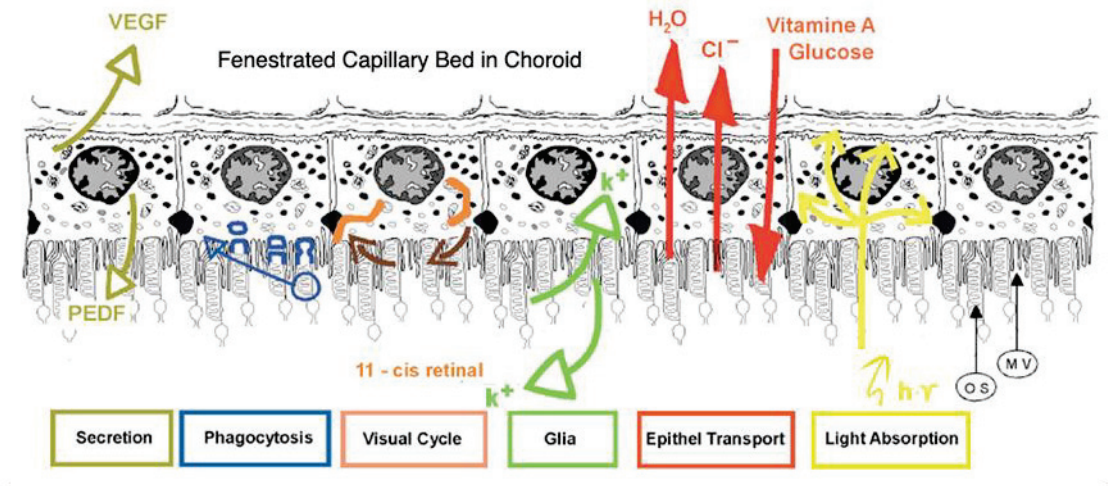


Figure 9. The principal RPE functions. RPE is involved in light absorption (yellow), regulated transport between choroid and neural retina (red), ions homeostasis (green), phagocytosis of old outer segments (blue) and secretion of factors (gold). RPE plays also a role in visual cycle, regenerating 11 cis retinal (orange). From Strauss, 2005.

The presence of numerous melanin inclusions in RPE cells makes them able to adsorb light in excess, that is focused by the lens on the retina, and to limit photo-oxidation. Indeed, the retina is supplied with oxygen by a dense network of blood vessels. Light in excess releases energy that can then generate ROS (reactive oxygen species), rousing oxidative stress. Photon absorption of by melanin is critical to reducing such phenomenon.

As an epithelium tissue, the RPE can also regulate molecule exchanges between the apical side, facing the neural retina, and the basal side, facing the chorio-capillaris, in both

directions. Ion and metabolite permeability is in fact different on the two sides, thanks to RPE cell polarity and to a strictly regionalized distribution of protein carriers and ion channels. Exchanged metabolites can be nutrients like glucose, that is brought from the blood to the photoreceptors as energy source. The transport of water is also very important. Photoreceptors produce large amounts of water as an end-product of their metabolic activity. This water, accumulated in the sub-retinal space, is efficiently removed by the RPE with its specialized aquaporins.

Another very important metabolite that is carried from the blood to the photoreceptors is vitamin A, the precursor of visual pigment. The RPE uptakes it from the blood and converts it into 11-cis retinal. Then, 11-cis retinal is transported in the sub-retinal space and up-taken by photoreceptors where it binds the opsins. When 11-cis retinal is converted by light into all-trans retinal, it is released by the opsins and quickly redistributed in the extracellular space, where specialized proteins capture it and deliver it to the RPE. There, the RPE enzymes LRAT, RPE65 and 11-Cis RDH convert the all-trans retinal into 11-cis retinal, making it available again for photoreceptors. This turn-over activity makes the RPE an important regulator of the visual cycle.

Another very important function of the RPE is the phagocytosis of the tip of photoreceptor outer segments that are continuously produced and shed. In addition to its absorbing function that limits light in excess, preventing oxidative stress, the RPE is essential to buffer an additive source of oxidative products: indeed, the conspicuous metabolic activity of photoreceptors generates large amounts of end products, among which are many ROS. Moreover, because of the presence of reactive visual pigments, the OS are frequently undergoing photo-oxidative damage. To maintain photoreceptors integrity, the OS are continuously renewed. The process is light-activated. Diurnal and nocturnal animals have the same rate of phagocytosis. New discs are produced by the inner segment side, while the oldest ones are in contact with the RPE. Around 16 days are needed to fully renew the complete length of the OS in rodents (Kevany & Palczewski 2010; LaVail 1983).

The RPE secretes many different factors, involved in extracellular matrix turnover, angiogenesis, crystalline formation and photoreceptor survival and maintenance (Kay et al. 2013). Indeed, RPE cells and photoreceptors are continuously communicating, in physiological as well as in pathological conditions. In particular, the RPE secretes growth factors, such as neurotrophins that are of vital importance for photoreceptors viability

(LaVail et al. 1998; Bazan et al. 2010). Alteration of this secretion can be at the base of retinal diseases.

1.10 Interphotoreceptor matrix

In the retina, two different extracellular matrices are found: the Interphotoreceptor Matrix (IPM) and the extracellular matrix (ECM). The IPM is a specialized extracellular matrix situated in the sub-retinal space between the RPE and the photoreceptors. It fills, then the space between the two layers and the space between photoreceptors. The ECM is a more "classic" matrix, surrounding the other cells of the retina.

The rod and cone IPM are quite different. The IPM surrounding cones is quite thick, whereas the IPM surrounding rods is thin. Using different lectins, it is possible to specifically stain the IPM surrounding cones or rods. For instance, PNA is the lectin currently used to stain the cone IPM, while WGA stains the IPM surrounding rods. A full description of the components of the two different matrices has been reported in Ishikawa et al. 2015 and Mieziewska et al, 1996.

The IPM contains no collagens, and other structural proteins, like fibulins, are not abundant. The most abundant protein component is IRBP (interphotoreceptor matrix retinol binding protein), already described above to be important in the visual cycle. Other protein components are enzymes, like acid phosphatase, aryl sulphatase, metalloprotease, etc. Different growth factors have been identified, e.g. TGFβ, pigment epithelium-derived factor (PEDF), insulin-like growth factor (IGF) and inhibin. Other reported proteins, present in the IPM, include complement H, mucin and aβ crystallins. The IPM is mainly composed of proteoglycans PGs (98%) and glycosaminoglycans GAGs (2%). Sixty percent of the GAGs are chondroitin sulphates, among which the 4-sulfate and 6-sulfate are the most represented. Proteoglycans are formed by a protein core, covalently bound with a GAG chain. Even here the most abundant GAGs-chains are represented by chondroitin sulphates, dermatan sulphates and heparan sulphates.

In the IPM, chondroitin sulphates proteoglycans could have not only a structural function but also a role in the signalling recognition. Indeed, chondroitin sulphates have been largely described in the Central Nervous system to be involved in the recruitment of specific growth factors (Djerbal et al. 2017). Some interesting components exclusively found in the IPM,

belonging to the family of chondroitin/dermatan derived proteoglycans, are versican, decorin, biglycan and SPACRCAN (a syaloproteoglycan associated with cones and rods).

II Orthodenticle homeobox 2

Orthodenticle homeobox 2 (Otx2) is a homeodomain transcription factor. In the following paragraphs, a description about the state of art for this gene in the retina over the lifetime course will be given.

2.1 Homeobox genes

The homeodomain family of transcription factors is involved in a diverse set of functions, that include body plan specification, pattern formation, cell identity and cell fate determination. An exhaustive review has been recently made by Bürglin & Affolter (Bürglin & Affolter, 2016). The term *homeobox* refers to a specific DNA region present in all homeobox genes, which is coding for 60 amino acids, that are, then, structured in a helix-turn-helix motif, able to recognize and bind to specific DNA elements (Gehring et al. 1990; Gehring et al. 1994). The homeodomain was identified in 1984 in the *Drosophila melanogaster* model (McGinnis et al. 1984; McGinnis et al. 1984b), but in few years it was recognized to be evolutionary conserved among different species of animals and plants (Tassetto et al. 2005). The first *homeobox* genes were so named because of their role in body plan organization (homeotic function). However, not all homeobox genes do have a homeotic function.

Thanks to full genome sequence availability, multiple alignments between homeobox genes have been made possible. Although a consensus sequence emerged from such alignments for the homeodomain, a certain flexibility for some positions has been reported. According to that, today, homeobox genes fall in at least 4 different super classes (Figure 10 Bürglin & Affolter 2016). Each superclass is further subdivided into subclasses and families. For instance, *Hox* genes belong to the *ANTP* superclass, *HoxL* class. The second class of *ANTP*, referred to as *NKL*, does not include *Hox* genes (Bürglin 2011). To date, 300 homeobox genes have been described in humans, among which 235 are functional and 65 are pseudogenes (Holland et al. 2007).

When homeobox genes were discovered, it was believed that their function was merely developmental, restricted to embryogenesis. However, the observation that different tissues maintained *homeobox* gene expression in adulthood suggested that homeodomain factors could also have a role at this stage. Further studies validated this hypothesis for some

of these factors. For instance, several *Hox* genes have been shown to have a role in adult hematopoiesis and leukemogenesis (*Hoxb3, Hoxb4*) (Argiropoulos & Humphries 2007; Adamaki et al. 2017), in skeleton regeneration (*Hox11*) (Rux & Wellik 2017) as well as in endometrial formation for female reproduction (*HoxA10*, *HoxA11*) (Du & Taylor 2016). The double requirement of these genes at embryonic and adult stage distinguishes two main categories of mutations: inherited mutations, that usually cause developmental related disorders and acquired mutations. Since most of the homeobox genes are involved in cell renewal or tissue regeneration in adulthood, acquired mutations and/or mis regulation can promote tumoral events (Haria & Naora 2014; Joo et al. 2016).

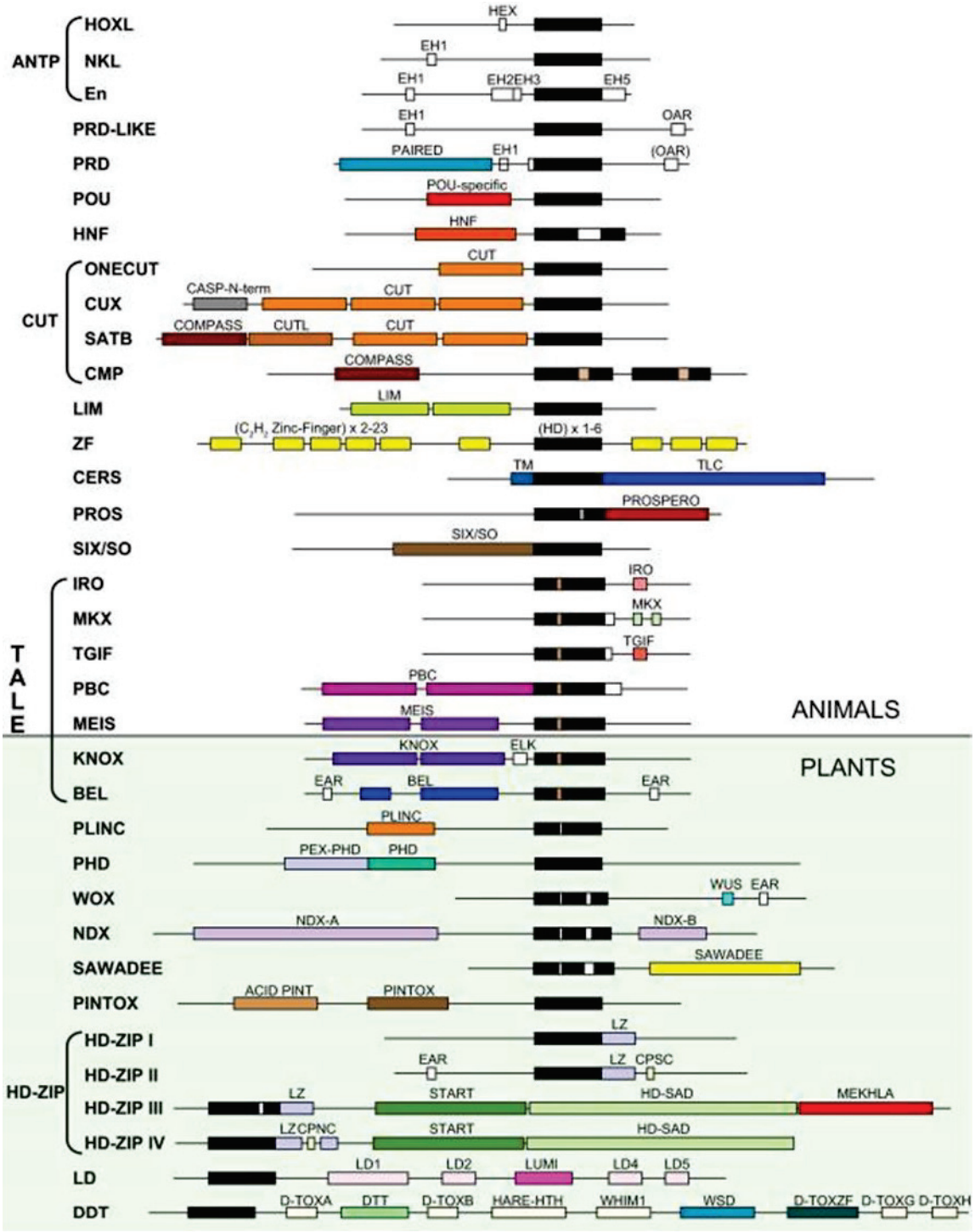


Figure 10. Diversity of homeodomain containing proteins. A schematic representation of homeodomain containing proteins in classes and super classes. The homeodomain is shown as a black box, conserved sequences as open boxes and structural motifs as coloured boxes. In the upper panel three different super classes of animal homeoproteins are represented, while in the lower part, a specific plant subclass is reported. The TALE family is shared by animals and plants. Not all the members appear in this schema. Some classes, like PRD-like and PRD do not belong to any superclass. Adapted from Bürglin & Affolter, 2016.

2.2 Homeobox gene function in the retina

During retinogenesis, homeodomain transcription factors play essential roles in the regionalization and patterning of the optic neuroepithelium, specification of retinal progenitors and differentiation of all the retinal cell populations. Homeodomain transcription factors control retinal cell fate by regulating the expression of target genes required for retinal progenitor cell fate decisions and for terminal differentiation of specific retinal cell types. The importance of homeobox genes during retinal development is demonstrated by the number of human eye diseases, including colobomas and anophthalmia, which are attributed to homeobox gene mutations.

Homeobox genes are involved in retinal development, differentiation and maintenance; their interaction network is complex. Here, before moving to a detailed description of Otx2 role in embryonic and adult retina, a short description of the most important retinal homeobox genes and their function in the retina will be given. A detailed table of selected homeobox genes with a role in the retina is given in Zagozewski et al. 2014.

Rax, the retina and anterior neural fold homeobox gene (Furukawa et al. 1997), is involved in the earliest phases of eye development. *Rax* null mice are eyeless, while *Rax* overexpression in Xenopus and Zebrafish promotes ectopic eye formation (Mathers et al. 1997; Terada et al. 2006). Rax is also important for photoreceptor specification, by regulating Otx2, a key factor for photoreceptor cell-fate acquisition (Muranishi et al. 2011) and differentiation, and by regulating some of the phototransduction genes (Nelson et al., 2009, Pan et al., 2010). Rax expression in the mouse neural retina disappears two weeks after birth.

Pax6 (paired-box gene 6) has a dual role in retinal neurogenesis and cell-fate specification, according to the spatial position of the retinal precursor cells (RPCs) where it is expressed (Oron-Karni et al. 2008). In the peripheral retina, Pax6 prevents the premature expression of Crx, avoiding a premature photoreceptor fate acquisition. In the central retina, Pax6 has the role to confer pluripotency to the retinal cell precursors (RPCs). Indeed, as a consequence of its ablation, amacrine cell is the only fate for differentiated progenitors. In the adult, Pax6 is expressed in amacrine cells and retinal ganglion cells, but its role has not been yet investigated there. As for *Rax*, several ocular diseases are also related to *Pax6*

mutations and/or mis regulation in humans (aniridia, microphthalmia, optic nerve hypoplasia, etc.) (Hill et al., 1991, Tzoulaki et al., 2005).

Crx (cone and rod homeobox factor) is a key gene for development, maintenance and terminal differentiation of photoreceptors (Chen et al. 1997). It is expressed in pre-committed precursors, under the direct regulation of Otx2 (Nishida et al. 2003). $Crx^{+/-}$ and $Crx^{-/-}$ mice show no obvious differences in retina lamination until post-natal stage P7. However, at P14, photoreceptor outer segments appear shorter in heterozygous $Crx^{+/-}$ mice and are absent in $Crx^{-/-}$ null mice, suggesting that terminal differentiation of the photoreceptors is impaired. At P21 the number of cells in the ONL is reduced in null $Crx^{-/-}$ mice. Immunostaining for the phosphorylated form of histone 3, a mitotic marker, suggested that the decreased number was not due to a block of cell division, while a staining for active caspase-3, a marker of cell death, confirmed that cells were undergoing apoptosis. Subsequent electroretinogram tests showed that these $Crx^{-/-}$ mice had a strong defect in light detection. Moreover, different photoreceptor specific genes (e.g. rhodopsin, recoverin, arrestin, transducin subunits), were strongly downregulated in $Crx^{-/-}$ null mice (Furukawa et al. 1999; Koike et al. 2007).

While the $Crx^{-/-}$ model clearly shows the developmental role of Crx, much less is known about its putative later roles. In adult mice, Crx expression is limited to the neural retina, where it is strongly activated in photoreceptors and weakly in bipolar cells. In humans, Crx seems to be expressed also in the adult RPE (Glubrecht et al. 2009; Bibb 2001). However, its role at the adult stage has not yet been investigated due of the absence of a conditional mutant allele. Even though, some information can be inferred from studying late-onset retinal diseases. In 2014 a study on 18 patients from 11 families reported different new mutations in *Crx* (Hull et al. 2014). While for some of them, mutations in the *Crx* gene were associated with a birth onset of the disease and were probably resulting from developmental defects, in 15 out of 18 patients, mutations were associated with childhood or adult onset of the disease, at an age ranging from 6 years to 50 years. The reported mutations were frameshift or truncating mutations, in a heterozygous context, and were associated with Cone-Rod Dystrophy, Rod-Cone Dystrophy, Leber Congenital Amaurosis or Macular Dystrophy. Recently, another mutation in *Crx* has been identified (c.766C>T; p.Gln256Ter) in two 35 and 57 old members of a family and associated with late onset of macular degeneration (Griffith et al. 2018). Although the mechanism by which these Crx

mutations act is not always understood at the adult stage, the late onset of photoreceptor disease in patients carrying these mutations indicates that proper Crx activity is required for long-term maintenance of photoreceptors. Therefore, *Crx* must have a role in the adult retina, which makes sense with its maintained expression throughout life.

2.3 *Otx2* gene organization and transcription

Otx2 (Orthodenticle homeobox gene 2) belongs to a family of related genes together with *Otx1* and *Crx,* the most divergent one (Germot et al. 2001). It is evolutionary related to Drosophila *Otd* (Simeone et al. 1992).

The human *OTX2* gene is located on chromosome 14q22.3 and is coding for a paired homeodomain transcription factor. It is organized in 5 exons with two 5' non-coding exons. In the mouse, there is a third non-coding exon (Figure 11). Transcription of *Otx2* mRNA can be initiated at three alternative promoters, producing three isoforms that differ in their 5' untranslated region but share the same coding sequence and 3' untranslated region (Courtois et al. 2003). For convenience, the three isoforms are named A, B and C, where A is the one produced by the distal promoter, B by the intermediary promoter and C by the proximal promoter. The proximal promoter is preferentially used during early embryogenesis, while the distal one starts to be dominant around the gastrulation stage. In the adult retina, only A and B isoforms are expressed. The switch between proximal and distal promoter is gradual and the ratio of isoform A / isoform C expression increases during development with a sustained expression of isoform A, taking over isoform C. The B promoter is preferentially used in adult retina (Fossat et al. 2005) giving up to 30% of the total *Otx2* mRNA in this tissue.

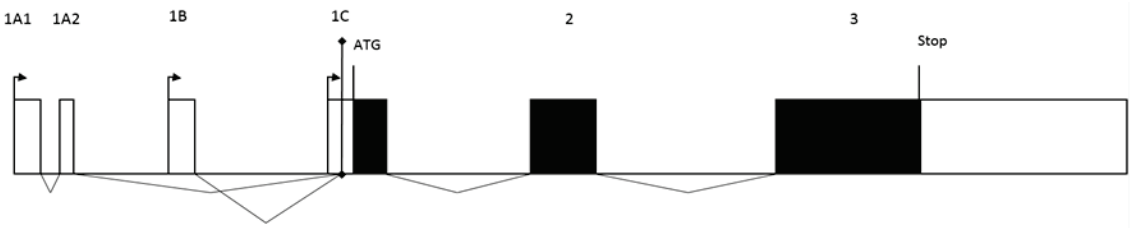


Figure 11. Schematic structure of the mouse *Otx2* gene. Exons are labelled and shown boxed. Arrows symbolize the transcription start sites. The 3' splice site common to A and B transcript is shown by an arrowhead. Translation start (ATG) and termination (Stop) codons are shown. *Otx2* coding regions are in black. Modified from Fossat et al., 2005.

2.4 *Otx2* expression and functions in the nervous system

Lack of *Otx2* expression is lethal. *Otx2* null embryos do not develop head and brain (Acampora et al. 1995; Matsuo et al. 1995). Indeed, *Otx2* is a master gene for neurodevelopment and has a critical function for forebrain and, later, midbrain induction (Rhinn et al. 1998; Acampora et al. 2001; Tian et al. 2002).

Otx2 expression is very dynamic (Figure 12) (Fossat et al. 2006; Beby & Lamonerie 2013) In the mouse, it starts at the morula stage (Kimura et al. 2001) and, then, covers the entire epiblast. During gastrulation, *Otx2* expression becomes restricted to the anterior part of the embryo, where it becomes critical for the specification of the rostral nervous system. A pivotal role of *Otx2* has been described in the establishment of anterior-posterior polarity. *Otx2* is necessary for the formation of the anterior visceral endoderm (AVE) where it is then expressed (Hoshino et al. 2015; Kimura et al. 2000). It plays an important role in the migration of the pre-committed AVE cells and formation of this compartment, an event that is critical for establishing the Anterior/Posterior (A/P) axis (Kimura et al. 2000; Kimura et al. 2001; Morris et al. 2012; Kimura-Yoshida et al. 2007). Next, *Otx2* expression becomes restricted to the anterior part of the embryo, where it is involved in the induction of the anterior neuroectoderm (Rhinn et al., 1999). Once the anterior neuroectoderm has been induced, *Otx2* becomes expressed itself in this compartment, where, it drives the specification of the forebrain and midbrain. Otx2 cooperates with Pax6 and Emx2 to induce the formation of the caudal forebrain that will develop in the archipallium (Kimura et al. 2005). *Otx2* induction in the anterior mesoderm (AME) is also required for head formation by regulating the Wnt antagonist Dkk1 and Lhx1 (Ip et al. 2014; Kimura-Yoshida 2005).

Otx2 also plays a pivotal role in the rostro-caudal regionalization and patterning of the brain. A competition between *Otx2* and *Gbx2*, the gastrulation brain homeobox 2, is fundamental

for the correct positioning of the midbrain-hindbrain boundary (MHB) (Kimura et al. 2001) (Acampora et al. 2001) (Joyner et al. 1999). This organizer region induces adjacent regions to become midbrain or hindbrain territories. In parallel, the correct positioning of the MHB has an impact on the rostro-caudal regionalization of the brain. After this boundary is established, *Otx2* has a function in the specification of the rostral part of the hindbrain, while *Gbx2* has a role in the specification of the caudal part of the hindbrain. Indeed, *Otx2* is expressed in the anterior-most part of the dorsal hindbrain, referred to as rhombomere 1 and will induce this region to become the future cerebellum (Di Giovannantonio et al. 2014; Su et al. 2014).

Otx2 has also an important function in the patterning of the diencephalon. There, Otx2 contributes together with Otx1 to establish and position the borders of the *zona limitans intra-thalamica*, an organizer that induces adjacent territories into specific diencephalic sub-regions: thalamus, pretectum and pre-thalamus (Scholpp et al. 2006; Scholpp et al. 2007).

In late embryogenesis, *Otx2* expression is found in several areas of the basal telencephalon including the anterior ganglionic eminence, the septum adjacent to the diencephalon, the striatum, the hippocampus and the ventral part of the olfactory bulb, and also in the diencephalon, mesencephalon, plexus choroid, pineal gland and cerebellum (Beby & Lamonerie 2013). *Otx2* is present in the medial habenula and interpeduncular nucleus, two components of the reward circuits connected by the *fasciculus retroflexus*.

In the adult, *Otx2* expression is maintained in many structures where it played a role during development. A strong presence of Otx2 has been detected at this stage in the thalamus, the superior colliculus, the choroid plexuses and the hypothalamus (Figure 12).

Otx2 is also important for the development of sensory organs, like the inner ear, the olfactory epithelium and the retina. Again, these structures retain *Otx2* expression at the adult stage.

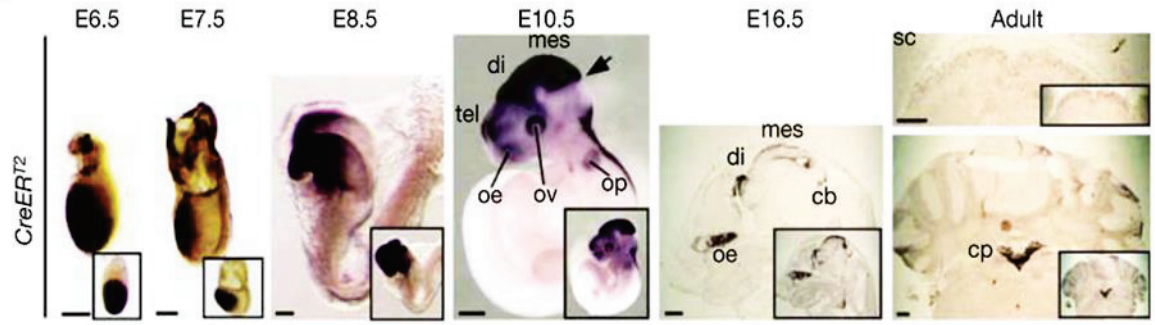


Figure 12. CreER^{T2} and Otx2 expression pattern in Otx2$^{+/CreERT2}$ mouse embryos. In situ hybridization of Otx2$^{+/CreERT2}$ embryos at embryonic day E6.5 – E10.5 (whole mount) and E16.5 (sagittal sections) and adults (transversal sections) at the level of the mesencephalon (top) and cerebellum (bottom) with *CreER^{T2}* and *Otx2* probes (insets). The arrow points to the isthmus. Cb, cerebellum; cp, choroid plexus; di, diencephalon; mes, mesencephalon; oe, olfactory epithelium; op, otic placode; ov, optic vesicle; sc, superior colliculi; tel, telencephalon. Adapted from Béby et al., 2013.

2.5 Otx2 roles in retinal development

Eye and retina formation are orchestrated by a network of genes that collaborate or inhibit each other at precise times and places. A general schema of vertebrate eye development is presented in **Figure 13** (Dash et al. 2016).

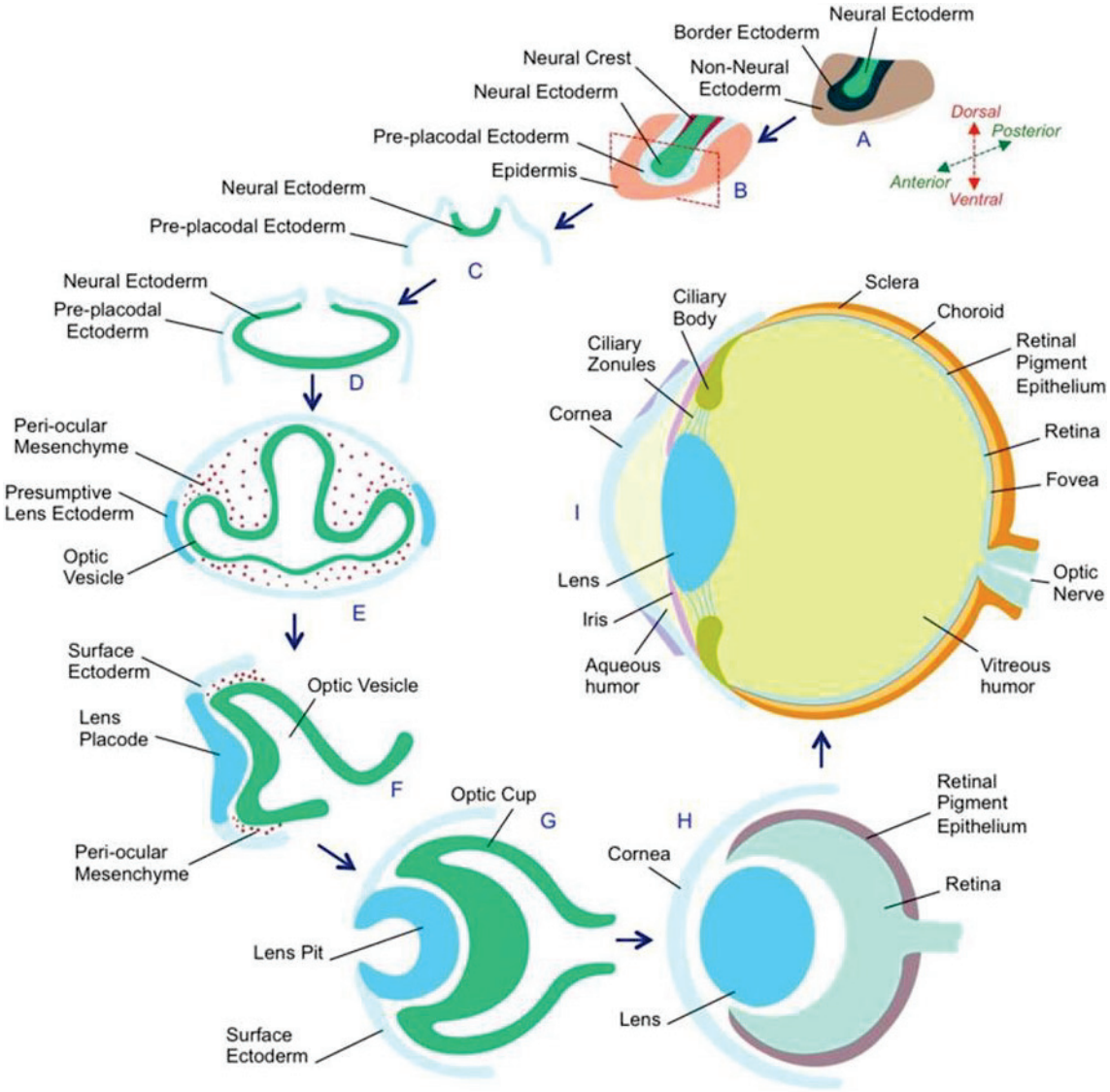


Figure 13 Schema of vertebrate eye development. The main steps of eye development, characterized by the appearance of specific structures, are presented (A-H). The eye-field starts to be formed during gastrulation in a central region of the anterior neural plate. Next, the eye-field is divided in two structures that developed into optic vesicles and, finally, optic cup. From Dash et al., 2016

The first morphological evidence of eye formation in vertebrates is a bilateral expansion of tissue from the early forebrain that forms the optic vesicles. However, a presumptive eye tissue (eye field) exists prior to optic vesicle formation.

Several eye-field transcription factors (EFTFs) are expressed in the anterior region of the vertebrate neural plate and are essential for eye formation. These EFTFs are Six3, Pax6, Rx1, Lhx2 and Six6 (Chuang & Raymond 2002). Ectopic expression of these genes can generate ectopic eyes. However, these ectopic eyes can only form in a competent area, located in the head region anterior to the hindbrain. This area is derived from the anterior neural plate, a domain expressing Otx2. Because Otx2 is necessary for eye formation, it could be a permissive factor that collaborates with the EFTFs in the eye-field, where these genes are endogenously expressed (Sinn & Wittbrodt 2013). Supporting this, additional studies show that EFTFs are under the joint control of noggin and Otx2 (Zuber et al. 2003). EFTFs activation would trigger a self-activation feedback and subsequent downregulation of Otx2 in the eye-field (Beby & Lamonerie 2013). The exact order and exact weight that each gene has on the induction of the eye-field remains to be determined.

Retinal identity is first established in a single, centrally positioned eye-field, marked by overlapping expression of Otx2 Six3, Rx3 and Pax6. Then, secreted factors of the Tgf-ß-, Fgf- and Shh-families, emanating from the underlying axial mesoderm, split the eye anlage into two bilateral symmetric retinal primordia that expand while they are forming. This expansion gives rise to the optic vesicles. The neural retina develops from the distal/ ventral portion of the optic vesicle, while the RPE emerges from the dorsal region (Hirashima et al. 2008; Fuhrmann 2010; Kagiyama et al. 2005). Cell elongation and proliferation are not needed for optic vesicle formation, but migration of retinal precursor cells (RPCs) is required for this step (Harris and Hartenstein, 1991; Svoboda and O'Shea, 1987) (Loosli et al. 2003; Winkler et al. 2000; Loosli et al. 2001).

At the optic vesicle stage, *Otx2* and *Otx1* are expressed in the presumptive layer that will be specified as the retinal pigmented epithelium (RPE). RPE specification starts at E9.5 and requires Otx2. Indeed, *Otx2* full or partial ablation at this stage is associated with optic vesicle malformation, with complete re-specification of the RPE into neural retina (identified by the expression of *Pax2*, *Pax6* and *Six3*) and optic stalk (Martinez-Morales et al. 2001). Supporting that, Otx2 overexpression in the avian neural retina can activate melanogenesis genes such as *Tyr* (tyrosinase) and *Tyrp-1,* in cooperation with the microphthalmia

transcription factor Mitf. However, Otx2 does not regulate *Mitf* expression and vice versa (Martinez-Moralez et al., 2003; Masuda et Esumi 2010; Reinisalo et al. 2012. Another role of Otx2 in RPE specification is the inhibition of the pro-neural factors Sox2 and Fgf8 (Nishihara et al., 2012).

At E10.5, *Otx2* expression is induced in the neuroblastic layer where a single type of retinal cell precursor will give rise to all future neuronal cell types of the retina. *Otx2* that was downregulated after the eye-field determination is activated in these RPCs by Rax (Muranishi et al. 2011). *Otx2* is expressed in the last cell cycle of RPCs, just before they become post-mitotic. Stronger Otx2 expression is found in cells facing the RPE, that are fated to become photoreceptors. Weak expression is also detected in the inner neuroblastic layer, which is later specified into inner nuclear layer cells. By a genetic approach, Nishida et al. (2003) demonstrated that Otx2 has a key role to specify photoreceptors. Using a conditional *Crx-Cre* mouse line, they knocked out *Otx2* specifically in each *Crx*-expressing cell as soon as *Crx* is induced, i.e. around E12.5. This resulted in the complete abolition of *Crx* expression, demonstrating that *Crx* is a direct Otx2 target gene. In the absence of Otx2, no photoreceptor cells developed while the retina showed an expansion of amacrine cells. These data show a direct role of Otx2 in orienting RPCs, that otherwise would become amacrine cells, towards the photoreceptor cell fate (Nishida et al. 2003).

Photoreceptor terminal differentiation is orchestrated by the key factor Crx but Otx2 also plays a role in this process. Two series of evidence support this. The first one is based on extended studies of *Crx* null mutant mice. As described above, *Crx* null mice do specify photoreceptors but these do not undergo full terminal differentiation and eventually degenerate (Koike et al. 2007). By comparing *Crx* null mice and *Crx* null mice carrying only one functional *Otx2* allele, Koike et al. showed that the downregulation of phototransduction genes observed in the absence of Crx, was much stronger when one *Otx2* allele was missing (Koike et al. 2007). This demonstrates that Otx2 is acting in cooperation with Crx to control photoreceptor terminal differentiation. The second evidence is based on regeneration studies. Muller glial cells are currently investigated for their capacity to re-differentiate into photoreceptors to restore damaged retinas. This ability is a characteristic of fishes and other vertebrates, but not of mammals. However, mammalian Muller glial cells can be reactivated to divide by Wnt signalling activation and converted into specific cell types by an appropriate cocktail of transcription factors. Xiong et al., (2019) used this

strategy to produce photoreceptors by delivering viral vectors expressing Crx and Nrl. The rate of fully differentiated photoreceptors was increased when Otx2 was added to the cocktail compared to a condition where only Crx and Nrl were overexpressed (Yao et al. 2018). This experiment emphasizes the role of Otx2 in photoreceptor terminal differentiation.

Otx2 has also a role in bipolar cell maturation. Nishida et al. (2003) showed that Otx2 depletion from *Crx*-expressing RPCs did not affect the number of bipolar cells, marked with Vsx2/Chx10. However, the position of these cells was compromised (Nishida et al. 2003). Later, Koike et al. (2007), using a mouse line expressing Cre under the *L7* bipolar specific promoter demonstrated that removing *Otx2* from P0 neural retina reduced the number of terminally differentiated bipolar cells. While the number of Chx10 positive cells appeared normal, the number of rod bipolar cells, marked with PKC alpha was decreased. Whether Otx2 depletion changed the fate of rod bipolar cells to other subclasses of bipolar cells has not been investigated. However, since Otx2 can regulate the *PKC alpha* promoter *in vitro*, this result suggests that bipolar-specific *Otx2* KO rather affects their terminal differentiation and maturation than it changes their fate.

Lineage studies have shown that *Otx2*-expressing precursors can form both photoreceptors and bipolar cells (Baas et al. 2000). The regulator that allows the cells to make a choice between the two fates is *Blimp1*. This gene, which is under the direct regulation of Otx2, has been shown to inhibit the bipolar fate, promoting, by contrast, a photoreceptor fate (Brzezinski IV et al. 2010; Brzezinski et al. 2013). Indeed, *Blimp1* ablation at early stage of embryogenesis leads to mature retinas with a strong increase of bipolar cell numbers, that form at the expense of photoreceptors. During embryogenesis, in *Blimp1* KO mice, bipolar cells start to differentiate earlier than in control mice, while photoreceptors cells are reduced at the same age. These data support the idea that Blimp1 is a repressor of bipolar markers. It acts by inhibiting the bipolar fate and simultaneously allowing then to adopt a photoreceptor fate. A schematic representation of Otx2 functions in retinal development is shown in Figure 14 (Beby & Lamonerie 2013).

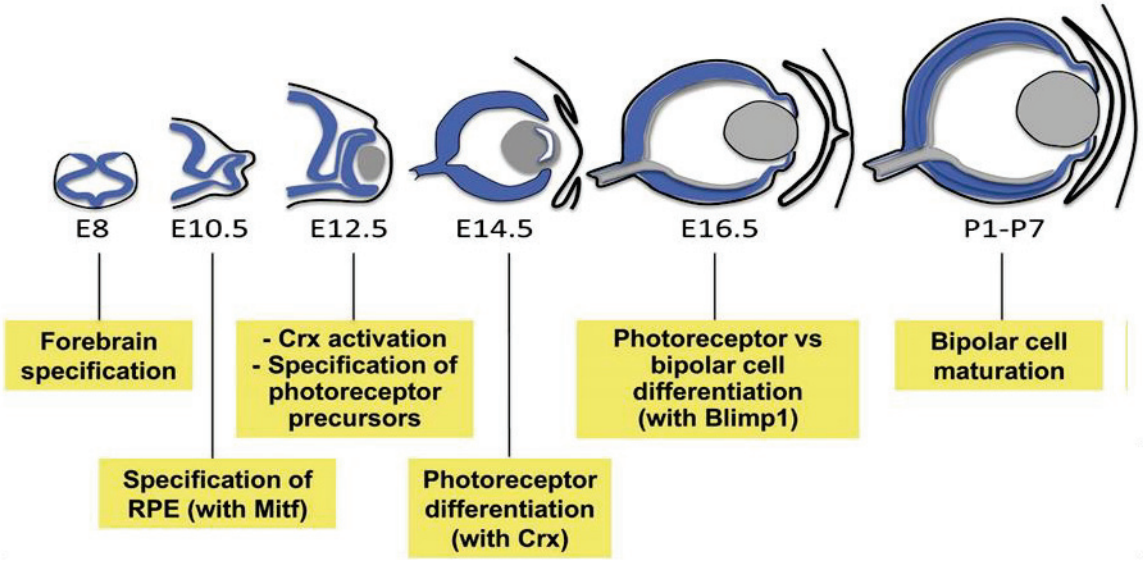


Figure 14. Temporal functions of Otx2 in the mouse retina. Schematic illustration of successive stages of eye development viewed in transverse sections of the indicated stages, with the corresponding Otx2 known functions. RGC: retinal ganglion cell layer; INL: inner nuclear layer; PR: photoreceptor cell layer; RPE: retinal pigment epithelium. Adapted from Béby et al., 2013

2.6 Otx2 role in the adult retina

In the retina, *Otx2* expression is maintained throughout life in RPE, photoreceptors and bipolar cells. Different studies, based on conditional *Otx2* ablation, have investigated the role of this homeodomain factor at this stage. To achieve conditional *Otx2* KO in all *Otx2* expressing cells at the desired time point, the self-knockout strategy was used (Fossat et al. 2006). It relies on a genetic combination where one of the *Otx2* alleles has its endogenous coding sequence replaced by the CreER^{T2} coding sequence, and the other allele has its coding exon 2 flanked by two *loxP* sites. The genotype is referred to as *Otx2*$^{CreERT2/flox}$. CreER^{T2} is a classic Cre recombinase fused with the modified ligand binding domain of the estrogen receptor, which is reactive to tamoxifen, an estrogenic agonist, but not to endogenous estrogen (Feil et al., 1997). In the absence of tamoxifen, the protein interacts with the HSP70 chaperone, which maintains it sequestered in the cytoplasm. In the presence of tamoxifen, the ER^{T2} moiety undergoes a conformation change. This dissociates CreER^{T2} from HSP70 and unmasks a nuclear localization sequence that allows CreER^{T2} to be translocated to the nucleus, where it can recombine *loxP* sites. A single pulse of tamoxifen is sufficient to activate CreER^{T2} recombinase activity, promoting the deletion of the floxed sequence and

then the KO of *Otx2*. When *Otx2* is knocked out simultaneously in RPE, photoreceptors and bipolar cells at P30, this causes the slow degeneration of both rods and cones. The degeneration starts 20 days after KO induction with a detachment of photoreceptor outer segments from the RPE. Thirty days after KO, photoreceptors in the ONL start to die by apoptosis, and after 90 to 120 days, photoreceptors have completely disappeared (**Figure 15**) (Béby et al. 2010).

The viability of other cell types is not affected but the number and the distribution of melanosomes in the RPE cells are modified as early as 20 days after KO. These appear smaller and less numerous and are all distributed on the apical side, suggesting that Otx2 has a role in RPE melanogenesis. Large vacuoles in the cytoplasm of RPE cells, appear after KO induction, reflecting an increased autophagy activity, but RPE cells do not die. Transcriptome analysis following KO induction has shown that 2 days after KO, several genes involved in RPE melanogenesis, pH regulation and visual cycle are downregulated (Housset 2013). This downregulation is observed until 8 days after KO, suggesting that RPE functionality is really compromised. Four days after KO, several genes involved in the Muller glial cells activation and inflammatory process are upregulated, indicating a reaction to retinal stress. However, these genes are probably not direct targets of Otx2, whereas they could be activated indirectly because of generic stress.

Surprisingly, although photoreceptors degenerate, no photoreceptor-specific genes were found to be deregulated. Redundancy between Otx2 and the related Crx, strongly expressed in adult photoreceptors, might explain this situation (Samuel et al, 2015). Together, these results suggest that in the adult retina, Otx2 mainly regulates RPE-specific genes, the absence of which causes subsequent retinal inflammation and selective degeneration of photoreceptors (Beby 2010, Housset 2013).

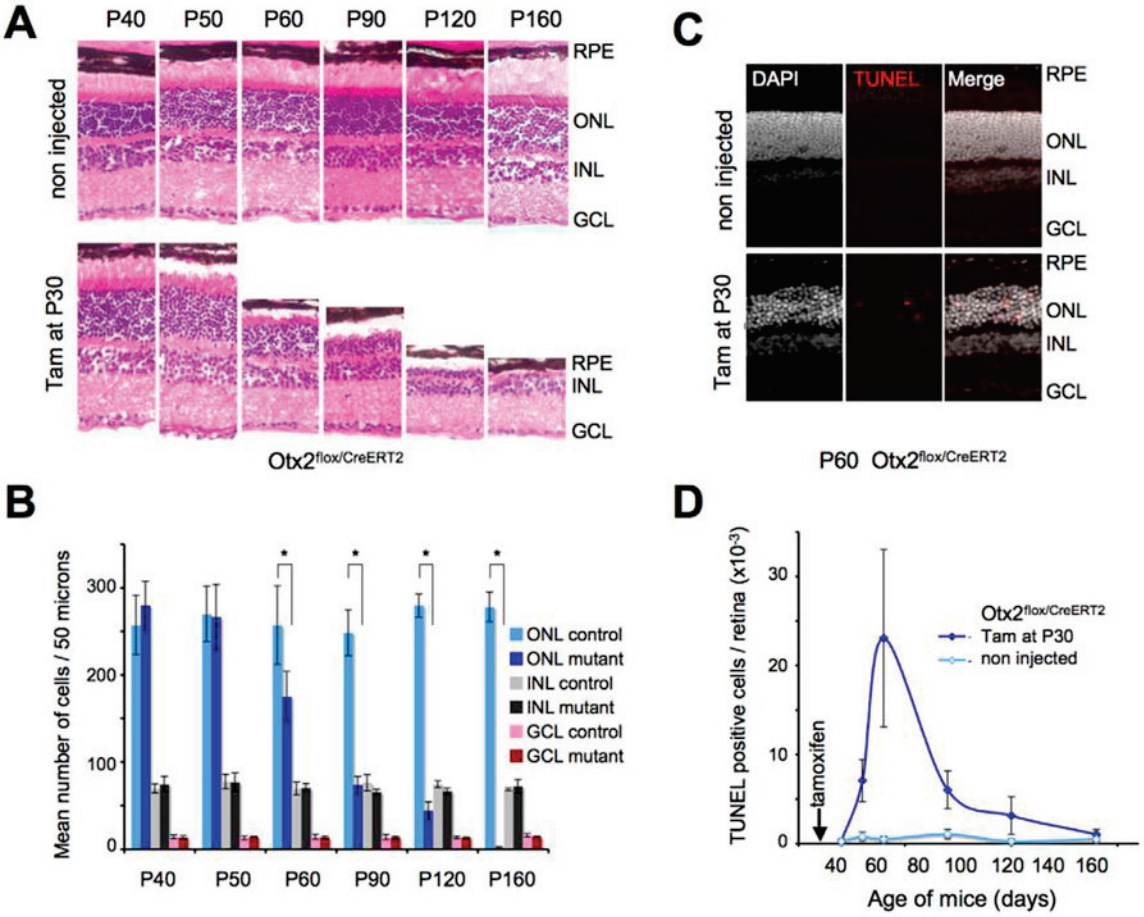


Figure 15. Loss of Otx2 leads to photoreceptor cell degeneration. A. Histology of control (non-injected) and mutant (Tamoxifen administrated at P30) series of $Otx2^{flox/CreERT2}$ retinas of the indicated ages. Sections are stained with Eosin and Haematoxylin. RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. B. Cell counts in the three layers retinal sections of control or tamoxifen treated (mutant) $Otx2^{flox/CreERT2}$ mice at indicated ages. Normalized fields of the same eye area were used. Mean cell number and standard deviation are indicated for each condition (*P,0.001). C. Detection of apoptotic cells in control or tamoxifen treated $Otx2^{flox/CreERT2}$ retinas 30 days after treatment. Left, middle and right panels show respectively DAPI staining, TUNEL labelled cells and superimposition of both images. D. Kinetics of apoptosis following Otx2 gene ablation. TUNEL labelled cells were counted on normalized sections of three independent mice for each stage, each corresponding to 0.063 mm2 of retinal area. Error bars are standard deviation. Adapted from Béby et al., 2013.

To prove this scenario, Housset et al. performed RPE-specific ablation of Otx2 by subretinal injection of a Cre-expressing lentiviral vectors in $Otx2^{flox/flox}$ mice. An IRES-GFP was inserted in the construct to visualize the transduced region. The regions where Otx2 was depleted from the RPE underwent photoreceptor degeneration at the same rate than in the full retina KO, despite rods and cones maintained endogenous Otx2 expression. Conversely, transduction of RPE cells with a lentiviral vector expressing Otx2 rescued photoreceptors of

tamoxifen injected *Otx2*$^{CreERT2/flox}$ mice, in the transduced area, while in the non-infected area, Otx2-depleted photoreceptors degenerated. These experiments show that Otx2 expression in the RPE is necessary and sufficient for photoreceptor viability (Housset et al. 2013).

Otx2 expression in the RPE has, thus, a non-cell-autonomous neuroprotective effect on photoreceptors. The most parsimonious hypothesis to explain this effect is that RPE-dysfunction, following *Otx2* knockout, indirectly affects photoreceptor viability. Indeed, since photoreceptors strictly depend on the nursing activity of the RPE for their nutrient supply, waste elimination and pigment recycling, it is likely that any dysfunction of the RPE threatens photoreceptor survival. However, it cannot be excluded that Otx2 KO also affects the expression of putative neuroprotective molecules normally secreted by the RPE, that keep photoreceptors alive. Identification of such signals, that would require the analysis of RPE-specific *Otx2* KO retinas, has not been investigated yet, because no truly inducible RPE-specific Cre driver line exists. The alternative would be to use the above Cre-expressing lentiviral system, but the limited extent of transduced retina makes it difficult to isolate KO RPE cells from surrounding normal ones.

Another possibility to consider is that *Otx2* KO in photoreceptors could affect RPE functionality. While it has been shown that *Otx2* ablation in all retina causes RPE dysfunction, it has not been investigated whether RPE melanogenesis defects are fully rescued when RPE Otx2 expression is restored by lentiviral administration in this layer. The same applies to the autophagy process. Therefore, it cannot be excluded that RPE dysfunction could be partially due to photoreceptor stress following *Otx2* ablation in the whole retina and not only to the absence of endogenous Otx2. Whether photoreceptor-specific *Otx2*-KO reproduce at least partially these RPE phenotypes remains to be investigated.

Does Otx2 play an intrinsic role in adult photoreceptors? Tamoxifen-injected *Otx2*$^{flox/CreERT2}$ mice show a defect in scotopic electroretinogram (ERG) as early as 4 weeks following *Otx2* KO (Housset et al. 2013). The abnormal electrical activity of RPE (c-wave) and photoreceptors (a-wave) indicates that KO mice have an altered vision that progressively evolves to blindness. When Otx2 expression is partially restored by injection of Otx2-expressing lentiviral vectors in *Otx2*$^{flox/CreERT2}$ RPE, photoreceptors survive, but no ERG tests are possible because more than 50% of the non-transduced retina degenerate. This

prevents the analysis of a potential role of Otx2 in photoreceptors. A strong argument to rule out a role of Otx2 in photoreceptors is that no change in photoreceptor-specific gene expression was found following *Otx2* ablation in the whole retina. On the other hand, ChIP-seq analyses have shown that Otx2 can bind to regulatory elements of genes involved in sensory perception of the light stimuli, vision perception, photoreceptors differentiation such as rhodopsin, recoverin, visual arrestin, phosphodiesterases PDE6B and PDE6G, supporting the idea that Otx2 could have a role in adult photoreceptors. Interestingly, the same genome regions are also bound by Crx, a member of the Otx family (Samuel et al. 2014). Therefore, it cannot be excluded that Otx2 and Crx act, at least partially, in a redundant way so that when Otx2 is knocked out, Crx could fulfil some of its functions. Such redundancy could mask the consequence of *Otx2* ablation on photoreceptor gene expression. Proteomic studies, showing that Otx2, but not Crx, interacts with some components of the phototransduction such as arrestin 1, add weight to the idea that Otx2 may play a direct role in photoreceptors (Fant et al. 2015). Other protein partners that specifically bind to Otx2 and not to Crx have been reported in Fant et al. 2015. These results suggest that Otx2 can have extra-nuclear functions and that Otx2 and Crx, redundant at the nuclear level, could have different functions in other photoreceptor compartments.

III Homeoprotein transfer as an alternative signalling mechanism

The concept of homeoprotein transduction has its origins in the 1990s, when it was shown for the first time that the 60 amino-acid homeodomain of Drosophila Antennapedia was able to cross the membrane of cultured mammalian neurons, changing their morphology (Joliot et al. 1991). Today, this mechanism is recognized to be evolutionary conserved. More than 150 homeoproteins have been shown to be able to transfer from cell to cell, being thus potential signalling proteins (Lee et al. 2019). This mechanism of transfer appears widely spread among multicellular organisms. In plants, KNOTTED1 homeoprotein uses its homeodomain to move between cells, trafficking between cellular plasmodesmata, the specialized structures of the cell-wall that are connecting neighbouring cells (Kim et al. 2005; Kim et al. 2002; Lucas et al. 1995). Engrailed is transferred between different territories of the wing imaginal disc during Drosophila development (Layalle et al. 2011).

3.1 Homeoprotein transfer: discovery and initial studies

Homeoprotein transduction was accidentally observed in a control experiment in 1991, when the homeodomain from Antennapedia was shown to be captured by mammalian neurons *in vitro* and to change their morphology and polarity (Joliot et al. 1991) (Dupont et al. 2015). A similar phenomenon has been observed in chicken motor neurons, where exogenous Antennapedia homeodomain changed their growth and branching, suggesting that homeodomain transfer could be generalized to other species and other cell-types (Bloch-Gallego et al. 1993).

The fusion of the Antennapedia homeodomain with the C-terminus of the GTPase Rab3, a potent inhibitor of prolactin secretion by pituitary gland cells in culture, conferred this chimeric peptide the ability to cross cell membranes, suggesting that homeodomain could be a vehicle for protein transfer (Perez et al. 1992). The transferred fusion maintained its inhibitory activity (Perez et al. 1994).

Dissection of the Antennapedia homeodomain sequence showed that the signal for internalization was included in the third helix, containing 16 amino-acids (43 to 58) in the C-terminal part of the homeodomain (Derossi et al. 1994). This peptide of 16 amino-acids was termed penetratin. Internalization of the Antennapedia homeodomain or of the penetratin peptide works at 37°C as well as at 4°C, suggesting that it is energy independent. Sequence

integrity of the Antennapedia homeodomain was shown to be critical for its binding to *HoxA5* promoter and mutations in the third helix completely abolished its capacity to regulate cell morphology (Le Roux et al. 1993). In particular, mutated forms that deleted the tryptophan in position 48 and the phenylalanine in position 49 (WF 48-49), two highly conserved positions of the homeodomain, lost both penetration and DNA-binding activity (Le Roux et al. 1993). These observations opened the way to develop penetratin-based systems to drive different molecules in specific cells (Derossi et al. 1998; Prochiantz 1999; Pooga & Langel 2015; Derossi & Prochiantz 1995). Together, these data showed overlapping DNA binding activity and penetratin-activity, both of which appear necessary for target cell uptake and regulation of gene expression (Le Roux et al. 1995).

After this initial *in vitro* characterization, the mechanisms of secretion and internalization and the functions of transferred homeoproteins have been investigated *in vivo*. It has been found that secreted homeoproteins bind specific receptor motifs present on the cell surface and/or in the surrounding extracellular matrix. Secreted homeodomain proteins can act in a paracrine way or travel long distances before being up-taken by remote cell targets. Among them, Otx2 has been shown to have a key role in different contexts such as visual cortex plasticity regulation and retinal cells survival. The discovery of this transfer ability has modified the traditional view of homeoproteins as transcription factors acting cell-autonomously, extending it to signalling molecules acting in an unexpected non-cell-autonomous manner.

3.2 Homeoprotein transfer: signals and mechanisms for secretion and internalization

The third helix of the homeodomain, which is essential to recognize and bind DNA, also confers Antennapedia its ability to be internalized by cells. The two amino-acids WF 48-49 that appear critical for Antennapedia internalization are also important for other homeoproteins: for instance, mutations introduced at these 2 amino-acid positions in Otx2 homeodomain impair its internalization (Ibad et al. 2011). The internalization process appears, at least for Antennapedia, to be independent of a protein receptor, as it is not affected by the temperature (Derossi et al. 1996; Dom et al. 2003). Biophysical studies showed that the homeodomain and penetratin can directly establish electrostatic interactions with anionic phospholipids of the membrane, and then can asymmetrically translocate across the membrane bilayer by hydrophobic interactions. Early studies

suggested that penetratin aggregated outside the membrane before translocation, but a recent NMR study has shown that it is asymmetrically inserted into the membrane bilayer without an aggregation (Su et al. 2008). According to this study, charged residues are involved in a first recognition step between penetratin and anionic phospholipids of the membrane and then, the tryptophan residue at position 48 destabilizes the phospholipid bilayer, allowing subsequent translocation. This process of internalization has been proposed to function in parallel with an endocytosis process, where an aggregation step precedes internalization. Whether this endocytosis is a non-specific process triggered by peptide aggregation or an alternative mechanism of internalization, cell-context dependent, is not clear yet. For instance, the En2 WF→SR mutant, where the conserved tryptophan-phenylalanine 48-49 dipeptide has been replaced by a serine-arginine dipeptide, is up-taken by mammalian neurons in culture, but remains in their cytoplasm, contrary to the wild-type isoform, which goes into the nucleus (Joliot et al. 1998). Such abnormal localization would be a consequence of non-specific endocytosis due to the aggregation of secreted mutant En2 homeoprotein in the medium. The fact that this En2 mutant, when added at a lower concentration to the medium, is not internalized by retinal explants of Xenopus, supports this view (Brunet et al. 2005). In short, homeoprotein internalization mostly relies on unconventional translocation across cell membranes, following interaction of the third helix of the homeodomain with negatively charged membrane lipids.

To act as a paracrine or long-distance signal, homeoproteins need to be secreted. While internalization studies were performed using purified peptides or proteins added to cell cultures (Le Roux et al. 1993; Chatelin et al. 1996), other strategies were needed to study the mechanism of secretion. The first evidence that homeoproteins can be produced and secreted by a source cell and internalized by a target cell was obtained in co-culture experiments using Engrailed 2 (En2). Cos7 cells, transiently transfected with an expression vector coding a Myc-tagged chicken *En2* (cEn2), were co-cultured with rat embryonic E15 cortical primary neurons for 48 hours. After fixation, detection with Myc antibody showed a strong staining of the neuron nuclei, suggesting that a direct transfer of Myc-tagged En2 had occurred between Cos7 and primary neurons. Mutation of the amino-acids WF 48-49 (WF mutant) motif did not impair En2 secretion, but affected its internalization, although not to the same extent as that of Antennapedia (Derossi et al. 1994) and Otx2 (Ibad et al.

2011). This demonstrates that the homeodomain sequence required for secretion is different from the sequence required for internalization.

Additional mutants, generated to identify the sequences that are required for secretion, have emphasized the importance of an 11 amino-acid region spanning across helix 2 and helix 3 (Joliot et al. 1998; Maizel et al. 1999; Dupont et al. 2007). While a purified Myc-tagged Δ11 cEn2 mutant protein, lacking this region, is normally up-taken by primary neurons when added to the culture medium, the same protein, transiently expressed by Cos7 cells, is no longer found in co-cultured primary neurons, demonstrating that the deleted region has a role in secretion (Joliot et al. 1998; Maizel et al. 1999). The mechanism of secretion has not been fully characterized. En2 has been detected *in vitro* and *in vivo* (in the developing rodent cerebellum and mesencephalon) in membrane fractions enriched in cholesterol and glycosphingolipids, and containing caveolin proteins (Joliot et al. 1997). Caveolin-coated vesicles are involved in a transport system that allows intracellular trafficking, but they are also involved in exocytosis and endocytosis. This observation suggested that En2 could use caveolin-coated vesicles to be secreted. The amount of protein present in these vesicles has been estimated at about 5% of the total En2 protein in the cells, suggesting that secretion concerns only a small fraction of the produced protein. When the 11 amino-acid region is deleted, En2 is no longer present in this compartment (Joliot et al. 1998). Supporting the hypothesis of a vesicle-based secretion system, a recent study showed that En2 is secreted by prostate cancer cells after accumulating into micro-vesicles; these uncharacterized vesicles arise from the nucleus and then fuse to cell membranes, allowing the secretion of their content (Punia et al. 2019). Since caveolins are present in the nucleus, the micro-vesicle transporting En2 could derive from a caveolin-enriched nuclear compartment (Fridolfsson et al. 2014; Han et al. 2016).

Homeoprotein secretion can be regulated by post-translational modifications. For instance, Engrailed can be phosphorylated by Casein Kinase 2. This phosphorylation inhibits En2 secretion, promoting cytoplasmic accumulation of the protein (Maizel et al. 2002).

The 11 amino-acid sequence described above is not always necessary nor sufficient for secretion. In a recent study, over 160 human homeoproteins have been screened for their ability to be secreted and internalized (Lee et al. 2019). Among them, 151 were positive to the screen, including Otx2. A global alignment between all analysed proteins confirmed the strong conservation of the 11 amino-acid sequence. However, while deletion of these 11

amino-acids was critical for the secretion of some homeoproteins, it did not completely abrogate the secretion of other ones, such as Otx2, suggesting that the requirement of this sequence of 11 amino-acids could be also protein specific.

This global alignment also identified different conserved hydrophobic positions outside the homeodomain. Mutations of the corresponding residues affected the secretion of some tested homeoproteins. For instance, mutation of the leucine at positions 143 or 192 affected the secretion of En2. This seems to be a general rule, as mutating these hydrophobic residues in other homeoproteins, including OTX2(L220E), OTX2(F258E), PAX6(L130E), and VAX1(L305E), also affected their secretion.

In conclusion, homeoprotein secretion appears to use an unconventional secretory pathway based probably on micro-vesicles formed in a caveolin-rich nuclear compartment, and requires an 11 amino-acid motif of the homeodomain as well as hydrophobic conserved residues out of the homeodomain, with a protein-specific relative requirement.

3.3 Homeoprotein *in vivo* transfer

Almost 30 years separate the first evidence of homeoprotein transfer (Joliot et al. 1991) from the recent large-scale analysis of transfer ability of 150 homeoproteins (Lee et al. 2019). In between, several studies have addressed the existence and significance of homeoprotein transduction *in vivo*. (reviewed in Joshi et al. 2011, Prochiantz & Di Nardo 2015; Prochiantz et al. 2014).

The importance of homeoprotein signalling has been shown in the developing and adult nervous system. Early studies have shown the role of exogenous En2 on Xenopus embryonic retinal axon growth and guidance. A gradient of this exogenous protein strongly repels growth cones of the axons originating from the temporal retina while, by contrast, it attracts nasal axons. This turning behaviour is induced by the internalization of the protein in the growth cones, where exogenous En2 regulates local protein translation, by promoting the phosphorylation of eIF4E and 4E-BP1, two components of the protein translation machinery (Brunet et al. 2005). The fact that transcription inhibitors do not have any effect on the axonal growth enforces the idea that exogenous En2 acts at post-transcriptional level.

A role of exogenous homeoproteins in establishing projection maps has been described. En2 and En1 are expressed in the optic tectum of chicken and amphibians and in the analogous superior colliculus of mammals, forming an anterior-low to posterior-high concentration

gradient. This gradient has been shown to be necessary to organize the retinotectal map between axons of new-born retinal ganglion cells and tectum, in Xenopus. Blocking exogenous En2 by tectal expression of a secreted single-chain antibody disrupts this topographic organization, with temporal axons projecting aberrantly because of altered response to chemotropic factors as ephrin-A (Wizenmann et al. 2009). The mechanism has been investigated. It has been found that exogenous En2 moves into the axoplasm of retinal ganglion cells where it modulates the sensitivity of response to ephrin-A by regulating the local translation of some mRNAs (Yoon et al. 2012). In particular, mRNA translation of lamin B and of proteins of the ATP complex 1 is increased by the exogenous En1 and En2, suggesting that Engrailed family can also play a role in neuron survival by promoting ATP synthesis (Fuchs et al. 2012; Stettler et al. 2012).

En2 and En1 are also expressed in adult midbrain dopaminergic neurons (mDAs) of the *substantia nigra pars compacta. En1*$^{+/-}$ mice progressively lose mDAs (Sonnier et al.,2007) suggesting a dose-dependent survival mechanism that may be relevant to Parkinson's disease, as this disease is characterized by the loss of the same mDAs. Based on the previously shown En1/2 ability to be internalized and to increase the ATP synthesis level, infusion of purified En1 was performed just above the *substantia nigra* of *En1*$^{+/-}$ *mice*. A neuroprotective effect was obtained with a rescue of 50% of mDAs (Joshi et al. 2011). To test whether exogenous En1 increased ATP synthesis, mice were infused with MPTP (1-methyl-4-phenylpyridinium), a toxin of complex 1, which is commonly used to induce Parkinson-like disease. When En1 was infused in pre-treated mice, the mDA cell death rate was reduced, confirming a neuroprotective effect of infused exogenous En1 (Alvarez-Fischer et al. 2011). Together these results showed that Engrailed members can act as exogenous signals at both embryonic and adult stages, playing different roles such as axon guidance or neuroprotection in a degenerating-mDA neurons mouse model.

Other studies investigating the role of exogenous Pax6 in zebrafish eye development (Lesaffre et al. 2007) and in oligodendrocyte precursor cell migration in the chick embryonic neural tube (di Lullo et al. 2011), or the role of exogenous Vax1 in retinal ganglion cell axon growth (Kim et al. 2014) extend the observation to more proteins, suggesting that homeoprotein transfer is a process more commonly used in living organisms than previously thought.

3.4 Otx2 *in vivo* transfer

A striking role of homeoprotein transfer in brain plasticity and maturation has been reported, concerning the Otx2 transcription factor. During postnatal development, the cortex goes through critical periods (CPs) of plasticity that allow environmental influence during a vulnerable time window. There are several CPs for different sensory circuits that differ in time and duration (opening and closure times). For the primary visual cortex, CP opening occurs at P20 and closure at P40 in mice. Blinding one eye before P20 or after P40 neither affects the spatial distribution of ocular dominance columns in the binocular cortex nor the binocular vision, while blinding one eye during CP promotes eye blindness and amblyopia (Hensch 2005). This suggests that ocular dominance is established during this CP of plasticity (Levelt & Hübener 2012). At the cellular level, CP onset corresponds to the maturation of parvalbumin GABAergic interneurons (PV cells) present in layers 3 and 4 of the visual cortex. A clear index of PV-cells maturation is the expression of the marker parvalbumin and the formation of a specialized extracellular matrix enriched in chondroitin sulphate chains, the perineuronal net (PNN). Disruption of the PNN, by infusing chondroitinase ABC (ChABC) in the cortex, dramatically affects CP opening and closure time (Hou et al. 2017; Miyata et al. 2018).

Otx2 is strongly involved in PV-cells maturation in the visual cortex. Sugyiama et al. (2008) showed that Otx2 starts to be detectable in PV-cells at the time when they become mature. At P19, one day before CP onset, Otx2 is weakly detectable in the forming PV-cells, that start to form their PNN. In the middle of the CP, Otx2 signal increases and so does the number of mature PV-cells, co-labelled with Otx2. Otx2 is then kept in these cells after CP closure. Interestingly, when young mice are raised in the dark, the CP onset is delayed because PV-cells do not mature. In these mice, Otx2 does not accumulate in the visual cortex and PV-cells do not mature, producing no PNN. Administration of recombinant Otx2 in one hemisphere of these dark-reared mice is sufficient to open the CP and to allow cells to express parvalbumin and form their PNN. After infusion, Otx2 is found accumulated in these cells, suggesting that it is transferred from the extracellular space.

When recombinant Otx2 is administrated before the normal day of the CP opening in the young P17 wild type mice, CP onset is also anticipated. These data suggest that Otx2 is critical for PV-cells maturation and that it accumulates in PV-cells in an experience-

dependent manner. In the absence of light exposure, no accumulation of Otx2 in PV-cells occurs, preventing the maturation of PV-cells. However, this study does not explain why Otx2 is retained in the PV-cells also after CP closure. Two different studies tried to answer this question by different approaches (Beurdeley et al. 2012, Spatazza et al. 2013). In the first one, it was shown that Otx2 interacts with negatively charged chondroitin sulphate chains of the PNN with a region of 15 amino-acids containing several basic positively charged residues. The two first amino-acids, arginine and lysine (RK), that are just flanking the N-terminus of the homeodomain are also necessary for this binding. When these two RK amino-acids are mutated into two alanine residues, the resulting synthetic AA-peptide no longer interacts *in vitro* with chondroitin sulphate. Accordingly, recombinant AA-Otx2 is much less actively internalized by PV-cells than wild type Otx2 and it has a trend to lose cell selectivity and enter into other cell types of the visual cortex. These observations suggest that the RK motif confers Otx2 the ability to be specifically recognized by the chondroitin sulphate chains of the PNN surrounding PV-cells. Without this binding, PV-cells are not able to sequester Otx2, which is then non-specifically internalized by other cell types.

As mentioned above, ChABC treatment affects CP opening and closure time. This treatment also affects the amount of Otx2 present in PV-cells, because these cells can no longer bind Otx2 and accumulate it. The consequence is a reduction of Otx2 level inside the PV-cells and the CP is re-opened. To exclude that the reopening of CP could also be a consequence of other pathways affected by ChABC treatments, mice have been treated by injecting high amounts of RK-peptide that compete with Otx2 on PV-cells surface, without touching sulphate chains. This treatment induces a reduction of Otx2 in the PV-cells and the reopening of CP, suggesting that this effect is really due to the reduced amount of Otx2 in PV-cells. The working hypothesis is that a first Otx2 threshold is required to open the CP while a second threshold, that is maintained by constant uptake of Otx2 from extracellular space, is required after CP closure to avoid further re-opening of the CP (Figure 16).

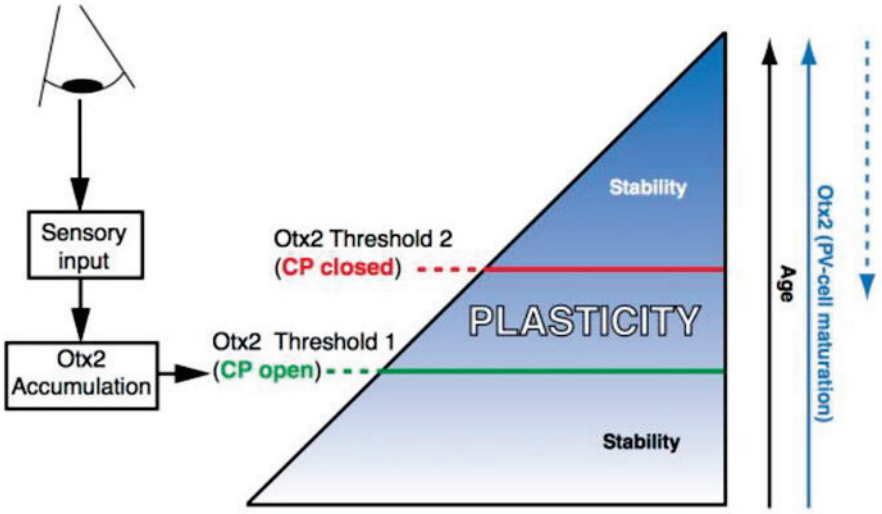


Figure 16. The double threshold hypothesis. Otx2 regulation of cortical plasticity. Early sensory experience initiates the accumulation of Otx2 in PV-cells to promote their functional maturation and plasticity onset. As the CP proceeds, PNNs condense tightly around PV-cells causing higher levels of Otx2 to accumulate and to structurally stabilize the local circuit. Adapted from Spatazza et al., 2013

The puzzling aspect of this set of data is that although Otx2 can be internalized by PV-cells, the *Otx2* gene itself is not expressed in the visual cortex (Sugiyama et al. 2008). Two external sources have been then hypothesized: the first one could be the retina; indeed, recombinant biotinylated Otx2, once injected in the eyes of mice, is accumulated in retinal cells but also in PV-cells, suggesting that it could move through the different synapses of the visual pathway, between the retina and the lateral geniculate nuclei, where *Otx2* is expressed, and the PV-cells of the visual cortex (Sugiyama et al. 2008). Two genetic approaches led to propose the choroid plexus as another source of Otx2 for PV-cells (Spatazza et al. 2013; Bernard et al. 2016). Otx2 is strongly expressed in the choroid plexuses, in all brain ventricles, of developing and adult mice, and it is released in the cerebrospinal fluid that circulates around the central nervous system and that can reach PV-cells in the visual cortex as well as other cortex regions. Knockdown of Otx2 in the choroid plexus, by injection of Tat-Cre protein in the brain ventricle of $Otx2^{flox/flox}$ mice, strongly affected the amount of Otx2 in the PV-cells. When this knockdown was induced after the normal closure of CP (P40), this was re-opened, confirming that a continuous Otx2 protein internalization in the PV-cells is required to inhibit the reopening of this plasticity (Spatazza et al. 2013). Two potential weaknesses of this study are that Otx2 knockdown in the choroid

plexus may have collateral effects on other cortex regions that also require Otx2 supply from this structure, and that Otx2 knockdown in the choroid plexus may also have a cell-autonomous effect and affects secretion of other active molecules such as growth factors (Johansson et al. 2013). To refute these arguments, a mouse line secreting single-chain anti-Otx2 antibodies specifically from PV-cells or from choroid plexus was used. In this line, secreted Otx2 is trapped outside PV-cells or in the cerebrospinal fluid, preventing its internalization. Again, a reduction of Otx2 in PV-cells was observed, followed by a reopening of CP plasticity (Bernard et al. 2016).

Several pieces of evidence show that internalized Otx2 stimulates PV-cells to produce and mature their PNN (Bernard & Prochiantz 2016; Beurdeley et al. 2012; Testa et al. 2019) while upon inhibition of Otx2 internalization, PNN tends to disappear and to be less organized. These observations suggest that up-taken Otx2 directly or indirectly regulates genes involved in PNN synthesis by a positive feedback, but a direct proof is still missing. Mechanisms explaining how Otx2 promotes CP opening at a first threshold and how, above a second threshold, it inhibits a reopening of a second CP, are not fully understood. What has been recently shown is that internalized Otx2 regulates the expression of Gadd45b, a factor involved in neurogenesis, genome stability and long-term memory, by stimulating gene demethylation. Recently Gadd45b has also been shown to regulate plasticity genes (Apulei et al., 2019). Gadd45b is highly expressed at the opening of the CP, while its expression declines after CP closure. Injections of recombinant Otx2 in the visual cortex before the opening time of the CP upregulate expression of Gadd45b. The same effect is observed when Otx2 up-take is inhibited in PV-cells of adult mice after the CP closure. These observations suggest that Otx2 works as an activator of Gadd45b before CP onset and as an inhibitor after CP closure. Bioinformatics analyses confirmed the presence of an Otx2-binding site in *Gadd45b* promoter and interaction was confirmed by ChIP assay. These data suggest that Otx2 internalization in PV-cells can regulate gene plasticity, through Gadd45, in an opposite manner at onset and closure of the CP.

Otx2 transfer has also been described in the retina. Two papers report that Otx2 can be transferred by photoreceptors to bipolar cells *in vivo* (Kim et al. 2015) and that can be up-taken by retinal ganglion cells (Ibad et al. 2011).

Otx2 haploinsufficiency affects bipolar cell viability in adult retina (Kim et al. 2015). Analyses that were done on P90 old mouse retina of wild type mice and heterozygous $Otx2^{+/GFP}$ retina

(Fossat et al. 2007), showed that bipolar cells were less numerous in the heterozygous mice suggesting that Otx2 haploinsufficiency promotes a slow and late degeneration of bipolar cells. Among all the different subpopulations of bipolar cells, the most affected are the T2-OFF bipolar cells. Looking at gene expression, these cells are not expressing *Otx2* but they are positively labelled for Otx2 protein, suggesting that they get it from an external source. Kim et al. (2015) demonstrated that conditional ablation of Otx2 in photoreceptors also abolished Otx2 detection in this subpopulation of bipolar cells, that subsequently died. This population was rescued by intraocular administration of purified recombinant Otx2, confirming that the exogenous protein is required for their survival. Looking into details, exogenous Otx2 localized into the mitochondria of these T2-Off bipolar cells. Analysis of the mitochondrial protein fraction, co-immunoprecipitated with an anti-Otx2 antibody, revealed that different subunits of the mitochondrial ATP synthase complex F0/F1 interact with Otx2; this suggests a role of Otx2 in ATP synthesis. Moreover, isolated mitochondria from mouse retina, produce more ATP when purified Otx2 is added to the culture medium. Sugiyama et al. (2008) showed that intraocularly injected Otx2 was also up-taken by retinal ganglion cells that do not express the *Otx2* gene (Fossat et al. 2007). To confirm this phenomenon and to investigate the role of exogenous Otx2 in these cells, Ibad et al. (2011) used cultures of dissociated adult retina from B6.Cg(Thy1-CFP)23Jrs/j mice, where CFP is endogenously expressed in retinal ganglion cells under the Thy1 promoter (Feng et al., 2000). By measuring CFP fluorescence over time, they observed that Otx2 addition to the medium improved retinal ganglion cell survival, suggesting a neuroprotective effect.

To demonstrate that intraocularly injected Myc-tagged Otx2 protein accumulates in retinal ganglion cells via the specific homeodomain internalization route, a mutant form of Otx2, where the conserved tryptophan 48 and phenylalanine 49, were replaced by tyrosine and leucine residues (WF→YL) was generated. This YL-Otx2 mutant protein, when injected in the eyes, was not detected in any retinal cell. Moreover, YL-Otx2 did not have any neuroprotective effect on retinal ganglion cell cultures, contrary to wild type Otx2. In addition, *in vivo* experiments showed that wild type Otx2, but not YL-Otx2 were protecting retinal ganglion cells against NMDA-mediated stress. Together, these observations suggest that retinal ganglion cells can internalize Otx2 through the homeodomain transfer pathway and use it as a neuroprotective factor. The source has not yet been identified, but bipolar cells, that establish synaptic contacts with ganglion cells, appear to be good candidates.

Finally, several regions of Otx2 homeodomain have been proved or supposed to be important in its cell to cell transfer. A schematic summary of all these regions and their corresponding references is shown in the Figure 17.

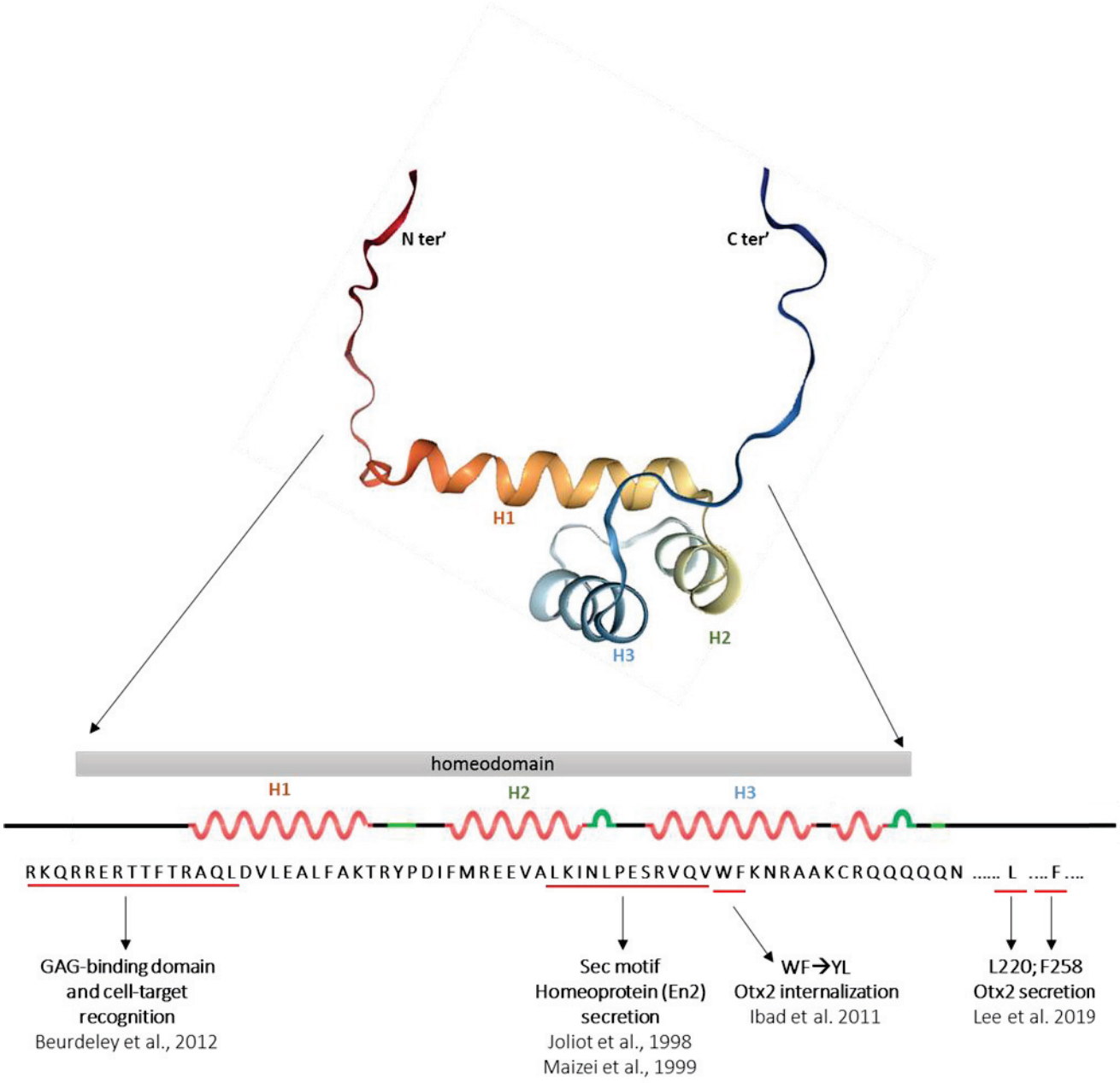


Figure 17. Otx2 signals involved in homeoprotein transfer. Top: 3D view of Otx2 homeodomain (PDB: 2DMS) representing the three helices (H1, H2, H3) involved in DNA binding activity. Bottom: simplified linear representation of the same homeodomain structure, with the corresponding aminoacid sequence underneath. Signals involved in Otx2 secretion and internalization, with corresponding references, are underlined. Two remote aminoacids important for Otx2 secretion are also shown (L220 and F258).

IV Objectives of the thesis

Otx2 is a fundamental factor in retinal development and maintenance in adults. Different mutations have been associated with different eye and retinal diseases as anophthalmia, microphthalmia and coloboma. Although the role of Otx2 transcription factor in retinal development is well studied, its role in the adult retina remains unclear. *Otx2* expression is maintained in Retinal pigmented epithelium, photoreceptors and bipolar cells throughout life. Previous works showed that *Otx2* ablation in the adult retina leads to photoreceptor degeneration, suggesting a role in their maintenance. It was then showed that RPE-restricted *Otx2* expression was both necessary and sufficient for photoreceptor maintenance, indicating a neuroprotective non-cell autonomous effect. This left the function of endogenous Otx2 in photoreceptors and the molecular mechanisms underlying RPE-Otx2 neuroprotective function unexplained.

The two main objectives of this thesis are to study the role of endogenous Otx2 in mature photoreceptors using a genetic approach that allows its ablation specifically in these cells at the stage of interest and to study the molecular mechanisms by which the Otx2 protein produced in RPE cells exerts a neuroprotective effect on PRs. According to the literature and our data, this neuroprotective effect could be the consequence of a direct transfer of Otx2 from the RPE to photoreceptors. Despite other possibilities explored, we have focused our efforts on this intriguing hypothesis.

MATERIALS AND METHODS

V Materials and Methods

Animals

All animal care and experiments have been carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EEC) and in accordance with the PEA ("Projet Experimentation Animale") ref. 201707051056715 guidelines, approved. The pre-mutant floxed-Otx2 line, referred to as Otx2^{flox} line, was generated previously in our laboratory (Fossat et al. 2006). The transgenic line Tg(Crx-CreER^{T2})1Tfur, referred to as *Crx*-CreER^{T2} (Muranishi et al. 2011), and the *Gt(ROSA)26Sor$^{tm14(CAG- TdTomato)Hze}$ Cre*-reporter line, referred to as Ai14 (Madisen et al. 2010) were purchased from the RIKEN BRC and the Jackson Laboratory, respectively. *Crx*-CreER^{T2};*Otx2$^{flox/flox}$* and *Crx*-CreER^{T2};*Otx2*$^{flox/flox}$*;Ai14*$^{+/+}$ genotypes were obtained as result of their breeding. Mice were maintained in 129/SV background and kept in 12:12 hours light/dark cycle from 7 am to 7 pm, except where differently specified. Mice were provided with food and water *ad libitum* and housed with cage enrichment. For all experiments, both sexes were indistinguishably used. Otx2 KO and/or TdTomato activation were induced by tamoxifen intraperitoneal injection of a solution 10 mg/ml of tamoxifen, dissolved in sunflower oil, at the desired stage. The dose was 50 µg/gram of body weight.

Genotyping

For DNA extraction, mouse biopsies were digested 30 minutes – 1 hour at 95°C in a 25 mM NaOH, 0.2 mM EDTA, pH 12, and equilibrated with the same volume of 40 mM Trizma-HCl, pH 5. 1-2 µl of this solution was used for PCR, using specific couples of oligonucleotides (Table I). PCR conditions: - initial denaturation 94°C for 5 minutes - 35 cycles including a denaturation step at 94°C for 30 seconds, an annealing step at 60°C for 30 seconds and an extension step at 72°C for 45 seconds, - a final extension at 72°C for 5 minutes. PCR samples were analysed by gel electrophoresis.

RNA preparation, RT-PCR and RNA-sequencing (RNA-Seq) procedure

For RNA-seq analyses KO was induced at post-natal stage P30 and mice were collected 2, 4 and 8 days after KO induction (P32, P34, P38). Controls were P30 uninjected mice. The experiment was performed in triplicate for each condition. Animals were sacrificed by

cervical dislocation and eyed collected in cold PBS and kept on ice. A hole was done at the level of the *ora serrata* and cornea was gently cut and removed with the lens. Retina was, then, carefully pulled out from eyecup and placed in TRI Reagent® for total RNA extraction. Around 3 µg of RNA was obtained for each eye. The quality and the amount of RNA were verified for each sample by gel electrophoresis. For semi-quantitative RT-PCR, cDNA first strands were synthesized using 1 µg of RNA, 5 units of MLV reverse transcriptase (Promega) and 100 ng of random hexamers. The product of the reaction was diluted in 50 µl (final concentration around 20 ng/µl, assuming reverse transcription efficiency to be 100%). One µl or RT reaction was used for the next amplification step, using Taq Polymerase and specific genes – oligonucleotides (Table II). An initial denaturation was performed at 94°C for 3 minutes, followed by 30 cycles with a denaturation step at 94°C for 15 seconds, an annealing step at 60°C for 30 seconds and an extension step at 72°C for 30 seconds. An additive long extension step was added at the end of the 30 cycles (72°C for 5 minutes). For RNA-seq analyses, 1 µg of RNA was sent to the GenomEast Platform (Illkirch, France). RNA-Seq libraries were prepared from 500 ng of total RNA using TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA), according to manufacturer's instructions. Briefly, following purification with poly-T oligo attached magnetic beads, the mRNA was fragmented using divalent cations at 94°C for 2 minutes. The cleaved RNA fragments were copied into first strand cDNA using a reverse transcriptase and random primers. Strand specificity was obtained by replacing dTTP with dUTP during second strand cDNA synthesis using DNA Polymerase I and RNase H. Following addition of a single 'A' base and subsequent ligation of the adapter on double-stranded cDNA fragments, the products were purified and enriched with PCR (30 seconds at 98°C; [10 seconds at 98°C, 30 seconds at 60°C, 30 seconds at 72°C] x 12 cycles; 5 minutes at 72°C) to create the cDNA library. Surplus PCR primers were further removed by purification using AMPure XP beads (Beckman-Coulter, Villepinte, France) and the final cDNA libraries were checked for quality and quantified using capillary electrophoresis. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14 For alignment, reads were mapped onto mm10 assembly of the mouse genome using STAR v.2.5.3a. HTSeq v.0.6.1p1 software (union mode) and Ensembl 93 software were used for gene expression quantification and annotation.

RNA-Seq data analyses

Differential gene expression analyses were done comparing each KO stage with control and comparing different KO stages between them. For each comparison, genes were sorted according to specific logFC (fold change) value and adjusted p-value. The list of ranked genes was, then, loaded and processed on Gene Ontology (www.geneontology.org), ImPALA (impala.molgen.mpg.de) and GSEA (software.broadinstitute.org/gsea/index.jsp) for gene enrichment and pathway analyses. Using, then, ClueGO (apps.cytoscape.org/apps/cluego), a Cytoscape plugin, it was possible to group genes in groups as the same as Gene Ontology. Each group was corresponding to a specific biological process or biological function with a corresponding GO identifier. The power of ClueGO anyway is to connect these different groups and do clusters generating a network between different biological processes that are known to be similar or correlated.

Cell cultures and transient transfection

HeLa and Cos7 lines were used for *in vitro* experiments. Cells were maintained in medium supplemented with 10% of Foetal Bovine Serum, 100 units/ml penicillin/100 µg/ml streptomycin and 2 mM L-glutamine. For transient transfection, 4 millions of cells were plated in a 10cm culture dish on day 1. The next day cells were around 80% of confluence. Plasmids coding for genes of interests and Polyethylenimine (PEI) were diluted in Optimem in a ration between 1:3 (w/w). This ratio in our hand ensured good gene expression and minimum cell mortality. Except where specified, transfected cells were incubated two days before being used for the next steps.

Protein extraction and western blot analyses

For protein expression analyses, transiently transfected cells were scraped from the surface of the plate with a silicon policeman, washed with Mg^{2+} and Ca^{2+} free PBS and incubated in lysis buffer (Tris 50 mM pH 7.5, NaCl 150 mM, Glycerol 10%, EDTA 5 mM, NP40 0.1%, protease inhibitors) 30 minutes on ice. A sonication (40% amplitude 10 cycles 2 sec ON; 1 sec OFF) step was applied to fragment DNA and complete cell lysis. Proteins were concentrated in the supernatant aqueous phase and separated from cell debris by a centrifugation step. Bradford assay was used to estimate concentration (Bio-Rad Protein

Assay Dye Reagent Concentrate, 450 ml #5000006). For western blot in denaturing conditions, a 4% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel were prepared. Protein samples were mixed with Laemmli Buffer and fresh DTT 1mM and denatured 10 minutes at 95°C before loading. Migration was performed for 2 hours a 100V. Separated proteins were transferred on PVDF membrane (Immobilion P, Millipore, IPVH00010) and blocked with a blocking solution [5% milk powder in TBST (TBS plus Tween 20 0.1%)], 30 minutes at room temperature. Milk concentration was decreased to 2% when primary antibodies were diluted to the solution. Membranes were incubated overnight at 4°C in slight agitation. The day after, membranes were washed 3 times in TBST, 10 minutes each and incubated 2 hours with a solution of HRP-conjugated secondary antibodies diluted in 2% milk powder in TBST. A list of primary and secondary antibodies used for western blot analyses is furnished in Table III.

After 3 washes of 10 minutes in TBST, membranes were incubated with HRP chemiluminescent substrates (Immobilon Western, Millipore, P90720) for signal revelation and pictures were acquired with Fusion FX7 machinery. Exposure time was adjusted according to the expressed gene and to the antibodies.

Slot blot to detect AP-activity in conditioned medium

Cos7 cells were transiently transfected with expression vectors carrying coding for SEAP (Secreted Alkaline Phosphatase) or SEAP fusion proteins (see next paragraphs). To verify if the protein was secreted a slot blot was performed to verify if specific SEAP activity was detectable in conditioned medium 1 and 2 days after transient transfection. The medium was collected from plates, centrifuged to remove debris and supernatant was filtered with 0.45 µm filters to remove any other contaminant cell. Part of the sample was heated at 65°C for 20 minutes to inactivate endogenous phosphatases and, then, serial dilutions of heated and not heated medium were loaded and fixed on a PVDF membrane (same reference as for western blot analyses) using the Vacuum blotting system (Core Life Science, 21052014). After samples were fixed, the membrane was incubated with an alkaline buffer (Tris 100 m/m pH 9.4, NaCl 100 mM and $MgCl_2$ 5 mM) for 30 minutes. AP-substrates NBT/BCIP were, then, added to the solution as substrates for SEAP and the membrane was incubated for 10 minutes at room temperature.

AP activity detection *in vitro*

The same transiently transfected COS7 cells were also checked for AP enzymatic activity by colorimetric staining. Conditioned medium was removed and cells washed extensively with Mg^{2+} and Ca^{2} free PBS deprived of $^{+}$. After, cells were fixed with PFA 4% in PBS for 10 minutes. Excess of PFA was removed, by extensively washing plates with PBS. Then, cells were left with a slight coverture of PBS and heated at 65°C to inactivate endogenous phosphatases. Cells were, then, incubated with an alkaline solution (Tris 100 m/m pH 9.4, NaCl 100 mM and $MgCl_2$ 5 mM) for 30 minutes. Finally, AP-substrates NBT/BCIP were added to the solution as substrates for SEAP and incubated for 20 – 30 minutes at 37°C or overnight at 4°C.

SEAP Reporter Gene Assay

Transcriptional activity of the tagged Otx proteins was assayed using the region -66 to +68 of the *IRBP* promoter (Fong and Fong, 1999) cloned into the pSEAP2-basic vector (BD Biosciences, Palo Alto, CA, USA) as previously described (Courtois et al. 2003). The pSEAP2-Basic (promoter-less) and pSEAP2-Control (SV40 promoter) vectors were used as negative and positive controls respectively. In standard assays, 10^5 HeLa cells per well were seeded in 24 wells plates were transfected by the PEI method with 0.2 µg IRBP-SEAP, 0.1 µg GFP- or Otx2 wild type- or tagged-Otx2 expression vector and 0.05 µg pTK-Luciferase. After 40 hours of incubation, secreted alkaline phosphatase (SEAP) activity was measured in a plate reader using a CSPD assay (Promega) and normalized with luciferase activity. Normalized SEAP background in the absence of Otx2 expression vector was taken as 1-fold activation. Three independent experiments were done in duplicate to generate each data.

Construct generation and recombinant AAV vector production

Different tagged forms of Otx2 were cloned under the RPE-specific *Vmd2* promoter and the resulting vectors were used to produce recombinant AAV (rAAV) 2/DJ serotype, using the AAV- DJ Helper Free Packaging System kit (Cell Biolabs Inc.). The AAV-derived expression vector, referred to as pTR-Vmd2-GFP (a gift of professor Hauswirth WW) contained the *Vmd2* minimal promoter (Esumi et al. 2004) upstream the coding sequence of the reporter GFP. Not I digestion was used to cut off the GFP coding sequence from this plasmid and the

remaining cohesive extremities, present in the plasmid backbone, were used for the next cloning steps of the other constructs of interest. In total, 4 different constructs were produced and ligated under the *Vmd2* promoter.

The first construct, an Otx2 fused at the N-terminus with an HA tag and at the C-terminus with 4 repeated in tandem FLAG tags [referred to as HA-Otx2-(M2x4)], was derived from a pcDNA3-based expression vector already present in the laboratory. In this plasmid a Not I site was present downstream the stop codon. To excise the full coding sequence and move it under the *Vmd2* promoter, an additive Not I site was inserted upstream the coding sequence and Kozak region. To do that, the original plasmid was linearized with EcoR I, present upstream the *HA-Otx2-(M2x4*) coding sequence. Complementary oligonucleotides, containing the new Not I flanked by cohesive EcoR I compatible extremities were, then, cloned into this linearized plasmid. Therefore, the newly obtained plasmid was again cut with Not I site and purified Not I fragment, corresponding to the Kozak region and *HA-Otx2-(M2x4)* sequence was ligated with the plasmid backbone containing *Vmd2* promoter.

The second construct corresponds to the double mutant YL-Otx2 (W85Y/F86L). The construct was produced by mutagenesis PCR, using *HA-Otx2-(M2x4)* as a template. PCR product contained cohesive Not I-compatible extremities at the 5' and 3' regions, used to clone it in the pSK+ plasmid for sequencing the PCR product and, then, to replace GFP under *Vmd2* promoter in the AAV-derived expression vector.

The third construct corresponds to a *SEAP*, Secreted Alkaline Phosphatase. As for the construct 1, the plasmid was already generated in the lab and cloned in a pSK+ backbone. The *SEAP* coding sequence was first PCR amplified using this bacterial vector as template and primers introducing an EcoR I and a Not I site in the 5' region of the coding sequence and an additive EcoR I site at the 3' end. The EcoR I digested PCR product was inserted into an EcoR I-digested pcDNA3 plasmid to obtain a eukaryotic SEAP expression vector that was used for further steps. The Not I site introduced in the 5' region and the one present in the pcDNA3 vector downstream the insert were used to clone the construct under the *Vmd2* promoter, replacing the excised GFP.

The fourth construct produced is coding for a SEAP-Otx2 fusion protein. In detail, SEAP was fused at the N-terminus of HA-Otx2-(M2x4). In the first step the *SEAP* sequence, lacking its translational stop codon, was amplified by PCR using the same template than for construct 3). During the PCR step, an EcoR I and a Not I site were introduced in the 5' region of the

coding sequence. At the 3' an additive EcoR I was introduced. In the acceptor pcDNA3 plasmid, the HA-Otx2-(M2x4) sequence is flanked by an EcoR I site at the 5' side and a Not I site at the 3' side. EcoR I digested PCR product was purified and ligated with the EcoR I digested acceptor plasmid generating a fusion *SEAP-Otx2* sequence under a *CMV* promoter. This vector was used for further tests. Finally, the Not I site at the 5' side of the SEAP and the one at the 3' side of HA-Otx2-(M2x4) were used to clone the final construct under the *Vmd2* promoter, replacing GFP in the AAV-derived expression vector.

Once produced and verified, these plasmids were transiently transfected into 293AAV cells together with an AAV-Helper plasmid and an AAV-DJ REP-CAP plasmid. Recombinant AAVs were, then, purified and titrated according to the manufacturer instructions (VPK- 400- DJ for purification kit reference; VPK-145-T for quantification kit reference, Cell Biolabs, Inc.). For each AAV, two starting 10 cm plates were transfected and used for particles production. The average of concentration obtained was 5x10^8 genome copies /µl.

Sub-retinal injection

Before sub-retinal injection, mice were anesthetized with a mix containing tiletamine, zolazepam, xylazine, buprenorphine 80mg/Kg (10 µl/g of weight). Mice started to sleep 2 minutes later. One drop per eye of Atropine 1% was then administrated. One minute later, one drop per eye of neosinephrine 1% was added on the eye to dilate pupils. Finally, one drop per eye of tetracaine 1% was administrated to complete local anaesthesia. Mice were then positioned on a heated platform to maintain body temperature around 37°C. For particles injection, mice were positioned under a binocular. AAV vectors were loaded in a Hamilton syringe and injected in the space between the RPE and the neural retina. For each mouse, only the right eye was injected with 10^9 gc in a maximum of 2 µl of AAV vector suspension. The extension of the bubble generated under the neural retina was used as an index on the efficiency of injection.

Immunocytochemistry and histological analyses

Eyes were collected and placed in cold PBS, where a hole was done at the level of the *ora serrata*. Then, eyes were fixed in 4% PFA in PBS for 2 hours at room temperature. Excess of PFA was removed by rinsing 3 times fixed eyes in PBS. Samples were cryo-protected overnight at 4°C in 20% sucrose in PBS and embedded in OCT. Samples were conserved at -

80°C until they were processed. Sections of 14 µm were prepared on the cryostat and slides were kept at -80°C until they were used.

For immunocytochemistry analyses, frozen slides were re-equilibrated at room temperature to air-dry them. Samples were washed, then, in PBST (PBS with 0.1% Triton X-100; 3 washes of 5 minutes), pre-incubated for 30 minutes in Blocking Solution (PBST with 10% Foetal Bovine Serum) and incubated overnight at 4°C with primary antibodies, diluted in Blocking Solution. After three washes in PBST of 10 minutes, slides were incubated 2 hours at room temperature with secondary antibodies diluted in Blocking Solution. For a complete list of primary and secondary antibodies and corresponding dilution see Table IV. Secondary antibodies were conjugated with fluorophores. A counterstaining of cell nuclei was obtained by incubating specimens with a solution of 1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS. Samples were rinsed in PBS and mounted. Images were acquired with the wide-field microscope "Axiofluo: AxioPlan Zeiss / Black and White Camera for fluorescence" or laser scanning confocal microscopes Leica SP5 and Zeiss LSM780. Photos were, then, analysed with the software Fiji (Fiji is ImageJ).

For histological analyses, slides were thawed, equilibrated at room temperature, incubated 30 minutes in PBS and post-fixed in Formalin for 20 minutes at room temperature. Excess of formalin was removed by 3 washes of 5 minutes in PBS. Specimens were then stained in the Haematoxylin solution for 50 seconds. Excess was removed by rinsing specimens in 3 following boxes containing tap water. A blueing step of 1 minute in 0.1% Sodium Bicarbonate solution was performed to shape nuclear haematoxylin staining. Specimens were dehydrated by a series of ethanol dilutions (3 washes of 1 minute in 50% ethanol; 2 washes of 1 minute in 70% ethanol) and stained 3 minutes with a solution of Eosin in 70% ethanol. The excess of eosin was removed by rinsing slides in 50% ethanol and quickly mounted with Mowiol solution. Pictures were acquired with the Axiocolor: AxioPlan Zeiss / Colour camera for coloration and analysed with ImageJ and Phenochart 1.0.8.

AAV-driven SEAP and SEAP-Otx2 expression analyses on retinal sections

Expression and localization of SEAP and SEAP-Otx2 constructs, expressed by the sub-retinal injected AAV under Vmd2 promoter, was verified by an NBT/BCIP colorimetric assay, based on the residual SEAP enzymatic activity. As for immunocytochemistry, eyes were collected

in PBS, fixed 2 hours at room temperature in PFA 4% in PBS, washed in PBS, cryoprotected in sucrose 20% in PBS (overnight at 4°C) and embedded in OCT. Sections of 14 µM were prepared with the cryostat and stored at -80°C. On the day of the assay, the slides were air-dried 1 hour at room temperature and washed 3 times for 5 minutes in PBS. Endogenous phosphatases were inactivated by heating specimens 20 minutes at 65°C. To ensure a better contrast of the NBT/BCIP staining in the RPE layer, a step of pigment bleaching was required. To do that, specimens were incubated with a Bleaching solution (Formamide 5%, SSC 0.5X, H2O2 7.5%), until the RPE appeared light brown under binocular (5 – 10 minutes). Slides were then washed 3 times for 5 minutes in PBS and, then, equilibrated 1 hour in alkaline solution NTMT (NaCl 100 mM, Tris 100 mM pH 9.5, Tween X-20 1%, MgCl2 50 mM). After, AP-substrates NBT/BCIP were added to the solution. Slides were incubated 10 – 15 minutes at 37°C or overnight at 4°C until the signal was well detectable and distinguishable from the background. Finally, slides were mounted. Pictures were acquired at the Axiocolor:AxioPlan Zeiss / Colour camera.

Light/Dark adaptation and behavioural tests

All behavioural tests were done on littermates of 7-10 weeks to minimize background effects. Before starting procedures, mice were acclimated in a behavioural room for 30 minutes.

For Light Dark Box (LDB) test, mice were individually tested for a total of 25 minutes in a dark/light box. The box is composed of 2 compartments: one, kept with bright light (140 lux), of the following dimensions 19.5cm width (W) X 29.5cm length (L) X 30cm height (H), is white painted and lacks a lid to close the top; the other, kept in the dark, has sizes of 19.5 W X 14.5 L X 30 H cm, and is black painted and fully enclosed. A small passage (5X5 cm) connects the two compartments and allows the mouse to change the compartment freely. Before testing, mice were acclimated 30 minutes in their cages in the testing room in the dark. Each mouse was placed in the centre of the lighted compartment, facing the dark side. Mice were video tracked for a total time of 25 minutes by EthoVision software. During the first 10 minutes, mice were let free to explore the light/dark box. Then, an object (white plastic cylinder of 5cm diameter, 4cm height) was placed in the right corner of the lighted compartment at the opposite side to the dark one for 5 minutes. Next, the same object was

moved to the right corner of the dark compartment at the opposite site of the lighted one. During the last 5 minutes, no object was present in the box. The video tracking system was used to monitor the behaviour and quantify the time each mouse spent in the light side versus dark side all over the test.

For the open-field test, each mouse was placed in a moderate-lighted (60 lux) square arena (40 W X 40 L X 30 H cm), surrounded by transparent walls to prevent animal escape. Animals were individually positioned in the centre of the arena and video tracked over 10 minutes by EthoVision software. Time spent in centre and in the periphery are measured (periphery was set as 5 cm) as well as distance mice travelled and their velocity. Anxiety-related behaviour is measured by the degree to which the mouse avoids the central area.

Table I: oligonucleotides used in genotyping procedure

Gene	Forward oligonucleotide	Reverse oligonucleotide	Additive oligonucleotide (if any*)
Floxed-Otx2	GAACAAACGTCCCTGTGGTG	AGGAGCCACAATTCCCATC	ACCAAATGAGGTATGGCCTTG
CreERT2	TGGCGATCTCGAGCCATCTG	ATGCTGTACAGATGCTCCA	
TdTomato	AAGGGAGCTGCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC	

Table II: oligonucleotides used in RT semi-quantitative PCR

Gene	Forward oligonucleotide	Reverse oligonucleotide
Tyrosinase	ATTGATTTTGCCCATGAAGCA	TTCCATCGCATAAAACCTGAT
Tyrp-1	TTCACTGATGCGGTCTTTGA	CGAAAATGGCAGCTACAAGT
HGPRT	ATGAGTACTTCAGGGATTTGA	TAAGCGACAATCTACCAGAG

Table III: primary and secondary antibodies for western blot

Primary antibodies

ANTIGEN	DILUTION	COMPANY	CAT. NUMBER
Otx2 (goat)	1/2000	R&D Systems	AF1979
HA (mouse)	1/5000	Covance/Eurogentec	MMS-101R
M2 (for FLAG tag) (mouse)	1/5000	Sigma	F3165

HRP conjugated secondary antibodies

ANTIGEN	DILUTION	COMPANY	CAT. NUMBER
Goat	1:10000	Thermo Fisher	A5420
Mouse	1/5000	Pierce	185413

Table IV: primary and secondary antibodies for immunocytochemistry

Primary antibodies

ANTIGEN	DILUTION	COMPANY	CAT. NUMBER
Otx2 (goat)	1/500	R&D Systems	AF1979
HA (mouse)	1/500	Covance/Eurogentec	MMS-101R
M2 (mouse)	1/500	Sigma	F3165
Arrestin (mouse)	1/10000	Santa Cruz	sc-166383
Rhodopsin (rabbit)	1/1000	Genetex	GTX129910
M-Opsin (rabbit)	1/1000	Abcam	AB5405
S-Opsin (rabbit)	1/1000	Abcam	AB5407
Chx10 (mouse)	1/250	Santa Cruz	sc-365519
Crx (sheep)	1/500	R&D Systems	AF7085
Crx (rabbit)	1/250	Sigma	HPA036762
Brn3A (goat)	1/250	Abcam	AB144
Sox9 (rabbit)	1/500	Merck Millipore	AB5535
Pax6 (rabbit)	1/500	Merck Millipore	AB2237
Syntaxin 1 (mouse)	1/1000	Santa Cruz	sc-12736
GS (mouse)	1/1000	BD Sciences	610517
Calbindin (mouse)	1/500	Sigma	C9848
ER (rabbit)	1/200	Thermo Scientific	RM9101-SO
DS-Red (rabbit)	1/500	Takara (OZYME)	632496
GFP (chicken)	1/1000	Avés Lab	GFP-1020

Secondary antibodies (conjugated fluorophore)

ANTIGEN	DILUTION	COMPANY	CAT. NUMBER
αRabbit (Cy3)	1/500	Jackson Immuno research	711-065-152
αRabbit (Cy5)	1/500	Jackson Immuno research	711-065-152
αRabbit (Alexa 488)	1/500	Jackson Immuno research	711-065-152
aGoat (Cy3)	1/500	Jackson Immuno research	705-165-147
aGoat (647)	1/500	Jackson Immuno research	705-165-147
aGoat (Alexa 488)	1/500	Jackson Immuno research	705-165-147
aMouse (647)	1/500	Jackson Immuno research	715-545-150
aMouse (Alexa 488)	1/500	Jackson Immuno research	715-545-150
aChicken (Alexa 488)	1/1000	Jackson Immuno research	703-545-155

All secondary antibodies were raised from Donkey

RESULTS

VI Role of Otx2 in photoreceptors of the mature retina

6.1 Characterization of the Crx-CreER^{T2};Otx2$^{flox/flox}$ mouse model

To study the role of the homeobox transcription factor Otx2 in photoreceptors of the mature retina, we used a conditional knockout (KO) mouse model, generated by crossing two previously published mouse line. The first one is a transgenic line, where expression of the tamoxifen-dependent CreER^{T2} recombinase is driven by a fragment containing ~2 kb of 5'-regulatory sequence and the core promoter of the photoreceptor-essential *Crx* (Cone-rod homeobox) gene (Furukawa et al., 2002). Although *Crx* was shown to be also expressed in a subset of bipolar cells (Glubrecht et al. 2009; Chen et al. 1997), this regulatory element is described to drive gene expression only in photoreceptors both at embryonic and adult stages (Koike et al. 2007; Muranishi et al. 2011; Kim et al. 2015). Therefore, the *Crx-CreER^{T2}* line appears to be a tool of choice for invalidating a gene only in photoreceptors. The second line is a floxed-*Otx2* mouse line (Fossat et al. 2006), where the coding exon 2 of *Otx2*, that encodes most of the homeodomain, is flanked by two *loxP* sites. Upon Cre-mediated recombination, the genomic sequence located between the *loxP* sites is deleted, which leads to knockout of the *Otx2* gene. The final result of the breeding is a *Crx-CreER^{T2};Otx2$^{flox/flox}$* mouse line (**Figure 18A**). We first analysed CreER^{T2} expression by immuno-detection on retinal sections (**Figure 18B**). CreER^{T2} expression was detected only in the photoreceptor layer, validating the driver line. To verify the efficiency of *Otx2* knockout, we then induced CreER^{T2} activity by a single intraperitoneal injection of tamoxifen and compared *Otx2* expression in control and KO retina 48h later. We found efficient *Otx2* KO in photoreceptors (**Figure 18C**). As expected, no recombination was observed in bipolar cells. Therefore, the *Crx-CreER^{T2};Otx2$^{flox/flox}$* genetic combination appears perfectly suited to study Otx2 function in photoreceptors.

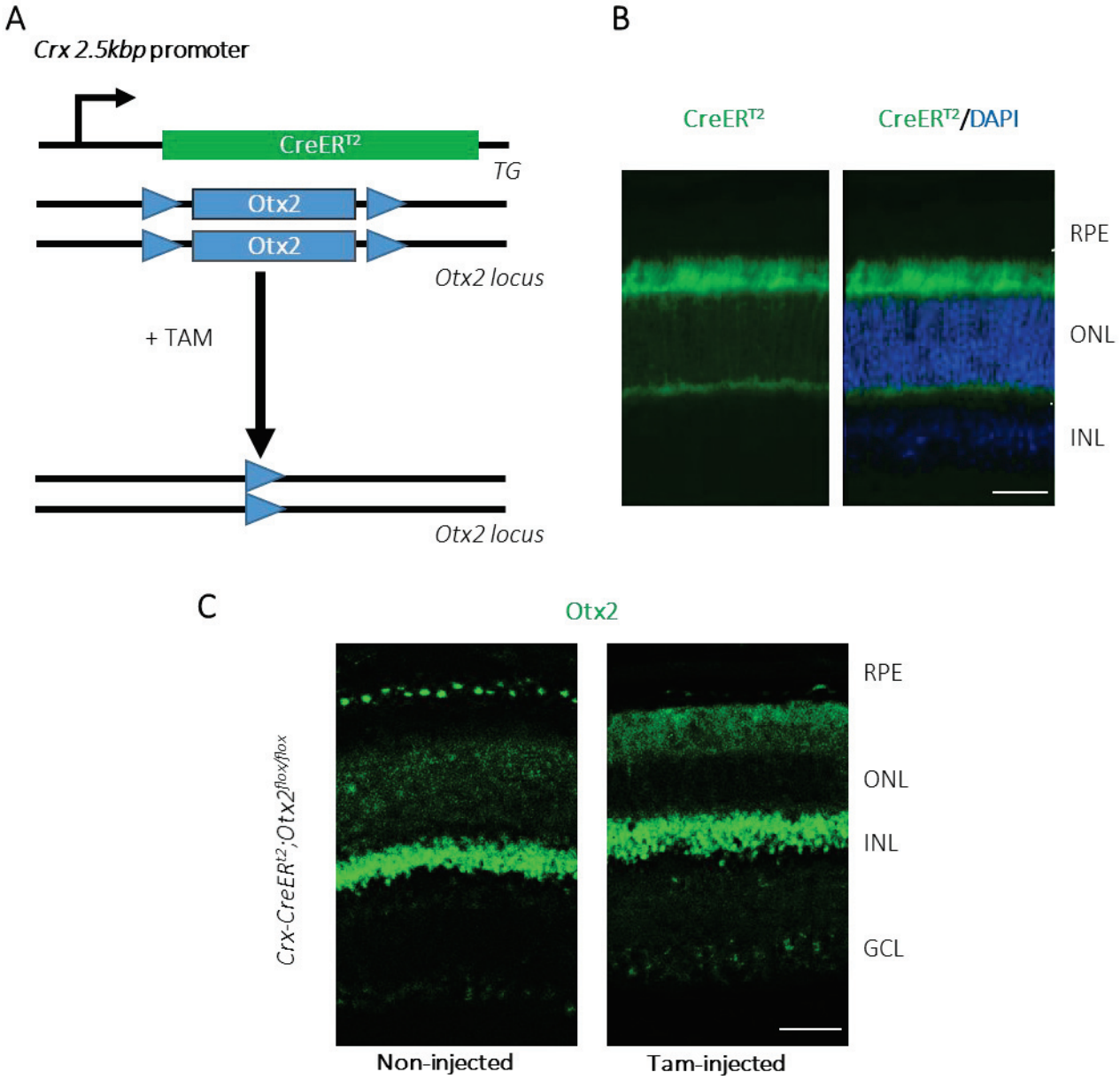


Figure 18. PR-specific Otx2 ablation. A) Schematic representation of the *Crx-CreER^{T2};Otx2$^{flox/flox}$* mouse line and knock out induction. The *CreER^{T2}* transgene (TG) is expressed under the control of the PR-specific *Crx* promoter. Both *Otx2* alleles bear two *loxP* sites (blue triangles) flanking the exon2. A single tamoxifen injection promotes *loxP* sites recombination and *Otx2* KO. B) ER immunostaining of adult retinal sections: CreER^{T2} is specifically detected in ONL C) Immunodetection of Otx2 in non-injected (left) and tam-injected (right) P32 mice. Otx2 is expressed in RPE, photoreceptors (ONL) and bipolar cells (INL) in *Crx-CreER^{T2};Otx2$^{flox/flox}$*. Tam-injected mice show specific Otx2 ablation in the ONL, 2 days after injection. RPE = Retinal Pigmented Epithelium; ONL = Outer Nuclear Layer; INL = Inner Nuclear Layer; GCL = Ganglion Cell Layer. Scale bar = 50 μm

In the course of theses analyses, we noticed that *CreER^{T2}* expression could be mosaic, although it was always restricted to the photoreceptor layer. Indeed, while in some mice almost 100% of photoreceptor cells were expressing the recombinase, in other animals the proportion of labelled cells could fall below 50%, with a columnar alternance of positive and negative photoreceptor cells. This suggested the possibility of stochastic epigenetic shutoff of the transgene in early retinal precursors. The mosaicism did not depend on the sex and, moreover, mice from the same litters exhibited variation of mosaicism. The consequence was that *Otx2* ablation was inefficient or absent in retinal regions where photoreceptors lacked CreER^{T2} recombinase (**Figure 19, upper panel**). To overcome this problem, we developed an imaging software *macro* to select animals with minimal mosaicism. The macro, based on Fiji software, was designed to automatically measure the relative area of photoreceptors showing recombinase expression. Briefly, retinal sections from each sample were stained for CreER^{T2} and at least 10 sections, covering all levels of the retina, were analysed with the tool we developed (**Figure 19, lower panel**). As CreER^{T2} recombinase accumulated in all the photoreceptors compartments (outer segments OS, inner segments IS, soma and outer plexiform layer OPL, **Figure 18B**), we first measured the regions corresponding to OS + IS. For each section, the ratio between OS + IS stained area and total OS + IS area was used as a first index of mosaicism. To have a second index, in parallel we did theses analyses also on OPL. An average of the two ratios on the 10 sections was then used to estimate the percentage of photoreceptors that were expressing recombinase. Using this tool, we preferentially selected mice expressing *CreER^{T2}* in almost 100% of photoreceptors, and if it was not the case, we set a threshold to select mice with a minimum of 75% positive photoreceptors.

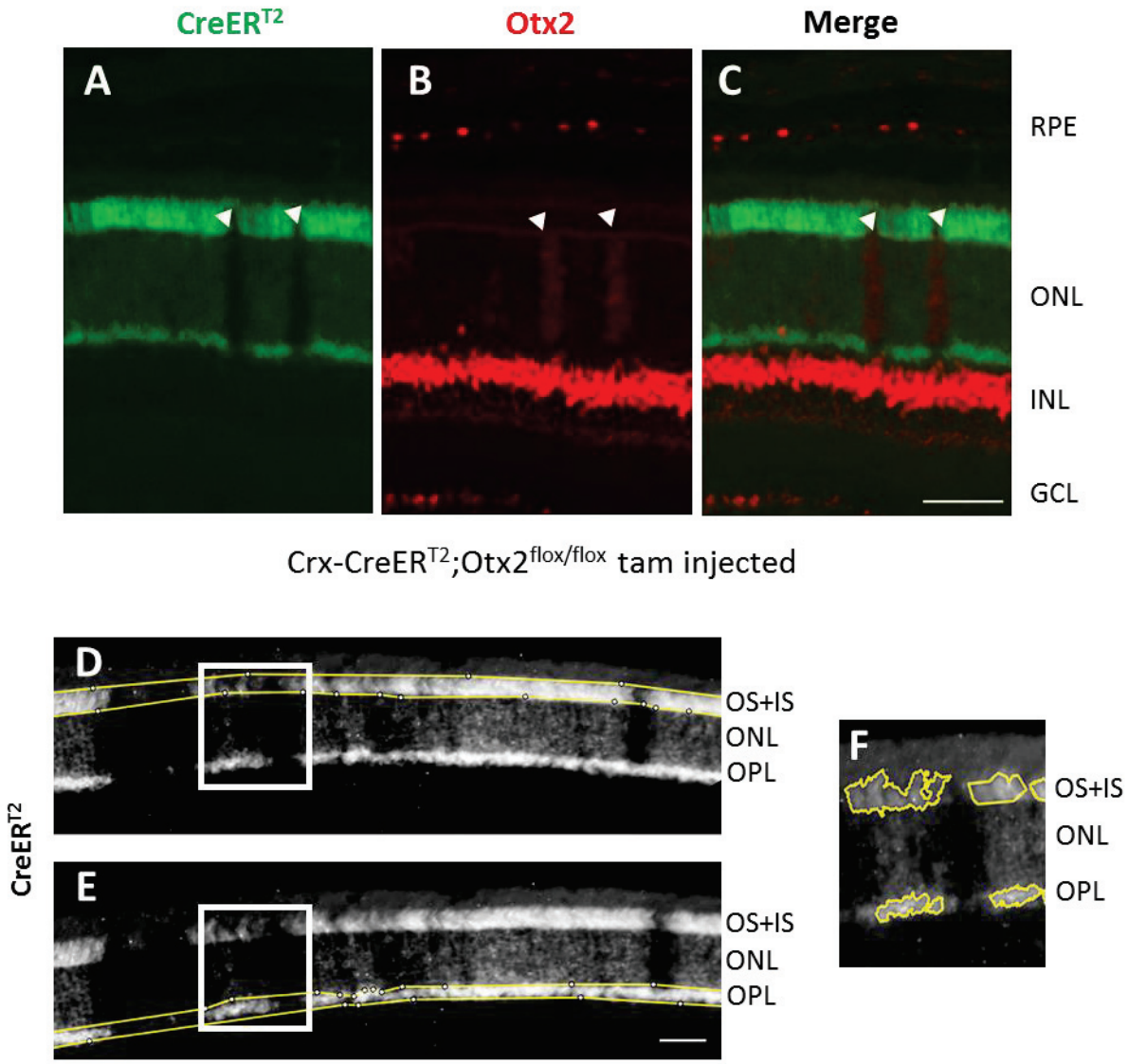


Figure 19. Mosaic expression of the Crx-CreER^{T2} transgene. A) CreER^{T2} expression, detected by immunostaining on retinal sections, showing mosaicism. B) In the ONL regions where CreER^{T2} is not expressed, Otx2 is not knocked out after tam-injection (arrowheads). C) merge. D-F) ImageJ tool to measure of CreER^{T2} mosaicism: total OS + IS (D) and OPL (E) area was measured on retinal sections stained for CreER^{T2}. CreER^{T2} stained areas (F) from each region were measured. Square (D-E) represents the area showed in F. RPE = Retinal Pigmented Epithelium; ONL = Outer Nuclear Layer; INL = Inner Nuclear Layer; GCL = Ganglion Cellular Layer; OS = Outer segment; IS = Inner Segment; OPL = Outer Plexiform Layer. Scale bar = 50 µm

6.2 *Otx2* ablation in photoreceptors does not affect cell viability in the retina

Having validated the *Cre-CreER*^{T2} driver line, we first asked whether PR-specific *Otx2* ablation affected photoreceptor viability since *Otx2* full KO in the adult retina has been shown to have an important impact on photoreceptor maintenance. Indeed, simultaneous Otx2 ablation in all *Otx2*-expressing populations (RPE, photoreceptors and bipolar cells) promotes a slow and complete degeneration of both rods and cones in about 3 months (Béby et al. 2010). Interestingly, in this condition, *Otx2* expression restricted to the RPE was sufficient to rescue photoreceptors and, conversely, when *Otx2* KO was restricted to the RPE, photoreceptors degenerated at the same pace as in full retina KO, despite their maintained expression of *Otx2* (Housset et al. 2013). These data strongly support the existence of a neuroprotective mechanism based on *Otx2* expression in the RPE, while they suggest that endogenous *Otx2* has no essential role in photoreceptor cell viability. To verify this hypothesis, we promoted *Otx2* KO at P30 by single tamoxifen injection and collected eyes 10, 30, 60 and 90 days after KO induction). For each stage, samples were collected and analysed in triplicate. Eyes from uninjected mice were collected at the same stage and used as controls. Histology was examined by Haematoxylin / Eosin staining of cryostat sections (Figure 20A). The thickness of the Outer Nuclear Layer (ONL) was measured to obtain an indication of photoreceptor maintenance. In the full KO reported in Béby et al. (2010) degeneration starts at P50, almost 20 days after KO induction with a detachment of the photoreceptors from the RPE. Cell death reaches a peak around P60 and ONL thickness is gradually reduced, to disappear completely at P120. In our model of photoreceptors-specific *Otx2* KO, we never observed rod and cone detachment from the RPE. The reduction of ONL thickness is low and comparable to that of control mice at the different time points, confirming that it is only a consequence of retina aging.

To further validate that, a TUNEL assay was performed at the same time points tested by haematoxylin/eosin staining. The assay confirmed that no cells were dying by apoptosis in the ONL of KO mice. (Figure 20B). These observations, together, confirm that endogenous *Otx2* does not play a role in photoreceptor maintenance.

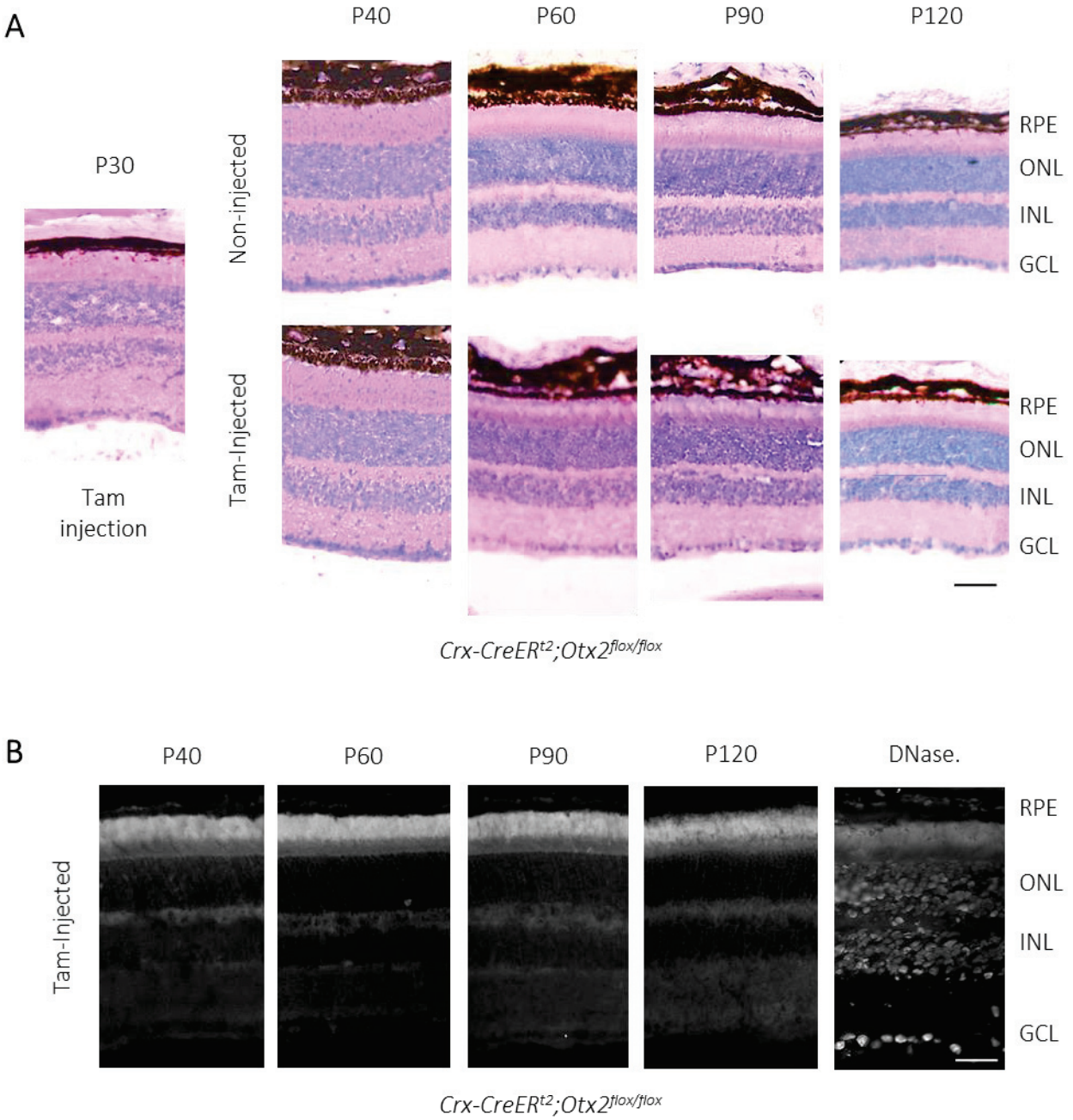
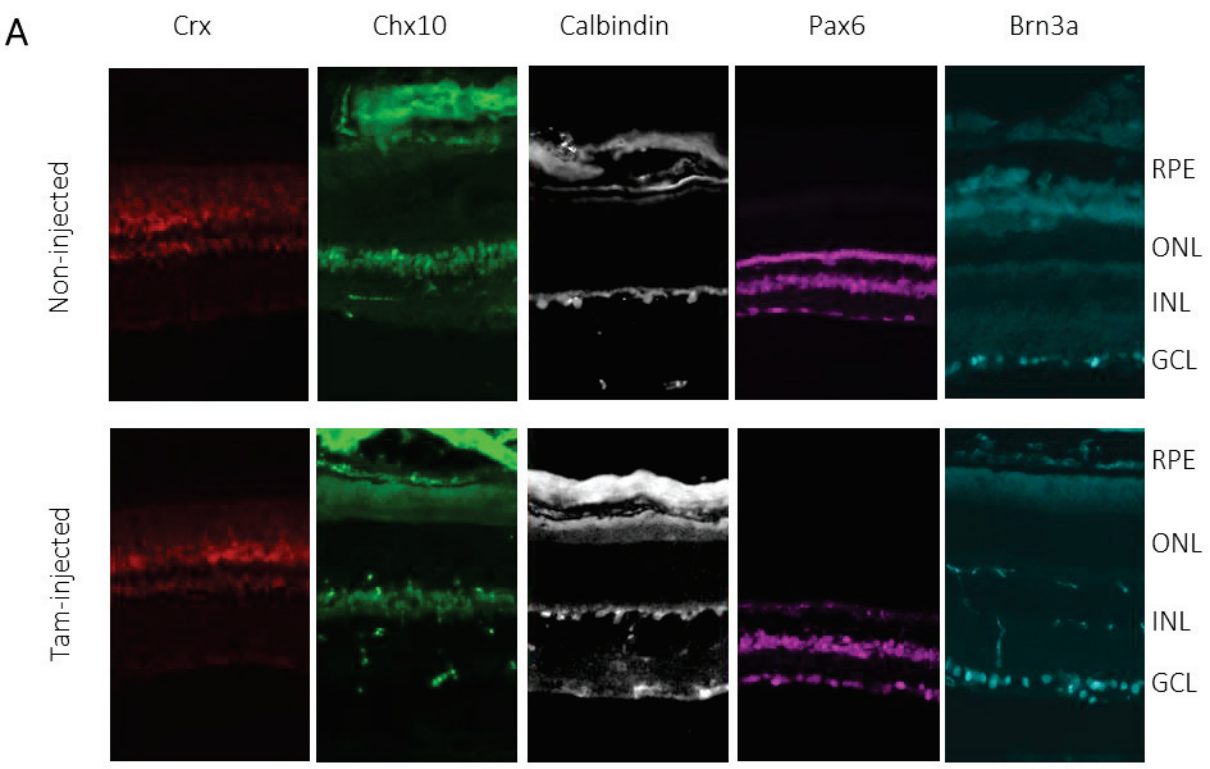


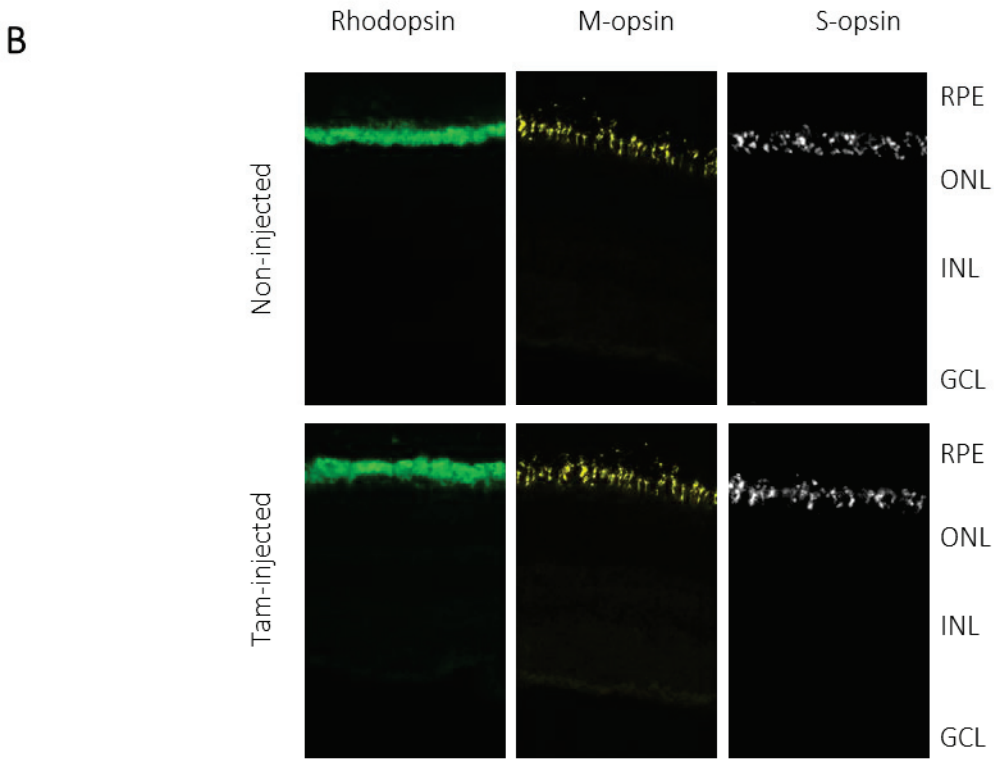
Figure 20. Otx2 expression in photoreceptors is not required for cell viability. A) Haematoxylin/eosin staining on retinal section of control *Crx-CreER^{t2};Otx2$^{flox/flox}$* not injected mice (top panel) and knock out *Crx-CreER^{t2};Otx2$^{flox/flox}$* mice, tamoxinfen injected at P30 at the indicated stages B) TUNEL staining of retinal sections of KO mice 10 (P40), 30 (P60), 60 (P90) and 90 (P120) days after tamoxifen injection (P30). DNAse treated retinal section of adult mouse (P32) was used as a positive control (right). RPE = Retinal Pigmented Epithelium; ONL = Outer Nuclear Layer; INL = Inner Nuclear Layer; GCL = Ganglion Cell Layer. Scale bar = 50 µm

6.3 *Otx2* ablation in photoreceptors does not modify cell identity in the retina

The absence of Otx2 in photoreceptors does not affect their viability. However, homeobox genes are well known to be involved in the acquisition, but also maintenance of cell identity (Holland et al. 2007; Bürglin & Affolter 2016). During embryogenesis, *Otx2* is required to specify a pool of retinal precursors toward photoreceptor/bipolar fate and its KO in such subpopulation promotes an identity switch, converting them into precursors of amacrine cells (Nishida et al. 2003). To test whether, in the *Crx-CreER*^{T2} KO line, cells of the ONL keep their photoreceptor identity and whether other retinal neuronal populations are affected by PR-specific *Otx2* ablation, we performed staining for different retinal populations markers in control and PRs-KO mice 30 days after tamoxifen injection (P60). The explored markers were: *Crx* (all photoreceptors), Chx10 (bipolar cells), calbindin (horizontal cells), Pax6 (amacrine cells), Brn3a (retinal ganglion cells) (Figure 21A). Specific markers for rods or cones were also evaluated. No difference appeared between control and KO retinas; in fact, the cells of the outer nuclear layer retained expression of major photoreceptors markers (*Crx*, rhodopsin and cones opsins) (Figure 21B), whereas no other populations markers were detectable in this layer. This confirmed that photoreceptor cell identity is not affected in the absence of *Otx2*. Moreover, no expansion of other cells populations was observed, suggesting that the classic cell proportion and lamination of the retina is maintained. Together, these results exclude a role of *Otx2* in the maintenance of cell identity of photoreceptor and other neuronal populations of the retina.



P60 *Crx-CreER^{T2};Otx2$^{flox/flox}$*



P60 *Crx-CreER^{T2};Otx2$^{flox/flox}$*

Figure 21. Loss of Otx2 expression in photoreceptors does not affect the identity of retinal cells. A) Expression of cell specific markers Crx (photoreceptors), Chx10 (bipolar cells); Pax6 (amacrine cells and retinal ganglion cells); Brn3a (retinal ganglion cells) in control upper panels and PR-specific Otx2 KO retinas (lower panels. B) Expression of rod (rhodopsin) and cones (S- and M- opsin) in P60 control (top panel) and tam-injected at P30 (lower panel) *Crx-CreER^{t2};Otx2$^{flox/flox}$* mice. Scale bar = 50 µm

6.4 Otx2 ablation in photoreceptors affects visual arrestin redistribution and light adaptation

The absence of Otx2 in photoreceptors neither affects their viability nor their identity. Yet, this factor is produced constitutively throughout life by these cells, suggesting that it plays a role. As photoreceptors are specialized neurons whose unique function is light detection and conversion into a nervous message, we set out to examine whether Otx2 ablation affects this function.

Several techniques are used to investigate different aspects of photoreceptor functionality. For instance, ERG response is used to have a general view of the light response in the retina, while the optomotor test is preferentially used to test visual acuity. Unfortunately, for practical and technical local reasons, we could not perform these tests on PR-specific *Otx2* KO mice. However, we could address the functionality of phototransduction and vision parameters, studying the movement of arrestin in KO retinas. This movement is an interesting readout of retina physiology and vision, as it provides simultaneous information on photoreceptor response and on light adaptation. In the dark, arrestin localizes in the soma and inner segments of photoreceptors. Prolonged illumination induces massive translocation of arrestin to the outer segment, where it binds and inactivate rhodopsin, preventing an over-stimulation of the phototransduction cascade. When light is off, arrestin goes back to the inner segment and other proximal cell compartments, while transducin, the principal activator of phototransduction, moves in the opposite direction, increasing the sensitivity of the photoreceptors. Defects of arrestin or transducin shuttling are susceptible to compromise light- and dark-adaptation.

6.4.1 Arrestin trafficking is impaired in Otx2 depleted photoreceptors

To study the movement of arrestin at convenient times, and to be able to collect at the same time light-adapted and dark-adapted mice, we shifted the 12 hours light-12 hours dark cycle of mice from 7 am - 7 pm to either noon-midnight or midnight-noon. Otx2 ablation was triggered at P30-P40 and mice were adapted to the new light/dark cycles. In order to exclude any circadian rhythm effect, a period of adaptation of at least 12 days was respected. The last day, mice were sacrificed and eyes collected 2 hours and 4 hours after light onset (light-adapted mice) or light offset (dark-adapted mice) (Figure 22). Dark-adapted mice were killed under red dim light. Cryostat sections were stained for arrestin and protein

localization was evaluated. At least 3 mice per condition were analysed. Two hours after light offset, arrestin was found in the inner segment and soma of photoreceptors in both control and KO, suggesting no change in dark-adaptation following *Otx2* ablation (Figure 23). Two hours after light onset, arrestin was almost fully localized in the outer segments in control mice, whereas in KO mice, the protein accumulated in inner segments and soma, with less than 50% of the protein in the outer segments. The situation was identical 4 hours after light offset and light onset. Indeed, no differences could be found between dark-adapted control and KO mice, while in the light-adapted mice, arrestin appeared mis localized in KO mice compared to control mice. To better understand if the protein movement was completely abolished or if the redistribution was only delayed, mice were also analysed 8 hours after light onset. The movement of arrestin was still impaired after light exposure and arrestin was partially accumulated in the soma and inner segments of the photoreceptors of the KO mice, while in the control arrestin was only found in the outer segments.

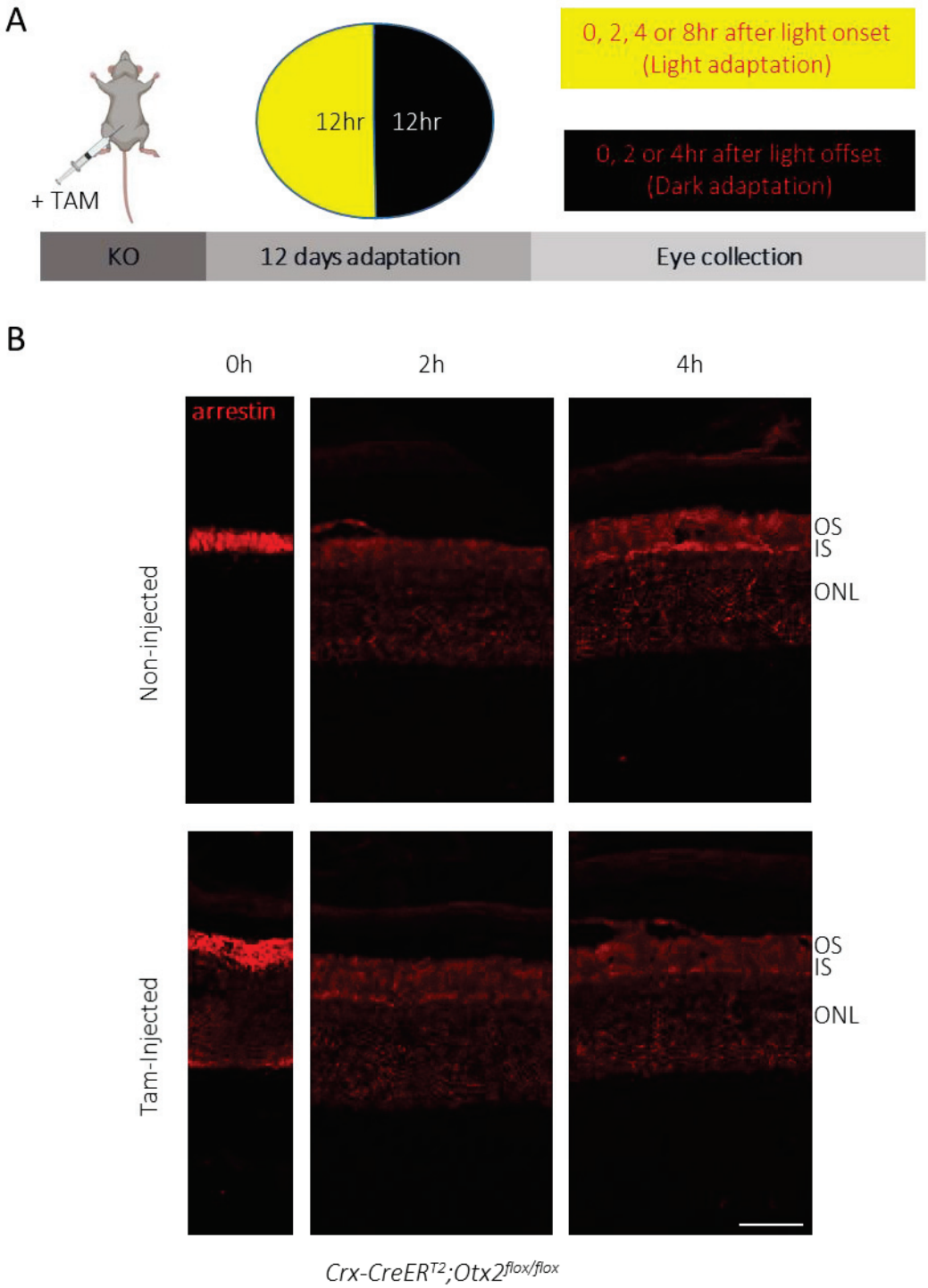


Figure 22. Arrestin localization is not affected by Otx2 KO in dark-adapted mice. A) Design of the experiment: mice were tam-injected or not at P30 to induce Otx2 KO in photoreceptors and adapted to a new light-dark cycle for 12 days. Retina were analysed 0, 2 or 4 hours after light offset or 0, 2, 4 or 8 hours after light onset. B) Arrestin localization in dark adapted mice: immunostaining on retinal sections of adult mice showing arrestin distribution 0, 2 and 4 hours after light offset in non injected (top panel) and tam-injected (lower panel) mice. OS = outer segments; IS = inner segment; ONL = outer nuclear layer. Scale bar = 50 µm

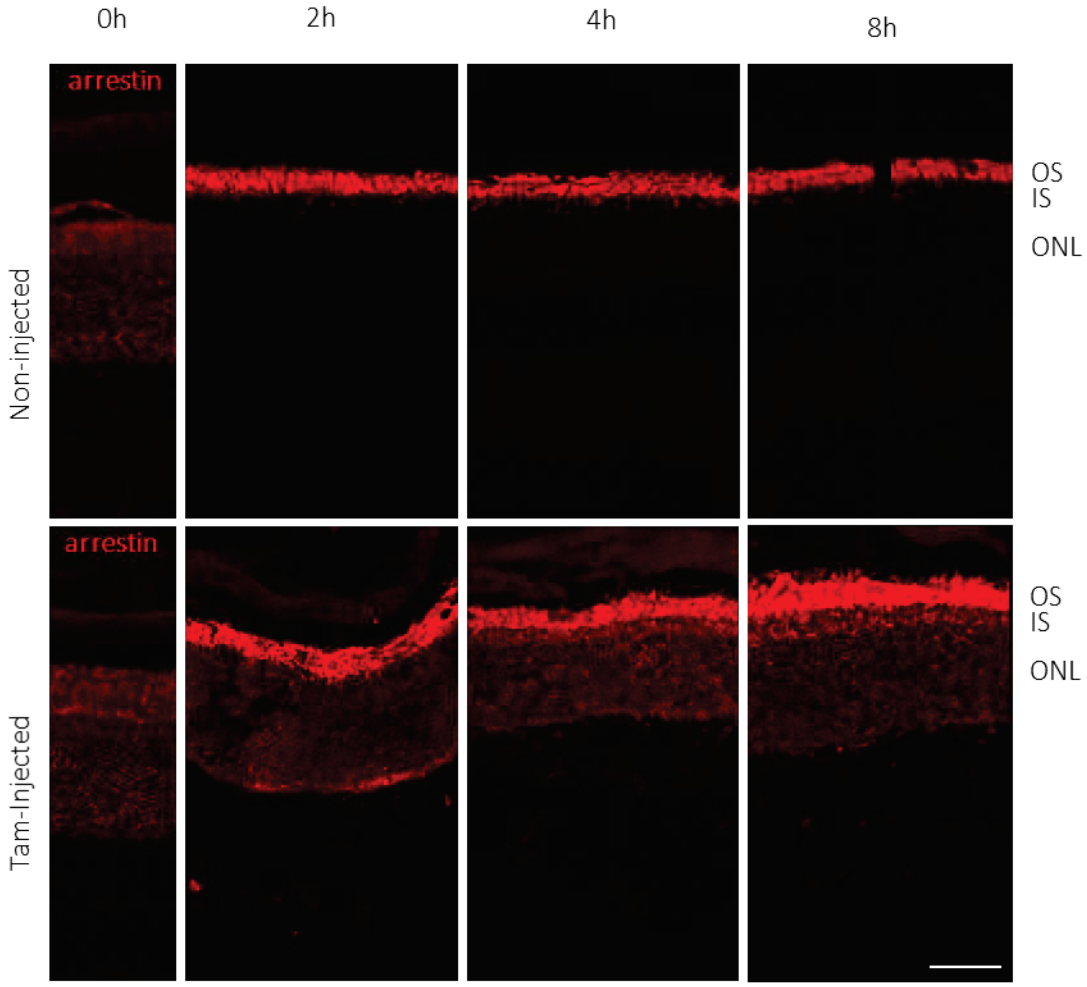


Figure 23. Arrestin localization is affected by *Otx2* KO in light-adapted mice. Arrestin localization in light adapted mice: immunostaining on retinal sections of adult mice showing arrestin distribution 0, 2, 4 and 8 hours after light onset in non injected (top panel) and tam-injected (lower panel) mice. OS = outer segments; IS = inner segment; ONL = outer nuclear layer. Scale bar = 50 µm

6.4.2 Behaviour tests confirm light adaptation defects in PR-specific Otx2 KO mice

To test whether the defect of arrestin localization observed in PR-specific *Otx2* KO mice had an impact on their light adaptation, we probed this adaptation using the light-dark box (LDB) test. Control and KO mice were preadapted in the dark for 30 minutes. Then, each mouse was individually positioned in a box with two communicating compartments, one brightly illuminated (140 lux) and one kept in the dark, and video-tracking was performed. After a short time of adaptation to the environment, an object was positioned alternatively in the light or in the dark compartment (Figure 24A). Mice, that are nocturnal animals, do prefer the dark compartment, but they are also curious, and tend to explore the light compartment, especially if novel objects are introduced. The test measures the proportion of time spent in the light compartment, when empty or with an object introduced. While performing the test, we noticed that already, during the adaptation time, KO mice spent almost all the time in the dark compartment, while controls were exploring the light one. When the object was positioned in the light compartment, control mice explored it, as expected, but KO mice stayed almost all the time in the dark compartment. At the end, KO mice were shown to spend more time in the dark box while control mice were exploring the environment for longer time (Figure 24A). The latency, the time that the mice spend in the dark before going for the first time in light compartment was the same, suggesting that KO mice went to the light but then they preferred to stay in the dark compartment, exhibiting a photophobic behaviour. Since the LDB test is a classic test used to evaluate the level of anxiety of mice, we performed an alternative anxiety test, the open field test, in dimmer light conditions (60 lux), to decipher whether the behaviour of KO mice was due to anxiety or to photophobia. In such light conditions, both control and KO mice showed similar normal exploratory activity in the open field test. Indeed, the time animals spent in the centre of the arena (Figure 24B), an index of anxiety level in the mice, was comparable between control and KO mice and not so much different from the time they spent in the corner of the arena (considered as the safe region of the arena). Locomotion and velocity were almost normal and comparable between control and KO animals, excluding the possibility of "freezing", one of the common behaviours that mice display when stressed. This shows that KO mice are not particularly anxious, but they strongly avoid bright light conditions, suggesting they have a defect in light adaptation that renders bright light painful or

unbearable for them. These data confirm that *Otx2* is involved in light adaptation, by directly or indirectly regulating arrestin localization upon prolonged light exposure.

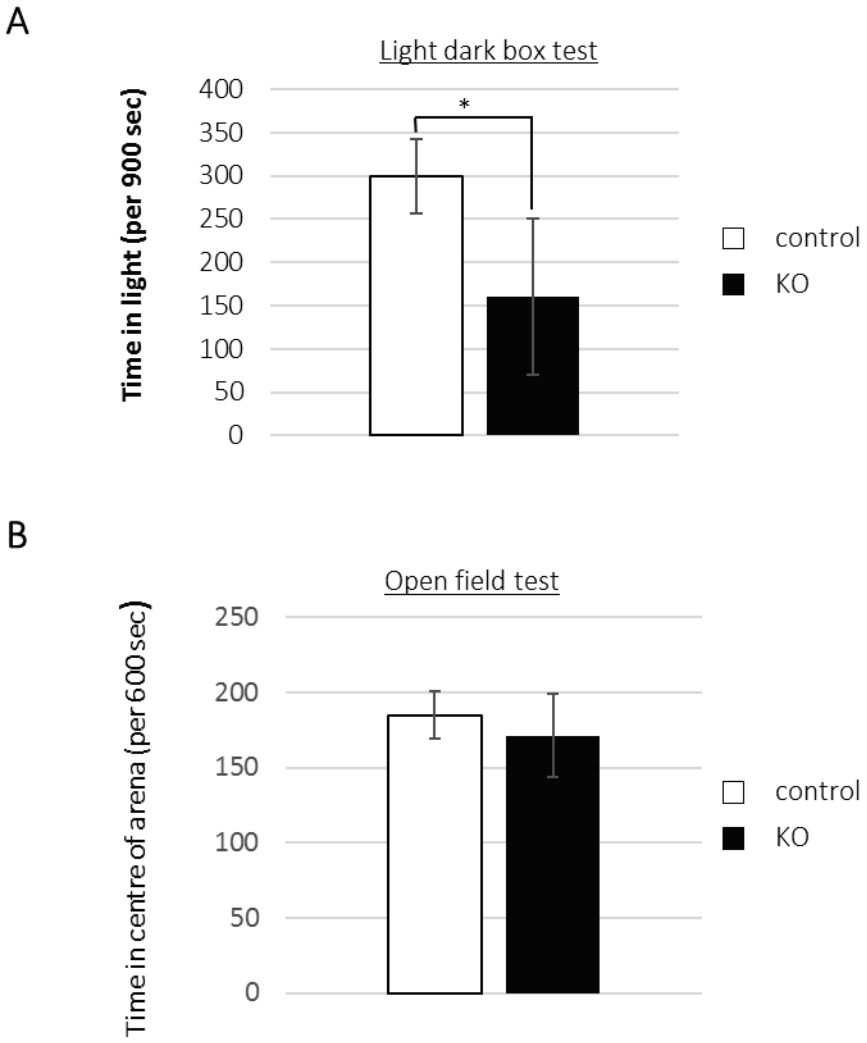


Figure 24. Loss of Otx2 expression in photoreceptors affects mouse behaviour. A) Light dark box test: average of the time spent by control (n = 5) and KO (n = 5) mice is reported during 15 minutes of the test (900 seconds), after a habituation time of 10 minutes (600 seconds). Test B) Open field test: average of the time spent by control (n = 5) and KO (n = 6) mice in the centre of the arena is reported. Error bars represent the standard deviation in the two groups. t-test: (*) = $p < 0.05$)

6.5 *Otx2* ablation in photoreceptors affects ECM and RPE gene expression

Since Otx2 is a transcription factor, we hypothesized that ablating its expression in photoreceptors would affect the regulation of its target gene network. To identify deregulated genes, we performed RNA-seq analyses following Otx2 ablation. RNA-seq results have been deposited and are available online (GEO DATASET GSE138097). KO was induced at postnatal day 30 (P30) and mice were sacrificed at P32, P34 and P38, i.e. 2, 4, and 8 days after tamoxifen injection. Uninjected P30 mice were used as "time zero" controls. Retinas were dissected and RNA extracted and subjected to high throughput sequencing. Experiments were done in triplicate. Analyses were done by comparing each KO stage with the P30 stage and different KO stages between them (P34 vs P32; P38 vs P32; P38 vs P34). In this way, we could establish the kinetics of gene expression across the 4 analysed stages. Genes were first sorted according to their statistical relevance (triplicate reproducibility) and assigned an adjusted p-value. Only genes having an adjusted p-value below 0.05 were considered statistically relevant. Genes between 0.05 and 0.01 are here represented as (*). Genes between 0.01 and 0.001 are defined as (**), while all the genes with a value below 0.001 were defined as (***). Then genes were ranked according to the magnitude of their expression variation across experimental stages. Genes having a logFC (fold change) value below or equal to -0.5 were considered as downregulated genes, while genes with a logFC value over or equal to +0.5 were considered as upregulated. After sorting genes according to these criteria, a list of downregulated or upregulated genes (see Annexes for the complete lists) for each comparison was produced and processed with Gene Ontology (geneontology.org) and confirmed with ImPALA (impala.molgen.mpg.de) and GSEA (software.broadinstitute.org/gsea/index.jsp) tools. The results obtained for the pair comparisons P32 vs P30, P34 vs P30 and P38 vs P30 were almost similar, indicating that gene expression changes observed 2 days after knockout were maintained over the whole time-course. A list of affected biological processes at P34, (the stage with more downregulated genes) is shown in Table V. Very few upregulated genes were found during analyses and, because of this limited number, it was not possible to find an enrichment for biological processes, pathways or particular functions in Gene Ontology database. Therefore, we did not investigate them further. More downregulated genes were found, in accordance with

the fact that Otx2 acts principally as a transcriptional activator. Among them, no photoreceptor marker genes, such as those involved in phototransduction were found, suggesting or that *Otx2* has no direct role on these genes or that a compensatory mechanism could substitute to *Otx2* function. These data are coherent with the study of Housset et al. (2013), where Otx2 ablation from all Otx2-expressing cells of the adult retina (RPE, photoreceptors and bipolar cells) did not identify photoreceptors-specific genes as direct targets of Otx2. A good candidate for a compensatory mechanism is *Crx*. During late retinal development, *Crx* is under direct regulation of Otx2 and its role is to promote activation of some phototransduction genes as rhodopsin, cone opsins, arrestin and transducin (Furukawa et al. 1999). This allows Retinal Cells Precursors to acquire photoreceptor fate. In the adult retina, as Housset et al., 2013 showed and our RNA-seq data confirmed, Crx is no longer under Otx2 control. Moreover, Otx2 and Crx in adult retina bind several identical regulatory regions (Samuel et al. 2014). Together these observations make plausible the hypothesis that Crx expression, that is retained after Otx2 ablation, could have a compensatory role. Further analyses will be needed to confirm this hypothesis.

Table V: biological processes affected 4 days after Otx2 KO in PRs

source	term_name
GO:MF	extracellular matrix structural constituent
GO:MF	glycosaminoglycan binding
GO:MF	heparin binding
GO:MF	extracellular matrix structural constituent conferring compression resistance
GO:MF	extracellular matrix binding
GO:MF	extracellular matrix constituent conferring elasticity
GO:BP	extracellular structure organization
GO:BP	extracellular matrix organization
GO:CC	extracellular matrix
GO:CC	collagen-containing extracellular matrix
GO:CC	extracellular region
GO:CC	extracellular region part
GO:CC	extracellular space
GO:BP	extracellular matrix assembly
GO:CC	extracellular matrix component
GO:CC	pigment granule membrane
GO:CC	melanosome membrane
GO:CC	pigment granule
GO:CC	melanosome
KEGG	Tyrosine metabolism
GO:BP	pigmentation
GO:BP	epithelium development
GO:BP	pigment cell differentiation
KEGG	TGF-beta signaling pathway
KEGG	Hedgehog signaling pathway
GO:BP	sensory organ development
GO:BP	camera-type eye development
GO:BP	eye morphogenesis
GO:BP	eye development
GO:BP	regulation of cellular component movement
GO:BP	movement of cell or subcellular component
GO:BP	regulation of cell motility
GO:BP	regulation of cell migration
GO:MF	oxidoreductase activity
GO:MF	oxidoreductase activity, acting on the CH-NH2 group of donors

On the other side, Gene ontology analyses consistently identified in the list of downregulated genes two groups of factors in all the comparisons we did. Using the software Cytoscape and the related application called ClueGO, it was possible to load the lists of downregulated genes, to sort out the list of corresponding GO (gene ontology) processes and to automatically interconnect related GO processes generating a map. From this initial map, containing all the affected processes, two interesting groups of downregulated genes appeared. One of them gathered many genes involved in the extracellular matrix assembly and functionality, suggesting a role of Otx2 in the control of the three-dimensional network of extracellular macromolecules that provide biochemical and structural support to photoreceptors and surrounding cells. Indeed, while several genes of this group are involved in the regulation of signal transduction and signal communication, in particular way in the TGFβ pathway regulation, the other genes have main roles in the structural organization of the ECM (collagens, fibronectins, glycosaminoglycans and peptidoglycans protein core genes) The interconnected processes belonging to these particular group have been isolated in ClueGo generating a specific map (Figure 25).

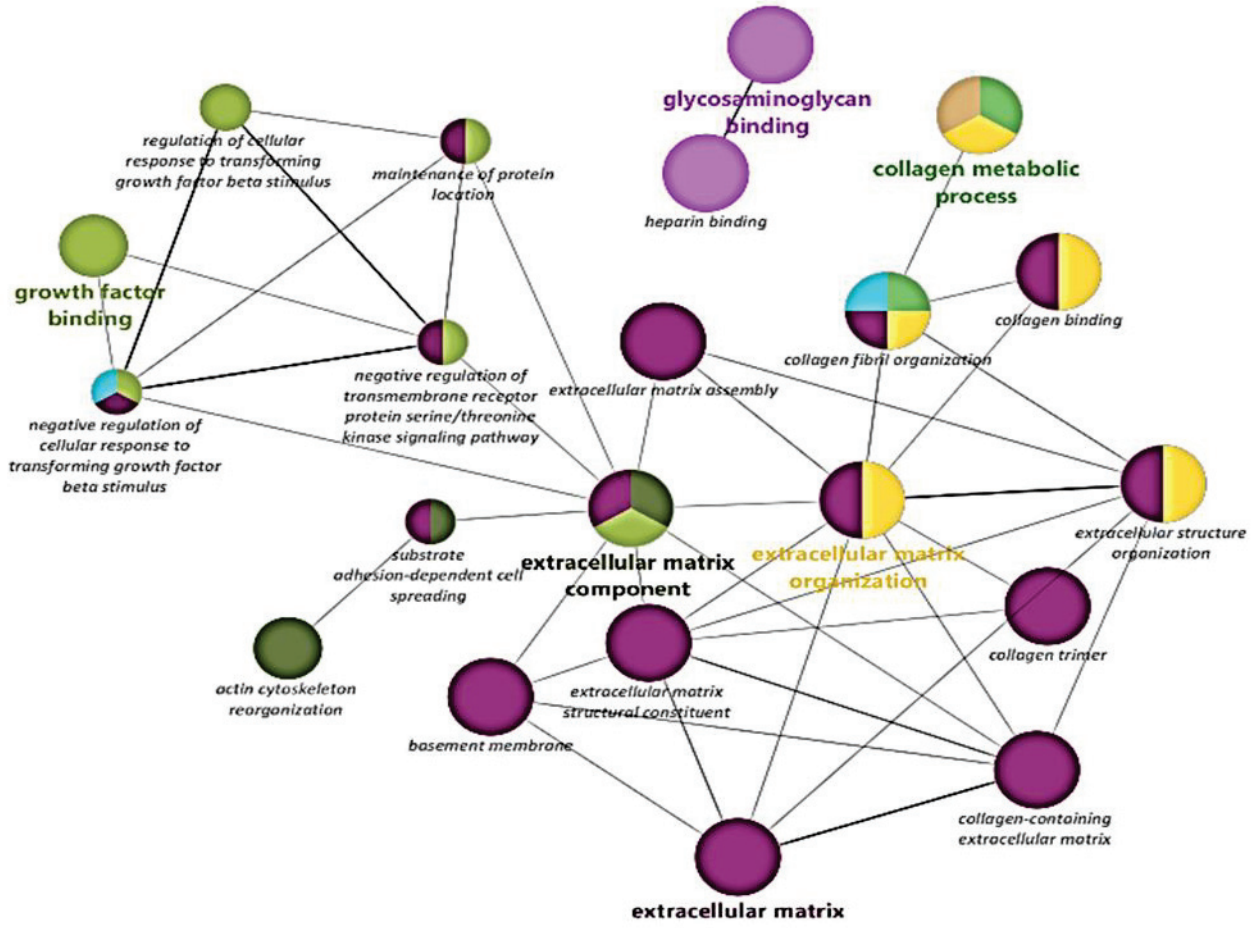


Figure 25. Otx2 expression in PRs is important to activate genes involved in extra-cellular matrix structural maintenance and in TGF-β response. Schematic representation of ECM-related processes that are affected by Otx2 ablation in PRs. P34 downregulated genes after tam-injection at P30 were analysed by Gene Ontology, and processes that were affected by Otx2 KO were isolated using the ClueGO tool, an extension of Cytoscape. Related processes were interconnected. Each labelled circle is a single Gene Ontology process. The thickness of the circle border is a statistical indication relative to the FDR and the circle area is proportional to the gene enrichment for each process. Similar processes are indicated with identical color.

Surprisingly, the other group of genes that were also present in the lists of downregulated factors, corresponded to RPE-specific genes involved specifically in the melanogenesis process (ClueGo map in Figure 26), while no other functions of the RPE appeared affected (visual cycle, pH regulation, nutrient transport, etc.).

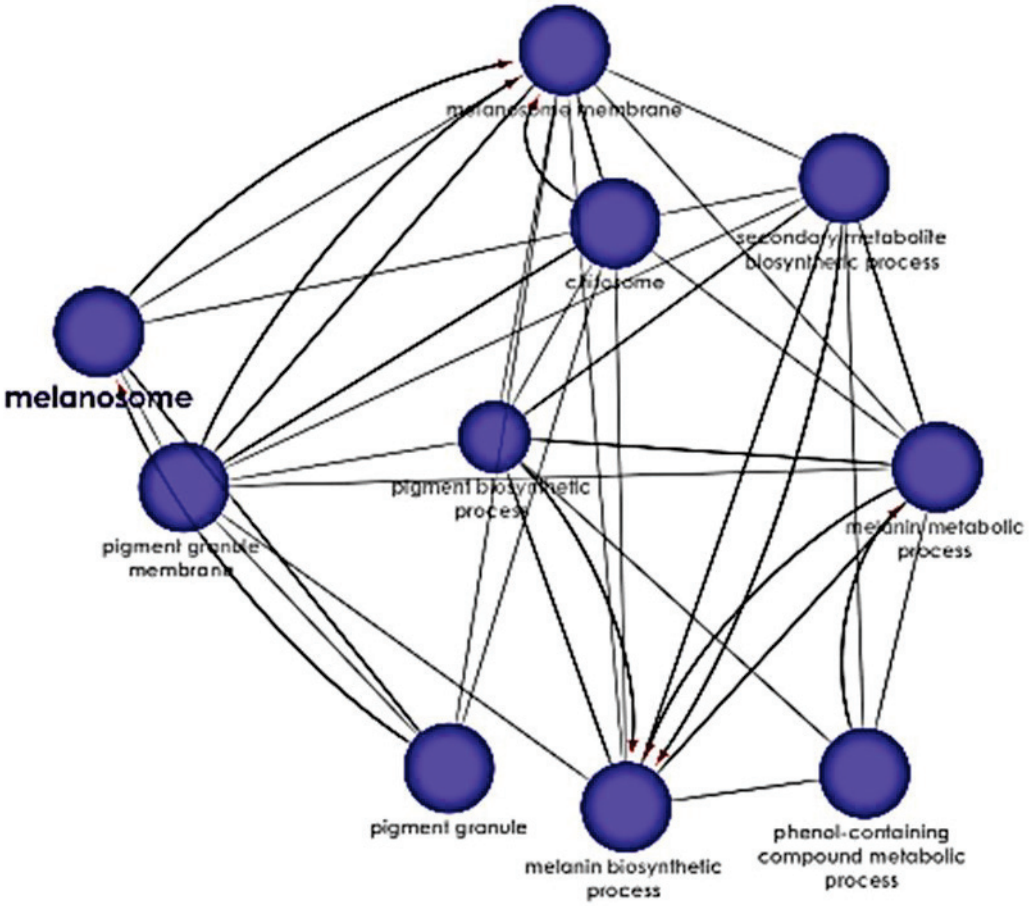


Figure 26. Otx2 ablation from PRs affects Otx2-target genes involved the RPE melanogenesis. Representation focusing on melanogenesis genes downregulated following Otx2 KO. P34 downregulated genes after tam-injection at P30 were analysed by Gene Ontology, and processes that were affected by Otx2 KO were isolated using Cytoscape ClueGO tool. Each labelled circle is a single Gene Ontology process. The thickness of the circle border is a statistical indication relative to the FDR and the circle area is proportional to the gene enrichment for each process.

Indeed, as shown by the heat map, several genes involved in the melanogenesis process were downregulated at all three KO stages (Figure 27A), with downregulation observed already 2 days after KO (Figure 27B). Downregulation of two of them, tyrosinase and tyrp-1, was confirmed by semi-quantitative RT-PCR (Figure 28).

The observation of a down-regulation of RPE-specific genes following Otx2 ablation in photoreceptors was unexpected, as in all our analyses, no CreER^{T2} expression was ever detected in the RPE (Figure 18B). Moreover, the melanogenesis genes that were deregulated, were known to be specific Otx2-targets, suggesting that Otx2 knockout in photoreceptor cells impaired in a way the function of Otx2 in the neighbouring RPE cells. This appeared to be quite specific, as other RPE-specific genes like *Mitf*, also involved in melanogenesis but not under the control of *Otx2*, were not affected.

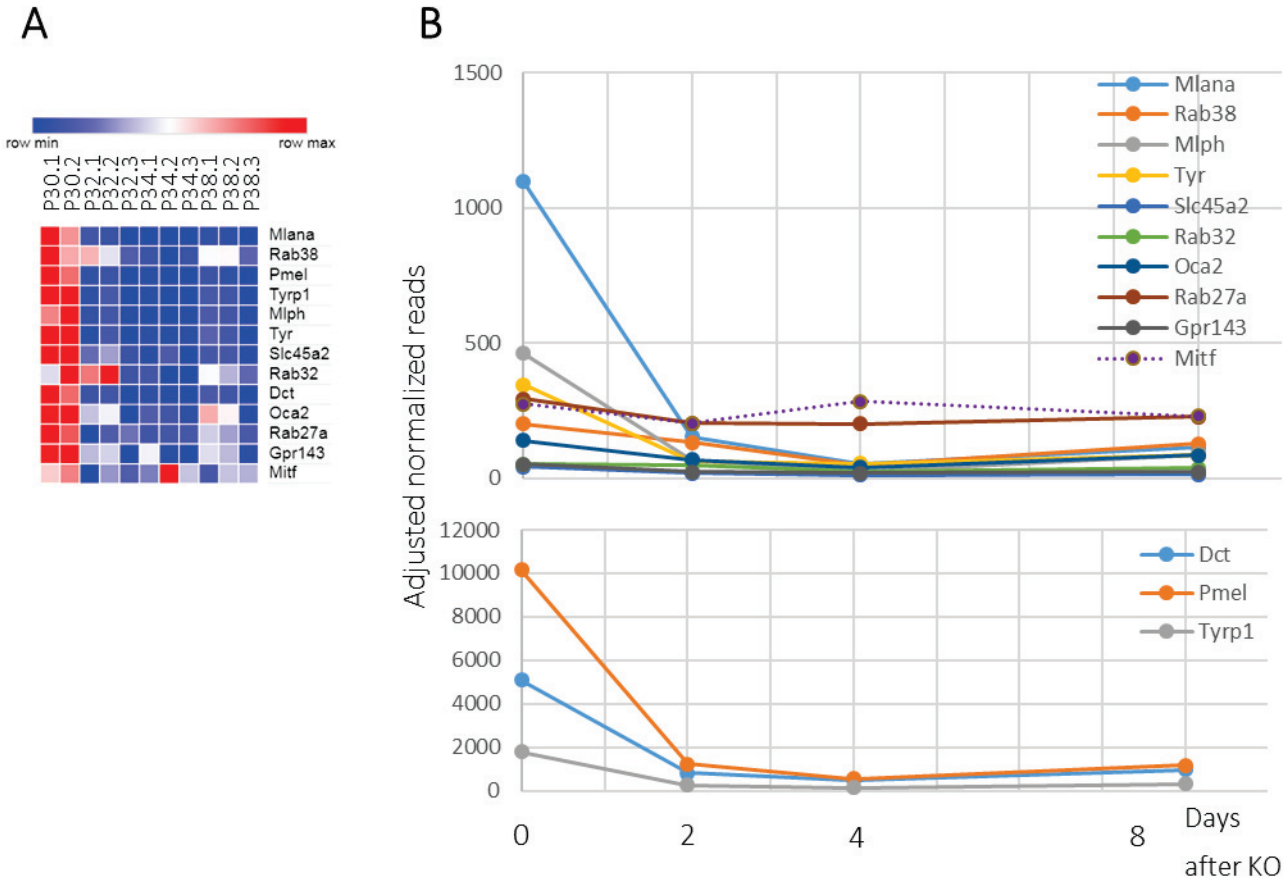


Figure 27. Temporal expression profile of RPE-specific genes following Otx2 knock out in PRs. A) Heat-map representation of the variation of expression of Otx2-target genes in the RPE, induced by Otx2 KO in PRs. Gene expression was analysed at the time of injection (P30) and 2, 4 and 8 days after KO (i.e P32, P34 and P38). The colour bar indicates the level of expression. B) Graphic representation showing the temporal expression profile of the indicated genes. Y-axis values represent an average of the replicates for each stage. Analyses were done in duplicate for P30 control mice (n = 2) and in triplicate for KO mice (n = 3) at the different stages. *Mitf*, which is not under control of Otx2 was not significantly affected by Otx2 KO.

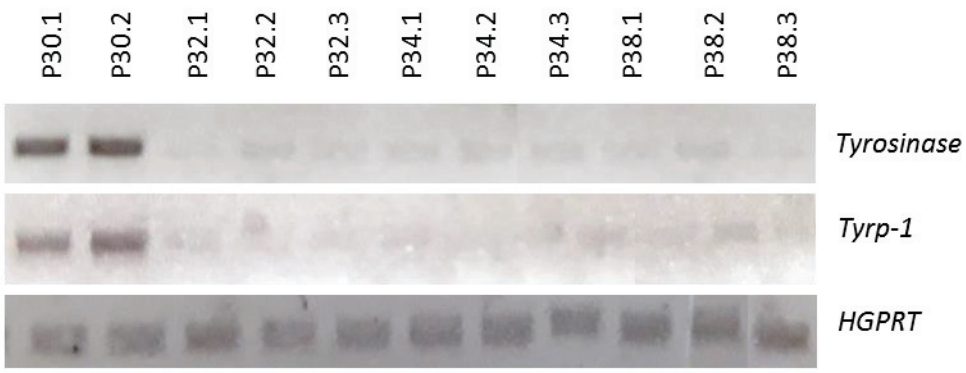


Figure 28. Validation of RNAseq data by RT-PCR. Gene expression of two Otx2-target genes involved in RPE melanogenesis, *Tyrosinase* and *Tyrp-1* was evaluated by semi-quantitative RT-PCR. PCR products were analysed by gel electrophoresis. *HGPRT* was used as normalizer. Expression of Tyrosinase and Tyrp-1 is decreased 2, 4 and 8 days after KO (P32, P34, P38) compared to P30 non injected mice. Analyses were performed in duplicate for uninjected P30 mice and in triplicate for KO stages.

A last interesting point we observed in GO analyses and by analysing the list of downregulated genes was the presence of 32 different genes with oxido-reductive function. Some of these enzymes were classified as cytochrome subunits, with a mitochondrial localization. Different cytochromes are members of the respiratory chain and have a role in ATP production. Other downregulated oxidoreductases were the Marc2 Mitochondrial amidoxime reducing component 2, the Far2 Fatty acyl-CoA reductase 2. Whether this deregulation has as consequence an oxidative stress and/or a reduction of ATP synthesized, should be investigated.

In conclusion, the major gene expression changes induced by photoreceptor-restricted Otx2 knockout concern genes involved in extracellular matrix assembly and function, Otx2-target genes of the RPE and genes that could induce oxidative stress and ATP synthesis reduction.

6.6 *Otx2* ablation in photoreceptors affects its own protein level in the RPE

Our RNA-sequencing results showing down-regulation of RPE-specific Otx2-target genes prompted us to look more precisely whether *Otx2* expression was affected also in the RPE after KO induction. In effect, confocal images of control and knockout retinas stained for *Otx2* showed decreased nuclear staining in the RPE of knockout mice. Moreover, we noticed that in the KO, diffuse *Otx2* staining was present in the outer segments of the photoreceptors (Figure 29). This labelling, considered at the beginning as an artefactual background, was found to be reproducible and specific in KO conditions. The simplest explanation for this partial loss of *Otx2* in the RPE would be a leaky expression of *CreER*^{T2} in this layer. Although *Crx* is not reported to be expressed in RPE of the mouse retina, it is possible that, as it happens for transgenes, the short 2.5 kb *Crx* promoter driving *CreER*^{T2} expression lacks some regulatory elements, that are preventing its expression out of photoreceptors. However, as shown in Figure 18B. CreER^{T2} was detected exclusively in the photoreceptors.

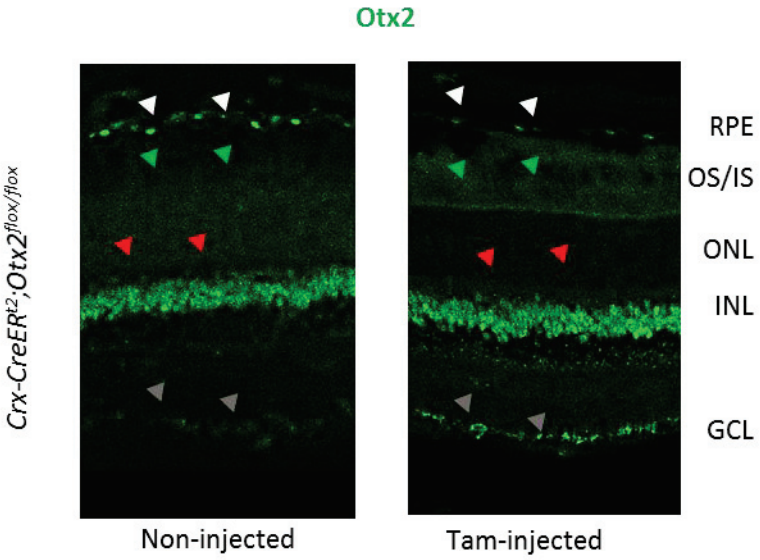


Figure 29. Otx2 ablation in photoreceptors affects Otx2 protein level in RPE. Immunodetection of Otx2 in the retina of non-injected (left) and tam-injected (right) P32 mice, 2 days after injection. Otx2 is expressed in RPE, photoreceptors (ONL) and bipolar cells (INL). Tam-injected mice show specific ablation of Otx2 only in the ONL, 2 days after injection. Otx2 protein level in RPE appears reduced in tam-injected mice (white triangle) and diffuse staining of OS and IS (green triangles) of PRs is seen after KO, despite photoreceptor nuclei are not labelled (red triangles). Staining appears in GCL after KO (grey triangles). RPE = Retinal Pigmented Epithelium; ONL = Outer Nuclear Layer; INL = Inner Nuclear Layer; GCL = Ganglion Cellular Layer; OS = Outer segment; IS = Inner Segment. Scale bar = 50 µm

Yet, we could not exclude that RPE cells express minute amounts of CreER^{T2}, sufficient to recombine *loxP* sites, but low enough to remain below the limit of detection of the immunostaining technique. To rule out this possibility, we functionally probed for Cre activity in the RPE. We crossed *Crx*-CreER^{T2} mice with the ultra-sensitive *Gt(ROSA)26Sor$^{tm14(CAG-TdTomato)Hze}$ Cre*-reporter line, also called Ai14 (Madisen et al. 2010). This mouse harbours the tandem Tomato (TdTomato) reporter gene inserted into the ROSA26 locus under the ubiquitous CAG promoter and flanked by enhancer element WPRE. Transcription is normally terminated by the presence of a stop cassette, but, following Cre-mediated recombination, the stop cassette is deleted, allowing strong expression of the bright TdTomato reporter (Figure 30A). In our model, the reporter was activated in almost all photoreceptors, which is consistent with the localization of the *CreER^{T2}* and with simultaneous loss of lost *Otx2* expression that became undetectable in the photoreceptor layer. In the RPE, no TdTomato labelled cells were found, confirming the absence of *CreER^{T2}* from this layer (Figure 30B). Together, *CreER^{T2}* staining and TdTomato localization suggest that the decrease of the protein level in the RPE is not due to partial knockout in RPE cells but results from an indirect effect triggered by *Otx2* depletion in the photoreceptors.

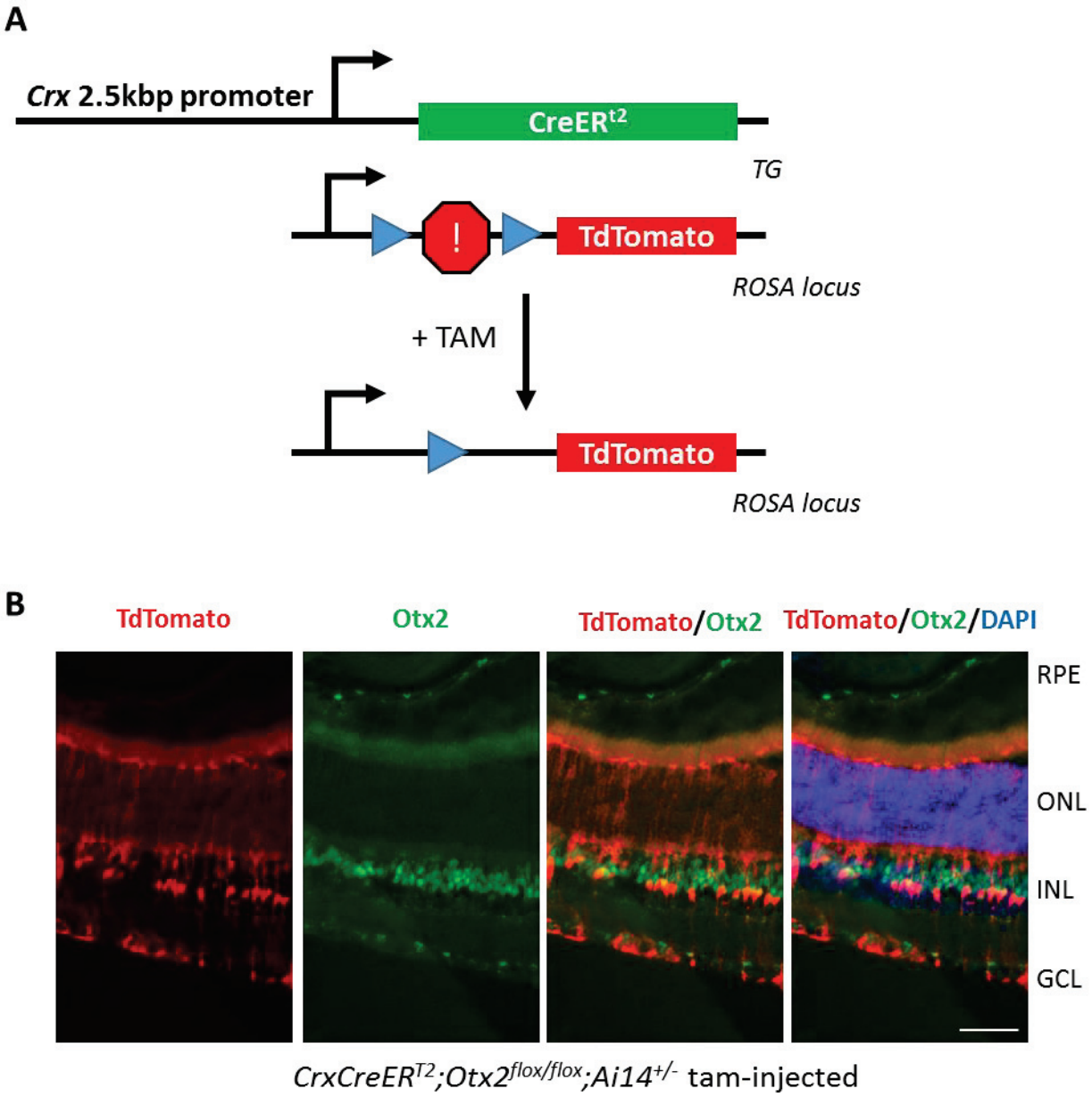


Figure 30. Activity of the *Crx-CreER^{T2}* transgene is restricted to ONL and INL cells. A) Schematic diagram of the Cre-reporter TdTomato line. The *CreER^{T2}* transgene (TG) is expressed under the PR-specific *Crx* promoter. The *TdTomato* coding sequence is inserted into the ROSA locus downstream a stop cassette (red octogon) flanked by two *loxP* sites (blue triangles). CreER^{T2} activation by tamoxifen promotes the recombination and excision of the stop cassette , allowing TdTomato expression. B) Immunostaining of retinal sections of P32 mice tam-injected at P30 with the indicated antibodies. Tamoxifen injection promotes Otx2 ablation specifically in the ONL. TdTomato predominantly stains the ONL, and also INL cells located just below Otx2-positive bipolar cells. The strong labelling of the outer limiting membrane and of the innermost part of the retina suggests the latter are Müller glial cells. No staining can be found in RPE cells. Scale bar = 50 µm

Studying the reporter line, we noticed that some cells of the Inner Nuclear Layer (INL) were also expressing TdTomato, after tamoxifen administration. These cells were identified as Sox9- and GS-positive cells (Poché et al. 2008; Riepe & Norenburg 1977), respectively nuclear and cytoplasmic markers of Muller Glial Cells (MGCs) (Figure 31). This observation is puzzling because Muller cells are not reported to express *Otx2* (Fossat et al. 2007). A likely explanation is that Muller cells, which take care of the visual cycle of cone photoreceptors, shuttle many proteins from cone outer segments. By doing so, they might have taken up $CreER^{T2}$ protein, which accumulates in the cytoplasm of cone cells in the absence of tamoxifen. However, since Muller cells normally do not express *Otx2*, its deletion in these cells should have no consequence and should not affect the quality of our results.

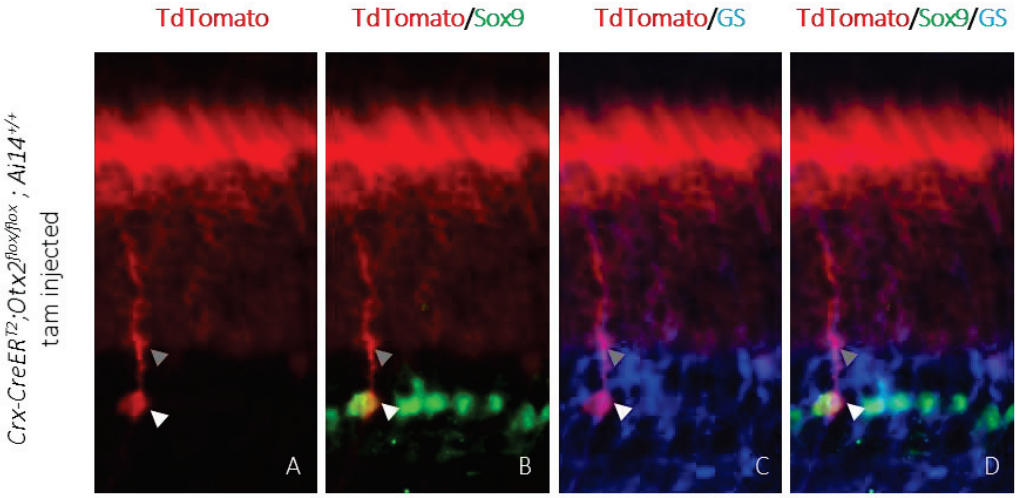


Figure 31. TdTomato reporter activation in *Crx-CreER^{T2};Otx2$^{flox/flox}$; Ai14$^{+/+}$* mice. Immunostaining of retinal section of adult (P30) mouse. TdTomato is predominantly found in photoreceptors (ONL) (A). TdTomato positive cells in INL are colabelled with Sox9 (B) at nuclear level (white triangle) and glutamine syntethase (C) at cytoplasmatic level (gray triangles), two markers of Müller Glial cells. D = triple channel merge. Scale bar = 50 µm

VII *Otx2* can be transferred from RPE to photoreceptors

7.1 *In vivo* expression of tagged-Otx2

Housset et al. (2013) showed that Otx2 expression in the RPE is both necessary and sufficient to prevent photoreceptors degeneration. Here, we also confirmed that Otx2 expression in RPE is sufficient for photoreceptors viability. Yet, the molecular mechanisms underlying this neuroprotection are still not known. One obvious possibility is that cell-autonomous Otx2 activity is critical for RPE cells to perform their PR-nursing functions and that Otx2 KO in the RPE, by affecting these functions, would indirectly cause the degeneration of photoreceptors. Another, not exclusive possibility is that RPE cells produce survival factors under Otx2 control, that are necessary for photoreceptor maintenance. Since we found that Otx2 KO in photoreceptors affects Otx2 protein level in the RPE and, since we observed a correlated downregulation of RPE-specific Otx2 target genes involved in melanogenesis, we reasoned that such survival factor could be Otx2 itself. Supporting this idea, a growing number of studies have documented the ability of homeodomain factors to act as signalling molecules. We then considered the hypothesis that Otx2 could be transferred from the RPE to photoreceptors, to protect them and ensure their long-term maintenance. To verify this hypothesis, we designed a strategy that consisted of specifically expressing a tagged-Otx2 protein in the RPE and test whether it could then be detected in photoreceptors. We needed to use a tagged form of Otx2 in order to discriminate transferred protein from endogenous protein in photoreceptors.

7.1.1 HA-Otx2-(M2x4) construct validation and *in vivo* analyses

Importantly, in our previous analyses of PR-specific *Otx2* KO retinas using an anti-Otx2 antibody, we did not observe any staining in photoreceptors, suggesting that, if a transfer existed, the amount of transferred protein was very low. For this reason, we needed a highly sensitive system and the selection of the tag was a critical issue. The first step was, then, to compare different tagged forms of *Otx2,* expressed by transiently transfected cell cultures and identify the one with the highest detection sensitivity. In particular, we generated an *Otx2* isoform fused with 2, 3 or 4 FLAG tags at the C-terminus (hereafter referred to as M2 tag, according to the antibody used for detecting the FLAG tag) and 1 HA tag at the N-terminus (Figure 32A). The isoform containing 4 FLAG tags, referred to as HA-Otx2-(M2x4), was detected with the highest sensitivity in Western Blot (Figure 32B) and chosen for further experiments. In a reporter gene assay, we verified that the presence of tags did not affect Otx2 transcriptional activity (Figure 32C). Finally, we showed that this tagged-protein expressed in transfected cells could also be easily detected in fixed cells with an anti-M2 antibody, confirming that the tag is readily detectable by immunostaining, at least in overexpressing cells (Figure 32D). As we reasoned that, during the transfer, Otx2 can be internalized by photoreceptors, we prepared in parallel a mutant version of the tagged-Otx2, that could be used as a negative control of protein internalization. This construct was based on a double-mutated form of *Otx2* (WF85/86YL, hereafter referred to as YL-*Otx2*), that was described to be defective for cell-penetration by Ibad et al (2011). The YL mutant was obtained by PCR mutagenesis of the HA-*Otx2*-(M2x4) construct.

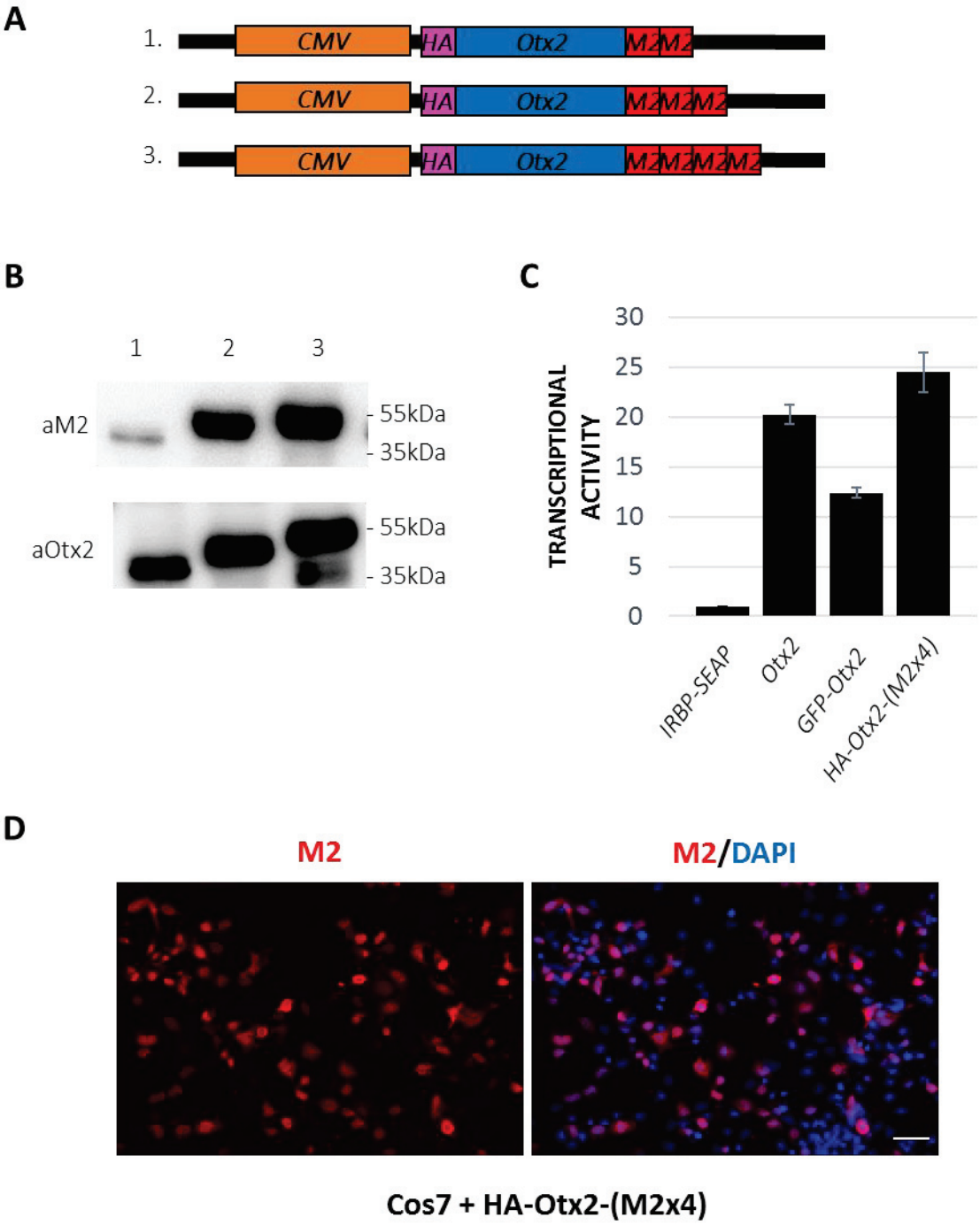


Figure 32. ***In vitro*** **selection and validation of tagged-Otx2 form for viral constructs.** A) Schematic diagram of the different tagged-Otx2 tested. B) Western blot analyses of protein extracts of Cos7 cells transiently transfected with the corresponding constructs with the indicated antibodies. C) Transcriptional activity of the indicated constructs on the IRBP promoter in a *SEAP* reporter assay. D) M2 immunostaining of Cos7 cells transiently transfected with HA-Otx2-(M2x4)-expressing vector. DNA staining with DAPI reveals all the cells. Scale bar = 50 µm

In order to achieve RPE-specific expression of wild type and YL-*Otx2* tagged proteins, both tagged *Otx2* wild type and YL-*Otx2* coding sequences were inserted into an AAV2-derived vector containing the RPE-specific *Vmd2* gene promoter (also known as *Bestrophin 1*, (Guziewicz et al. 2013)). As a positive control to monitor RPE cell-specific expression, we used a *Vmd2-GFP* construct inserted in the same AAV2-derived vector (Figure 33A). AAV-vector particles were produced and injected in the sub-retinal space of the right eye of P30 mouse and the non-injected left eye was used as a negative control. Seven days later, KO was induced or not by tamoxifen injection. We performed different tests to estimate the time necessary to achieve sufficient vector expression and concluded to collect eyes 3 weeks after vector administration. Mice injected with control GFP vector confirmed that in our conditions, expression from the *Vmd2* promoter was tightly restricted to the RPE: retinal regions infected by AAV-derived vectors showed GFP expression exclusively in this layer. The efficiency of RPE transduction ranged between 20% and 50% of total RPE, depending on the performance of the sub-retinal administration and quality of purified AAV derived vectors. Unfortunately, while GFP expression was clearly detectable in the RPE of injected eyes (Figure 33B), no clear staining could be observed for HA-*Otx2*-(M2x4) or YL-*Otx2*.

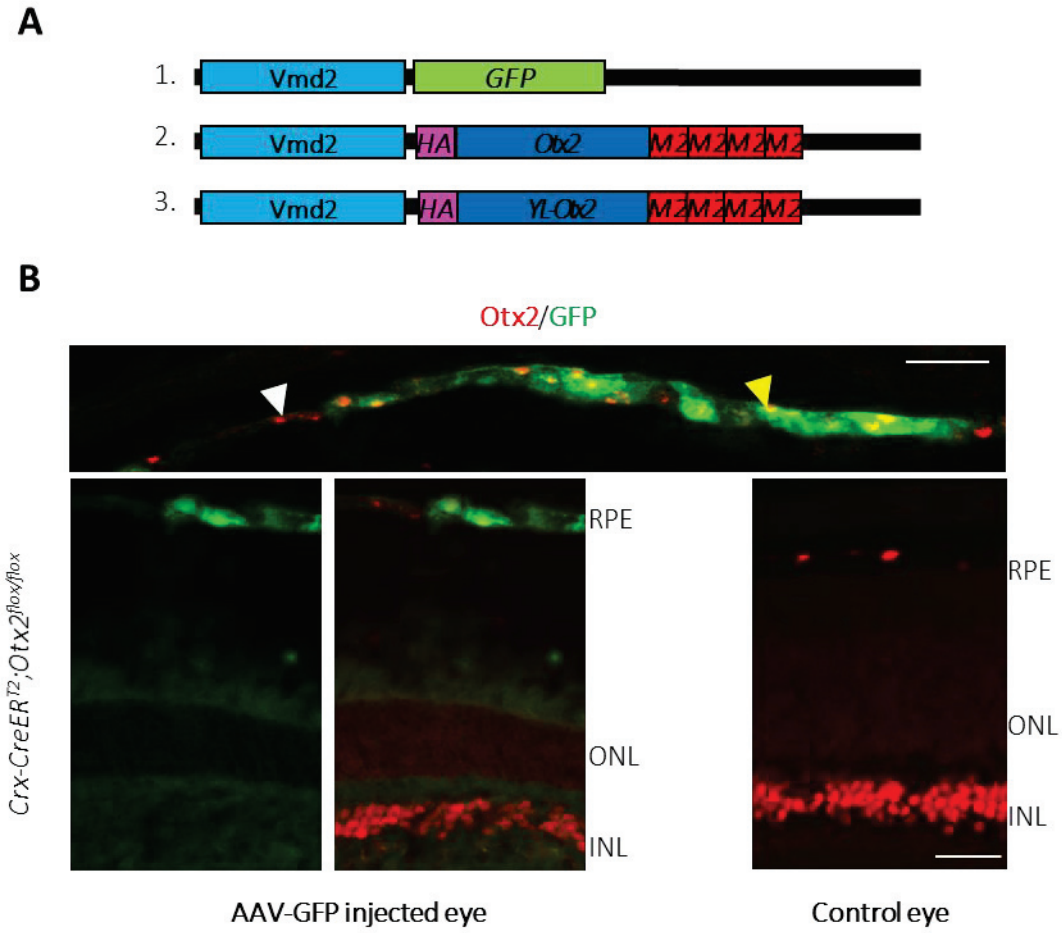


Figure 32. RPE-specific expression of AAV constructs. A) Schematic diagram of constructs used for the production of AAV derived particles. B) Staining for GFP of retinal section of an eye injected with AAV-GFP showing RPE-specific expression, co-labelled with Otx2 (yellow triangle). Non-infected regions (white triangle) only express Otx2 on a low magnification view (top panel). Lower panels: detail of Otx2 and GFP staining of retinal vertical sections of injected (left) and control (right) eyes. Scale bar = 50 µm

7.1.2 SEAP-Otx2 construct validation and *in vivo* analyses

Because *in vivo* immuno-detection of FLAG tag or HA tag failed due to high background, unexpected from *in vitro* tests, we decided to fuse an enzyme to Otx2*,* in order to achieve maximal signal to noise, thanks to the signal amplification allowed by enzymatic conversion of a chromogenic substrate. Alkaline Phosphatase (AP) is an interesting enzyme for such purpose. It has been already used to identify secreted proteins (Hoffman & Wright 1985), including homeodomain-containing proteins like *Otx2* (Beurdeley et al. 2012). We fused the *HA-Otx2-(M2x4)* coding sequence to that of *Secreted Alkaline Phosphatase* (*SEAP-Otx2*), a C-terminal truncated version of AP that cannot be anchored to internal cell membranes. The expression of the construct in Cos-7 cells was analysed by western blot with an anti-*Otx2* (Figure 34A), showing a product of the expected size. To test whether the SEAP enzymatic activity was maintained in the fusion protein, we performed a slot blot assay. Conditioned medium from cells expressing either SEAP-Otx2 fusion protein or SEAP alone (positive control) or Otx2 without SEAP-tag (negative control) was collected and heat-treated or not to inactivate the activity of endogenous phosphatases. Increasing amounts of medium of each condition were spotted on a membrane that was, then, incubated with NBT/BCIP for 10 minutes. The heated samples appeared almost similar to the not heated one, suggesting that most of the signal resulted from exogenous SEAP activity. The negative control did not show substrate conversion, confirming that very little or no endogenous phosphatase activity was present in the medium. SEAP and SEAP-Otx2 media yielded identical dose-dependent staining, confirming that SEAP-Otx2 fusion retained full enzymatic activity (Figure 34B). Additional proof was obtained by directly staining transfected cells. After heat inactivation of endogenous phosphatase activity, NBT/BCIP staining could be detected in SEAP and SEAP-Otx2 expressing cells, but not in cells transfected with an *Otx2*-expression vector (Figure 34C). To rule out the possibility of non-specific staining by endogenous phosphatases in these experiments, transfections mixes were spiked with an expression vector encoding the bright fluorescent TdTomato reporter. Only red cells appeared stained after NBT/BCIP incubation, confirming that detected AP activity was limited to the co-transfected exogenous SEAP, and was not due not to endogenous phosphatases. Interestingly, the cell area stained in SEAP-Otx2 transfected cells appeared close to the nucleus, while that stained in SEAP transfected cells appeared more diffuse in the cytoplasm

or accumulated only on one side of the cells (Figure 34D). Such a difference of localization suggested that, while SEAP alone can be accumulated on one cell-side where its secretion is ongoing, the SEAP-Otx2 fusion protein, containing a nuclear localization sequence in Otx2 part, needs an active, Otx2-dependent, secretion mechanism to be transported outside the cell. Together, these observations validate the SEAP-Otx2 fusion as a relevant and sensitive tool to probe for Otx2 transfer *in vivo.*

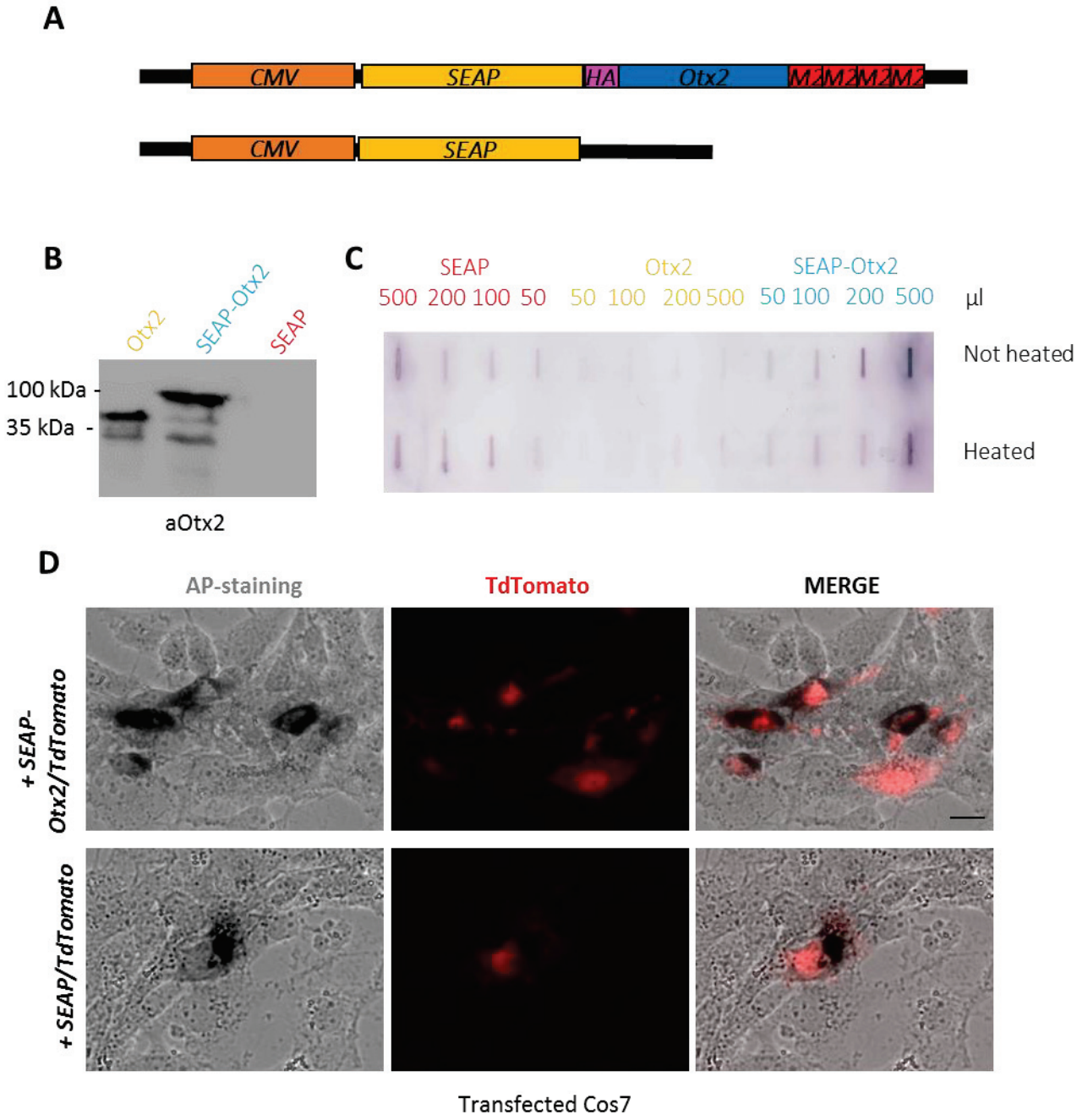


Figure 34. ***In vitro*** **validation of SEAP-Otx2 functionality.** A) Schematic diagram of DNA constructs. B western blot on Cos7 transiently transfected with the indicated constructs probed with anti-Otx2 antibody. C) Slot-blot assay: AP-staining of serial dilution of conditioned medium from Cos7 cells transiently transfected with the indicated expression vectors D) AP-staining of Cos7 cells transiently transfected with SEAP-Otx2 (top panel) or SEAP (lower panel) expression vector. Reaction was spiked with TdTomato to monitor transfection efficiency. The AP-staining in SEAP-Otx2 transfected cells appears surrounding nuclei, while it appears localized on one side of the SEAP-transfected cells. Scale bar = 20 µm

In order to achieve RPE-specific expression of SEAP and SEAP-Otx2 fusion protein, both sequences were inserted into an AAV2-derived vector under the RPE-specific *Vmd2* promoter mentioned in the previous paragraph (Figure 35A). AAV-vector particles were produced and injected in the sub-retinal space of the right eye of P30 mouse and the non-injected left eye was used as a negative control. Seven days later, KO was induced or not by tamoxifen injection and mice were sacrificed 3 weeks later. Expression and localization of SEAP alone, detected by NBT/BCIP staining, was strong in the RPE, where it is expressed under the *Vmd2* promoter. Because of spontaneous secretion, SEAP staining was also detected in the interphotoreceptor matrix, around the outer and inner segments. No obvious difference appeared between control and tam-injected mice. By contrast, SEAP-Otx2 was also found in the RPE, but it diffused into the photoreceptors until their synapses in the outer plexiform layer (Figure 35B). This meant that the SEAP-Otx2 fusion protein was secreted by the RPE and up-taken by photoreceptors, where, once internalized, it was able to reach their synaptic terminals. By comparing control and tam-injected mice, it appeared that the SEAP-Otx2 signal in the OS+IS and in the OPL was stronger in the KO mice than in the controls. Untreated left eyes used as a negative control did not show any SEAP staining (Figure 35C).

Together these data suggest that, once secreted by the RPE, SEAP-Otx2 but not SEAP can be up-taken by photoreceptors and can reach their synaptic terminals and that the process of internalization of SEAP-Otx2 by photoreceptors appears to be accelerated following *Otx2* ablation in these cells.

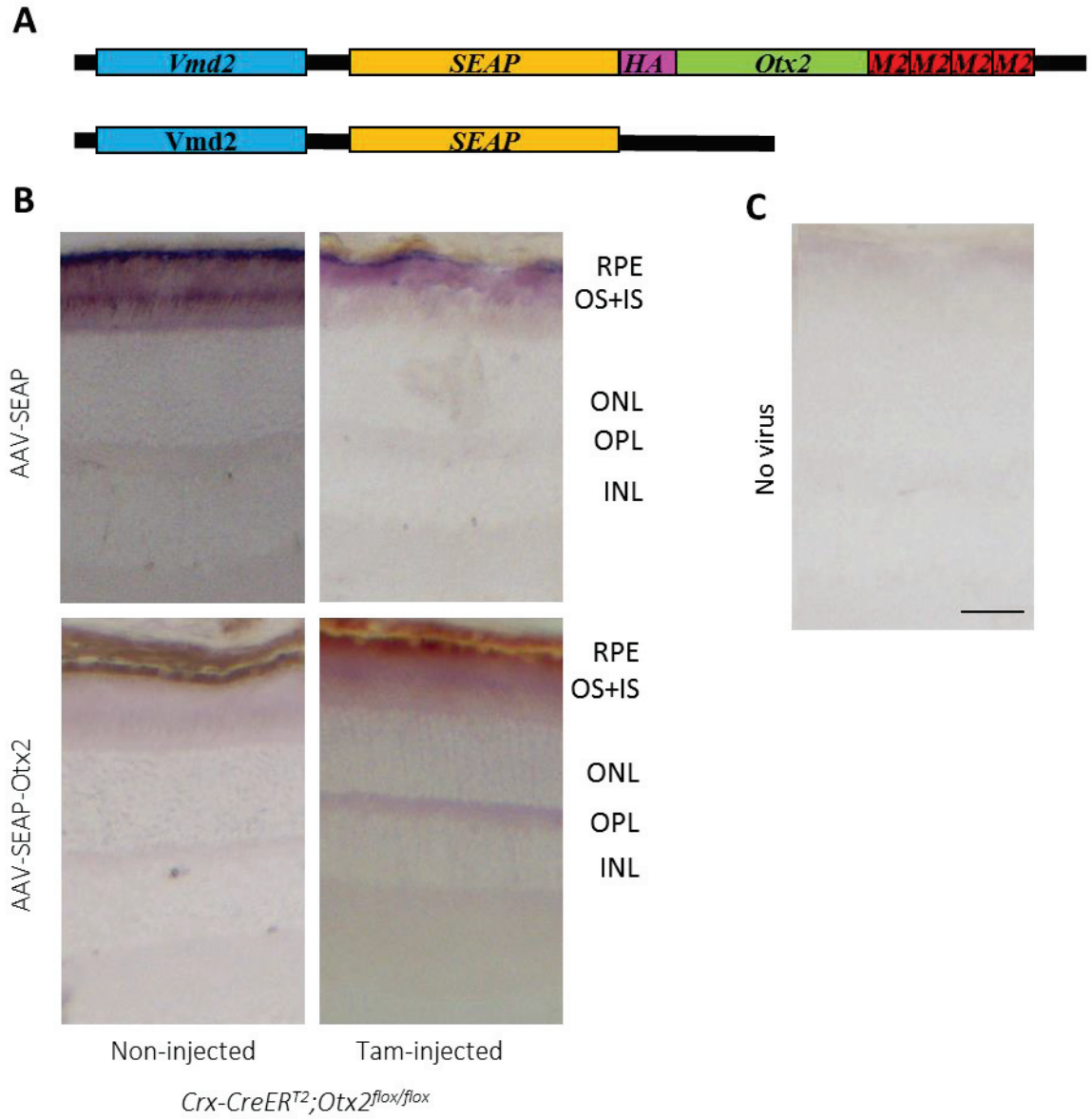


Figure 35. ***In vivo*** **detection of Otx2 transfer from RPE to photoreceptors.** *A)* Structure of the DNA constructs used to produce AAV-derived particles expressing SEAP-Otx2 or SEAP that were administrated to *Crx-CreER^{T2};Otx2$^{flox/flox}$* mice at P30. One week later, Crx-CreER^{T2};Otx2$^{flox/flox}$ mice were tam-injected or not to promote Otx2 KO in photoreceptors. Eyes were processed for analyses 7 weeks later. B) AP staining of retinal sections from control or KO eyes injected with the indicated vectors. C) AP staining of a retinal section from eyes that did not receive AAV vector. RPE = retinal pigmented epithelium; OS = outer segment; IS = inner segment; PNL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer. Scale bar = 50 µm

Discussion and perspectives

VIII Discussion and perspectives

Using photoreceptor-specific conditional ablation of *Otx2*, this study has uncovered novel functions of Otx2 in mature photoreceptors. On one hand, it confirmed that endogenous Otx2 does not play any key role in photoreceptor viability and identity and in the regulation of photoreceptor-specific genes, as it was previously observed in the mouse model of full-retina conditional *Otx2* ablation. On the other hand, here, endogenous Otx2 has been shown to actively contribute to light-induced relocalization of visual arrestin, with its absence impairing light adaptation. Moreover, this work demonstrates that Otx2 can be internalized by mature photoreceptors. The data presented here strongly suggest that exogenous Otx2 is released by RPE cells and transferred to photoreceptors.

8.1 OTX2 KO in PRs does not affect cell viability, cell identity and photoreceptor gene regulation

Loss of photoreceptors is the main consequence of different retinal diseases, such as age-related macular degeneration, Leber congenital amaurosis and cone-rod dystrophy.

Complete Otx2 ablation in the retina has been shown to dramatically affect photoreceptor viability (Béby et al. 2010). The same effect was observed when Otx2 was knocked out specifically in the RPE and, in a context of full retina KO, Otx2 expression restricted to the RPE preserved Otx2-deprived photoreceptors from degeneration (Housset et al. 2013). Otx2 expression in the RPE thus appears necessary and sufficient for photoreceptor viability, suggesting that photoreceptor survival depends on a non-cell-autonomous role of Otx2 expressed in the RPE and not on their endogenous expression of Otx2. Our data, obtained on a different model, where Otx2 is specifically knocked out from photoreceptors, confirm that endogenous Otx2 does not play a pivotal role in photoreceptor cell maintenance.

Switch or loss of cell-identity at the adult stage is not a classical phenomenon. However, this can occur in non-neural and neural context, when important factors are knocked out and/or overexpressed in specific cell populations (Deneris and Hobert, 2014). For example, tamoxifen-inducible dopaminergic targeting of Nurr1 in early adulthood leads to progressive and severe decreases in the expression of the dopaminergic neuron gene battery, encoding tyrosine hydroxylase, dopamine transporter and Vmat2 (Blaudin De Thé et al. 2016; Kadkhodaei et al. 2013). While the level of dopamine in the striatum is strongly affected, the

number of cells in the mutant is unchanged, suggesting that Nurr1 expression is required in this context for the maintenance of dopaminergic cell identity, not for their viability. In a similar way, 5-HT neurons require a sustained expression of *En2* (Fox & Deneris 2012), *Pitx3* (März et al. 2013) or *Lmx1b* (Song et al. 2011). In a non-neural context, it has been shown that β-cells of the adult pancreas, that are necessary to produce insulin, can be regenerated by *in vivo* reprogramming of α-cells, the cells that secrete glucagon (Chung & Levine 2010). One way to reprogram α-cells into β-cells is to ectopically overexpress a single transcription factor, Pax4, in these α-cells (Collombat et al. 2009). These studies show that in some contexts, knockdown or overexpression of a single transcription factor can perturb cell identity. This also applies to Otx2 during development. Indeed, during retinal development, premature ablation of *Otx2* in photoreceptor and bipolar cell precursors promotes a switch of fate to amacrine cell precursors (Nishida et al. 2003). For all these reasons, we investigated the effect of *Otx2* ablation in adult photoreceptors on their cell identity by labelling retinal sections with cell-specific markers of the main types of retinal neurons. However, no evidence of a switch of identity has been observed, confirming that *Otx2* expression does not play a pivotal role in photoreceptor cell identity.

RNA-seq analyses showed that *Otx2* ablation in photoreceptors does not affect the regulation of phototransduction genes. Similar results were obtained by Housset et al. (2013) after ablation of *Otx2* in all retinal compartments that maintain Otx2 expression (RPE, photoreceptors and bipolar cells) (Housset et al. 2013). There, only bipolar-specific and RPE-specific genes were found to be deregulated, while photoreceptor-specific genes were not. While our results are coherent with an unaltered cell identity, they contrast with the fact that Otx2 in the adult neural retina has been shown to bind regulatory elements of genes involved in phototransduction, such as cone opsins and rod opsin (Samuel et al. 2014). However, in the same work, it was shown that Crx, an Otx2 related protein strongly expressed in photoreceptors, binds the same regions, suggesting that there can be functional redundancy between the two factors. In order to better discriminate the real functions of the two transcription factors, it would be necessary to study the effects of *Crx* conditional ablation and of double *Crx*/*Otx2* KO in the retina and photoreceptors. To date, no *floxed-Crx* mouse line is available, probably because the germline *Crx* KO was found to be not lethal (Furukawa 2001) and because of the cost to interest trade-off to generate a new recombinant floxed line for a non-essential gene.

8.2 *Otx2* ablation in photoreceptors affects arrestin 1 relocalization and makes mice photophobic

Viability and cell identity are not affected by PR-specific *Otx2* KO. At the gene expression level, no deregulation has been observed for phototransduction genes. These data support the idea that photoreceptors functionality should also be unaffected. However, phototransduction is a sophisticated cascade, where protein-protein interactions and post-translational modifications are very important. Bleached rhodopsin is partially blocked by phosphorylation and definitively inhibited by arrestin binding. Moreover, although phototransduction is a well-studied phenomenon, the factors involved for each step have not all been identified. Some of these factors, that would play a role in photoreceptors functionality could be in the list of the uncharacterized downregulated genes following *Otx2* KO in photoreceptors. Several studies suggest that Otx2 is also detectable in other cell-compartments than the nucleus, interacting with mitochondrial proteins and cytoplasmic proteins (Kim et al. 2015; Fant et al. 2015). In the adult mouse retina, Otx2 is reported to interact with the elongation factor Eef1a1, the splicing factor U2af2, the intermediate filament subunits Krt16, Krt42, and Krt72, the cytoskeleton-associated protein Map2 or the motor protein Kif2a (Fant et al. 2015) and with visual arrestin. This suggests that Otx2 function is not limited to transcriptional regulation.

For all these reasons, we decided to check whether photoreceptors were functional after Otx2 KO. Since we could not perform classical ERG and optomotor tests that record the retinal electrical activity in response to light stimuli and the visual acuity, respectively, we looked at the well-described molecular movements accompanying dark- or light-adaptation. Indeed, one of the mechanisms involved in visual adaptation, is the continuous light-dependent trafficking of different proteins as visual arrestin (expressed in rods and cones Deming *et al.*, 2015), transducin and recoverin between outer segments, inner segments and soma of photoreceptors. Because visual arrestin is also an Otx2 interactor, we tested whether its re-localization during dark and light adaptation was impaired. While in the dark, the localization of the protein was not affected by *Otx2* KO in photoreceptors, with a physiological accumulation in the soma and inner segments of the photoreceptors, after light exposure the localization was impaired. Upon light exposure of dark-adapted retina, visual arrestin normally moves from the soma and inner segments to the outer segments.

In the PR-specific *Otx2* KO model, at least 50% of total protein was blocked in inner segments and soma 8 hours after light exposure, suggesting a potential defect of arrestin transport, a condition that could affect light adaptation. This hypothesis was reinforced by the results obtained in the Light-Dark Box test, where KO mice appeared photophobic after being adapted in the dark. Open-field tests excluded the hypothesis that KO-mice were anxious.
A model that could fit with the arrestin localization defect and the behaviour of PR-specific *Otx2* KO mice is that, while after dark adaptation, arrestin is correctly distributed, when exposed to the light, arrestin translocation to the outer segment is slowed down. For that reason, KO mice do not quickly adapt to light and prefer to stay in the dark. Interestingly, when KO mice are routinely manipulated to clean or change cages, to start breeding, etc., they do not show obvious light avoidance. Since the light intensity in the mouse facility is much lower than that of the light compartment of the light-dark box, and since manipulations are done in the middle of the light period, it is probable that at some point, arrestin reaches a sufficient level for adaptation to constant light. It would be interesting to study the behaviour of KO mice during routine manipulation under stronger light intensities.

In the dark, rod photoreceptors reach very high sensitivity and can detect single photons. When after dark adaptation, rods are exposed to light stimuli, this high sensitivity could saturate and hyperactivate the phototransduction cascade, with a toxic effect on the cells. The relocalization of arrestin in outer segments in bright light is thought to have a neuroprotective function. It would block the phosphorylated rhodopsin and quench all rod activation, leaving only cones active, which would protect rods from phototoxicity (Chen et al. 1999). Interestingly, visual arrestin seems to be involved in cone light adaptation but also neuroprotection, suggesting that both functions can be reached by the same mechanism (Deming, Pak, Shin, et al. 2015; Brown et al. 2010). The defect of arrestin relocalization observed in PR-specific *Otx2* KO mice might affect this neuroprotective activity. The expected phenotype would be a higher rate of rod death or degeneration following high-intensity light exposure. This could be tested by comparing the rate of rod death in control and PR-specific *Otx2* KO mice adapted in the dark and then exposed to bright light for different durations.

The mechanisms of arrestin localization defect are not yet clear, but they must depend on Otx2 nuclear and/or extra-nuclear function. In the future, it will be interesting to study how *Otx2* KO impacts on arrestin trafficking.

Since it was shown that arrestin and Otx2 interact in the adult retina (Fant et al. 2015), one hypothesis is that Otx2 directly binds to arrestin and helps to move it to the outer segment under light conditions. However, a stoichiometric interaction 1:1 should be excluded, because arrestin is very abundant in the outer segments and Otx2 should also accumulate in these compartments after light exposure, which has never been observed.

Another hypothesis is that Otx2 indirectly regulates arrestin trafficking by regulating genes involved in its trafficking. The literature about arrestin transport is still scarce and contains some apparent discrepancies. It is known that arrestin recruitment to the outer segment requires functional bleached rhodopsin. It is also recognized that arrestin trafficking requires an intact cytoskeleton and both, microfilaments and microtubules, are required (Reidel et al. 2008a; Peterson et al. 2005). Little is known about motor proteins required for arrestin trafficking. In mammals it has been shown that the class II kinesin encoded by *Kif3a* is necessary for arrestin distribution, reinforcing a direct role of microtubules (Marszalek *et al.*, 2000). In Drosophila, non-muscle myosins that are transporting proteins along the microfilaments appear also necessary for correct arrestin distribution (Strissel & Arshavsky 2004; Hardie et al. 2012; Lee & Montell 2004). In mammals this does not appear to be the case, at least for some of them, like myosin VIIa (Reidel *et al.*, 2008). However, other non-conventional myosins could be involved. In PR-specific *Otx2* KO retinas, we found no downregulation of microtubules-associated proteins involved in protein trafficking as kinesin or dynein. By contrast, *Myl9*, coding for the non-muscle light chain myosin 9 (class II) (Dasbiswas et al. 2018; Park et al. 2011) is downregulated. In the future, it will be interesting to knock down this protein in photoreceptors and study whether it affects arrestin trafficking. Another myosin gene, *Myh11*, it is also downregulated in PR-specific *Otx2* KO mice. This is coding for a smooth muscle myosin chain, a class of protein not reported to be commonly implicated in trafficking. However, its expression in the retina is not well characterized, and its role is unexplored. For that reason, it can be interesting to investigate its expression in the retina and, if it is expressed in photoreceptors, to knock it down and test whether it has any effect on arrestin localization.

It has been first proposed that arrestin redistribution was not depending on active transport, but on diffusion, that does not require ATP, (Nair et al. 2005). Recent insights changed this view. Indeed, the requirement of the cytoskeleton was the first evidence that a diffusion model could not be applied and lately, it was demonstrated that ATP is definitely necessary for protein translocation (Orisme et al. 2010). In retinal cells, Otx2 has been shown to interact with subunits of the F0/F1 ATP-synthetase complex (Kim et al. 2015) and this interaction is required to produce a sufficient amount of ATP. In a model where *Otx2* is knocked out in high-demanding ATP cells as are the photoreceptors, it can be hypothesized that ATP concentration is decreased and could impact on arrestin translocation. While the cells still produce enough ATP to survive, they would not reach a threshold for correct arrestin translocation.

At the gene expression level, RNA-seq obtained from PR-specific *Otx2* KO mice collected between 2 and 8 days after KO, no clear mitochondrial defects emerged. However different genes involved in oxido-reductive processes are downregulated, such as *Maob, Gldc, Loxl1, Cox7c, Suclg2*) suggesting general stress that could indirectly affect mitochondrial functionality (Ott et al. 2007; Barot et al. 2011; Guo et al. 2013). According to this stress hypothesis, GFAP staining on this KO-model (not shown) confirms the activation of Muller Glial Cells 10-20 days after KO.

8.3 Otx2 is transferred from RPE to photoreceptors, a process that is accelerated in PR-specific *Otx2* KO mice

As mentioned above, Otx2 expression in the RPE is necessary and sufficient for photoreceptor viability (Housset et al. 2013). Different hypotheses can justify this neuroprotective non-cell-autonomous function of Otx2. RPE has an important photoreceptor nursing activity, protecting against light-induced oxidative stress, providing metabolites, eliminating waste end-products and contributing to the visual cycle (Strauss, 2005). Several genes implicated in some of these RPE functions are under Otx2 control (Housset et al., 2013). Therefore, the simplest hypothesis is that after KO in the RPE, the global RPE functionality is compromised and this irreversibly affects photoreceptors viability. Accordingly, 12 RPE genes, that are downregulated after the ablation of *Otx2* from all retina, are associated with retinal diseases. Among them, *A2M* is associated with

anophthalmia, microphthalmia and retinitis pigmentosa, *Trf* with age-related macular degeneration and *Trpm1* (expressed also in bipolar cells) with congenital stationary night blindness.

Another critical function of the RPE is to continuously secrete neurotrophins, to preserve photoreceptors integrity. Then, it can still be a possibility that some of them are under Otx2 control and that RPE-specific *Otx2* KO promotes photoreceptor degeneration because survival signals are lost. Knocking out *Otx2* specifically in the RPE could help to identify good candidates that could act in this way. The strategy used in Housset et al. (2013) to perform *Otx2* KO specifically in the RPE was based on the lentiviral mediated expression of Cre recombinase in a *floxed-Otx2* line. Despite the KO was RPE specific, the percentage of RPE cells infected and, then, knocked out was variable. Such condition made gene expression study not possible, because of a potential variability in knocked-out cell number. To date, an efficient inducible, RPE-specific *Cre*-expressing line that would allow a significant and reproducible KO is missing. Setting up these tools could have different benefits, for instance, to help to understand whether photoreceptor viability also relies on the secretion of neurotrophic factors, regulated by Otx2 in the RPE. Melanogenesis gene promoters could be used in such a purpose.

Otx2 has been shown *in vitro* (Lee et al. 2019) as *in vivo* (Spatazza *et al.*, 2013;) to also act as a signalling factor and to have neuroprotective properties (Ibad *et al.*, 2011; Kim *et al.*, 2015). These recent findings open the possibility that Otx2 could be directly transferred from RPE to photoreceptors, to preserve their viability. Our gene expression RNA-seq data show that *Otx2* ablation in the photoreceptors clearly affects RPE-specific Otx2-target genes, that are involved in melanogenesis, suggesting a potential down-regulation of Otx2 gene expression in the RPE. When *Otx2* is knocked out, the exon 2 region is deleted in photoreceptors, but a mutant mRNA splicing exon 1 to exon 3 is still produced. Instead, RPE and bipolar cells express the complete mRNA. The number of reads of exon 1 and exon 3 of *Otx2* can be used to monitor whether its own expression is deregulated after KO. However, no differences were observed between control and KO mice, suggesting that the total amount of *Otx2* mRNA extracted from full retinas is conserved and then no deregulation should occur in the RPE (and bipolar cells). By contrast, the level of Otx2 protein in the RPE appears to be reduced after KO and some staining simultaneously appears at the level of outer and inner segments. These two observations, downregulation of Otx2 target-genes

and apparent decrease of Otx2 protein level in the RPE, support the possibility that Otx2 is secreted by the RPE and up-taken by photoreceptors. For that reason, we prepared some AAV derived particles driving expression of tagged-Otx2 specifically in the RPE. In the first series of experiments, we tested GFP as control and constructs but, while we could easily detect GFP in the RPE, it was not possible to detect the construct HA-Otx2-(M2x4), due to technical limits. In the second series of experiments, we used RPE-specific expression of SEAP and SEAP-Otx2 in wild type mice. With this strategy, we demonstrated that SEAP alone is strongly expressed in the infected RPE and is spontaneously released in the interphotoreceptor matrix where it is blocked around outer and inner segments. SEAP-Otx2, also secreted in the extracellular space from the RPE, was detectable in outer and inner segment, but also in the soma and in the outer plexiform layer. This suggests that the presence of Otx2 in the SEAP-Otx2 fusion allows the protein to be internalized by photoreceptors and to travel into photoreceptor cells, down to their synaptic terminals.

As observed by RNA-seq data and Otx2 protein level in RPE, KO in photoreceptors seems to increase the amount of secreted Otx2. Coherently with these observations, comparing PR-KO with control mice, it appears that the level of SEAP-Otx2 produced in RPE is decreased, whereas it is increased in outer and inner segments and in the outer plexiform layer. All together these observations strongly suggest that Otx2 can be spontaneously secreted by RPE cells and internalized by photoreceptors where it might act in a neuroprotective manner and that ablation of *Otx2* from PR increases the amount of transferred protein.

To strengthen these results, it will be necessary to have an efficient quantitative test to measure *Otx2* mRNA and protein levels in the RPE before and after KO. Single-cell mRNA sequencing analyses could be used to compare the amount of *Otx2* RNA still present in RPE cells of control and KO mice. In parallel, single-cell protein analyses could help to exactly measure the protein level of Otx2 in control and KO mice. While in the photoreceptors, Otx2 protein level should dramatically decrease after KO, single-cell RNA-seq could verify whether *Otx2* mRNA level is conserved.

Despite our data strongly support the idea that the Otx2 sequence confers the ability of SEAP-Otx2 to be internalized by photoreceptors and that RPE is the physiological source of exogenous Otx2 for photoreceptors, the utilization of a secreted alkaline phosphatase as a tag could still influence this phenomenon and compromise our results. To definitively prove that Otx2 is spontaneously secreted by RPE cells, it will be necessary to produce new tagged-

Otx2, using, for instance, a non-secreted alkaline phosphatase. This could be achieved by deleting the 14 N-terminal amino-acids, provided it does not affect the AP activity.

The first set of experiments with HA-Otx2-(M2x4) could have not worked for different reasons: for instance, the amount of produced protein in the RPE is not sufficient to be detectable; the antibodies used to detect FLAG and HA tags are not adapted for *in vivo* staining in adults, where the developed extracellular matrix causes high background; the titre of AAVs and the serotype do not allow efficient infection of retinal cells *in vivo*. The first hypothesis appears contrasting with the fact that GFP was easily detectable, suggesting a sustained protein expression under the *Vmd2* promoter. All constructs sharing the same promoter, it is puzzling that while GFP is detectable, HA-Otx2-(M2x4) is not. However, there could be a difference of stability, with faster degradation of tagged-Otx2. The other two hypotheses are more plausible. Therefore, to overcome the detection problems, alternative tags, for instance, the V5 tag used in Lee et al. (2019), could be tested. For the last point, high-titre AAV 2/5 production, a serotype reported to infect retinal cells with maximal efficiency, has been requested from a specialized platform.

Despite our experiments suggest that there is a transfer of Otx2 protein from the RPE to photoreceptors, they do not prove any role of exogenous Otx2. To investigate this, there are different possible strategies. The first, based on (Bernard et al. 2016) would be to use a mouse line that conditionally expresses single-chain (sc-Fv) secreted anti-Otx2 antibodies in mature photoreceptors, to block exogenous Otx2. These authors have developed a *ROSA26:lox-stop-lox-sc-Fv-Otx2* transgenic line where a stop cassette blocks the production of these secreted antibodies, which is recombinase dependent. By crossing this mouse line with the *Crx-CreER*^{T2} line it would be possible to activate in a time-dependent manner the production of these antibodies specifically in the photoreceptors. These antibodies, quickly secreted, do not have an impact on the amount of endogenous protein, impairing then only the exogenous source. The neuroprotective role of exogenous Otx2 could thus be addressed by comparing the photoreceptors survival and activity in control and sc-Fv-Otx2-expressing mice.

To date, it appears that secreted homeoproteins are not only implicated in the gene regulation process in their target cells but also in local protein translation or mitochondrial ATP production (Brunet et al. 2005; Kim et al. 2015).

To assess whether exogenous Otx2 also acts as a nuclear factor it could be interesting to perform gene expression analyses after induction of secreted antibodies. Comparing RNA-seq data obtained here after the ablation of endogenous *Otx2* with gene expression data obtained in an "exogenous Otx2 ablation" context would help to discriminate the functions of the two sources at the gene expression level. Combining then the sc-Fv-expressing line, *floxed-Otx2* line and *Crx-CreER*^{T2} transgene line could be a strategy to deplete at the same time endogenous and exogenous Otx2 sources, to observe whether there are cooperative functions between exogenous and endogenous Otx2 or whether they work autonomously. Proteomic analyses could also be done in similar conditions to discriminate whether exogenous and endogenous Otx2 have different partners in the nucleus and in other cell compartments.

While an inducible *RPE-Cre* line is not available to promote the secretion of these antibodies by RPE cells, it could be possible to obtain it by using lentiviral or AAV vectors driving *Cre* expression only in the RPE. Assessing photoreceptor survival and activity in these conditions would add weight to the idea that RPE is a source of secreted Otx2, that exerts a neuroprotective function on photoreceptors.

A second strategy would be to use the self-conditional *Otx2* KO model (Fossat et al. 2006) to induce *Otx2* KO in all the retina and then, to induce expression of tagged-Otx2 only in the RPE, and detect whether the tagged protein can be found in photoreceptors. The WF→YL mutant (Ibad et al. 2011) that was shown to be not internalized by retinal ganglion cells, would be an interesting negative control. The main risk of this strategy is that these two amino acids are in the homeodomain and their mutation may affect Otx2 transcriptional activity. For this reason, in a condition of full retina KO, the expression of this mutant in the RPE might not be able to rescue RPE functionality. Photoreceptors degeneration would then occur not as a consequence of missing internalization but as a consequence of RPE dysfunction.

Recently, additional sites have been identified in Otx2 protein sequence that, when mutated, block the secretion of the protein *in vitro* (Lee et al. 2019a). The position of these sites outside the homeodomain suggests that they should not have an impact on Otx2 transcriptional activity, but only on its secretion. Expression of these mutants in the RPE

should then preserve gene regulation but abolish its secretion. Whether secreted Otx2 has a neuroprotective role could then be estimated by assessing photoreceptor fitness.

8.4 Otx2 ablation in photoreceptors has an effect on interphotoreceptor and extracellular matrix genes

The extracellular matrix (ECM) is made by different proteins and complex carbohydrates, locally secreted and, then, assembled into a meshwork that surrounds the neighbour cells. The main ECM components are the proteoglycans, as well as collagens and elastin, fibres forming proteins, and other structural proteins such as fibronectin, fibrillin, laminins. Minor components are extracellular proteases and other proteins such as growth factors. The ECM is not only an amorphous compartment that plays a structural function, but it is directly involved in signalling pathways (Mecham RP., 2012).

In the retina, the specialized interphotoreceptor matrix (IPM) fills the subretinal space between photoreceptors and RPE, while the retinal ECM surrounds the other cells. The IPM structure has some specificities that distinguish it from the retinal ECM: it is devoid of collagens and elastin; the only structural proteins making fibres are the fibulins. IRBP, the transporter protein essential for the visual cycle, is only found in the IPM. Proteoglycans and glycosaminoglycans formed by chondroitin sulphate chains are very abundant in both matrices but versican, decorin and biglycan, seem to be exclusive in the IPM.

Four days after *Otx2* ablation in photoreceptors, we observed more than 50 down-regulated genes related to ECM, suggesting that both ECM structure (collagens, fibrillins, etc.) and signalling (TGF beta related factors) could be impaired. The presence of versican, decorin and biglycan in the list of downregulated genes suggested that IPM could also be affected.

A role of Otx2 in the ECM has recently been suggested. Otx2 has been shown to bind the chondroitin sulphate chains of perineuronal nets (PNN), a special matrix surrounding parvalbumin cells in the visual cortex. Chondroitin sulphate chains are known for their ability to interact with signalling molecules and to facilitate the recognition of their target cells (Mizumoto et al. 2013). In parvalbumin cells, Otx2 interaction with sulphate chains of glycosaminoglycans promotes its internalization and subsequent maturation of the surrounding PNN (Beurdeley et al. 2012; Bernard & Prochiantz 2016). When Otx2 internalization is blocked, the PNN disappear, suggesting that internalized Otx2 directly

regulates PNN and chondroitin sulphate related genes. The similar regulation of ECM and IPM genes by Otx2 in the mature retina observed in our study indicates that this could be a general function of Otx2 in neurons.

Since the ECM participates both in the rigidity of tissues and to the signalling activities between cells, these might be perturbed in Otx2 mutant retina. We did not observe any change of retinal tissue cohesion following Otx2 ablation, but our data are in good agreement with a modification of the propagation of signalling activities. Indeed, we showed that Otx2 secreted by the RPE is internalized by the photoreceptors and that the process is accelerated by Otx2 ablation in photoreceptors. Whether chondroitin sulphate chains or other proteoglycans and glycosaminoglycans are required to sequester Otx2 for this transfer is not known. However, contrary to the situation of parvalbumin cells, where a lack of Otx2 triggers a negative auto-regulatory feedback loop ending with the disappearance of PNN, down-regulation of chondroitin sulphate and glycosaminoglycans following *Otx2* KO in photoreceptors facilitates the uptake of exogenous Otx2. Therefore, ECM modifications can have opposite consequences for Otx2 trafficking. This difference could result from the specific organization of the retina with its IPM intimately associating RPE and PR cells. A simple explanation would be that the IPM contains components that are secreted by PR cells under the control of Otx2, which limit Otx2 diffusion across the IPM. Following Otx2 KO in PR cells, the concentration of these components in the IPM would decrease, allowing facilitated diffusion of RPE-derived Otx2, and possibly of other factors. A thorough analysis of IPM composition in control and Otx2 PR-specific KO retinas might reveal the nature of molecules that limit Otx2 diffusion as well as the signalling factors of which Otx2 is a part.

IX Conclusions

In conclusion, this study yielded two main results (**Figure 36**): the first one is that endogenous Otx2 in photoreceptors has a key role for light adaptation. Indeed, its depletion induces arrestin mis localization and photophobic behaviour during light exposure. On the other side this work confirms that Otx2 expression in photoreceptors does not play a pivotal role in their viability, cell identity and for photoreceptor-specific gene regulation. These last two points could be the consequence of a compensatory mechanism based on the redundant transcriptional activity of Crx. An exogenous source of Otx2 could be used by photoreceptors to maintain their viability. In line with this idea, the second main result is that Otx2 can be internalized by mature photoreceptors and that RPE can be the physiological source. Moreover, it appears that Otx2 secretion is increased when the endogenous Otx2 source is lost by photoreceptors, suggesting that exogenous Otx2 can compensate some of the endogenous Otx2 functions. One hypothesis is that endogenous and exogenous Otx2 are both required in the mitochondria to produce a sufficient amount of ATP. When endogenous Otx2 is depleted in photoreceptors, unknown molecular signals are released from photoreceptors to communicate to the RPE to increase secretion of Otx2 (and maybe) also of other neurotrophic factors. Each RPE cell is facing at least 10 different photoreceptors cells. Therefore, the secretion of Otx2 could be sufficient for maintaining photoreceptors viability, but probably not sufficient to maintain their functionality. As one of the consequences, visual arrestin, which is also ATP dependent, is not efficiently transported, generating visual adaptation defects and photophobic behaviour in the PR-specific *Otx2* KO model. Whether non-muscle myosins such as Myl9, identified here as one of Otx2 target genes, are required in this transport, remains to be clarified.

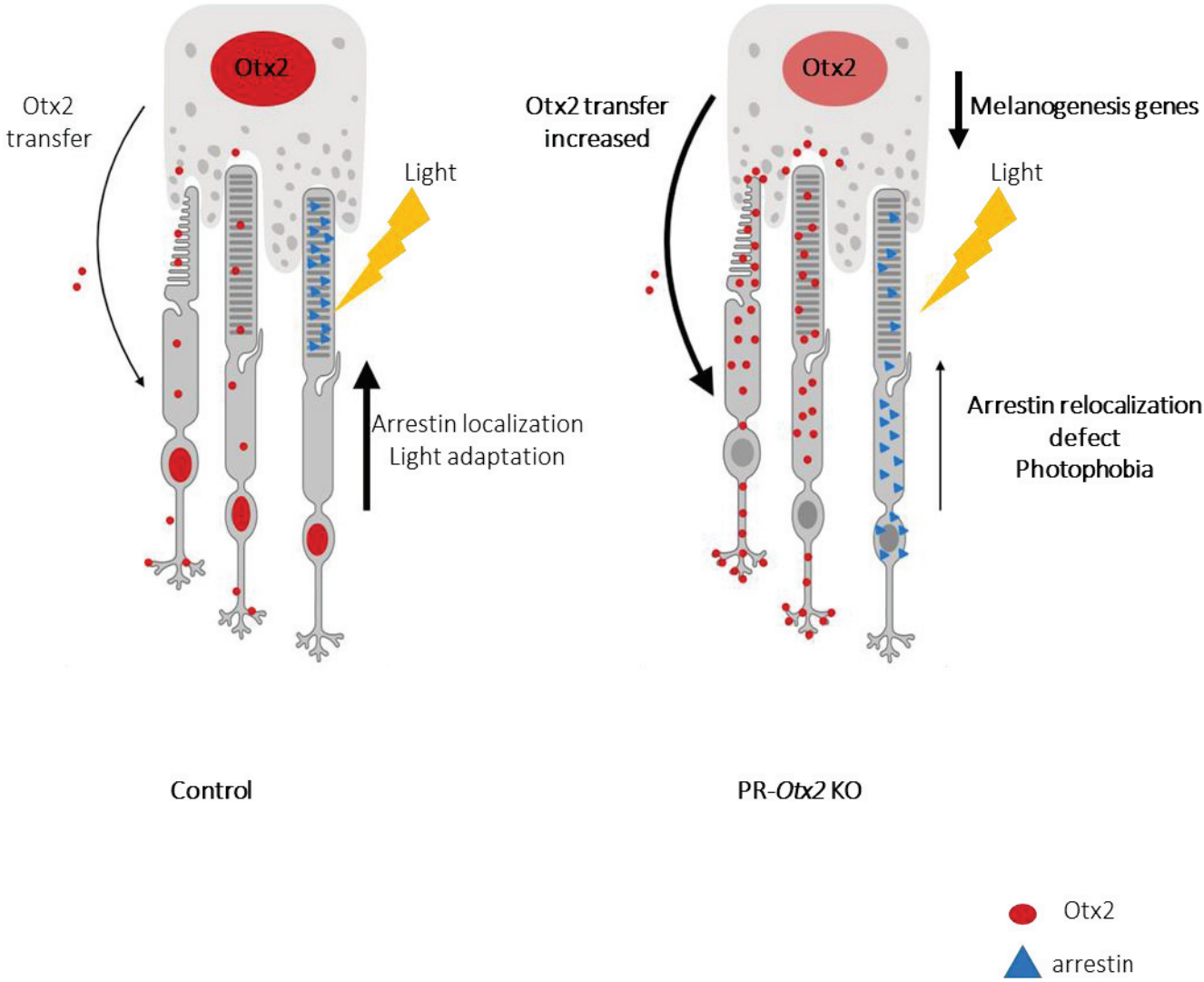


Figure 36. Schematic model of Otx2 functions in mature PRs. Endogenous Otx2 is implicated in arrestin light-induced relocalization from soma and inner segment to outer segments Otx2 KO in PRs impairs the correct accumulation of the arrestin in outer segments with a negative effect on light adaptation. RPE constitutively secretes Otx2, that is taken up by PRs. Otx2 KO in PRs increases this transfer, partially depleting RPE of this transcription factor, consequently affecting the expression of melanogenesis Otx2 target genes (downregulation) in this cell type.

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
Myh11	Vmn1r15	Sod3	A330074K22Rik	Myh11	A330074K22Rik
Tagln	Frat2	Vcan	Pcdh9	Tgm1	Cyp2j9
Tgm1	1700030C10Rik	Angptl2	Pcdh11x	Gsta3	5930430L01Rik
Mlana	Dusp1	Penk	Cdh12	Tagln	Ctif
Gpnmb	Nr4a1	Cldn19	L1cam	Myl9	Tln2
Angptl2		Ltbp1	Map1a	Rps3a1	Cdkl5
Myl9		Myh11	Kndc1	Mlana	Adcy1
Wnt16		Dio3	Tusc5	Gpnmb	Hydin
Pmel		Gsta3	Cdk14	Ubc	Fggy
Matn3		Mrc2	Grin2a	Pmel	Faah
Emilin2		Col18a1	Caln1	Matn3	Wsb2-ps
4930435M08Rik		Tgm1	Kif5a	Emilin2	Spta1
Gsta3		Serpine1	Mctp1	Wnt16	Mettl4
Rps3a1		Ism2	Cdkl1	4930435M08Rik	Kif5a
Bfsp2		Efemp1	Rbfox1	Bfsp2	Map1b
C2		Bace2	Sik1	Purg	Map1a
Pld5		Tagln	Lancl3	Angptl2	Odc1
Thbd		Matn2	Hydin	Edn3	Lancl3
Npsr1		Myl9	Srgap1	Thbd	Pcdh11x
Tyrp1		Emp3	Tmem151b	C2	Gm47021
Slc20a2		Edn3	Gpr158	Pld5	Cdh12
Tyr		Mlana	Pgr	Acta2	Impg1
Slc38a8		Atp1a2	Hecw2	Hpse	Gm5898
Bace2		Mgst1	Ar	Fam20a	Gm21955
Tspan10		Rarres2	Stc1	Gm14268	Slc9a7
Mlph		Slc38a8	Kcnq3	Slc38a8	Srgap1
Ccdc3		Rhoj	Atp2b3	Bace2	
Dcn		Wnt16	Tfap2d	Tyrp1	
Lyz2		Gpnmb	Htr5a	4930458D05Rik	
4932438H23Rik		Bgn	Pcdha6	Shmt1	
Acta2		Pmel	Prlr	Slc43a1	
Crybg1		Col9a2	D130079A08Rik	Zfp566	
Smtn		Fam129a	Onecut3	Smtn	
Slc11a1		Crhbp	Ppp1r1c	Adh1	
Ucp2		Pld5	Gm8983	Ociad2	
Papss2		Npsr1	Srl	AI464131	
Palmd		Iqgap2	Pou4f1	Npsr1	
C130021I20Rik		Bmp2	Ndst4	Serpine1	
		Wfdc1	Pcdha12	Ctsk	
		Emilin2	Klf12	Papss2	
		Col1a2	Alk	Gm44678	
		Tmprss11e	Gm49092	Hbb-bs	
		Rps3a1		Mlph	

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Wif1		Gm38021	
		Zic4		Tyr	
		Acta2		Mmp14	
		Zic1		Ucp2	
		Mdk		Wdr95	
		Notum		Gm26917	
		Klhdc7a		Stac	
		Dsg2		Cmtm3	
		Gpx3		Wif1	
		Matn3		Gxylt2	
		Mecom		E230020A03Rik	
		F5		Cnn1	
		Clec18a		Dcn	
		Tyrp1		Mdk	
		Rab38		Dmgdh	
		Defb9		Lrrc66	
		Mlph		C130021I20Rik	
		Thbs2		Gm28229	
		Stac		Dpp4	
		Ociad2		Slc24a5	
		Ifi30		Gjb6	
		Papss2		Palmd	
		Gldc		Tspan10	
		Idh2		Hbb-bt	
		Lox		Pdgfc	
		Cldn1		Nbl1	
		Bfsp2		Nradd	
		Ddr2		Gsx2	
		Gfpt2		Uap1l1	
		Smtn		Bgn	
		C2		Eva1c	
		Fbn1		Slc16a10	
		Ucp2		Atp1a2	
		Lyz2		Lox	
		Thbd		Nkd2	
		Ackr4		D130019J16Rik	
		Cnn2		Selenop	
		Fgfr1		Rny1	
		Dse			
		D630039A03Rik			
		Nid2			
		Nkd2			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Mrc1			
		C4b			
		Fbln7			
		Slc4a5			
		Pkdcc			
		Colec12			
		Aebp1			
		Tnfrsf21			
		Col9a1			
		Tyr			
		Zic2			
		Galnt12			
		Otx1			
		Sned1			
		Rapsn			
		Trip6			
		Mrgprf			
		Dio3os			
		Plin4			
		Igfbp4			
		Serping1			
		Gja1			
		Ces1d			
		Slc13a4			
		Dsg1a			
		BC067074			
		Fbln2			
		Crtap			
		Prelp			
		Tfeb			
		Cdk2			
		Suclg2			
		Col9a3			
		Ctsc			
		Adh1			
		Rcn3			
		Tspan10			
		Mdfic			
		Gm14268			
		H2-Aa			
		Ptgds			
		Bambi			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Fam107a			
		Tst			
		Boc			
		4930458D05Rik			
		Slc26a4			
		Aldh1a3			
		2310034O05Rik			
		Timp3			
		Id3			
		Slc4a11			
		Perp			
		Ephx1			
		Fbn2			
		Zfp185			
		Slc24a5			
		Optc			
		F11r			
		Frrs1			
		Tgfbr3			
		Fbln1			
		Nbl1			
		Fkbp9			
		Veph1			
		Bmp4			
		1810022K09Rik			
		4930523C07Rik			
		Crocc2			
		Adamts9			
		Cald1			
		Selenop			
		Cd74			
		Olfml2a			
		Cyba			
		P3h1			
		Chmp4c			
		Clec14a			
		Tmem176a			
		P2rx6			
		Fmod			
		Slc16a12			
		Dcn			
		Piezo1			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Gm7694			
		Tbx22			
		Gm44250			
		Flna			
		Gm26917			
		Ltbp2			
		Rapgef3			
		Nfatc4			
		Cmtm3			
		Arhgef5			
		Loxl3			
		Lamb2			
		Pon2			
		Rdm1			
		Cpz			
		Gypc			
		Nxn			
		Cyp4f15			
		H2-Eb1			
		Best2			
		Tlcd1			
		Blnk			
		Ccnd2			
		Ifitm2			
		Gas1			
		Eps8l2			
		Ctsh			
		Rhbdf1			
		Renbp			
		Eya1			
		Pon3			
		Ces5a			
		Ecm1			
		Pdgfrl			
		Efemp2			
		Ackr3			
		Acads			
		Cd82			
		Ankrd44			
		Tcim			
		Ajuba			
		Gpc4			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Mfap4			
		C1s1			
		Lrig3			
		Agpat2			
		Tmem176b			
		Slc6a13			
		Fbln5			
		Apoe			
		Bdh2			
		AI464131			
		Cgnl1			
		Gm45407			
		Nectin3			
		Ehd2			
		Slc25a34			
		Dpp4			
		Ggt5			
		Pcolce			
		Gprc5c			
		Osgin1			
		Atp10d			
		Cobll1			
		Thap6			
		Gm38021			
		Slc45a2			
		1700055D18Rik			
		Slc11a1			
		Pear1			
		Htra1			
		Josd2			
		Shmt1			
		4930594M22Rik			
		Ahnak			
		Plekhg3			
		Slc22a8			
		Gm43637			
		Fcgrt			
		Dct			
		Nedd9			
		Stx11			
		Ctsk			
		Abcc6			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Pdlim4			
		Fzd8			
		Tmem132b			
		Tmc6			
		Ptgr1			
		Cavin2			
		Syngr2			
		Gli2			
		Ager			
		Dok1			
		Pqlc3			
		Stab1			
		Plekhg2			
		AC152827.1			
		Echdc2			
		Tmem63a			
		Cd63			
		Ppfibp2			
		Vangl1			
		Slc2a1			
		Pgf			
		Kdelr3			
		Vstm4			
		A730049H05Rik			
		Hpse			
		Zic5			
		Wdr86			
		Sema3b			
		Pde3b			
		Sult1a1			
		Slc6a12			
		Itpripl2			
		Cldn2			
		Sqor			
		Iyd			
		Uap1l1			
		Mfrp			
		Abcc4			
		Gm42716			
		Nek8			
		Crybg1			
		C1qtnf6			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Il17rc			
		Nradd			
		Mxra8			
		Tmem140			
		Stk26			
		1500015O10Rik			
		Aldh1a7			
		Antxr1			
		Vill			
		Megf6			
		Tspo			
		Slc35f3			
		Scube1			
		1600023N17Rik			
		Abi3bp			
		Ttc16			
		Fmo1			
		Gpr137b			
		Ano1			
		D630024D03Rik			
		Igfbp5			
		Gm42555			
		Trim63			
		Wtip			
		Fam20a			
		Ptpn14			
		Gli3			
		Stard8			
		Hes1			
		Folr1			
		Itpr3			
		Dll1			
		Tns1			
		Tnfrsf19			
		Gm30698			
		Mfap2			
		Hhip			
		Smco4			
		Fkbp10			
		Gm44037			
		1700124L16Rik			
		P4ha2			

2 days KO DW genes	2 days KO UP genes	4 days KO DW genes	4 days KO UP genes	8 days KO DW genes	8 days KO UP genes
		Rab32			
		Cavin1			
		Lrrk1			
		2610035F20Rik			
		Lama5			
		Pla2g4a			
		Gm25835			
		Aqp5			
		Ephb4			
		Shc1			
		Gsn			
		Timp1			
		Clec3b			
		Cldn7			
		2810030D12Rik			
		Lgals1			
		Igf2bp1			
		Ntf3			
		Ogn			
		Gm44678			
		Slc35g1			
		Ahcy			
		Slc39a12			
		Serpinh1			
		Rbm47			
		Maob			
		Tead2			
		Pmp22			
		Slc4a4			
		Gje1			
		Rassf9			
		Six5			
		Gm20383			
		Plekhf1			
		Mboat1			
		Ch25h			
		Tmem150a			
		Snora73b			
		Wls			
		Adamts2			
		Loxl1			
		Gm12689			

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