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Régulation transcriptionnelle du facteur de transcription spécifique des bâtonnets, *Nrl*

Transcriptional regulation of the rod-specific transcription factor, *Nrl*

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The work presented here was performed under joined PhD-supervision between the Neurobiology-Neurodegeneration and Repair Laboratory, N-NRL, at the National Eye Institute, NIH, USA and the Institute of Cellular and Integrative Neurosciences, UPR 3212, Strasbourg, France. Most of the graduate work was performed at N-NRL and was supported by intramural research program of the National Eye Institute.

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Ms Marie-Audrey Kautzmann was a member of the European Doctoral College of the University of Strasbourg, during the preparation of her PhD, from 2009 to 2012, class name Charles Darwin. She has benefited from specific financial supports offered by the College and, along with her mainstream research, has followed a special course on topics of general European interests presented by international experts. This PhD research project has been led with the collaboration of two institutes: N-NRL, NEI, NIH, USA and INCI, UPR 3212, CNRS, University of Strasbourg, France.

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We will stand in the presence of riddles, but not without the hope of solving them. And riddles with the hope of solution - what more can a scientist desire?

Hans Spemann 1927

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Marie-Audrey KAUTZMANN

Régulation transcriptionnelle du facteur de transcription spécifique des bâtonnets, *Nrl*

Résumé

Le facteur de transcription Nrl (Leucine zipper de la rétine neurale) associé à d'autres facteurs de transcription, active l'expression de gènes des bâtonnets tels que le photopigment *Rhodopsine*,.et est impliqué dans la Rétinite Pigmentaire. Ainsi, le gène *Nrl* constitue un modèle intéressant pour la compréhension des programmes contrôlant le développement et l'homéostasie des photorécepteurs.

Ce travail de thèse vise à caractériser les mécanismes régulateurs de l'expression de *Nrl* au cours du développement rétinien. Avec l'électroporation *in vivo* de vecteurs rapporteurs portant des portions distinctes du promoteur *Nrl* dans la rétine de souris nouveaux-nés, nous avons identifié des séquences minimales de promoteur nécessaires à une expression spécifique dans les photorécepteurs. Nous avons identifié ROR comme facteur requis pour cette expression, et montré que les facteurs de transcription OTX2, CRX et CREB s'accrochent aussi directement à des régions régulatrices particulières du promoteur.

Nous avons construit un virus adéno-associé (AAV) contenant un promoteur *Nrl* minimal de 0.3 kb, et montré que ce dernier est adapté à la délivrance de gène spécifiquement dans les photorécepteurs.

Nous avons également montré que NRL, CRX et NR2E3, les régulateurs principaux de la *Rhodopsine*, ont une expression rythmique au cours de 24 h, et que l'expression cyclique de *Nrl* peut être due à l'activation par ROR, un composant moléculaire de l'horloge circadienne. Enfin, nous avons identifié un nouveau facteur de transcription, NonO, au niveau de la région du promoteur proximal de la *Rhodopsine*, et démontré que ce facteur, en combinaison avec NRL et CRX, active le promoteur de la *Rhodopsine*. L'invalidation de *NonO* au cours du développement rétinien a mis en évidence son implication pour le développement et l'homéostasie des bâtonnets.

Rétine, photorécepteurs, Leucine zipper de la rétine neurale, rétinogenèse, transcription, horloge circadienne, Rhodopsine

Résumé en anglais

The Neural Retina Leucine zipper transcription factor (Nrl) plays a central role in rod photoreceptor development and homeostasis. Nrl, combined with other photoreceptor-specific transcription factors, activates expression of rod-specific genes such as the visual photopigment, *Rhodopsin*. Moreover, mutations in *Nrl* have been associated with Retinitis Pigmentosa. Thus, *Nrl* gene is an interesting model for understanding genetic programs controlling photoreceptors development and homeostasis.

This thesis work aimed at characterizing regulatory mechanisms of *Nrl* expression during retinal development. Using *in vivo* electroporation of reporter vectors carrying distinct portions of *Nrl* promoter into neonatal mouse retina, we identified the minimal sequences necessary to drive reporter gene expression specifically in photoreceptors layer. We identified ROR as being required for this expression and showed that OTX2, CRX and CREB transcription factors also directly bind to the defined regulatory regions.

Based on these results we designed a novel adeno-associated virus (AAV) vector containing a minimal *Nrl* promoter fragment of 0.3 kb, and showed that it is well-suited for gene delivery specifically into photoreceptors.

We also showed that NRL, CRX, and NR2E3, the main transcriptional regulators of *Rhodopsin*, display rhythmic expression over 24 h. and that *Nrl* might undergo cyclic activation by RORwhich is part of the photoreceptor circadian clock. Finally, we investigated the role of a novel *Rhodopsin* transcriptional regulator, NonO, identified in the *Rhodopsin* proximal promoter region. We demonstrated that NonO co-activates *Rhodopsin* promoter along with NRL and CRX. By knocking down this gene during retinal development we provided evidence for its role in rod development and homeostasis.

Retina, photoreceptors, Neural retina leucine zipper, retinogenesis, transcription, circadian clock, rhodopsin

RECURRENT ABBREVIATIONS

AAV	Adeno-associated virus
BMAL1	Brain and mucle ARNTL protein 1
bZIP	basic motif leucine zipper
CAG	CMV early enhancer/chicken b-actine promoter
CLOCK	Circadian locomoter output cycles kaput
CMV	Cytomegalovirus
CREB	cAMP response element binding-protein
Crx	Cone rod homeobox
DAPI	4',6-diamidino-2-phenylindole
EGFP	Enhanced Green Fluoresceent Protein
EMSA	Electrophoretic mobility shifht assay
GCL	Ganglion cell layer
HEK	Human embryonic kidney
INL	Inner nuclear layer
Maf	Musculo Aponeurotic Fibrosarcoma
NonO	Non-POU domain containing octamer binding protein
Nr2e3	Nuclear receptor subfamily 2, group E, member 3
Nrl	Neural retina leucine zipper
ONL	Outer nuclear layer
OS	Outer segment
Otx2	Orthodenticle homolog 2

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PER	Period
PR	Photoreceptor
REVERBα	Nuclear receptor subfamily 1, group D, member 1 (Nr1d1)
RK	Rhodopspin Kinase
RORβ	RAR-related orphan receptor b
RORE	ROR response element
RP	Retinitis pigmentosa
RPE	Retinal pigmented epithelium
S-cone	Short wavelength-sensitive opsin-expressing cone
TF	Transcription factor

RÉSUMÉ DE LA THÈSE EN FRANÇAIS

Chez les Eukaryotes, la transcription d'un gène repose sur la présence d'éléments régulateurs autour de ce gène: le promoteur basal, ainsi que des séquences situées en aval et/ou en amont du promoteur. Ces séquences sont le siège d'accrochage direct de facteurs trans-activateurs ou facteurs de transcription (FT), qui travaillent en synergie avec des co-transactivateurs, ne liant pas l'ADN directement mais agissant par le biais des FT. Ces régulateurs sont essentiels à l'accrochage de la machinerie d'initiation de la transcription sur l'ADN. Cette machinerie est constituée d'un large complexe protéique, dont l'unité principale est l'ARN polymerase II. L'intervention de FT influencera le niveau de transcription d'un gène donné, mais également déterminera l'expression ou non de ce gène en ayant des fonctions activatrice ou inhibitrice. Le mode d'action des FT est vraisemblablement de recruter des protéines co-régulatrices de la transcription qui altèrent localement la conformation de la chromatine, permettant ainsi l'accès de l'ARN polymerase II au site d'initiation de la transcription. Une autre possibilité est le recrutement par les FT de co-activateurs qui possèdent des fonctions enzymatiques affectant la chromatine. De par sa structure primaire, la chromatine est hautement condensée par l'action des histones; l'accès aux gènes nécessite donc une altération transitoire de cette structure histone-ADN. Des enzymes telles que les transférases de groupements acétyl, méthyl ou de phosphates, permettent de marquer les gènes transcriptionnellement actifs et de déstabiliser ces complexes histones-ADN pour permettre l'ouverture de la chromatine et l'accès aux séquences régulatrices du promoteur pour initier la transcription. Certains FT permettent d'activer leur gène cible de façon spatio-temporelle et confèrent aux cellules dans lesquelles ils sont exprimés une identité cellulaire. En d'autre terme, c'est la fine régulation de l'expression de gènes spécifiques qui permettra de générer des types cellulaires variés constituant un tissu donné.

La diversité des types de neurones peut être étudiée en utilisant un tissu modèle : la rétine. Son accessibilité et son répertoire cellulaire bien défini font de la rétine un tissu de choix pour l'étude des mécanismes sous-jacents au développement de types cellulaires spécifiques. La rétine est un fin tissu nerveux situé au fond de l'œil, et est composée de six types de neurones et un type de glie organisés en couches bien distinctes. La lumière traverse l'ensemble de ces couches pour être perçue au niveau de

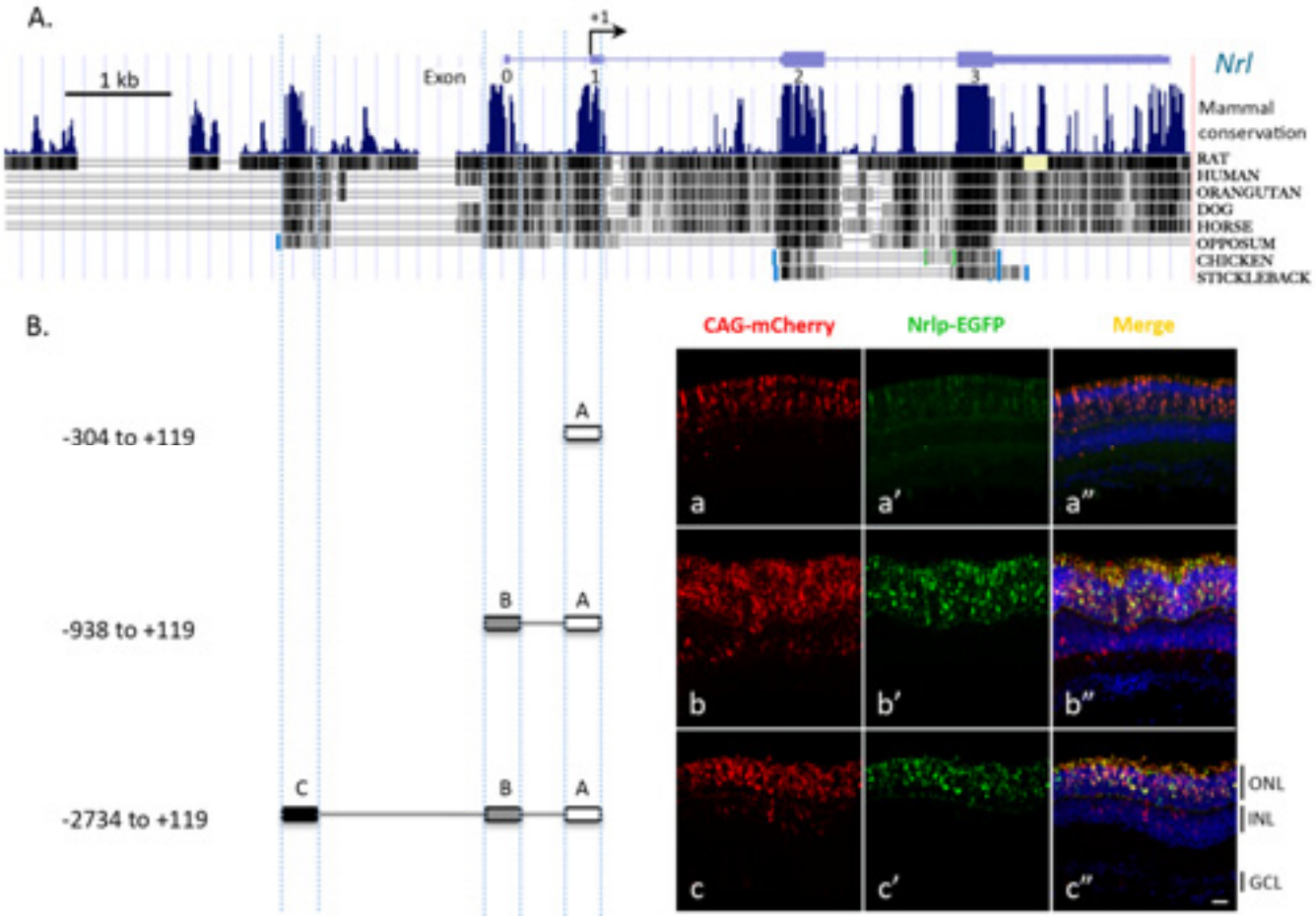

Figure 1'. Expression de séquences du promoteur/enhancer de *Nrl* au cours du développement de la rétine

Séquences génomiques en amont du promoteur/enhancer de *Nrl* représentant trois régions conservées (dénommées cluster A, B, C) au cours de l'évolution. Le diagramme de conservation de la séquence du promoteur de *Nrl* montre l'homologie de séquence entre divers vertébrés. Un ou plusieurs clusters du promoteur de *Nrl* ont été utilisés pour générer des constructions d'ADN contenant le gène rapporteur fluorescent de la GFP. Des sections représentatives de la rétine de souris transfectées *in vivo* au jour post natal (P) 0-2 ont été analysées à P14 pour évaluer l'expression de la GFP. La construction d'ADN CAG-mCherry, qui s'exprime dans toutes les cellules transfectées, a été utilisée pour indiquer l'efficacité de l'électroporation *in vivo*. Barre d'échelle : 20 µm. ONL, Couche Nucléaire Externe ; INL, Couche Nucléaire Interne ; GCL, Couche des cellules ganglionnaires.

la couche la plus externe, composée des photorécepteurs (PR). Les PR, qui possèdent des pigments visuels (opsines) sensibles aux photons, sont chargés de traduire l'influx lumineux en message nerveux et de le transmettre aux autres couches de la rétine qui relaient ce signal par le biais des axones des cellules ganglionnaires jusqu'aux centres d'intégration dans le cerveau. On distingue deux types de photorécepteurs, sur la base de leurs caractéristiques morphologiques et fonctionnelles, les cônes et les bâtonnets. Chez l'Homme, il existe trois types de cônes dissemblables par l'opsine qu'ils expriment. Les cônes sont responsables de la vision en forte intensité lumineuse et de la détection des couleurs, alors que les bâtonnets, qui ne possèdent qu'un type d'opsine, la Rhodopsine, fonctionnent en faible intensité lumineuse et sont responsables de la détection des mouvements et des formes. Les bâtonnets ont un rôle trophique pour les cônes (Leveillard, Mohand-Said et al. 2004) et sont particulièrement sensibles aux altérations d'expression génique ainsi qu'à la qualité de l'environnement extracellulaire (Parapuram, Cojocaru et al. 2010). De ce fait, l'élucidation de la genèse et du maintien fonctionnel des photorécepteurs de type bâtonnets est nécessaire pour une meilleure élaboration de stratégies thérapeutiques pour le traitement des affections rétiniennes conduisant à la cécité.

Chez les Mammifères, la genèse des différents types neuronaux composant la rétine s'effectue selon un ordre précis (Young 1985). L'intervention binaire de facteurs extrinsèques et intrinsèques est critique pour le développement rétinien (Livesey and Cepko 2001), cependant, il semblerait que les facteurs intrinsèques soient prédominants dans l'acquisition de la spécificité du type cellulaire. Parmi les facteurs intrinsèques agissant au niveau des cellules post-mitotiques, Nrl (Neural retina leucine zipper) (Swaroop, Xu et al. 1992), un facteur de transcription de type bZIP, joue un rôle primordial dans la détermination des précurseurs des photorécepteurs post-mitotiques à se différencier en bâtonnets plutôt qu'en cônes (Oh, Khan et al. 2007). Nrl est le facteur clé responsable de la formation de la lignée des bâtonnets, en effet, l'ablation du gène *Nrl* résulte en la formation d'une rétine composée uniquement de photorécepteurs de type cône (Mears, Kondo et al. 2001). L'expression ectopique de Nrl chez des animaux $Nrl^{-/-}$ résulte en une rétine composée uniquement de bâtonnets (Oh, Khan et al. 2007). Ces derniers se révèlent fonctionnels d'après l'électrorétinogramme (ERG) en conditions

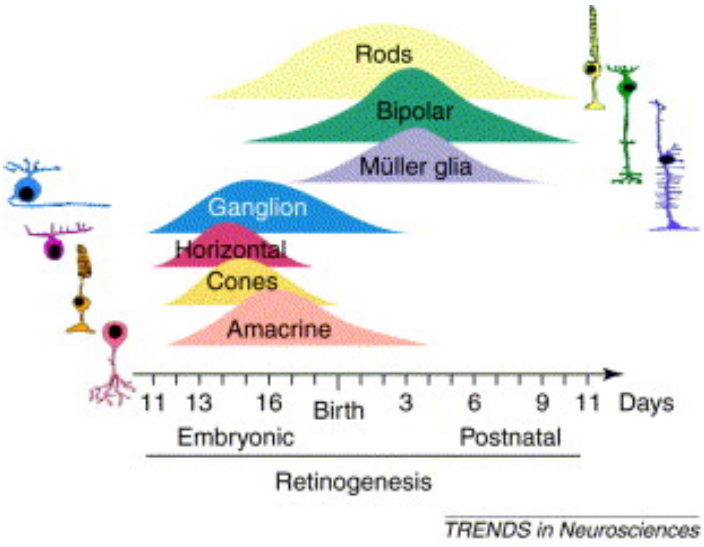

Figure 2'. Ordre de naissance des cellules de la rétine de souris

La neurogenèse rétinienne suit un ordre histogénique précis. Les cellules ganglionnaires et horizontales sont les premières à se différencier, suivies des phases de naissance juxtaposées des photorécepteurs de type cône, des cellules amacrines, des photorécepteurs de type bâtonnets, des cellules bipolaires et enfin des cellules gliales de Müller. Chaque courbe reflète la proportion relative de chaque type cellulaire produit, plutôt qu'un nombre absolu de cellules nées. (D'après Marquardt *et al*. 2002)

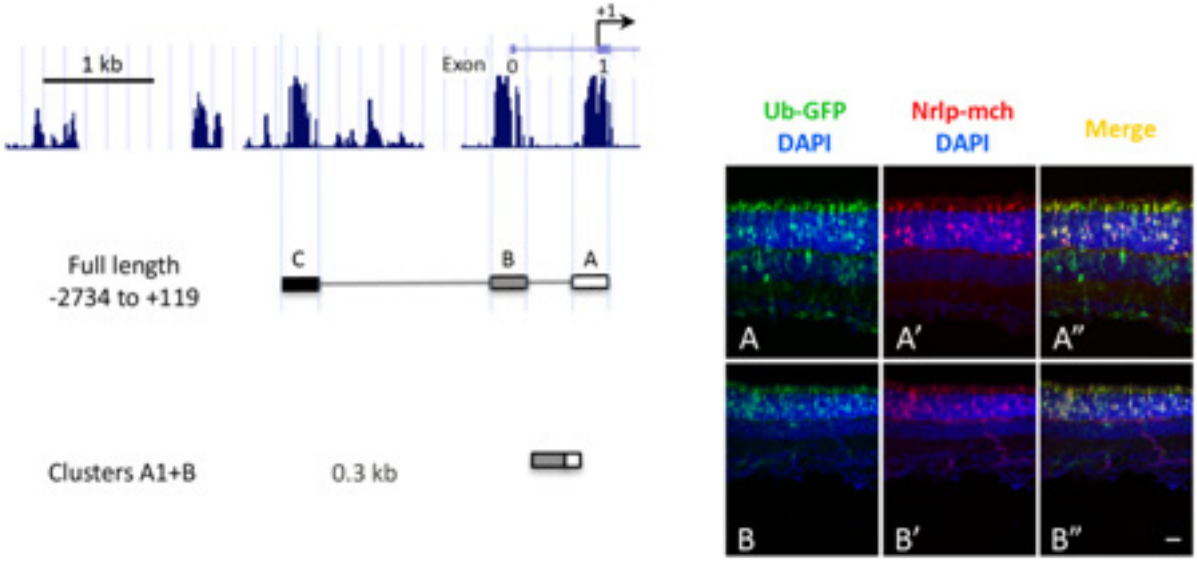

Figure 3'. Identification d'une région minimale du promoteur de *Nrl* transcriptionnellement active

La dissection du promoteur de *Nrl* a permis de mettre en évidence la fonctionnalité du cluster B (−938 à −657) en association avec la région A1 (−35 à +16) du cluster A. La combinaison de ces deux éléments de promoteur résulte en une séquence de 0.3 kb qui montre une activité transcriptionnelle similaire à celle du plus long fragment (−2734 à +119). Barre d'échelle, 20 µm.

scotopiques. Ces données suggèrent que Nrl est un facteur non seulement essentiel mais aussi suffisant à la genèse des photorécepteurs de type bâtonnet. De plus, Nrl est impliqué dans le maintien de l'homéostasie des bâtonnets, puisqu'il agit avec d'autres cofacteurs tels que Crx et Nr2e3 pour activer l'expression de la Rhodopsine (Rehemtulla, Warwar et al. 1996) et de plusieurs autres gènes spécifiques des bâtonnets (Pittler, Zhang et al. 2004; Oh, Cheng et al. 2008). Une altération de l'expression des gènes des photorécepteurs, dont notamment *NRL*, résulte en une dégénérescence de ces cellules à l'origine d'un certain nombre de pathologies telles que la Rétinite Pigmentaire (Bessant, Payne et al. 1999; Kanda, Friedman et al. 2007; Hernan, Gamundi et al. 2011). Ainsi, un fin contrôle de l'expression du gène *NRL* semble crucial pour le développement et le fonctionnement correct des photorécepteurs de type bâtonnet. De fait, *NRL* constitue un bon modèle pour comprendre les programmes génétiques contrôlant le développement des photorécepteurs.

Au cours de ma thèse, je me suis attachée à caractériser les mécanismes régulant l'expression du gène *Nrl*. Une partie de ce travail a donné lieu à une première publication dont je suis le premier auteur (Kautzmann, Kim et al. 2011). L'analyse des régions du promoteur m'a permis de cibler trois régions principales fortement conservées chez les vertébrés (Figure 1'). J'ai exploré les propriétés de ces régions en termes de régulation transcriptionnelle, en utilisant la technique d'électroporation *in vivo* qui consiste à faire pénétrer des acides nucléiques dans la rétine d'animaux nouveaux nés, correspondant à la période où la prolifération des bâtonnets est maximale (Figure 2'). L'injection de vecteurs rapporteurs contenant des segments du promoteur de *Nrl* de différentes tailles a permis d'identifier une région de 0.3 kilobases suffisante pour une expression du rapporteur fluorescent spécifiquement dans les bâtonnets (Figure 3'). Une analyse des séquences de promoteur transcriptionnellement actives a permis de mettre en évidence l'accrochage direct de facteurs de transcription RORβ, CRX, OTX2 et CREB sur des sites consensus prédits (Figure 4').

La définition d'un promoteur *Nrl* minimal, fonctionnel et spécifique pour l'expression dans les bâtonnets, a ouvert une perspective d'utilisation de ce promoteur en thérapie génique en utilisant des rAAV (virus recombinants adéno-associés). J'ai testé

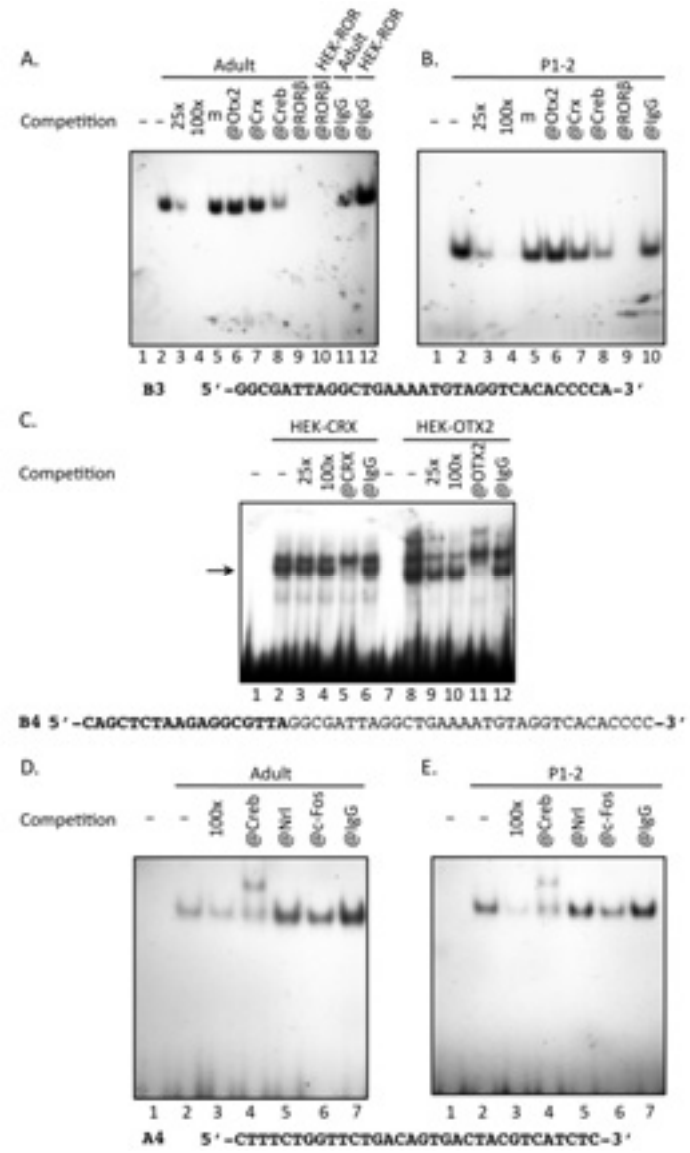

Figure 4'. RORβ, CRX, OTX2 et CREB s'accrochent aux éléments de séquence du promoteur /enhancer de *Nrl*

Autoradiogrammes de gels pour des oligonucléotides radiomarqués avec du ^{32}P et retardés avec des protéines nucléaires extraites de rétines de souris adultes (A et D) et nouveaux-nés P1-2 (B et E), ou extraites de cellules HEK293 transfectées avec les constructions d'ADN *CMV-RORβ* (A, lignes 10 et 12), *CMV-CRX* ou *CMV-OTX2* (C). L'ajout de 25 ou 100 ng d'oligonucléotides spéciques non-marqués entre en compétition avec l'oligonucléotide marqué et réduit son signal. L'oligonucléotide non-marqué mutant (m), comportant cinq substitutions de nucléotides dans la séquence consensus d'accrochage, n'entre pas en compétition. Des supershifts ont été réalisés avec des anticorps contre OTX2, CRX, CREB, RORβ, et ded IgG non dirigés contre ces facteurs (A and B), avec des anticorps anti-CRX ou -OTX2 (C), et avec des anticorps contre CREB, NRL, c-Fos, et d'IgG normaux (D and E). Les oligonucléotides B3, B4 et A4 sont indiqués. B4 contient 18 nucléotides supplémentaires à l'extrémité 5' de B3, lui conférant ainsi une meilleure séquence de reconnaissance aux facteurs de transcription de transcription à homéodomaine.

la fonctionnalité de ce promoteur de *NRL* à exprimer le gène rapporteur suivant son clonage dans le génome du vecteur viral AAV8 (dérivé de l'Adenovirus Associated Virus), et ai montré que ce promoteur pouvait être activé chez de jeunes souris (10 jours post-natal). De plus, deux types de rétines de souris ont été infectés *in vivo*, des rétines sauvages et des rétines de souris NRL KO composées uniquement de cônes. L'infection par les AAV8-NRLp-EGFP a montré une expression spécifique du gène rapporteur au niveau de la couche des photorécepteurs, et une activation du promoteur dans la rétine des NRL KO (Figure 5'). Cette dernière observation peut être expliquée par le fait que les photorécepteurs se différencient à partir d'un pool commun de précurseurs et que ces cellules expriment donc des facteurs de transcription communs aux deux types de photorécepteurs. Sur le même registre de thérapie génique, nous avons cherché à mettre en évidence la plasticité des photorécepteurs post-mitotiques dans leur capacité à changer leur spécificité cellulaire. Pour cela, nous avons utilisé des AAV pour réintroduire NRL sous le contrôle du promoteur ubiquitaire CMV dans la rétine de souris NRL KO. Trois âges distincts du développement de la rétine ont été choisis, P4, P10 et adulte. Par des expériences d'immunomarquage, nous avons démontré l'expression de la Rhodopsine pour chaque âge testé (Figure 6'); ces résultats nous indiquent donc que NRL est un facteur de transcription qui a la capacité de moduler la spécificité cellulaire aussi bien dans des cellules post-mitotiques en cours de développement que dans des cellules déjà matures. L'utilisation du promoteur *NRL* avec des rAAV ouvre une base d'étude pour le développement de futurs vecteurs viraux ciblant spécifiquement les photorécepteurs.

Nrl ne représente pas seulement un intérêt d'étude au niveau développemental, mais aussi du point vue circadien. En effet, il a été montré que la rétine des vertébrés possède une horloge circadienne capable d'entraîner des fonctions rythmiques sur 24 h. Une horloge circadienne se définit comme un oscillateur endogène qui permet aux organismes de s'adapter et d'anticiper les changements journaliers induits par la rotation de la Terre. Les rythmes générés par l'horloge circadienne sont auto-entretenus, en soit, ces rythmes persistent sur 24 h même en l'absence de donneurs temps ou zeitgebers. Ils sont générés par des boucles d'auto-régulations transcriptionnelles impliquant des facteurs de transcription dits facteurs horloge tels que *Clock*, *Bmal1*, *Period1-3*, *Rorβ*,

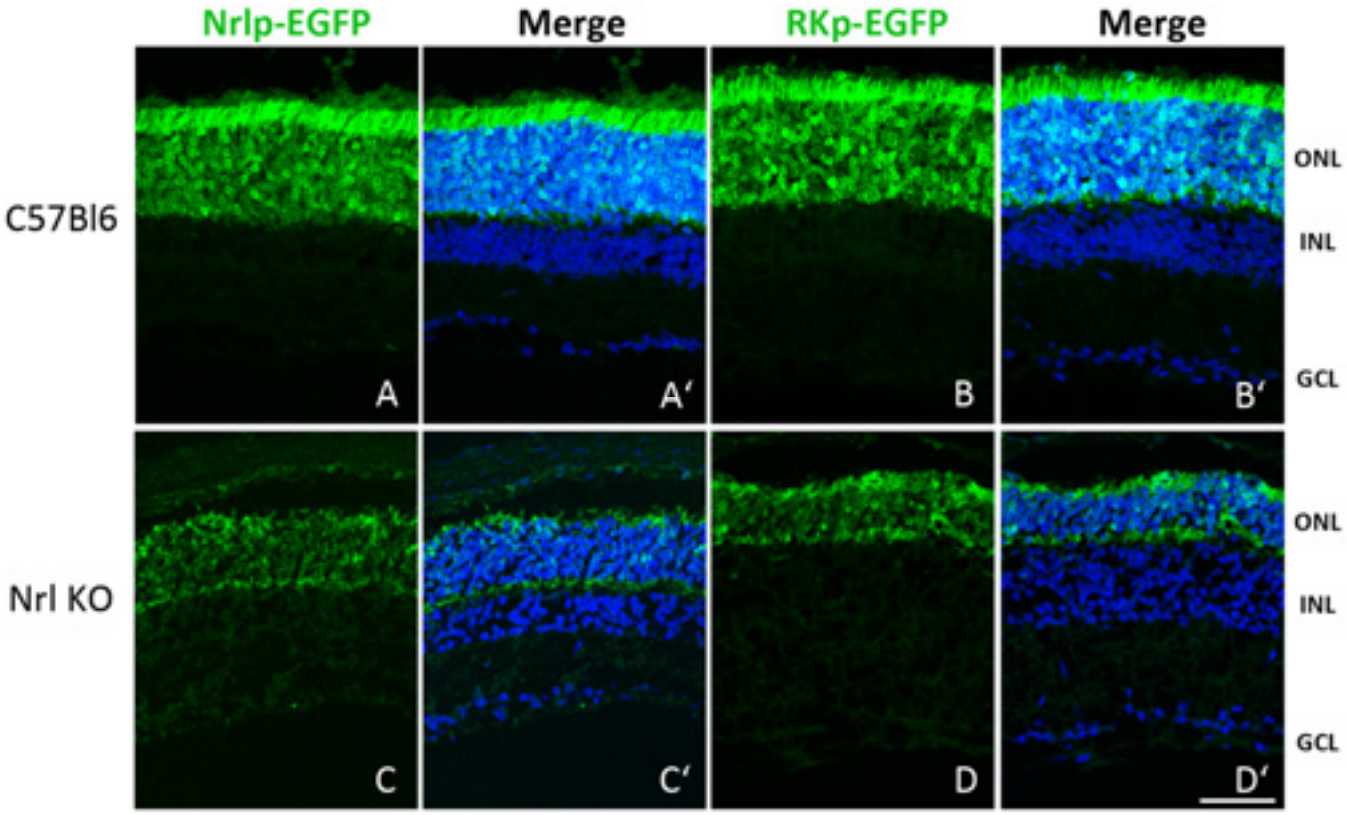

Figure 5'. Expression de la GFP dans des rétines de souris infectées avec des virus recombinants adéno-associés

Des rétines de souris de type sauvage (C57Bl6; A-B') et transgénique (Nrl KO; C- D') ont été injectées à P10 avec 1µL de constructions virales AAV8-Nrlp-EGFP (A, A', C, C'; concentré à 3^{e12} particules virales/mL ; *Nrl* promoteur murin de 0.3 kilobases) et AAV-RKp-EGFP (B, B', D, D'; concentré à 1^{e12} particules virales/mL ; Rhodopsin kinase promoteur humain de 1.2 kb). Trois semaines après l'injection, les rétines montrent une expression de la GFP uniformément sur la couche des photorécepteurs (couche nucléaire externe, ONL). Aucune expression de la GFP n'a été détectée dans la couche nucléaire interne (INL) ou dans la couche des cellules ganglionnaires (GCL). Barre d'échelle, 50 µm.

RevErbα, qui régulent aussi d'autres gènes cibles, entraînant ainsi les fonctions rythmiques de la cellule. Le zeitgeber le plus puissant est le cycle jour/nuit, perçu chez les Mammifères exclusivement par la rétine. Le rôle de l'horloge rétinienne est probablement d'adapter la rétine aux changements d'intensité lumineuse subits au cours de 24 h, de l'ordre de $1x10^6$ fois le niveau basal. Parmi les fonctions rythmiques de la

rétine, nous pouvons citer des phénomènes d'ordre physiologique, tels que la sensibilité visuelle et le traitement de l'information lumineuse qui se manifeste au niveau des variations de l'ERG, ainsi que la régulation du pH extracellulaire caractérisés chez le lapin (Brandenburg, Bobbert et al. 1983; Vaughan, Nemke et al. 2002), d'un point de vue moléculaire, les voies de signalisation de l'AMPc et phospho-CREB chez le Xénope et le poulet (Liu and Green 2002; Ivanova and Iuvone 2003), l'expression rythmique de l'ARN messager du gène de la *melanopsine* chez le poulet (Chaurasia, Rollag et al. 2005), ou encore de gènes impliqués dans la phototransduction, tels que la transducine chez le rat (Brann and Cohen 1987) et les opsines (Bowes, van Veen et al. 1988; von Schantz, Lucas et al. 1999; Li, Chaurasia et al. 2008). La variation journalière d'opsine étant l'une des sorties de l'horloge de la rétine, le contrôle de cette fonction rythmique peut être investiguée. Nous avons vérifié que l'expression de la Rhodopsine présente effectivement un rythme journalier dans la rétine de rat et montré qu'il en va de même pour l'expression de ses FT régulateurs principaux, Nrl, Crx, Nr2e3 et RevErbα. C'est pourquoi, sur la base de la caractérisation du promoteur de *Nrl* que j'avais réalisée, nous avons choisi d'aller un plus loin dans la compréhension de la régulation rythmique de ce gène.

Des expériences complémentaires liées à l'observation de l'existence d'un site « RORE » (Retinoid related orphan nuclear receptor response element, séquence cible des facteurs de transcription ROR et RevErβ) au niveau du promoteur *Nrl* m'ont permis de montrer que la fixation du facteur RORβ est requise pour l'expression du gène *Nrl* dans les bâtonnets (Figure 7'). RORβ se révèle être un candidat intéressant pour l'étude de la régulation de *Nrl* du point de vue développemental parce qu'il est requis pour l'induction du lignage des photorécepteurs (Jia, Oh et al. 2009), mais également d'un point de vue circadien. En effet, RORβ est aussi un constituant moléculaire de l'horloge circadienne et

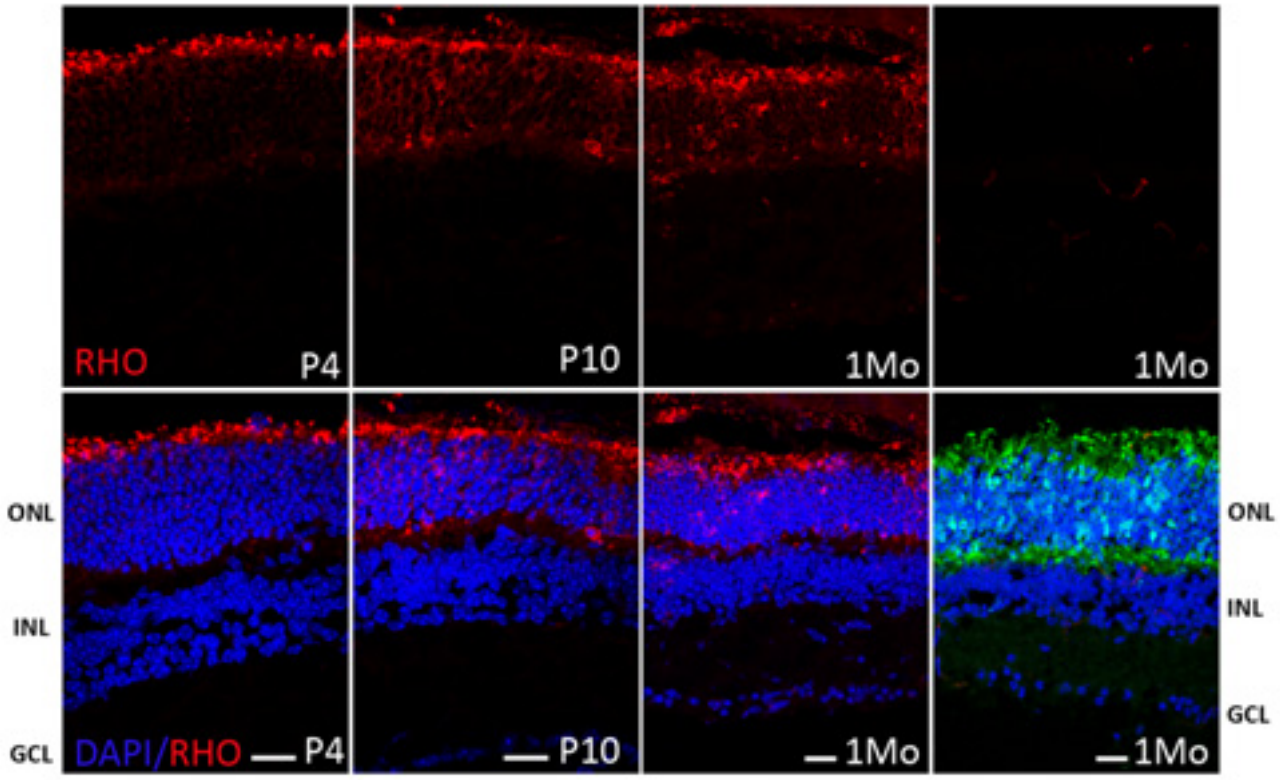

Figure 6'. L'expression de la Rhodopsine est rétablie suite à la ré-introduction de NRL dans des rétines Nrl KO

Des rétines de souris transgéniques Nrl KO ont été injectées à P4, P10 et 1 mois avec 1µL de constructions virales AAV8-CMV-NRL et AAV-RKp-EGFP. L'évaluation de l'expression de la Rhodopsine et de la GFP s'est effectuée trois semaines après l'injection. L'immunomarquage anti-Rhodopsine (RHO) sur des rétines Nrl KO injectées avec AAV8-CMV-NRL a permis de détecter la présence de cette protéine spécifiquement dans la couche des photorécepteurs (ONL) à tous les âges testés. L'injection de AAV-RKp-EGFP n'a pas montré de marquage positif, par contre nous observons une immunoréactivité des vaisseaux sanguins due à l'anticorps souris RHO employé. ONL, couche nucléaire externe ; INL, couche nucléaire interne, GCL, couche des cellules ganglionnaires. Barre d'échelle, 20 µm.

les résultats du laboratoire indiquent qu'il jouerait un rôle important dans la genèse des rythmes dans les photorécepteurs [Figure 8', (Sandu, Hicks et al. 2011)]. La régulation circadienne de *Nrl* pourrait s'expliquer également par la transactivation rythmique du promoteur de *Nrl* via ce même site RORE par RevErbα, un composant essentiel de l'horloge . Des expériences de transactivation du promoteur de *Nrl* ont révélé une réponse dose-dépendante à l'activation par le facteur RevErbα ainsi qu'une activation transcriptionnelle par le facteur RORβ (Figure 9'). Toutefois, aucune activation par les facteurs CLOCK et BMAL1 n'a été démontrée malgré la présence d'une séquence « E box » dans la région déterminante pour l'expression de NRL dans les bâtonnets. L'ensemble de ces résultats nous a conduits à proposer que NRL pourrait être un facteur contrôlé par des composants de l'horloge moléculaire, RevErbα et RORβ cependant pas par les facteurs CLOCK et BMAL1. Ces facteurs de l'horloge pourraient servir de relai dans la régulation rythmique des fonctions des photorécepteurs en stimulant notamment Nrl qui lui-même, en association avec Crx et Nr2e3, pourrait activer ses propres gènes cibles dont la Rhodopsine, au moment adéquat du cycle jour/nuit.

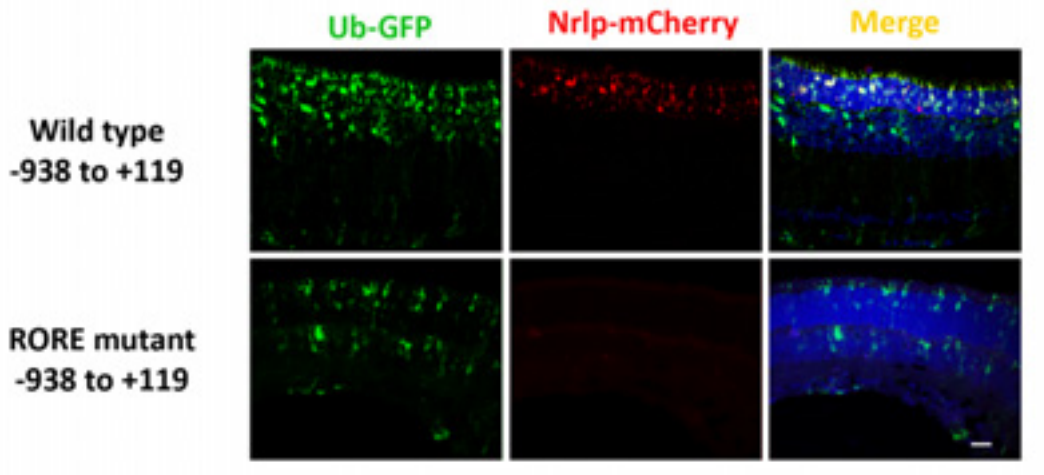

Figure 7'. RORβ est nécessaire à l'expression de *Nrl* au cours du développement

La transfection *in vivo* de promoteur *Nrl* WT montre une forte expression du gène rapporteur mCherry dans la couche des photorécepteurs. La mutagenèse dirigée contre le site RORE (substitution de deux nucléotides dans la séquence wild type, AAAATGTAGGTCA) abolit l'expression de mCherry. Vert, Green Flurorescent Protein ; rouge, fluorescence du mCherry ; bleu, DAPI. Barre d'échelle, 20 µm.

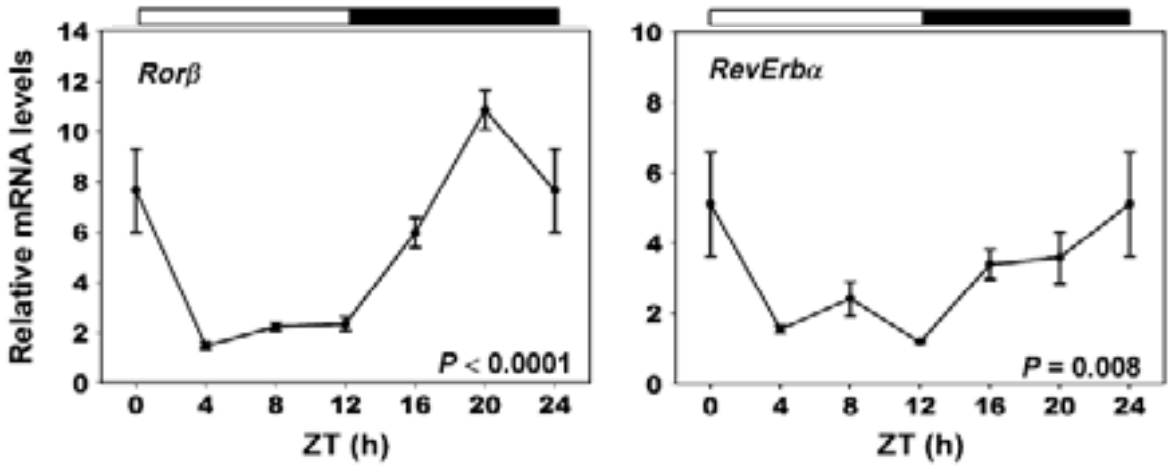

Figure 8'. Profiles d'expression d'ARNm de RORβ et RevErbα dans la couche des photorécepteurs de rat

Analyse en qPCR des profiles d'expression de gènes horloge sous des conditions lumière/obscurité (barres blanche et noire respectivement) de la couche de photorécepteurs isolée par coupe au vibratome. Les valeurs représentent la moyenne ± l'erreur type (SEM ; n = 5). Les variations significatives ($P < 0.05$) au cours du temps sont indiquées. Les données pour ZT0 et ZT24 sont dupliquées. (D'après Sandu *et al*. 2011, EJN)

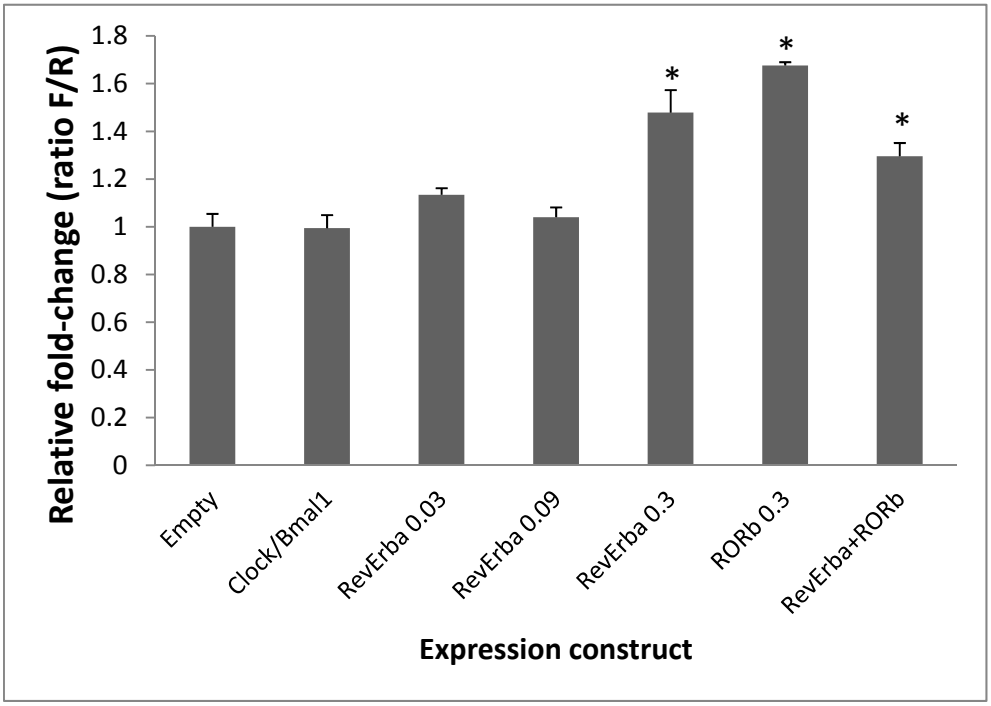

Figure 9'. Transactivation du promoteur de Nrl avec RORβ et/ou RevErbα

Des vecteurs d'expression *pSV-CLOCK*, *CMV-BMAL1*, *CMV-RORβ* et *CMV-RevErbα* ont été transfectés dans des cellules HEK293T avec le promoteur murin de *Nrl* (-938 à +119) cloné en amont du gène rapporteur luciferase (0.5 µg). Différentes concentrations (0.03 - 0.3 µg) du vecteur d'expression RevErbα ont été testées, de même que l'activation par RORβ et RevErbα ensembles (0.15 µg chaque) ou seuls (0.3µg) sur le promoteur de *Nrl*, ainsi que la co-activation de CLOCK et BMAL1 (0.1 µg chaque) La quantité totale d'ADN pour chaque condition est de 1 µg, les quantités d'ADN étant équilibrées avec un vecteur vide. Les valeurs représentées correspondent au rapport de luciferase avec le contrôle interne de la transfection, *CMV-Renilla* (1 ng). Ces valeurs sont relatives au niveau basal d'activité du promoteur obtenu par la transfection du vecteur vide contrôle. Les barres d'erreur représentent les erreurs type (SEM). Les astérisques représentent les valeurs $P < 0.05$.

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PREFACE

Tissue-specific expression of genes is an essential mechanism for the generation of different cell type. Precise levels of gene expression as well as temporal activation during development are events tightly controlled primarily by transcriptional regulators acting on *cis*-regulatory elements of promoters. The well-defined cellular repertoire offered by the retina is appropriate to understand molecular mechanisms responsible for establishment of neuronal cell type diversity. I focused my work on the transcriptional regulation of rod-photoreceptor genesis and homeostasis. Fine-tuning rods' transcriptome both in development and adulthood is a major area of interest, as over- or under- expression of genes may lead to photoreceptor degeneration as in many retinal dystrophies.

Neural Retina Leucine zipper (*Nrl*) transcription factor is a key determinant of rod differentiation and homeostasis. During my graduate research, I have used *Nrl* as a model gene to understand the genetic events leading to rod generation and to dissect molecular mechanisms driving daily rhythmicity in photoreceptors. In a bibliographical introduction, I will first depict the general mechanisms leading to transcription of typical genes. Then, I will describe cell fate-determination in the retina, influenced by control of *cis*-regulators. I will expand on *Nrl* and its pivotal role as a regulator of rod differentiation and maintenance. Subsequently, four chapters of results will be presented: the first chapter will describe the regulatory elements responsible for *Nrl* regulation during development; the second will describe the experimental design and results obtained using a minimal *Nrl* promoter/enhancer in adeno-associated virus; the third chapter will focus on the daily variation of expression levels for specific photoreceptor-genes including *Nrl*; and the last chapter will place NRL in the global protein complex leading to *rhodopsin* expression, by introducing a new transcription factor, NonO. Finally, I will discuss our findings and present a coherent view of what my studies have contributed to the field.

INTRODUCTION

A. General mechanisms of transcriptional regulation

1. Transcription initiation

1.1 Promoter description and function

In Eukaryotes, synthesis of messenger RNA from protein encoding genes requires regulatory elements that generally include a core promoter and upstream – or downstream – activator-binding sequences necessary for anchoring the *trans*-acting factors. These factors work with co-activators, which may not bind directly DNA, to direct transcriptional initiation by the RNA polymerase II apparatus (Maniatis, Goodbourn et al. 1987; Tjian and Maniatis 1994). The promoter is essential for efficient synthesis of a gene transcript by enabling the binding of the transcription initiation complex machinery. The core promoter corresponds to a region typically localized around 40 base pairs (bp) upstream of the transcription start site (TSS), while the proximal promoter represents a region that includes the core promoter and the further upstream and/or downstream elements (Figure 1). The proximal promoter contains DNA sequence elements that bind transcription factors, which then modulate levels of transcription, whereas the core promoter is necessary for the formation and binding of the pre-initiation complex (PIC). The PIC is composed of multiple factors regulating the initiation of transcription and stabilizing the enzyme responsible for the transcription, RNA polymerase II (Pol II), on the promoter. The PIC is recruited to the core promoter at a site typically located immediately upstream (25 - 30 bp) of the TSS. This site known as the TATA box [consensus DNA sequence, TATA(A/T)A(A/T)] allows binding of the first general transcription factor TFIID through its TATA-binding protein (TBP) (Tjian 1996; Naar, Lemon et al. 2001) (Figure 2). Some promoters lack a TATA box element and are defined as TATA-less promoters. In this case, transcription initiation is accomplished through other DNA elements in the core promoter, these include INR (initiator), DPE (downstream promoter element), BRE (TFIIB Recognition Element), MTE (Motif Ten Element), DCE (Downstream Core element), and/or XCPE1 (X Core Promoter Element 1) (Juven-Gershon and Kadonaga 2010). The INR encompasses the TSS and is a recognition site for the binding of TFIID, TBP-associated factors and Ying Yang 1 (YY1), a zinc finger transcription factor important for the regulation of gene transcription. Association of all these factors in addition to Pol II is enough to initiate the transcription in a TBP-

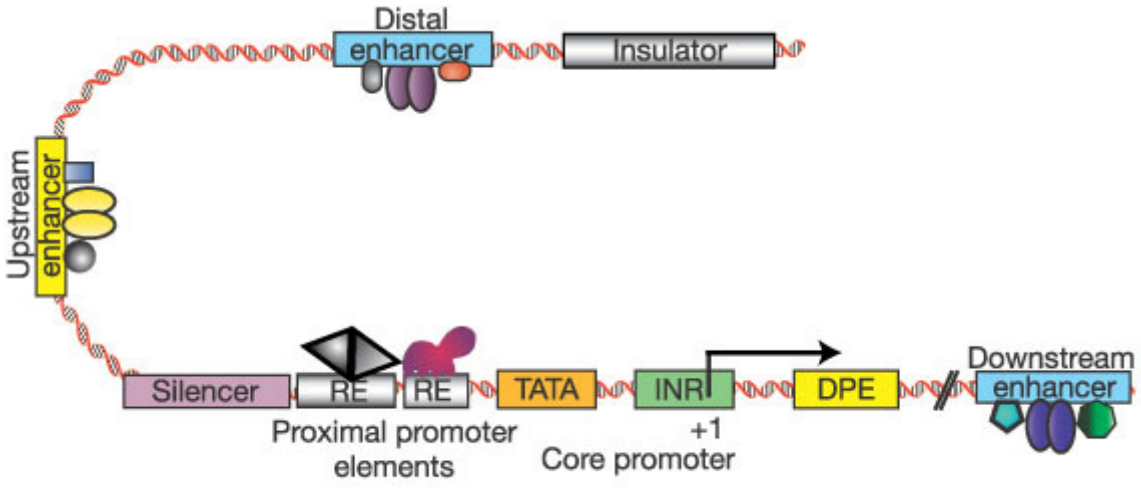

Figure 1. Scheme of extensively diversified metazoan regulatory modules

A complex arrangement of multiple clustered enhancer modules interspersed with silencer and insulator elements which can be located 10–50 kb either upstream or downstream of a composite core promoter containing TATA box (TATA), Initiator sequences (INR), and downstream promoter elements (DPE). Modified from Levine and Tjian, Nature, 2003.

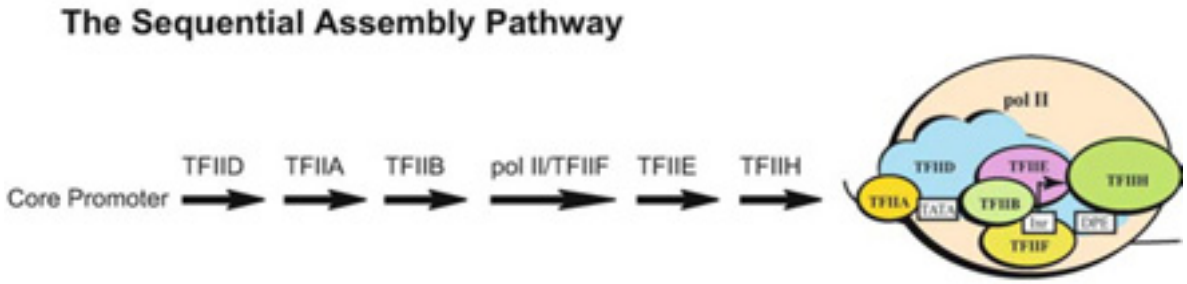

Figure 2. Pathway for the pre-initiation complex assembly

Stepwise assembling of the pre-initiation complex (PIC) occurring via the binding of TATA binding protein from TFIID at the core promoter, followed by the sequential recruitment of other general transcription factors (TFII) , completed by the stabilization of RNA polymerase II on this multi-protein complex. From Thomas and Chiang, Crit Rev Biochem Mol Biol., 2006.

independent manner (Usheva and Shenk 1994). The DPE is generally located from +28 to +33 relative to the INR and is classically found in TATA-less promoters (Figure 3). TFIID recognizes DPE sequence and binds to it to initiate basal machinery anchoring. Therefore, DPE could be considered as a "downstream TATA box" for TFIID binding (Kadonaga 2002). The DCE is a downstream regulatory element relatively frequent in TATA box containing promoters and appears to be distinct from the DPE. The XCPE1 motif is located around the TSS and acts in association with *trans*-acting factors such as Specificity protein 1 (Sp1) (Tokusumi, Ma et al. 2007). Sp1 acts as a basal transcription factor on 'Sp1 sites' (GC-boxes, CACCC-boxes and basic transcription elements) that represent constitutive promoter elements supporting the regulation of expression of thousands of genes (Tan and Khachigian 2009). XCPE1 appears to collaborate as well with sequence-specific activators on CpG islands which, when methylated by 5-methylcytosine will lead to gene silencing.

1.2 Recruitment of the initiation complex

TFIID is the first general transcription factor to make contact with the chromatin on the TATA box element via its TBP. TFIID serves as a foundation for the recruitment of other TFII proteins to the TATA box (Figure 2). Indeed, TFIID is stabilized by TFIIA, and then addition of TFIIB and TFIIH will complete this multi-factor structure. Finally, Pol II binds to this complex, facilitates supplemental general/basal transcription factors, TFIIE and TFIIF (Thomas and Chiang 2006). Besides the general transcription factors, the TBP-associated factors (TAFs) stabilize TBP on the TATA sequence, which is in general only a few base pairs long, by making contact with the INR and the DPE. TFIID is in fact a multi-protein complex composed of TBP and 12-15 distinct TAFs (Dynlacht, Hoey et al. 1991; Tanese, Pugh et al. 1991). TAFs are thought to interact with activators binding upstream DNA sequences, and to create a bridge between these activators and the basal machinery complex (Figure 4). Data suggest that TAF subunits can function as direct promoter-recognition factors, as coactivators capable of transducing signals from enhancer-bound activators to the basal machinery, and even as enzymatic modifiers of other proteins (Albright and Tjian 2000). Mediator is a multi-subunit co-activator complex that seems to facilitate the binding and/or function of Pol II at the core promoter (Kim, Bjorklund et al. 1994) (Figure 5).

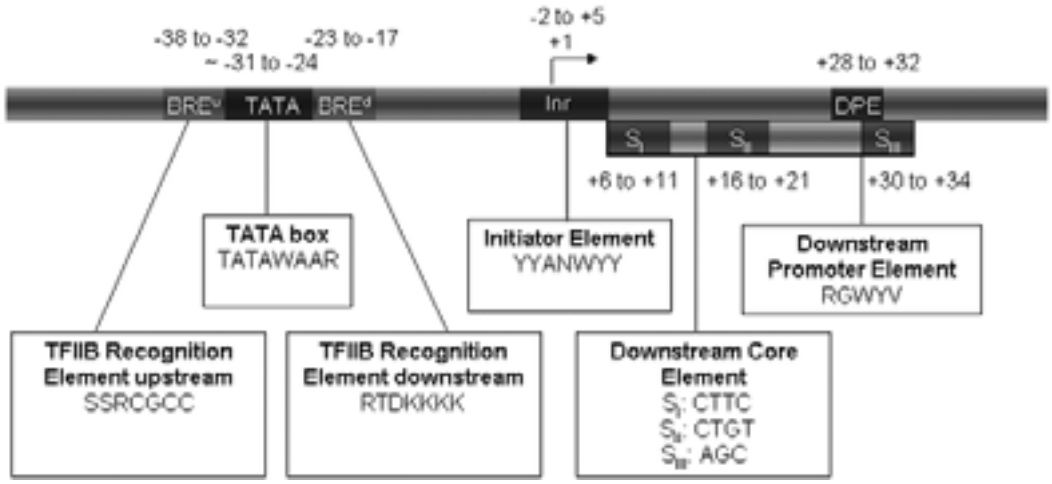

Figure 3. Core promoter elements

This diagram shows com-mon core promoter ele-ments with their con-sensus se-quences and relative position to the transcription start site (+1). The core promoter can show considerable variability as there are no universal elements. Each of the motifs is found in only a fraction of core promoters with different combinations. The TATA box, Inr, DPE, and DCE are recognition sites for binding of TFIID, whereas BREu and BREd interact with TFIIB. From Baumann *et al.*, Mol. Biotechnol., 2010.

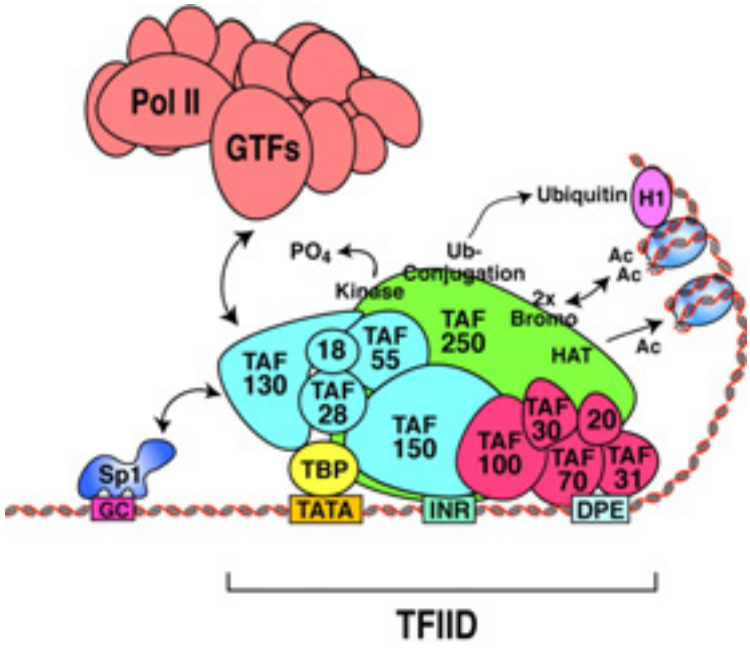

Figure 4. Functions of TAFs

Various functions of individual TBD-associated factors (TAFs) subunits in facilitating the process of transcriptional activation through the subunit TBP of TFIID. Modified from Näär *et al*, Annu Rev Biochem, 2001.

In metazoans, there are several Mediator-related complexes: TRAP, CRSP, ARC/DRIP, SMCC and hMed that are recruited to DNA template via interactions with sequence-specific transcriptional activators. The function of these complexes is similar to the TAFs, to create bridges between distal activators and the PIC. It is not until the carboxy-terminal domain (CTD) of Pol II is phosphorylated by the Mediator complex (Malik and Roeder 2010) and TFIIH (Oelgeschlager 2002) that Pol II is released from the proximal promoter for an efficient elongation of the transcript (Levine 2011).

2. Regulation of the transcription

2.1 Role of enhancers

Promoter and enhancers appear to be similar in their functions. Indeed, despite the operational distance – enhancers can activate a promoter from a distance reaching 50kb and even when located in intronic regions – enhancers facilitate gene activation by increasing the rate of transcription through the binding of multiple regulatory proteins. While the promoter is necessary for initiation of the transcription, enhancers can regulate the temporal and tissue-specific expression of differentially regulated genes (Maniatis, Goodbourn et al. 1987). Enhancers are classically highly conserved during evolution and regulate gene expression in specific tissues or during the development, or as a response to a specific signal or several together. Enhancers modulate transcriptional activity through the binding of several transcription factors on *cis*-regulatory sequences (Blackwood and Kadonaga 1998). Looping of chromatin is a phenomenon observed in active genes where enhancers are bringing bound DNA-sequence activators to a close proximity to the PIC (Lieberman-Aiden, van Berkum et al. 2009). Enhancers are central to transcription regulation but also to disease formation, indeed, chromosome rearrangements, deletion or point mutations in enhancers can cause abnormal phenotypes. For example, deletion of a 6 kb region spanning a remote element located 20 kb upstream of *Atoh7* (*Math5*) gene necessary for retinal ganglion cell development and formation of optic nerve, causes nonsyndromic congenital retinal nonattachment, a severe form of blindness characterized by a lack of optic nerve (Ghiasvand, Rudolph et al. 2011). Opposite to enhancer elements are silencers, which are not characterized as extensively as enhancers. The role of silencers is to down-regulate the transcription by

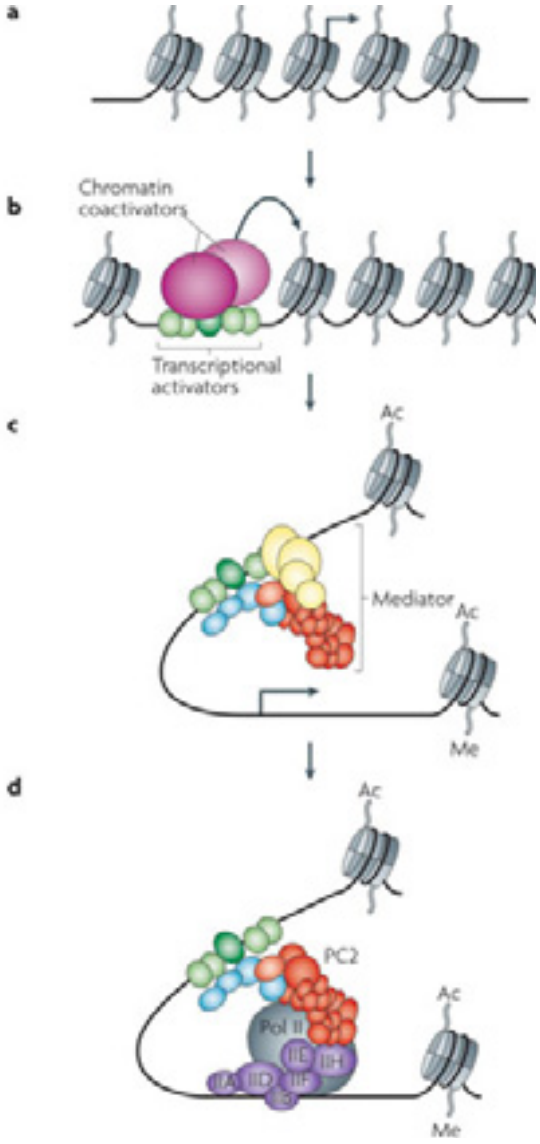

Figure 5. Current models of transcriptional activation involve multiple steps

(a)Transcriptionally inert chromatin, in which DNA is tightly packaged with nucleosomes. (b)Transcriptional activators bind to their cognate sites in the regulatory region of the gene and recruit series of chromatin co-activators that can covalently modify nucleosomes (c)Chromatin is characterized by distinct covalent modifications, such as acetylation (Ac) and methylation (Me), and by a relative dearth of nucleosomes. The activators then recruit Mediator. In some cases, the intact Mediator that consists of the core and the kinase module might be recruited at this stage. Modified from Malik and Roeder, Nat Rev Genet., 2010.

recruiting repressor transcription factors to their sites to interfere with the PIC assembly or by preventing the binding of activators to their sequences (Ogbourne and Antalis 1998). Silencers and enhancers are working in balance to activate or repress the transcription of specific genes in a spatial and temporal manner.

2.2 Transcription factors and chromatin remodeling

2.2.1 Mode of action

Transcription factors (TFs) are proteins that bind to specific DNA sequences found in enhancer or promoter region. TFs are generally composed of three major domains: the DNA-binding domain, responsible for recognition of specific DNA sequences. The second domain is the *trans*-activating domain accountable for the activation or repression of the targeted gene transcription and in certain cases can be necessary for the proteins interaction, however this function can be done by the protein-protein interaction domain, which allows TFs to interact with TAFs or other regulators to modulate the transcription (Figure 6). TFs can recruit co-regulator proteins that are altering locally chromatin conformation and thus enabling Pol II to access to the initiation site, or else, TFs are interacting with co-activators that are themselves enzymes altering chromatin structure. As a matter of fact, chromatin is the highest condensed structure of the DNA. The fundamental unit of chromatin is the nucleosome, which consists of 147 bp of DNA wrapped around histone proteins. As a result, some mechanisms are required for activators to access to the DNA and for the PIC to navigate through the chromatin, which appears then as a direct actor of the transcription regulation. Two types of mechanisms are responsible for chromatin decondensation; the first is the nucleosome dissociation releasing the chromatin. The second, which is better characterized, is the recruitment of proteins with enzymatic activities that will modify chromatin structure. Nucleosome remodeling complexes will destabilize histone-DNA interactions in an ATP-dependent manner, subsequently revealing chromatin segments that were masked, to allow binding of activators and the transcriptional machinery to enhancer and promoter regions. The first chromatin-remodeling complex identified is SWI/SNF in yeast and possesses a DNA-stimulated ATPase activity through its Swi2/Snf2p subunit, altering nucleosome structure and facilitating transcription factors to bind to their cognate sites via the protein-protein

Figure 6. Transcription factor domains

Scheme of the amino acid sequence of a prototypical transcription factor that contains a DNA-binding domain (DBD), a protein-protein interaction domain (PPD) and a transactivation domain (TAD). The order of placement and the number of domains may differ in various types of transcription factors.

interaction domain found in SWI/SNF (Naar, Lemon et al. 2001) (Figure 7). Another class of chromatin modifying enzymes is the histone acetyltransferases (HAT), whose function is to tag specific lysine residues with an acetyl group on the amino-terminal tails of histones 3 and 4 of the core of histone proteins. Acetylation will neutralize the basic charge of the lysine and destabilize the histone-DNA interaction (Marmorstein and Roth 2001). In general, hyperacetylation of histones is correlated with actively transcribed genes, while hypoacetylation of histones (accomplished by histone deacetylase, HDAC) is associated with repressed genes. Gcn5 was first identified as a co-activator that bridges activator proteins with basal transcription factors, but has since been found to exhibit HAT activity. Gcn5 is part of two distinct complexes, the Spt-Ada-Gcn5-acetyltransferase (SAGA) and the adaptor (ADA) co-activators. In human, hGCN5 and P/CAF (the homologs of yeast Gcn5) and SAGA have been found to interact with several TAFs but not with TFIID. It seems that TAFs are coordinating and ensuring the assembly and the integrity of multiple co-activator complexes, and function independently in transcription regulation through interaction with TFIID. Interestingly, TFIID also harbors HAT and protein kinase activities via one of its sub-unit TAF250 (Dikstein, Ruppert et al. 1996; Mizzen, Yang et al. 1996), suggesting a possible role for enzymatic modification of downstream targets upon TFIID recruitment. Two additional HATs, p300 and CBP (protein of 300 kDa and CREB-binding protein), have over 90% of sequence homology and are conserved in metazoans (Ogryzko, Schiltz et al. 1996). p300 and CBP catalyze the acetylation of all four core histones, and are reported to acetylate a large number of proteins, including themselves (Wang, Tang et al. 2008).

Therefore, p300 and CBP appear to be general transcriptional integrators. Other chromatin modifications during gene transcription are phosphorylation that rather serves as a covalent tag than a direct alteration of chromatin (Banerjee and Chakravarti 2011) and methylation which either activates or represses gene transcription (Kouzarides 2007).

2.2.2 Sub-family of basic motif leucine zipper

TFs play a pivotal role in contributing to the initiation of transcription and further in regulating expression levels of specific genes.

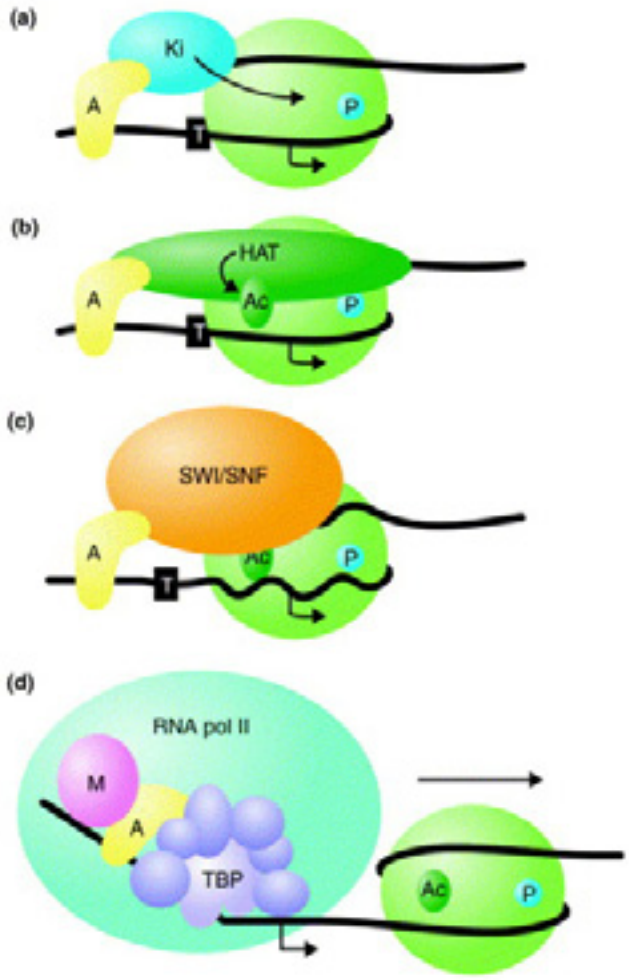

Figure 7. Suggested sequence of coactivator function in transcription initiation.

Nucleosome (large green circle) positioned over the TSS (subtended arrow), masking the TATA box (T). (a) Once bound to an enhancer, activator(s) may recruit a kinase (Ki) that phosphorylates histone H3 at Ser 10 (circled P). (b) HAT coactivator complexes bridge the activator AD and phospho-Ser-10. Followed by acetylation of lysines in histone (circled Ac), weakening contacts between DNA and histones. Acetylated chromatin allows activator to interact with chromatin remodeling complexes such as SWI/SNF (c), which make contacts to activator, and to acetylated chromatin. ATP-dependent remodeling affects the path of DNA around the histone octamer, thus exposing the adjacent TATA box. (d) TBP/TFIID binds the TATA box and induces a bend in DNA. This bending provokes repositioning of the nucleosome in a manner also dependent on histone acetylation. Nucleosome repositioning exposes the transcriptional start site and allows access to RNA pol II and GTFs. In some instances, recruitment of Mediator (M) by activator precedes and facilitates pol II entry. This latter event can also come under the control of signaling pathways linked to the cell cycle. Modified from Featherstone, Curr Opin Genet Dev., 2002.

TFs can be grouped in families based on structural homology in their DNA-binding domain. In this chapter, we focused on one specific family of TFs, the Maf sub-family. Maf stands for MusculoAponeurotic Fibrosarcoma; the structural organization and sequence homology of these proteins allowed classifying this family into the AP1 superfamily of leucine zipper containing transcription factors (Figure 8). Maf proteins are divided into two subgroups: the large Mafs, including c-Maf, MafA/L-Maf, MafB/Kreisler and Nrl proteins, comprise an acidic transactivation domain in their N terminus (Figure 9). The large Mafs are major regulators of tissue-specific gene expression and cellular differentiation in mammals; for example, mouse model studies revealed their importance for mammalian gene regulation of the retina (Nrl) (Mears, Kondo et al. 2001), the lens (c-Maf), brain (MafB), kidney, pancreas (c-Maf, MafA and MafB), bones (c-maf) and in hematopoietic cells (MafB) (Blank 2008). The small Mafs include the highly homologous MafF, MafG and MafK factors. In contrast to the large Mafs, the small Maf family members lack a recognizable transactivation domain. Maf proteins contain two specific domains; the bZIP domain common to all members of the AP1 family, the extended homology region (EHR) specific for Maf proteins, rich in glycines and containing repetition of histidine residues. Proteins with a bZIP DNA binding domain such as Jun, Fos, ATF/CREB, c/EBP, GCN4 or Maf, can bind palindromic sequences only when they are forming a dimer. The bZIP domain is constituted of two distinct subregions required for DNA binding. In the N-terminal part the bZIP contains a region rich in basic residues that are in direct contact with the DNA. The C-terminal part of the protein represents the “Leucine Zipper”, which is a protein-protein domain characterized by presence of leucine residues every seven amino-acids necessary for the dimerization. Maf proteins form homodimers or heterodimers and bind to palindromic region of the DNA called MARE (Maf response element, TGCTGAC(G)TCAGCA) containing consensus sites for fixation of AP1 and CREB proteins (Kataoka, Noda et al. 1994). The specific flanking regions TGC and GCA at one or the other end of the core are crucial for recognition of the MARE by Maf proteins (Kerppola and Curran 1994).

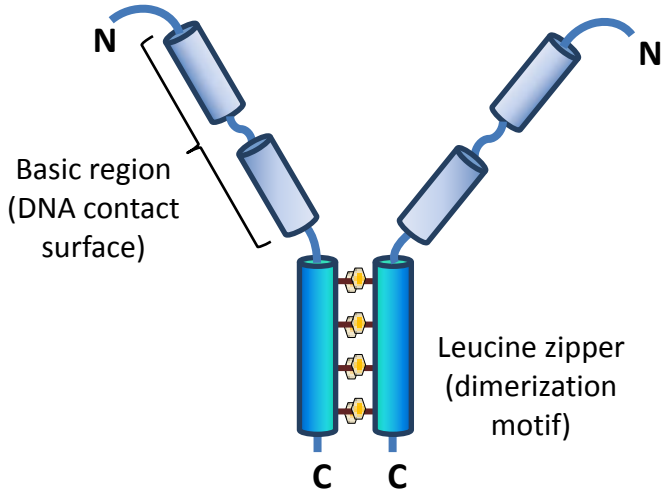

Figure 8. Model for a dimeric bZIP protein

Two bZIP polypeptides join via a Leucine zipper to form a Y-shaped molecule in which the stem of the Y corresponds to a coiled pair of α-helices held by the leucine zipper. The arms of the Y are the respective basic regions of each polypeptide; they act as a linked set of DNA contact surfaces. Source http://web.virginia.edu

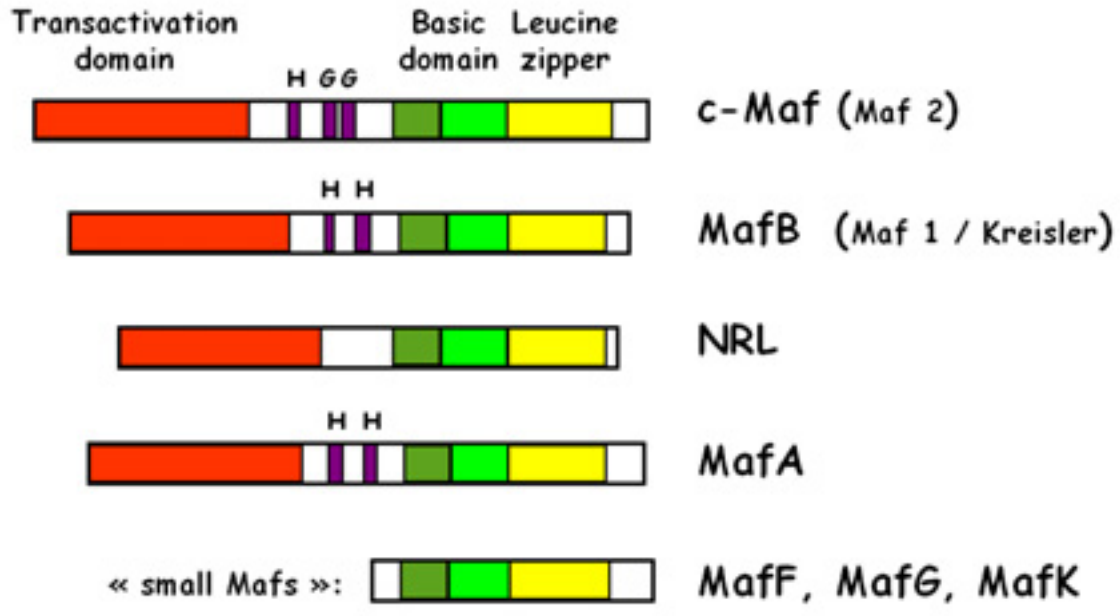

Figure 9. Various members of the Maf family transcription factors

Overview of the Maf transcription factor family. H: Histidine repeat; G: Glycine repeat.

B. Anatomy and development of the retina

1. Cellular composition of the retina

The visual system enables organisms to perceive light and to process images that will be treated in the visual cortex in the brain. The eye is the first component of this system, and is composed of various anatomical structures working together to project images on the retina (Figure 10). The retina is a thin tissue (500 µm in humans and 200 µm in mice) sitting on the back of the eye. It is a highly organized tissue composed of neurons and glia. The first description of the retina anatomy comes from the work of Santiago Ramon y Cajal in the late 19th century "Structure of the Mammalian Retina" (Figure 11). In this piece of classical work, he described several types of cells in the retina and classified them according to their morphology and arborization. He used the technique of Golgi silver staining to expose the details of retinal structure. The retina was then used by Cajal to support the neuron doctrine, providing clues to identify the direction of the signal transmission at the interneuronal contacts. This tissue, given its relatively simple structure, facilitated the understanding of signal flow from photoreceptors to retinal ganglion cells (Figure 12). Hence, for Cajal the retina was a model to decipher the connections between neurons allowing transduction of signal. Nowadays, the retina is still used as a neuronal model based on its anatomical accessibility and a well defined cell repertoire (Figure 13).

1.1 Outer Nuclear and Plexiform layers

The outer nuclear layer (ONL) contains the cell bodies of the photoreceptors (PRs) rods and cones, so named based on their morphological structure. The rod nuclei are more numerous and are placed on different levels throughout the retina, while the cone nuclei are fewer and placed on the sclera side, close to the outer limiting membrane. The proportion of rods versus cones in the ONL seems to be correlated with the lifestyle of species; a diurnal animal will generally contain a higher number of cones compared to rods and vice versa for a nocturnal animal. For example, mouse and rat, two nocturnal rodents have a percentage of cones below 3% over the total PRs population, while the grass rat *Arvicanthis ansorgei*, a diurnal rodent has around 33% of cones (Bobu, Lahmam et al. 2008).

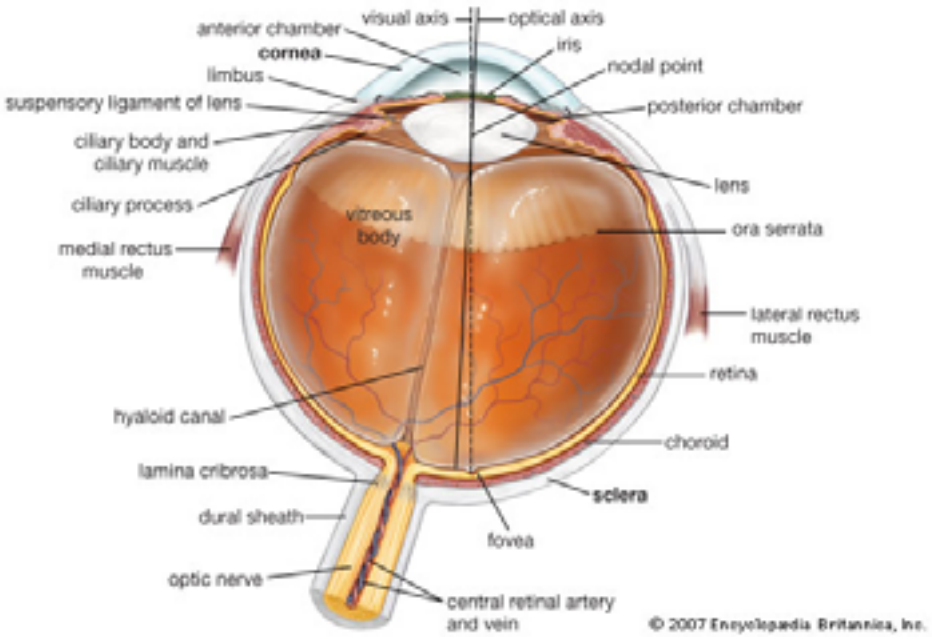

Figure 10. The human retina

A horizontal cross section of the human eye, showing the major parts of the eye. From Encyclopædia Britannica, Inc, http://www.britannica.com/

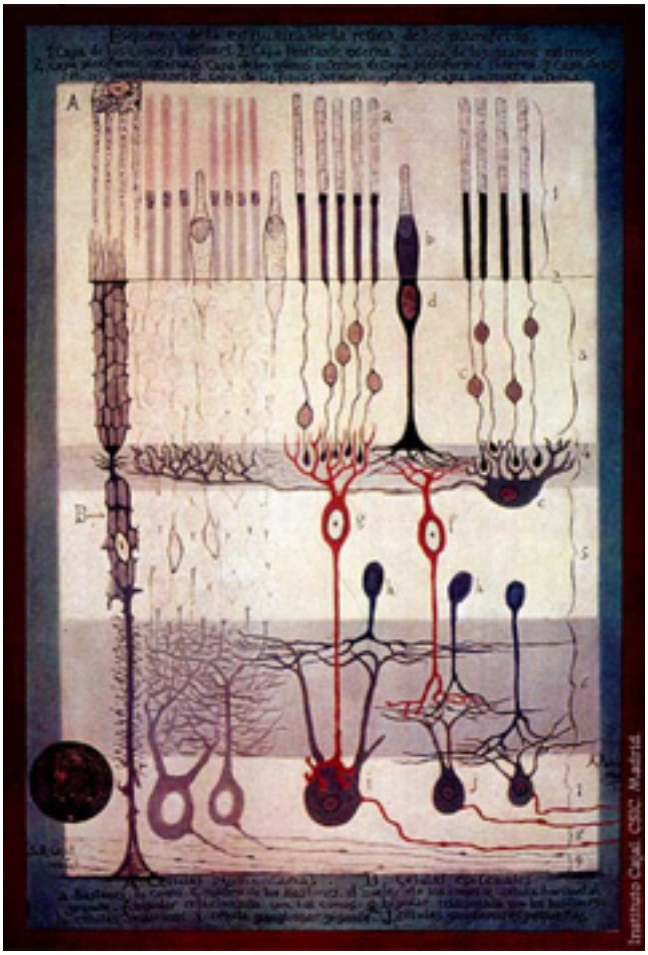

Figure 11. The mammalian retina drawing by Cajal

1. Rod and Cone layer, 2.-Outer nuclear layer, 3. Granule layer, 4. External plexiform layer, A: Pigmented cells, B: epithelial cells . From "Structure of the Mammalian Retina" century 1900, by Santiago Ramon y Cajal.

This percentage is even higher in another diurnal rodent species, the sand rat *Psammomys obesus* (Saidi, Mbarek et al. 2011). The cone/rod ratio of the diurnal primate human is 5-6%. The rods are in majority and are distributed on the whole surface of the retina, except in one particular region called the fovea, which is constituted exclusively of cone photoreceptors.

Rods and cones have the same basic structure with four distinct sections: the outer segment, the inner segment, the cell body and the synaptic terminal.

The outer segment is the truly receptive part of the PR as it is photosensitive. It is composed of disk membranes containing the visual pigments (cone and rod opsins) of the PRs. In rods the disks result from the internalization of the cell membrane and are isolated from the plasma, while the cone disks originate from the successive folding of the cell membrane and are in continuity and opened to the extracellular space. Outer segments of PRs are in fact highly specialized primary cilia responsible for the photosensitivity. The proteins involved in the phototransduction cascade are synthesized in the inner segment and fill up the outer segment via the connecting cilium, which constitutes a passage for the proteins traveling to and from the outer segment.

The inner segment contains mitochondria, endoplasmic reticulum, Golgi apparatus and ribosomes, structures required for proper functioning of the translational machinery and trafficking of proteins targeted to the outer segment. Opsin molecules are synthesized in the endoplasmic reticulum, glycolysated in the Golgi and docked in lipidic membranes that will form the disks of the outer segment.

The cell body is located at the transition between the inner segment and the axon. The cell body contains almost exclusively the nucleus leaving reduced room for cytoplasm.

Both rod and cone axon terminals contain vesicles filled with an excitatory neurotransmitter, the glutamate. The rod axon terminal is called spherule, and is smaller than the cone pedicle, each of these structures form an invaginated synapse with bipolar and horizontal cells in the outer plexiform layer.

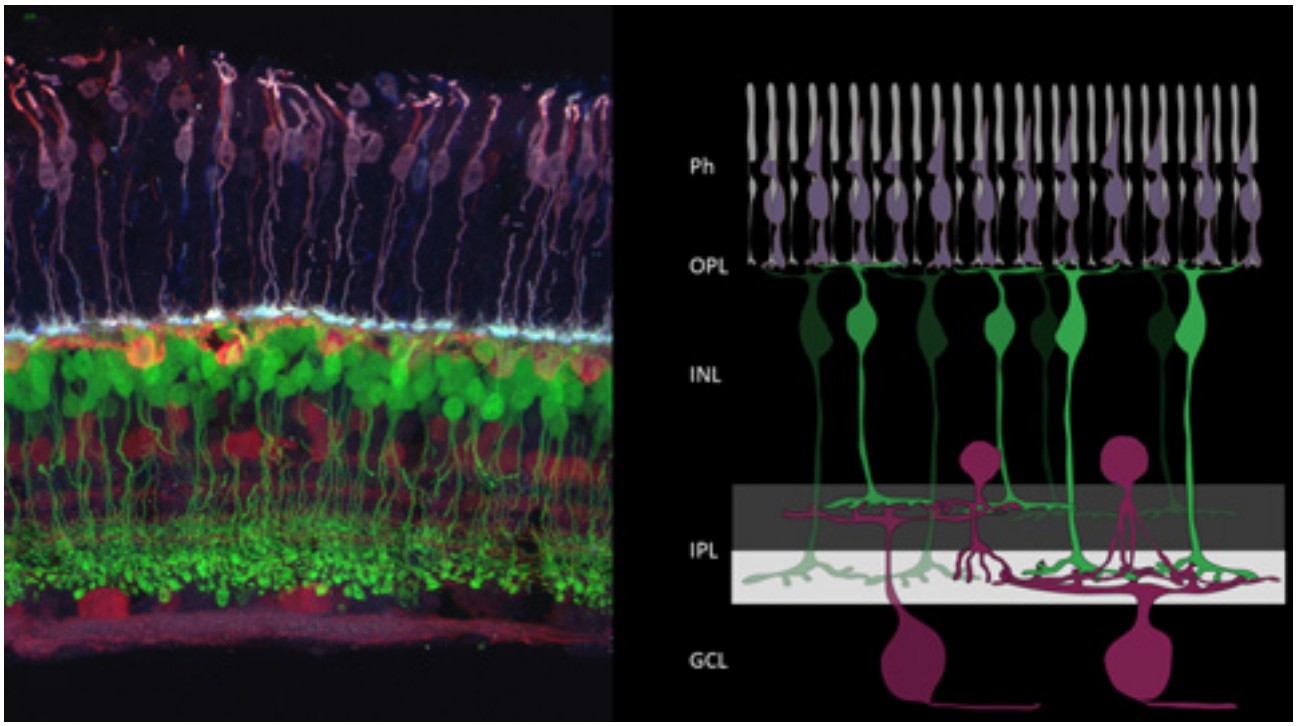

Figure 12. Retinal organization

Rod and cone photoreceptors form an outer nuclear layer that contacts bipolar cells and horizontal cells. Bipolar cells relay information from the outer retina to the inner retina where they contact retinal ganglion cells and amacrine cells. The cell bodies and synaptic connections between the various retinal cell types are organized into distinct layers. Ph: photoreceptors. OPL/IPL: Outer/Inner Plexiform Layers. INL: Inner Nuclear Layer. GCL: Ganglion Cell Layer. From Rachel Wong laboratory, Josh Morgan.

The outer plexiform layer is the layer where the first synapses of the retina are made. This layer contains the axon terminals of the rods and cones that are contacted by the axon terminals of horizontal cells (for rods), by the dendrites of horizontal cells (for cones) and by dendrites of bipolar cells (for both rods and cones) (Kolb and West 1977; Nelson 1977). Two types of synapses are found in the outer plexiform layer: ribbon synapses between the PRs and ON-bipolar cells or horizontal cells, and flat synapses between PRs and OFF-cone bipolar cells.

1.1.1 Cone photoreceptors

The cones are responsible for the bright light (photopic) and color visions (Figure 14). They are less light sensitive than rods but allow finer detail perception. Different categories of chromatic vision emerged from evolution; hence monochromatic vision is when only one color can be perceived and polychromatic vision, two or more colors. The ability to detect colors is due to the presence of visual pigments. In vertebrates, cones can have up to four spectrally different visual pigments or opsins, forming then one type of cone depending on the opsin expressed in this cell (Figure 15). Each opsin belongs to one gene family: *Short wavelength sensitive 1* (*SWS1*), allowing detection from ultraviolet to violet; *Short wavelength sensitive 2* (*SWS2*), from violet to blue; *Rhodopsin type 2* (*RH2*), *middle wavelength sensitive, green range; Middle wavelength sensitive* (*MWS*), for green, and *Long wavelength* (*LWS*) for red (Jacobs 2009). Human and Old Primates have typically three types of cones named after their spectral sensitivity maxima **S**hort (blue cone), **M**edium (green cone) and **L**ong (red cone) (Nathans, Thomas et al. 1986; Jacobs 1998; Yokoyama 2000). This trichromatic vision results from the split of the M/L opsin into two distinct types of pigments, the green and the red opsins (Linberg, Cuenca et al. 2001).

Several retinal diseases are related with cone dysfunction. These pathologies are called cone-rod dystrophies resulting in the primary loss of cone cells followed by rod cells (Hamel 2007). Some cone dystrophies may be part of other syndromes such as the Bardet Biedl syndrome (Mockel, Perdomo et al. 2011), which is a ciliopathy causing among other symptoms retinal dystrophy (rod-cone or cone-rod). Seven BBS proteins (BBS1, 2, 4, 5, 7, 8 and 9) participate in the formation of a stable protein complex named

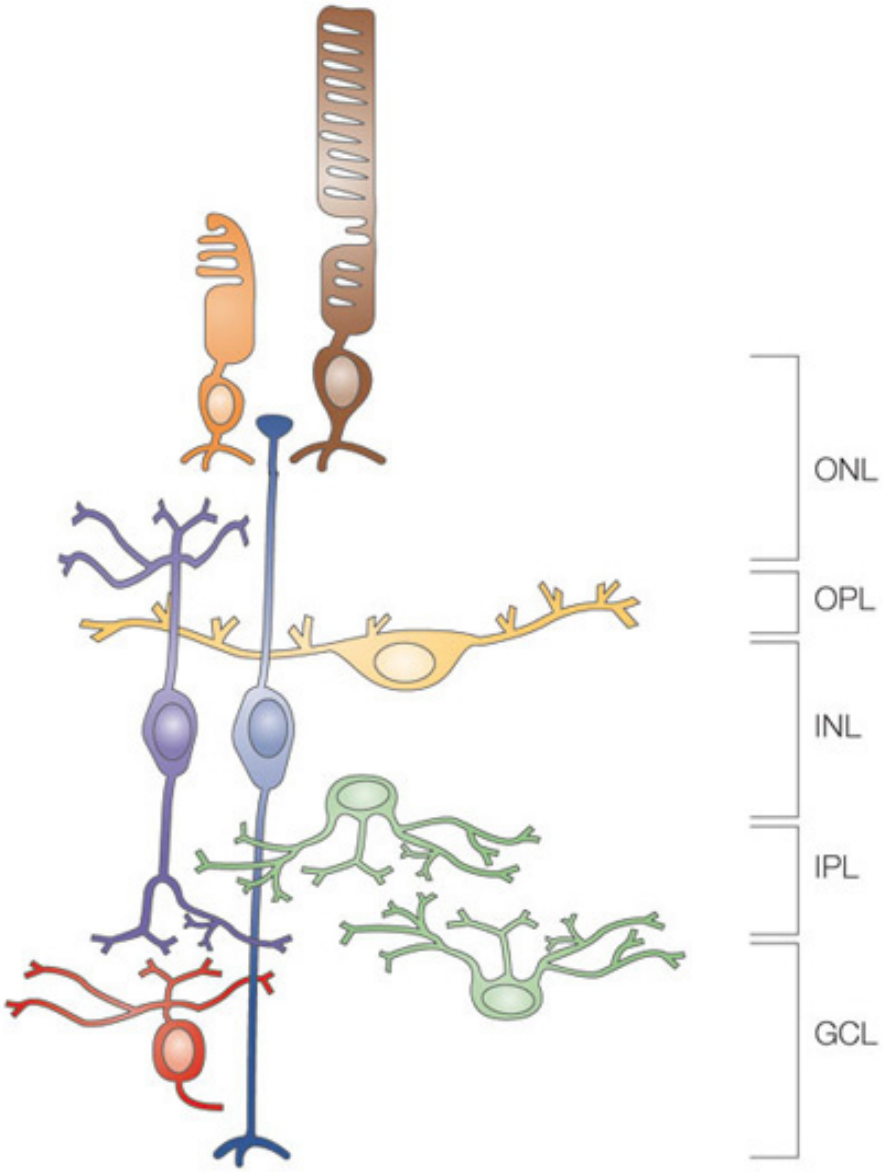

Figure 13. The mature retina

Rod cell (brown) and cone cell (orange) bodies are located in the outer nuclear layer (ONL) and have downward processes that synapse with horizontal (yellow) and bipolar (violet) processes in the outer plexiform layer (OPL). Horizontal, bipolar, Müller (blue) and amacrine (green) cell bodies are located in the inner nuclear layer (INL). In addition to upward processes that end in the OPL, bipolar cells have downward processes that terminate with amacrine and ganglion process in the inner plexiform layer (IPL). Müller glia, which provide support functions in the retina, have upward and downward processes that terminate at the outer edge of the ONL and the inner surface of the ganglion cell layer (GCL), respectively. From Dyer and Bremmer, Nat Rev Cancer, 2005.

the BBSome involved in vesicular trafficking to the ciliary membrane (Nachury, Loktev et al. 2007). Mutations in one of these genes cause basal body and/or cilium dysfunction at the origin of the syndrome. Other cone dystrophies can be non-syndromic, such as Stargardt disease caused by mutation in *ABCA4* gene and affecting the phototransduction cascade (Briggs, Rucinski et al. 2001).

1.1.2 Rod photoreceptors

Contrary to the cones, rod photoreceptors can function in less intense light (Figure 14). Rods are responsible for the dim light perception (scotopic vision in gray scale) and peripheral vision. One rod cell is enough to respond to one single photon, making it 100 times more sensitive than cones. Rhodopsin, the visual pigment of the rod, belongs to the G protein-coupled receptor family. This protein family consists of seven transmembrane helices connected to each other by protein loops. The chromophore of Rhodopsin is the 11-*cis* retinal. This latter is isomerized when a photon of light strikes the Rhodopsin protein and will trigger the phototransduction cascade.

Mutations in *Rhodopsin* are among the most common cause of Retinitis Pigmentosa (RP). RP, a form of rod-cone dystrophy, is a group of retinal degenerative diseases that are characterized by the loss of rod photoreceptor cells, followed by cone degeneration. Patients with RP experience night blindness, the progressive loss of peripheral vision and eventually central vision as cone-cell viability is compromised by rod-cell death. More than 120 point mutations in *Rhodopsin* have been identified, some of them have been linked to recessive RP and congenital stationary night blindness, but the vast majority cause autosomal dominant RP (Mendes, van der Spuy et al. 2005).

1.2 Inner Nuclear and Plexiform Layers

The inner nuclear layer (INL) is the layer of the interneurons that mediate and modulate the flow of information between the photoreceptors and the ganglion cells. INL contains the cell bodies of bipolar cells, amacrine cells, horizontal cells, interplexiform cells and Müller glial cells. Nuclei distribution within the INL is the following: bipolar cells are located in the middle of the INL; most of the amacrine cells are found in the innermost

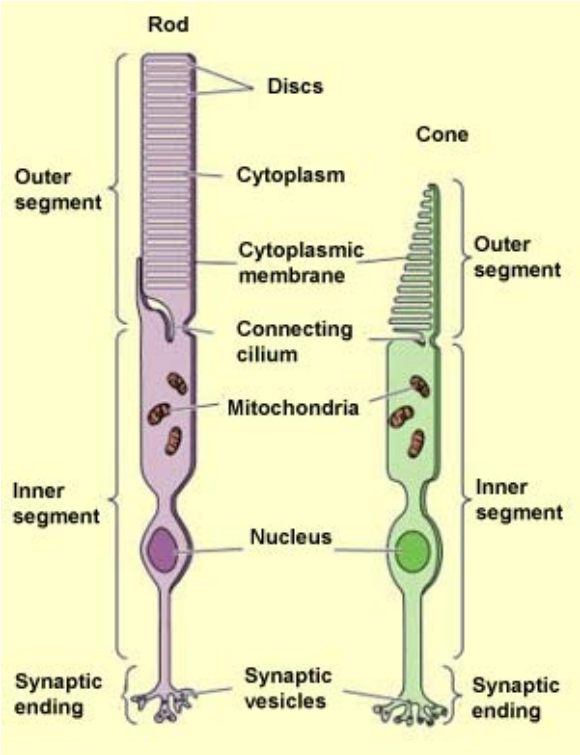

Figure 14. Rod and cone structures

Both types of photoreceptors are composed of an outer segment, an inner segment a cell body, an axon and a synaptic terminal characteristic for each type of photoreceptor. From http://thebrain.mcgill.ca

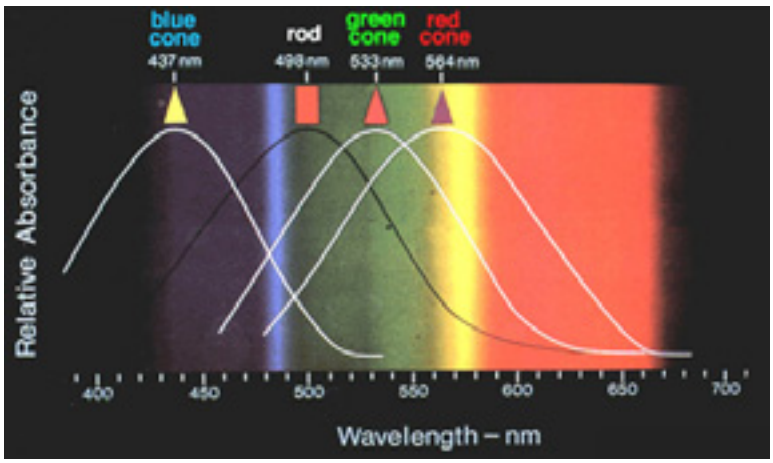

Figure 15. Absorption spectra of visual pigments in humans

Specific wavelength of each type of visual pigment from the human retina. From Bowmaker and Dartnall, 1980

cell rows of the INL, some amacrine cells can also be found in the ganglion cell layer; horizontal cells occupy the outermost rows of the INL and interplexiform cells share the same location than the amacrine cells, at the inner part of the INL.

The inner plexiform layer (IPL) consists of the synapses between amacrine cells, ganglion cells, horizontal cells and bipolar cells.

1.2.1 Bipolar cells

Bipolar cells are the second type of neuron relaying the signal after the PRs. Their role is to transfer the neuronal signal coming from the PRs to the amacrine cells and ganglion cells. All bipolar cells have their dendrites branching in the ONL contacting one or more PRs, while their axon joins the IPL to contact amacrine cells and ganglion cells. Two categories of bipolar cells are distinguishable based on their coupling with rod or cone cell, these bipolars are then called rod bipolar or cone bipolar cells. Rod bipolar cells are always of the ON type, they are depolarized by light stimulation, while the OFF bipolar cells are hyperpolarized by light (Werblin and Dowling 1969). The dendrites of OFF bipolar cells form flat contacts with cone cells, whereas ON bipolar cells form triads with a central invaginated bipolar cell dendrite and two peripheral horizontal cell processes (Kolb 2003).

1.2.2 Amacrine cells

There is a high variety of amacrine cells in the IPL. These cells do not have an axon but can have a long axon-like projection. Amacrine cells receive inputs from bipolar cells and ganglion cells. The diversity of amacrine cell types comes from their morphology and from the range of neurotransmitters in these cells (Pycock 1985). Indeed, depending on the cell subtype, amacrine cells can release glycine or GABA, representative of an inhibitory signaling. Acetylcholine, or GABA plus acetylcholine can be found in the same cell, reflecting the excitatory or inhibitory function of the cell depending on the context. Dopaminergic amacrine cells type 1 make the bridge between rod bipolar cells and ganglion cells. Finally, nitric oxide seems to be used as a neurotransmitter or neuromodulator since NADPH-diaphorase activity is found in amacrine cells that contain nitric oxide synthase (NOS) immunoreactivity (Eldred 2001).

1.2.3 Horizontal cells

Two types of horizontal cells are found in the mammalian retina, HI-H1 (or B type) and HII-H2 (or A type). HI-H1 subtype makes contact with cone cells; HII-H2 branches out with cones on the dendritic part, and with rods on the axonal part. The horizontal-cone cell connection is inhibitory, horizontal cells using GABA as neurotransmitter. The role of horizontal cells is to provide an inhibitory feedback to the PRs and an inhibitory feed-forward to the bipolar cells and hence to reinforce perception of contrasts.

1.3 Ganglion cell layer and Optic Nerve

The retinal ganglion cells (RGCs) are the third order of neurons carrying the visual information. The midget retinal ganglion cells, the parasol cells, the bistratified cells and the photosensitive ganglion cells are amongst the different types of RGCs found in the ganglion cell layer. This cell type classification is based on their morphologies, projections and functions. As an example, the photosensitive RGCs contain a photopigment called melanopsin, which allows the RGCs to respond directly to the light signal, even in absence of rod and cone photoreceptors. These particular cells have a function in setting and maintaining circadian rhythms (Berson, Dunn et al. 2002; Hattar, Liao et al. 2002). RGCs collect the visual information in the IPL through their dendrites and send it to the brain via their axons that are gathering in a bundle to form the optic nerve that leaves the ocular globe. On their way to the brain, the neuronal fibers are myelinated; in primates this phenomenon occurs not until the optic nerve exits the ocular globe. The optic nerves of each eye reach the brain; the fibers from the nasal optic field partially decussate and form then the optic chiasma and then project to the lateral geniculate body; a small fraction of RGCs project to the supra-optic and hypothalamic structures, participating in non-visual processes like the pupillary reflexes and circadian rhythm regulation.

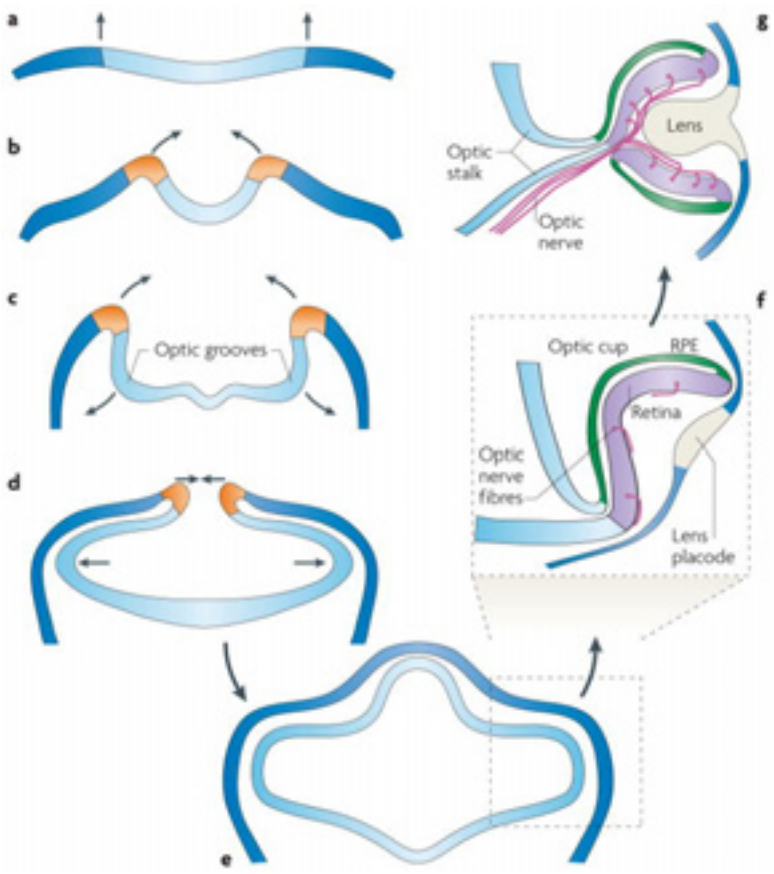

Figure 16. Development of the vertebrate eye cup

(a) The neural plate is the starting point for the development of the vertebrate eye cup. (b) The neural plate folds upwards and inwards. (c) The optic grooves evaginate. (d) The lips of the neural folds approach each other and the optic vesicles bulge outwards. (e) After the lips have sealed the neural tube is pinched off. At this stage the forebrain grows upwards and the optic vesicles continue to balloon outwards: they contact the surface ectoderm and induce the lens placode. (f) The optic vesicle now invaginates, so that the future retina is apposed to the future retinal pigment epithelium (RPE), and the ventricular space that was between them disappears. Developing retinal ganglion cells send axons out across the retinal surface. The surface ectoderm at the lens placode begins to form the lens pit. This section is midline in the right eye, through the choroid fissure, so only the upper region of the retina and the RPE are visible. g | The eye cup grows circumferentially, eventually sealing over the choroidal fissure and enclosing the axons of the optic nerve (as well as the hyaloid/retinal vessels; not shown). The ectodermal tissue continues to differentiate and eventually forms the lens. From Lamb et al., *Nat Rev Neurosci*., 2007.

1.4 Non-neuronal cells of the retina

1.4.1 Retinal pigmented epithelium

The ONL is adjacent to the monolayer of cells, called the retinal pigment epithelium (RPE), which plays a key role in PR homeostasis and survival. The outer segments of the PRs are embodied into the microvillies formed by the RPE: this organization is essential for the enzymatic cascade triggered by the photic izomerization of the retinal (the phototransduction cascade will be described below). The RPE plays a protective role for the PRs with the melanin granules by forming a pigmented shield that absorbs the straight light. RPE has also a supportive role for the PRs, by participating in the recycling of outer segments, storage and metabolism of vitamin A, and by producing cytokines required for retinal development and homeostasis (la Cour and Tezel 2005).

1.4.2 Glial cells

There are typically three types of glial cells in mammals: Müller cells, astrocytes and microglial cells. Astrocytes and microglial cells are found more sparsely, whereas Müller cells are the most numerous kind of glial cells found in the retina. Astrocytes are found in the GCL and IPL and contact RGCs and capillaries. Microglia cells are phagocytic cells found in the nerve fiber layer, but can migrate anywhere in the retina in pathologic condition. Müller cells form a scaffold for the retina by extending their cell body through the whole retina thickness, with their nucleus positioned in the INL. The role of Müller glia cells is to regulate the extracellular environment of the retina by controlling the amount of potassium released by the cells following the light signal, they also uptake the glutamate from the extracellular space (Bringmann, Pannicke et al. 2006). The Müller glia cells play a fundamental role in response to a retinal injury, by proliferating and secreting some extrinsic factors that will interact directly with the surrounding cell types (Dyer and Cepko 2000). In fish and birds, it is well-established that Müller glia cells can serve as a source to generate new neurons in damaged retina (Fischer and Reh 2001; Anthony, Klein et al. 2004). Indeed, Müller cells seem to acquire the ability to de-differentiate, proliferate, and become neuronal progenitors in acutely damaged retinas. This finding presents a therapeutic potential to regenerate injured retina.

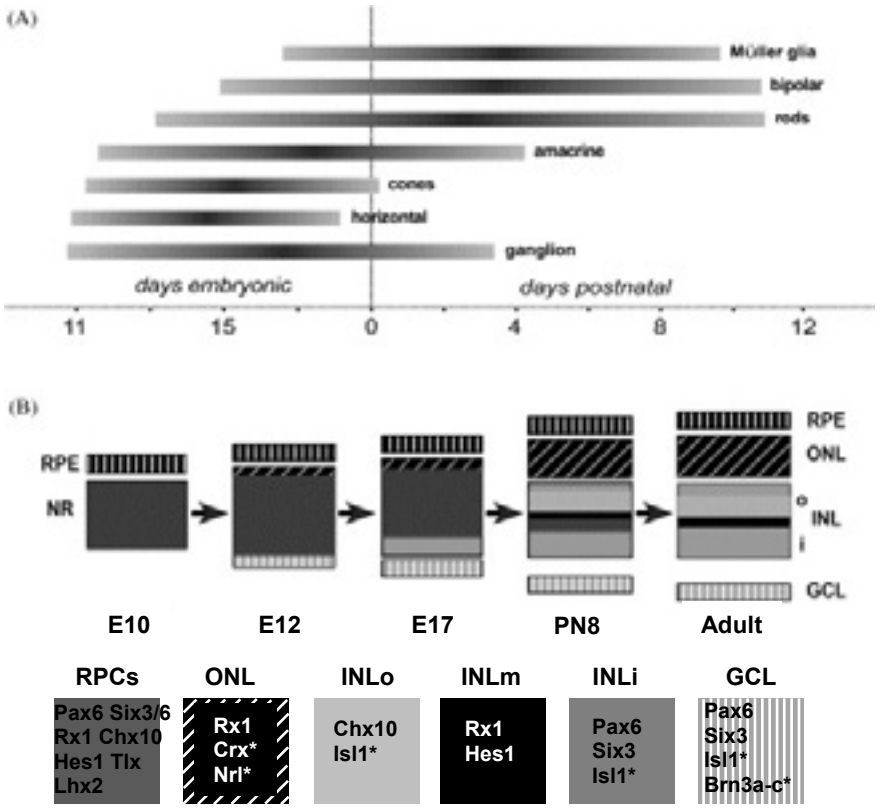

Figure 17. Retinal cell diversification

Retinal neurogenesis proceeds in a fixed histogenetic order: Ganglion cells and horizontal cells are born first, followed by cone-photoreceptors, amacrine cells, rod-photoreceptors, bipolar cells and Müller glia cells. The prenatal (E) and postnatal days (PN) refer to the respective stages of mouse development. After Young (1985). (B) A set of transcription factors is initially coexpressed in mitotic retinal progenitor cells (RPCs). However, with advancing retinogenesis, their expression domains start to segregate so that finally each retinal layer expresses a unique combination of these factors (given below). Also indicated are some examples of postmitotic transcription factors, such as Crx (marked by an asterisk). ONL, INL, GCL: outer nuclear, inner nuclear, and ganglion cell layer; INLi,m,o: inner, middle, outer INL; RPE: retinal pigment epithelium. E: embryonic day (of mouse development). Modified from Marquardt, Prog Retin Eye Res., 2003.

2. Retinogenesis

2.1 Development of the eye cup in vertebrates

The retina formation begins from the neural ectoderm, the source of the entire nervous system of an individual (Figure 16). The optic vesicle formation is the second step in the process of neural retina development. In parallel, occurs the development of the lens that will exert an effect on the optic cup shape by forcing it to invaginate. The inner wall of the optic cup will define the retina, while the outer wall develops into the RPE, forming a single-layer pigmented epithelium. At this point, the RPE will not undergo further changes other than growing. By the time the optic cup is formed, the retina is already undergoing cellular differentiation, forming two nuclear layers in the inner wall of the optic cup, called inner and outer neuroblastic layers. Cells start then to divide and proliferate. Daughter cells that are not leaving the mitosis cycle continue to proliferate, whereas the ones leaving the cell cycle start their differentiation.

2.2 Multipotent progenitors

Six early expressed transcription factors are involved in defining retinal identity: Rx1, Pax6, Six6, Six3, Tlx, and Lhx2 have been shown to stimulate retinoblast proliferation (Zuber, Gestri et al. 2003) (Figure 17). Suppression of these TFs during the development leads to either a reduction of the retinal progenitor cell (RPC) proliferation (Pax6 and Tlx) (Marquardt, Ashery-Padan et al. 2001; Miyawaki, Uemura et al. 2004), or to micropthalmia (Rx1) (Andreazzoli, Gestri et al. 1999); whereas overexpression of some of these factors is associated with stimulation of RPC proliferation (Rx1 and Pax6) (Casarosa, Amato et al. 2003; Zaghloul and Moody 2007) and giant eye development (Six6) (Zuber, Perron et al. 1999) or induction of Müller glia cell differentiation (Rax) (Furukawa, Mukherjee et al. 2000).

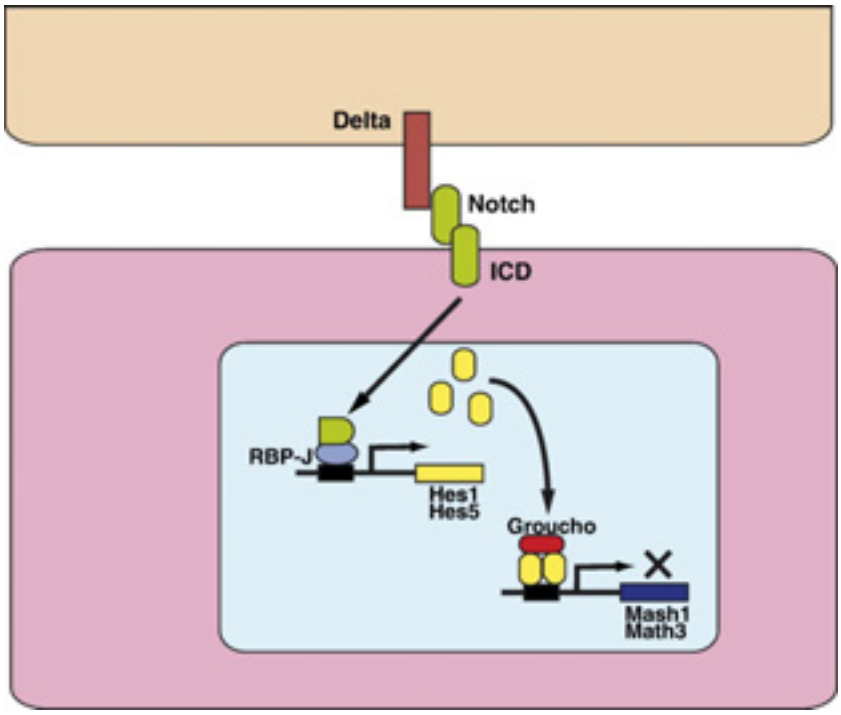

Figure 18. Schematic representation of Notch signaling.

Activation of Notch by its ligand Delta leads to the cleavage and translocation of the intracellular domain (ICD) of Notch. Notch ICD binds to the RBP-J complex and activates expression of Hes genes. Hes proteins interact with the corepressor Groucho and repress neural bHLH genes such as Mash1 and Math3, which are required for neuronal cell fate specification in the developing retina. From Ohsawa and Kageyame, Brain Res., 2008.

2.3 Retinal precursor cells

2.3.1 Role of extrinsic factors in retinal development

a. Secreted factors

The differentiation of certain retinal cell types generated from RPCs may be influenced by the action of secreted factors. Among these are the Ciliary Neurotrophic Factor (**CNTF**) and Leukemia Inhibitory Factor (**LIF**), both involved in changing the fate of rod precursors into bipolar-like cells, resulting in the loss of rod markers in these precursor cells (Ezzeddine, Yang et al. 1997). The Epidermal Growth Factor (**EGF**) and Transforming Growth factor-α (**TGF-α**) are both binding to the EGF receptor (EGFR) and were found to have mitogenic effects on the RPCs (Anchan, Reh et al. 1991). **Retinoic acid** is highly produced in the developing retina, likely by both RPCs and postmitotic cells, and promotes rod photoreceptor development (Kelley, Turner et al. 1994). Another factor supporting photoreceptor differentiation, is the Fibroblast Growth Factor (**FGF**), which was shown to have an effect in stimulating cells to express opsins, and therefore to differentiate into photoreceptors (Hicks and Courtois 1992).

b. The Notch pathway

There is a high contribution of cell-cell interactions for cell fate specification. This is explained by the presence of cell surface proteins, such as receptors and membrane-bound ligands that are working together to transduce the signal pathway to modulate retinal development. The Delta-Notch pathway has been extensively characterized for its role in retinogenesis (Perron and Harris 2000). Notch1 (Neurogenic locus notch homolog protein 1) is a transmembrane receptor activated by one of its ligands, Delta (Figure 18). Both elements are widely expressed in the developing retina and are associated with retinal cell differentiation. It is suggested that the Notch pathway inhibits neuronal differentiation, and rather supports RPC proliferation as well as gliogenesis (Furukawa, Mukherjee et al. 2000). There is a balance between Notch and Delta expression in adjacent cells that will make them become neurons or keep them in proliferation state. This phenomenon is called lateral inhibition, in which a pool of cells expressing more Delta on their surfacewill inhibit the neighboring cells from exiting the mitogenic cycle to

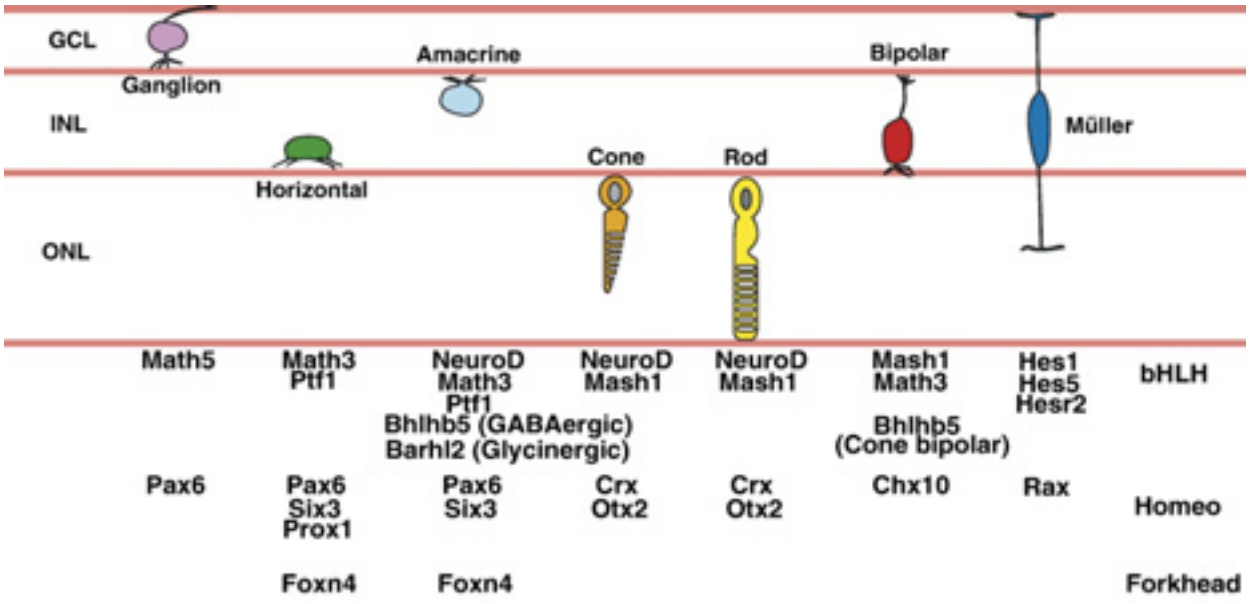

Figure 19. Regulation of retinal cell fate specification by transcription factors

Combinations of multiple transcription factors, such as bHLH-type and homeobox-type factors, are required for proper specification of retinal cell types. From Ohsawa and Kageyame, Brain Res., 2008.

become neurons, by enhancing Notch expression in those cells. A high expression of Notch in cells will contribute to glia development (Louvi and Artavanis-Tsakonas 2006).

Importantly, isolated embryonic retinal cells cultured with postnatal retinal cells do not preferentially adopt the cell fate of these postnatal RPCs. Therefore, RPC differentiation is influenced by other mechanisms than extrinsic signaling to adopt diverse cell type identity during retinogenesis.

2.3.2 Contribution of intrinsic cues in cell fate determination

- Basic helix-loop-helix transcription factors

Notch signaling activates the expression of Hes1 and Hes5 (E(spl)/hairy homologue) (Figure 18), two basic helix-loop-helix (bHLH) transcription factors that will in turn modulate the expression of downstream targets (Ohtsuka, Ishibashi et al. 1999). Some bHLH factors like Neurogenin and Mash1 act on keeping the proliferation of RPCs, whereas others are expressed in postmitotic neurons or differentiated neurons like the neurogenic differentiation factor 1 (NeuroD) (Figure 19). NeuroD plays a role in neuron and glia cell fate determination, and regulates amacrine cell type development over bipolar cell differentiation (Morrow, Furukawa et al. 1999). The achaete-scute homologue 1 (Ascl1; also known as Mash1) factor is expressed in amacrine cells, rod photoreceptors and late-born retinal cells and later is restricted to the INL (Cepko 1999). Math5 (also called Atoh7) is required for retinal ganglion cell development (Brown, Kanekar et al. 1998) and regulates the transcription factor Brn3b, which in turn is necessary for the terminal differentiation of retinal ganglion cells (Liu, Mo et al. 2001).

2.4 Immature retinal cell specification

There is a highly conserved histogenic order in retinal neurogenesis (Figure 17). In mice, the RPC genesis starts around embryonic day 9,5. There is an overlap of births with retinal ganglion cells produced first, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and finally Müller glia cells (Young 1985; Livesey and Cepko 2001). Upon becoming postmitotic, newly generated neurons have to express various post-mitotical TFs to induce their terminal differentiation; however, these TFs must also be expressed throughout the whole life of the cell for a normal

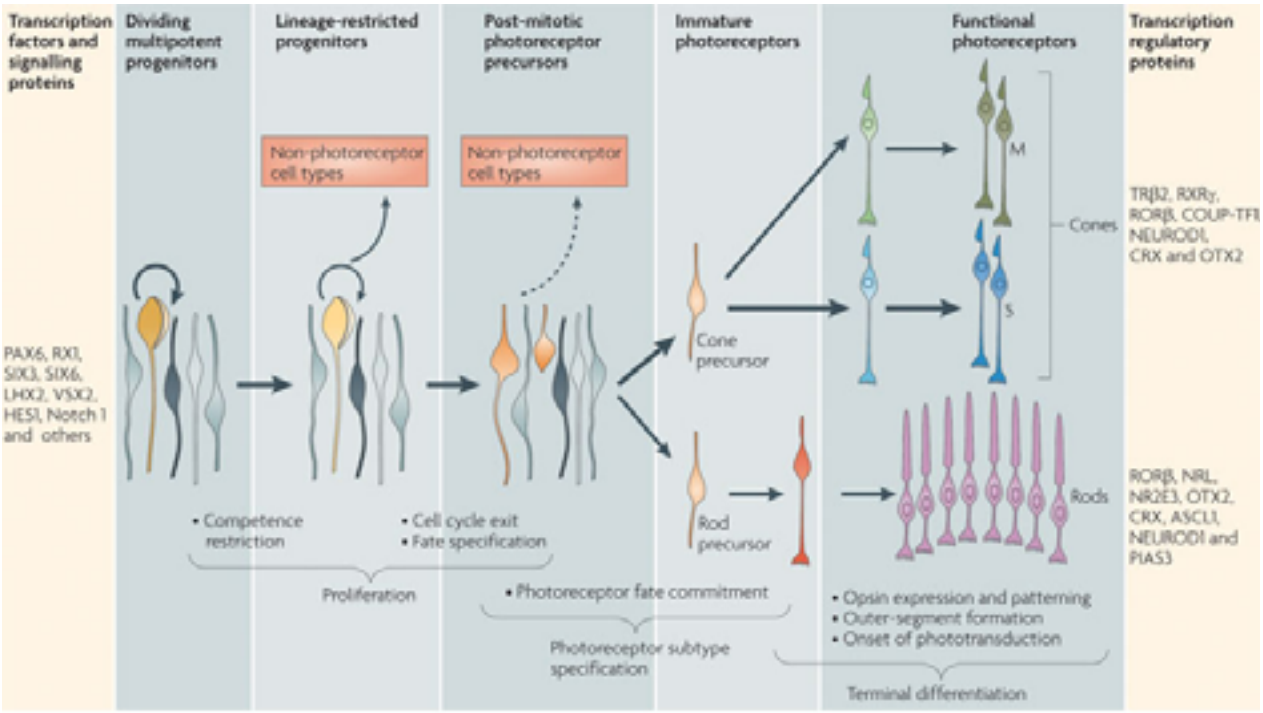

Figure 20. Stages of photoreceptor development

Multipotent retinal progenitor cells (RPCs) divide and produce additional multipotent progenitors (thick circular arrow) or progenitor cells that become restricted in their competence to generate various cell types (thin circular arrow). Some of these proliferating cells become restricted to a lineage that will give rise to at least one photoreceptor cell and possibly to non-photoreceptor cells. After cell cycle exit, postmitotic precursors can remain plastic. During cell type specification of photoreceptors, precursors are directed to become cones or rods that eventually express photopigments (M opsin and S opsin in cones, and rhodopsin in rods), and form outer segments and synapses. From Swaroop *et al*., Nat Rev Neurosci. 2010.

function of the mature neuron. Hence, post-mitotical TFs appear essential for both maturation and maintenance of homeostasis of the cells.

2.4.1 Photoreceptor fate commitment

Studies of gene ablation brought to light the complex mechanisms accountable for a particular cell type development. TFs are typically responsible for regulating cell fate specification; however, it is rather combinations of multiple TFs than action of a single one that leads to the maturation of a particular cell type (Figure 20). However, some exception can be made, for example, in the rod versus cone cell fate specification, the basic-leucine zipper Nrl (Neural retina leucine zipper) is a transcription factor preferentially expressed in precursor cells that will become rod photoreceptors (Swaroop, Xu et al. 1992). Deletion of *Nrl* induces the loss of rod photoreceptors that are converted into S-cones (Mears, Kondo et al. 2001) (Nrl transcription factor will be further detailed in chapter D). In retinogenesis, the role of Nrl is to activate the rod-pathway and to repress the cone-specific genes in association with other TFs, such as the nuclear receptor subfamily 2, group E, member 3 (Nr2e3) (Oh, Cheng et al. 2008), and the cone rod homeobox (Crx) (Mitton, Swain et al. 2000). Nr2e3 (formerly Photoreceptor-specific Nuclear Receptor, PNR) (Kobayashi, Takezawa et al. 1999) supports the rod-photoreceptor lineage by inhibiting the expression of cone genes (Cheng, Khanna et al. 2004; Peng, Ahmad et al. 2005; Cheng, Aleman et al. 2006), and Nrl and Crx, activating rod genes (Chen, Rattner et al. 2005). Mutations reported in *Nr2e3* are associated with enhanced S-cones syndrome (ESCS), characterized in patients by an increased number of the S-cone subtype. Electroretinographies of patients show an increased S-cone mediated sensitivity and extinction in rod response. Moreover sensitivity of L/M cones is reduced in variable degrees (Haider, Jacobson et al. 2000). These data suggest that the precursors of photoreceptors develop into an S-cone default pathway unless additional factors influence the pluripotent cells to adopt a particular photoreceptor-subtype fate. Another transcription factor involved in the photoreceptor pathway is the homeobox gene *Crx*. Crx is a transcription factor contributing to the photoreceptor maturation and homeostasis (Furukawa, Morrow et al. 1997; Hennig, Peng et al. 2008). In *Crx* deficient mice, the ONL is formed of photoreceptors that lack outer segments and whose neuronal activity is impaired (Furukawa, Morrow et al. 1999). This implies that Crx is critical for

photoreceptor maturation although it does not specify the photoreceptor cell fate. Another homeobox gene necessary for the photoreceptor lineage is the paired-type homeodomain transcription factor *Otx2*. Otx2 is early expressed in the transcriptional cascade leading to photoreceptor cell fate (Swaroop, Kim et al. 2010). Otx2 transactivates Crx and is a key regulator of photoreceptor development; indeed, deletion of Otx2 leads to a retina deprived of photoreceptors, and an increase of amacrine-like cells, suggesting that the precursors for photoreceptors changed their fate in absence of Otx2 to become amacrine cells (Nishida, Furukawa et al. 2003). RORβ, an orphan nuclear receptor, acts for both rod and cone differenciation. Lack of RORb in developing mouse retina results in the absence of rods and features an excess of primitive S cone-like nphotoreceptors that are not functional. In *RORb*$^{-/-}$ mice, *Nrl* is not expressed, suggesting that *RORb* lies upstream of *Nrl* in the photoreceptor cell fate lineage (Jia, Oh et al. 2009). Cone-photoreceptor lineage is highly influenced by the expression of thyroid hormone receptor. Indeed, in the Thyroid hormone receptor β2 (TRβ2)-deficient mice, while the rod photoreceptors seem normal, the only cone subtype found in the retina of these animals, is the S-cone subtype. No M-cone immunoreactivity is found, suggesting that TRβ2 has a dual role in cone pathway development, by stimulating M-cone development and repressing S-cone generation (Ng, Hurley et al. 2001; Ng, Ma et al. 2009; Ng, Lu et al. 2011).

2.4.2 Other retinal cell fate commitment

Both the homeobox gene *Chx10* and two bHLH, Mash1 and Math3, seem to play a major role in the bipolar cell-type specification. Mutations in *Chx10* result in a proliferation of RPCs and a bipolar-less retina (Burmeister, Novak et al. 1996). The double knock-out animals for *Mash1* and *Math3* genes present a total absence of bipolar cells and an increase of Müller glia cells, suggesting that Mash1 and Math3 are necessary for bipolar cell development and inhibition of gliogenesis (Tomita, Moriyoshi et al. 2000; Hatakeyama, Tomita et al. 2001).

Amacrine cell development seems to be induced by the expression of a set of bHLH factors such as Math3, NeuroD, Pax6 and Six3, shown to promote amacrine cell genesis (Inoue, Hojo et al. 2002). In the transcriptional cascade leading to the amacrine cell specification, Sox2 is found upstream of Pax6 expression. Sox2 transcription induces

Pax6 expression, which will in turn activate NeuroD, and both factors will promote the amacrine cell fate (Lin, Ouchi et al. 2009).

Horizontal cells and amacrine cells seem to have linked development, since both cell types are sharing common transcription factors. Misexpression of Math3 and Pax6 showed an increase in both amacrine and horizontal cells (Inoue, Hojo et al. 2002). Deletion of Foxn4 results in a total disappearance of horizontal cells, likely due to the fact that Foxn4 is expressed early in the retinogenesis and likely affects Math3 expression (Li, Mo et al. 2004).

One critical regulator of the RGCs development is the bHLH factor Math5. Deletion of this factor results in an almost total disappearance of RGCs that are converted into other cell types (Wang, Kim et al. 2001; Le, Wroblewski et al. 2006). Downstream of Math5, expression of Brn3b is likely required for formation of RGC axons and axon path-finding and is essential for early retinal ganglion cell differentiation (Gan, Wang et al. 1999; Erkman, Yates et al. 2000).

2.5 Mature and functional photoreceptors

After specification of the cell type, photoreceptors will start to express specific genes that are involved in their functioning, such as genes for the phototransduction cascade and genes that will confer them their typical morphology. Then, axonal growth and synapse formation between photoreceptors and bipolar and horizontal cells will establish the connections necessary for the signal transduction. Finally, outer segment development will intricate their structure within the RPE for establishment of the phototransduction (Swaroop, Kim et al. 2010; Hao, Kim et al. 2012).

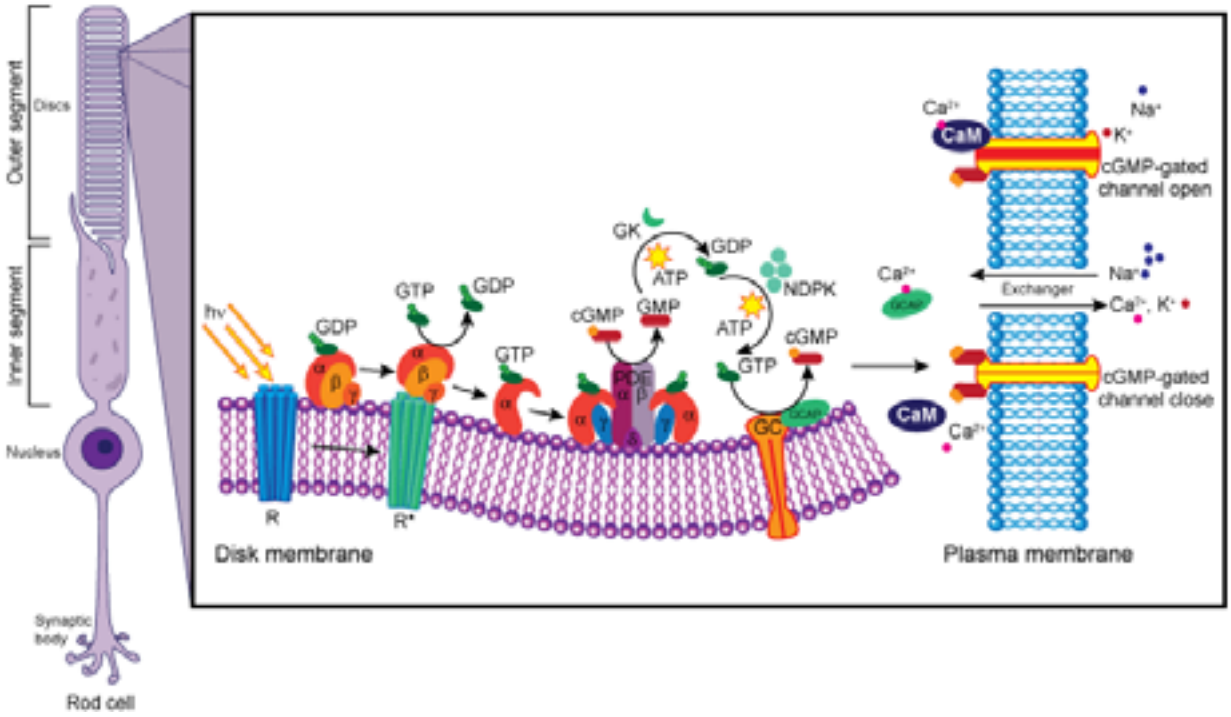

Figure 21. The visual phototransduction cascade

In the dark, opsin is bound to 11-cis-retinal to form inactive rhodopsin (R) in the disc membranes. Basal activity of the guanylyl cyclase (GC) keeps cGMP levels high. The binding of Ca^{2+}-bound calmodulin (CaM) confers high affinity for cGMP to cGMP-gated channels in the plasma membrane, allowing these channels to remain open. Both Na^+ and Ca^{2+} enter the channels resulting in high Ca^{2+} levels and Ca^{2+}-bound guanylate cyclase-activating protein (GCAP). Light (Hν) results in photoisomerization of 11-cis-retinal to all-trans-retinal, forming activated rhodopsin (R^+), which binds and activates the heterotrimeric G protein, transducin ($\alpha\beta\gamma$). The GTP-bound transducin α subunit activates cGMP phosphodiesterase (PDE), which hydrolyzes cGMP to GMP, reducing the cGMP concentration and the binding of cGMP to the cGMP-gated channels. The probability of channel closing increases proportionally to light intensity, reducing Ca^{2+} influx. Intracellular Ca^{2+} is further depleted by activity of the Na^+–Ca^{2+}, K^+ exchanger. Low intracellular Ca^{2+} leads to active GCAP, which in turn activates GC to synthesize cGMP from GTP supplied by the guanine nucleotide cycle. This comprises guanylate kinase (GK) and nucleoside diphosphate kinase (NDPK). Release of Ca^{2+} from CaM leads to its dissociation from the cGMP-gated channels conferring a lower affinity for cGMP and further closure of the channels. From http://mutagenetix.utsouthwestern.edu

C. Physiology of the retina

1. Phototransduction

The visual phototransduction is the process by which the light signal is converted into an electrical message by the different cells composing the retina. The phototransduction cascade has been more extensively investigated in rods than in cones. In the dark, the photoreceptors are depolarized due to the opening of the cGMP-gated sodium channels allowing an inward current, resulting in neurotransmitter release. Lightning induces the hyperpolarization of photoreceptors that will activate/inhibit downstream cells and allow transduction of the signal. The first step of the signal transduction is taking place in the rod OS, when a single photon strikes the photopigment rhodopsin. The photopigment is composed of an opsin covalently bound to a chromophore called 11 *cis*-retinal, which is the photosensitive molecule of the rhodopsin. When a photon hits this molecule, the 11 *cis*-retinal is converted into all-*trans* retinal. This change of conformation leads to a change of rhodopsin into metarhodopsin II, which in turn activates a trimeric G-protein called transducin. Activated-transducin exchanges its alpha-subunit-bound GDP for GTP. Then, this complex activates a phosphodiesterase located in the disk membrane that hydrolyzes cGMP into 5'-GMP. The lowering of cGMP concentration throughout the outer segment causes the sodium channels to close, and provokes hyperpolarization of the cell, therefore, lessening the release of the glutamate neurotransmitter. A decrease of the glutamate release by the photoreceptors causes depolarization of ON-bipolar cells (rod and cone ON-bipolar cells) and hyperpolarization of cone OFF-bipolar cells (Figure 21). Bipolar cells excitation is relayed to the third order neuron in the visual signal pathway, the ganglion cells. These ganglion cells are the only retinal cells able to generate action potentials that travel via the optic nerve to one of the main visual relay structure, the lateral geniculate nucleus in the thalamus, which is the first structure to process the visual signal. Neurons then leave the lateral geniculate nucleus to connect the primary visual cortex.

Besides the obvious role of the retina in the visual signal formation and transduction, this neural tissue plays a substantial function in the light/dark information essential for the setting of our biological clock.

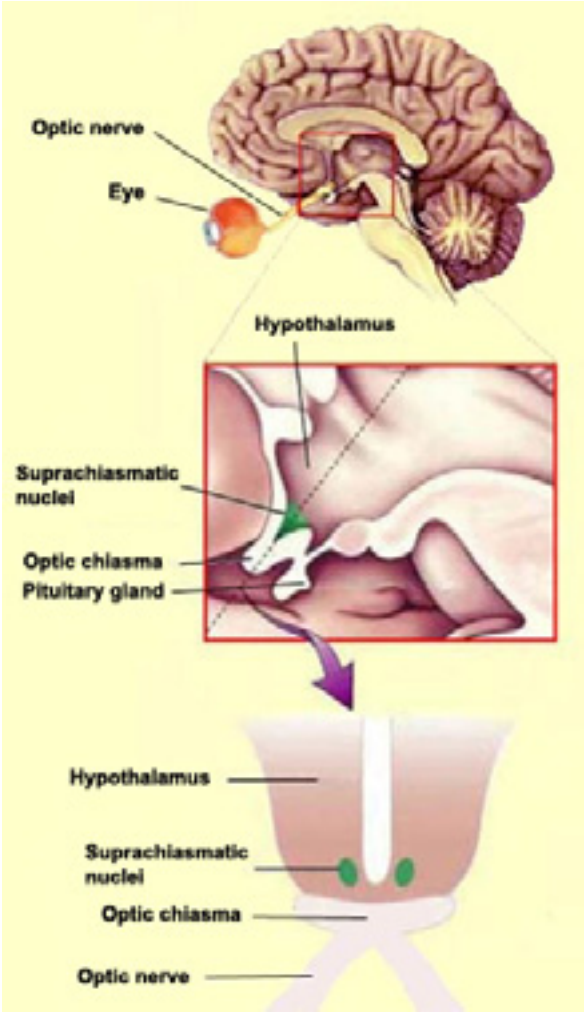

Figure 22. Location of the suprachiasmatic nuclei

The master clock regulating the circadian cycles is located in two small structures in the brain, at the base of the hypothalamus, called the suprachiasmatic nuclei. From http://thebrain.mcgill.ca

2. The retina and generation of rhythmic functions

2.1 The circadian system

Circadian rhythms in physiology and behavior exist in virtually all living organisms and allow adaptation to and anticipation of daily changes induced by earth rotation. These rhythms are controlled by circadian clocks, endogenous time-keeping systems able to generate ~24 hour rhythms even in the absence of external time-giving cues or zeitgebers. The most powerful zeitgeber is the light/dark cycle, which in mammals is perceived exclusively by the retina. In addition to the photoreceptors, the classical photosensitive cells of the retina, there is another class of cells sensitive to light intensity variations: a small group of retinal ganglion cells expressing the photopigment melanopsin (Hattar, Liao et al. 2002). Axons of these cells, called intrinsically photosensitive ganglion cells (ipRGC), project via the optic nerve and optic chiasm to the suprachiasmatic nuclei (SCN), which is considered as the “master clock” of the entire organism (Hannibal and Fahrenkrug 2002). The SCN are a paired structure of the hypothalamus, composed of distinct groups of neurons receiving and interpreting information on environmental light and dark conditions and day length, both important for the entrainment of the master clock (Ralph and Menaker 1988; Ralph, Foster et al. 1990) (Figure 22). SCN coordinate brain and peripheral secondary clocks by various output signals such as hormonal cues or neuronal efferences. In particular, they direct the pineal gland to secrete the hormone melatonin which is considered one of the main molecular outputs of the clock. This hormone is secreted by the pineal gland, with a higher expression during the night, and its expression is related to the length of the night, which varies depending on the season (short in summer and long in winter). Although the pineal gland was found to be the principal site to produce melatonin, the retina produced as well this horamone rhtymically, giving to this tissue an importance in rhythm generation. The melatonin in the retina has a neuromodulatory role in acting on several aspects of retinal physiology. For instance, melatonin is suggested to be a chemical mediator for dark-adaptation in the retinal network (Besharse, Iuvone et al. 1988); in non-primate vertebrates, melatonin is responsible for the dark-adaptive migration of the photopigment in the OS (Cheze and Ali 1976). Moreover, this hormone

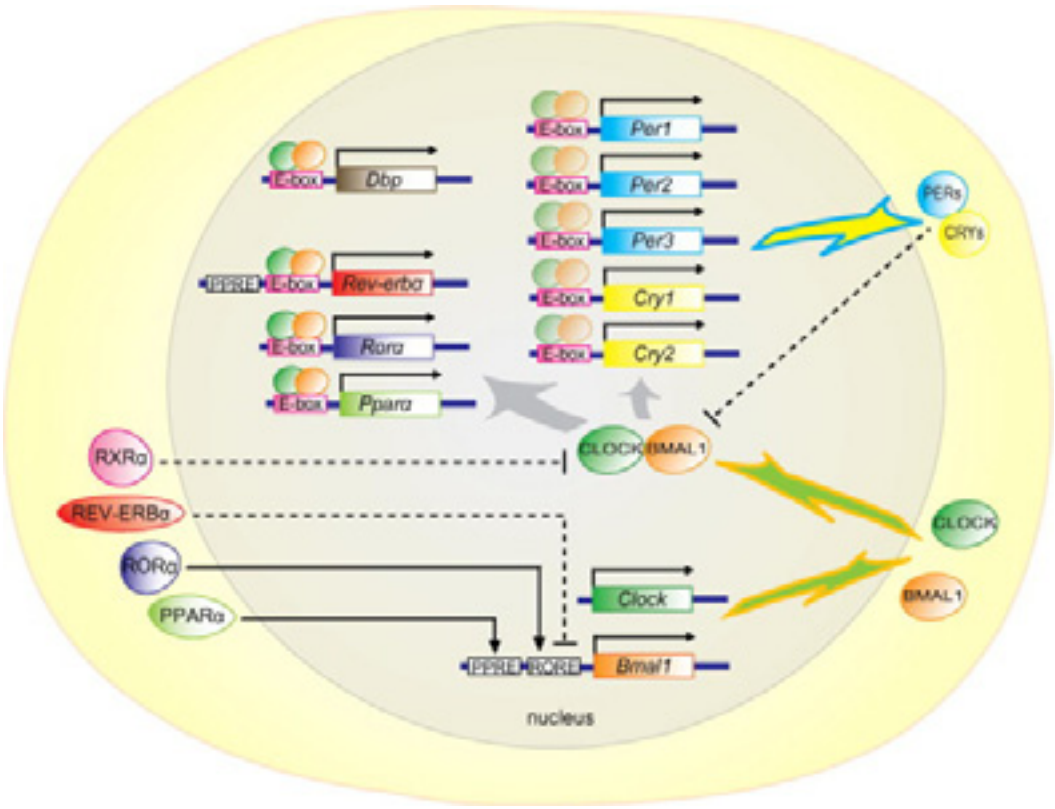

Figure 23. The molecular circadian clock machinery

This diagram depicts the positive (CLOCK:BMAL1) and negative (PER:CRY) limbs of the circadian clock gene feedback loop that is present in virtually all cells throughout the body. REV-ERBα and RORα represent components of a secondary feedback loop that either activate (solid line with arrow) or inhibit (dashed line) *Bmal1* activity. The CLOCK:BMAL1 transcription complex has the ability to directly regulate the activity of genes (clock controlled genes) critically involved in energy metabolism, including *Dbp* and *Pparα*. In turn, PPARα can directly bind to *Bmal1* and influence activation of the positive limb of the feedback loop. BMAL1, brain muscle arnt like factor; CLOCK, circadian locomotor output cycles kaput; CRY, cryptochrome; DBP, albumin D-element binding protein; PER, period; PPARα, peroxisome proliferator-activated factor alpha; REV-ERBα, reverse erythroblastosis virus alpha; RORα, retinoic acid receptor-related orphan receptor alpha; RXRα, retinoid X receptor alpha. From Laposky et al., FEBS Lett., 2008.

is involved in the rod OS disc shedding in *Xenopus* and rat retina (Besharse, Dunis et al. 1984; White and Fisher 1989).

At the molecular level, the clock machinery relies on interactions of specific clock genes, working together in positive and negative regulatory loops during a period close to 24 hours, and this mechanism is quite similar between SCN and the secondary clocks (Figure 23). The positive loop is constituted of CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (ARNTL aryl hydrocarbon receptor nuclear translocator-like), two bHLH-PAS factors forming a heterodimer binding to E-box sequence (core consensus sequence, 5'-CANNTG-3') found in the promoter of target genes. Among the genes regulated by CLOCK/BMAL1, there are *Dec1-2*, *RevErbα-β* and *Rorα-γ* plus the main factors involved in the negative loop, *Per1-3* and *Cry1-2*. PER (Period) and CRY (cryptochrome) proteins are forming dimers in the cytosol and enter the nucleus to inhibit CLOCK/BMAL1 dimer and hence to repress their own expression. PER and CRY also undergo proteasome-mediated degradation. Thus, when their levels are low enough, their activation by CLOCK/BMAL1 can re-start and expression of PER and CRY proteins rises again.

2.2 The retinal clock

Almost 30 years ago, the first demonstration that the vertebrate retina contains an endogenous oscillator was made in *Xenopus*. In this initial study, the authors showed *in vivo* that the retina displays oscillations in N-acetyltransferase activity in constant conditions, substantiating that the retina is a peripheral clock controlling the rhythms of local functions and can work independently of the master clock located in the SCN (Besharse and Iuvone 1983). Existence of an oscillator in the retina is likely to prepare the retina to the > 1000 000 fold-change in light intensity encountered throughout the light/dark cycle. Several rhythms have been described in the vertebrate retina; some of them will be described further in the next paragraphs. Among the rhythmic functions of the retina, we can cite the visual sensitivity and ERG response (Brandenburg, Bobbert et al. 1983) and extracellular pH (Dmitriev and Mangel 2001) both characterized in rabbit; sensitivity to light-induced photoreceptor degeneration in rat (Vaughan, Nemke et al. 2002); melatonin biosynthesis in photoreceptor cells in chicken (Hamm and Menaker 1980), dopamine content and release from amacrine and interplexiform cells in fish

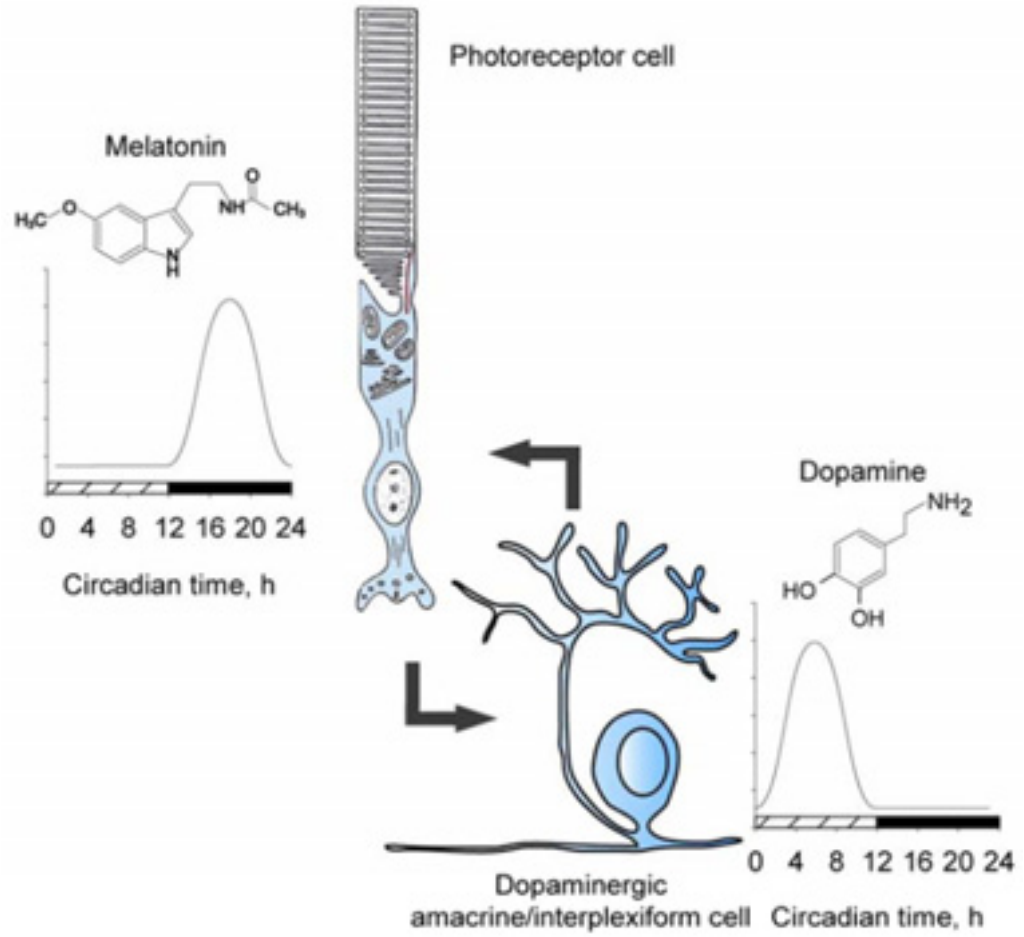

Figure 24. Melatonin and dopamine rhythmic synthesis in the retina

Melatonin and dopamine play opposing roles in the regulation of retinal adaptive physiology Dopamine functions as a humoral signal for light, producing light-adaptive physiology, while melatonin has dark-adaptive effects. The synthesis and release of both melatonin and dopamine are under circadian control, with melatonin released at night and dopamine during the daytime. Melatonin inhibits the release of dopamine through an action on melatonin receptors and dopamine inhibits the synthesis and release of melatonin from photoreceptor cells by acting on dopamine receptors present on photoreceptors. Thus, the melatonin synthesizing photoreceptors and dopamine secreting inner retinal neurons form a cellular feedback loop that regulates circadian retinal physiology. From Tosini *et al.*, Bioessays, 2008.

(Ribelayga, Wang et al. 2002); rod–cone dominance in quail (Manglapus, Uchiyama et al. 1998); rod–cone coupling in goldfish and mouse retina (Ribelayga, Cao et al. 2008); cAMP (Ivanova and Iuvone 2003) and pCREB (Liu and Green 2002) signaling pathways in photoreceptors of chicken and *Xenopus*, respectively; transducin mRNA in rat (Brann and Cohen 1987); iodopsin mRNA (Pierce, Sheshberadaran et al. 1993) and melanopsin mRNA (Chaurasia, Rollag et al. 2005), both studies in chicken; nocturnin mRNA, characterized in *Xenopus* and mouse (Green and Besharse 1996; Wang, Osterbur et al. 2001) (Iuvone, Tosini et al. 2005). *Bmal1* function within the retina is required to generate circadian rhythms for the inner retinal visual processing. Deletion of *Bmal1* affects rhythmicity of genes normally rhythmic in LD, suggesting that *Bmal1* plays an important role in light-dependent gene regulation (Storch, Paz et al. 2007). Virtually all the layers oscillate in an independent manner (C. Jaeger, unpublished data) and express the same clock genes considered to be part of the core clockwork found in the SCN (Tosini and Fukuhara 2002).

Rhythmic functions have been particularly characterized in photoreceptors, supporting existence of a circadian clock in the ONL. Experiments that led to this conclusion are described below.

2.2.1 Melatonin and Dopamine rhythms

The initial characterization of a circadian clock in the photoreceptors was made with *Xenopus* isolated photoreceptors kept in culture. Although these cells were disconnected from the SNC, they showed sustained circadian oscillations of melatonin release for many days in vitro. Moreover, addition of dopamine in the culture reset the melatonin rhythm, meaning that the photoreceptors cells possess the receptors, identified as the D2/D4-like receptors, to respond to the dopamine release and this symbolizes a setting for the entrainment of the clock (Cahill and Besharse 1993; Tosini and Dirden 2000). In addition to that, AANAT (the enzyme catalyzing the rate limiting step in melatonin synthesis) is more or less regulated by a circadian clock, likely located in photoreceptors, further strengthening melatonin rhythmicity. Thus, melatonin is secreted by the photoreceptors at night, whereas the dopamine, produced by the amacrine/interplexiform cells, is found during the daytime in the retina (Figure 24). There are inhibitory effects of the melatonin on the dopamine release through the action

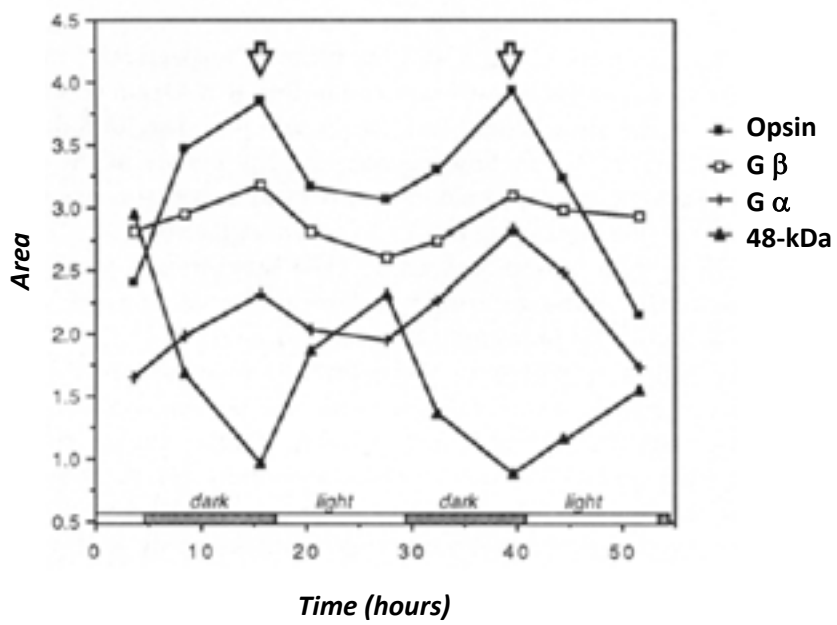

Figure 25. Light–dark cycling of opsin, Gα, Gβ, and 48-kDa protein mRNA levels

Graphic representation of total retinal RNA isolated from WT mouse and labeled with specific cDNA probes for opsin, Gα, Gβ, and 48-kDa protein. Area of each slot is marked on the ordinate. The isolation times are indicated on the abscissa. Highest levels of opsin (black box), Gα (+) and Gβ (white box) mRNAs and lowest levels of 48-kDA protein (black triangle) mRNA occur 1.5h before lights on (3.30 a.m. on day 1 and 3.30 a.m. on day 2, arrows). Modified from Bowes *et al*., Exp. Eye Res., 1988.

on MT2-like melatonin receptors (Ribelayga, Wang et al. 2004). Thus, there is a cellular feedback loop formed by the photoreceptors releasing melatonin and the amacrine/interplexiform cells, to regulate circadian retinal physiology (Iuvone, Tosini et al. 2005; Tosini, Pozdeyev et al. 2008).

2.2.2 Disc shedding

Another aspect of the retinal circadian functions found in photoreceptors, is the phagocytosis of the outer segment of these cells by the RPE. Both rod and cone outer segments are constantly renewed. The degradation of visual pigment-containing discs implies the formation of new discs that are pushing the old ones at the tip of the OS. Next, the disc detaches from the OS and is phagocytosed by the adjacent RPE; this phenomenon is materialized by the formation of phagosomes in the RPE cells. The quantification of the number of phagosomes in rod photorecetors during a circadian cycle shows a rhythmic apparition of these structures, with a peak of disk shedding at the beginning of the light period in a light/dark cycle (LaVail 1976; Bobu and Hicks 2009). In chicken retina, the peak of disk shedding in cones is observed at the beginning of the dark period (Young 1978). Disc shedding persists in constant darkness, supporting the fact that this output is driven by a circadian oscillator.

2.2.3 Circadian expression of photopigments

A few studies have demonstrated the rhythmic expression of visual pigments in the mammalian retina. The work of Bowes et al. is the first to show in the mouse retina a differential level of expression of opsin transcript between the day versus the night, with the highest level of the opsin mRNA reached at night (Bowes, van Veen et al. 1988) (Figure 25). The ryhthmic expression of rhodopsin transcripts was shown to be persistent under constant darkness, as well as the S-/UV-opsin expression, with maximal values around the subjective light/dark transition (von Schantz, Lucas et al. 1999). Although these data clearly show the daily regulation of opsin expression by a circadian clock, the mechanisms by which these rhythmic functions are regulated remain unknown. One hypothesis for the circadian regulation of opsin comes from the work of Li and collaborators, where they studied the expression of the long-wavelength cone opsin (LC opsin) in zebrafish. LC opsin mRNA expression profile shows rhythmic variation in LD and

DD conditions with a peak at the (subjective) light/dark transition. Expression of *Clock* and *Per3* mRNA in the LC opsin-expressing cells suggest the presence of clock machinery within these cells. cAMP could be one of the vector of the circadian system to impose rhythms to the cells. Measurements of cAMP showed rhythmic variation in constant condition, and knockdown of CLOCK abolished the *cAMP* and *LC opsin* mRNA fluctuations, meaning that CLOCK may regulate the circadian rhythms of LC opsin mRNA expression via cAMP signaling pathways (Li, Chaurasia et al. 2008).

2.2.4 Clock gene expression in photoreceptors

Techniques of cellular isolation permit studying expression of genes in individual cell-types. Laser capture micro-dissection is one of these techniques, where it is possible to dissect out the photoreceptor layer and to study in a specific mode the molecular parameters affected to these cells. Analysis of clock gene expression in isolated-photoreceptors in light/dark cycle showed a significant variation in the transcript amount for *Clock*, *Per1*, *Per3* and *Casein kinase I*ε, whereas *Bmal1* mRNA variation was not significant. In dark/dark conditions only *Clock* and *Per3* remained significantly variable. These data are placed in correlation with analysis of the clock gene expression in the whole retina, where in LD, all the genes cited above showed significant variability during the cycle, while in DD, the variability remained in *Clock*, *Bmal1*, *Per2* and *Per3*. During 24 h, changes in mRNA levels of clock genes in photoreceptors indicate that these cells potentially contain a circadian clock working independently to other retinal layers (Schneider, Tippmann et al. 2010). A recent study used another technique to isolate photoreceptors, performing vibratome-dissection; the authors isolated rat photoreceptors and quantified mRNA amounts of clock genes during LD and DD conditions. They showed significant variations of transcript for *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *Ror*β and *RevErb*α genes in LD condition, whereas in DD, none of the genes' transcript remained significant except for *Ror*β and *RevErb*α. The clock outputs *Aanat* and *c-Fos* remained strongly rhythmic in LD and DD conditions (Sandu, Hicks et al. 2011). Thus in spite of some differences, the data of this and previous studies are globally in agreement. They suggest that the photoreceptors have the components of a functional clock in LD; however, lack of rhythmicity of genes' expression in DD suggests that the

clock located in photoreceptors may require additional settings provided by other retinal layers to entrain the photoreceptor clock in constant condition.

D. *Nrl* a model gene to decipher the mechanisms regulating rod-development and homeostasis in the mammalian retina

The discovery of the Neural Retina Leucine zipper transcription factor was made from a cDNA library of human adult retina. Swaroop and his collaborators identified a gene specifically expressed during different developmental stages of the retina and in a retinoblastoma cell line (Swaroop, Xu et al. 1992). Dissection of the murine *Nrl* gene mapping to chromosome 14 like the human ortholog, reveals that this gene spans a genomic region of about 6 kb and shares ~ 90% of homology with human *NRL* gene. *Nrl* is composed of three exons and has an additional predicted exon (non-translated) in the 5'-upstream region. The first methionine of *Nrl* is detected in the second exon and leads to an open reading frame of 237 amino acids. Hence, the first exon is untranslated and may be required for the recruitment of a complex participating to the initiation of transcription. Analysis of the 5'-untranslated region helped identifying some of the critical regulatory elements that might play a role in the transcription of *Nrl*. Among these elements, a non-canonical TATA box, a CAAT box, two E boxes and putative transcription factor binding sites for octamer and AP2 proteins were identified (Farjo, Jackson et al. 1993).

NRL can form homodimers and heterodimers with c-Maf, Fos and Jun, and binds to an AP-1 like sequence $TGCN_{6-8}GCA$ designated as NRL Response Element (NRE) (Kerppola and Curran 1994; Kerppola and Curran 1994). The work of Rehemtulla and collaborators was the first to show the functional role of NRL as being part of a retinal protein complex able to specifically transactivate the promoter of *rhodopsin* gene (Rehemtulla, Warwar et al. 1996). NRL was also shown to interact with the TATA-Binding protein (TBP) through 35 amino acid residues identified in NRL protein as the minimal transactivation domain (Friedman, Khanna et al. 2004).

These data suggest that NRL could play an active role in the formation or stabilization of the transcription initiation complex at the promoter of target genes. NRL protein undergoes post-translational modifications (PTMs), such as phosphorylation, which result in the detection of several isoforms in retinal immunoblots (Swain, Hicks et al. 2001). The role of PTMs is thought to modulate the activity of transcription factors. Thus, alterations in NRL-mediated transactivation were observed with NRL proteins carrying mutated residues p.S50 and p.P51. These residues are important sites for NRL phosphorylation: it was shown that mutations at these residues – identified in patients with autosomal dominant retinitis pigmentosa (Bessant, Payne et al. 1999; Kanda, Friedman et al. 2007) – result in reduced number of NRL isoforms by immunoblotting. Moreover, p.S50 and p.P51 mutants displayed a significantly higher capacity to transactivate *rhodopsin* promoter as compared to the WT protein, conferring them a gain-of function (Kanda, Friedman et al. 2007). Interestingly, p.S50 and p.P51 residues are located within NRL minimal transactivation domain, which is important for the interaction of NRL with TBP [see above, (Friedman, Khanna et al. 2004)], suggesting that phosphorylation status affects NRL ability to bind TBP and/or other components of the general transcription machinery. Recently, another type of PTM affecting NRL protein has been demonstrated: the sumoylation of NRL with two small ubiquitin-like modifier (SUMO) molecules attached to the Lys-20 residue of this protein. Mutant NRL proteins for SUMO sites exhibit lesser transactivation properties on the target promoter of Nr2e3 (Roger, Nellissery et al. 2010). Hence, it appears that PTMs modulate the activity of NRL to activate promoters of specific target genes that contribute to rod development and function.

1. Transcriptional regulation of the rod-photopigment Rhodopsin

1.1 Multi-factorial complex formation for *Rhodopsin* expression

Rhodopsin is one of the most critical genes for the rod-photoreceptor. Fine-tuning its expression is a critical step for proper development and functioning of the rods. Mutations in *Rhodopsin* are associated with autosomal dominant or recessive Retinitis Pigmentosa, an inherited retinal dystrophy leading progressively to vision loss (Humphries, Farrar et al. 1990; Olsson, Gordon et al. 1992). In mice, disruption of *Rhodopsin* gene leads to a decline with age of the total number of photoreceptors,

ultimately resulting in a thinning of the ONL. In these mice, rod-outer segments appear shorther and no immunoreactivity for Rhodopsin is found. Moreover, electroretinograms (ERG) display a reduced cone response, but no rod response is detected (Humphries, Rancourt et al. 1997). These data put emphasis on the importance of an accurate regulation of *Rhodopsin* for rod cell homeostasis, as proven also by using transgenic mouse strains overexpressing *Rhodopsin* (Tan et al, IOVS 2001). Among the classical factors known to positively regulate *Rhodopsin*, NRL is the first transcription factor identified in a retinal protein complex that binds to a sequence spanning the NRE found in *Rhodopsin* promoter and to stimulate a *Rhodopsin* promoter-reporter gene construct in retinal cell cultures (Kumar, Chen et al. 1996; Rehemtulla, Warwar et al. 1996). The leucine zipper domain of NRL is important for protein-protein interaction; it is via this domain that NRL binds to CRX homeodomain (Mitton, Swain et al. 2000). CRX binds three distinct regions in *Rhodopsin* promoter and regulates other rod-specific gene promoters (Chen, Wang et al. 1997; Lerner, Gribanova et al. 2001; Pittler, Zhang et al. 2004). Therefore, NRL in synergy with CRX is required for elevated expression of *Rhodopsin*. Transactivation of *Rhodopsin* by NRL and CRX is reinforced by action of NR2E3 on the target promoter. Combination of these three factors and others enhances more significantly *Rhodopsin* promoter activity than the action of single transcription factors or combinations of only two of them. Interestingly, the authors demonstrate the interaction *in vivo* of NR2E3 with NR1D1, also known as RevErbα. The highest level of *Rhodopsin* promoter-activation occurs when NRL, CRX and NR2E3 are combined with RevErbα (Cheng, Khanna et al. 2004). Recently, NRL was found to interact with c-Jun N-terminal kinase 1 (JNK1) and HIV Tat-interacting protein 60 (Tip60), a histone acetyltransferase, to enhance Nrl transcriptional activity on *Rhodopsin* promoter. JNK1 phosphorylates NRL at serine 50 and increases its stability on the promoters of *Rhodopsin* and *Ppp2r5c* (protein phosphatase 2, regulatory subunit B', gamma; a gene involved in balancing phosphorylation signals that are critical for cell proliferation and differentiation (Kim, Jang et al. 2012). Therefore, NRL, in synergy with JNK1 and Tip60, may be required to precisely control spatiotemporal photoreceptor-specific gene expression during retinal development.

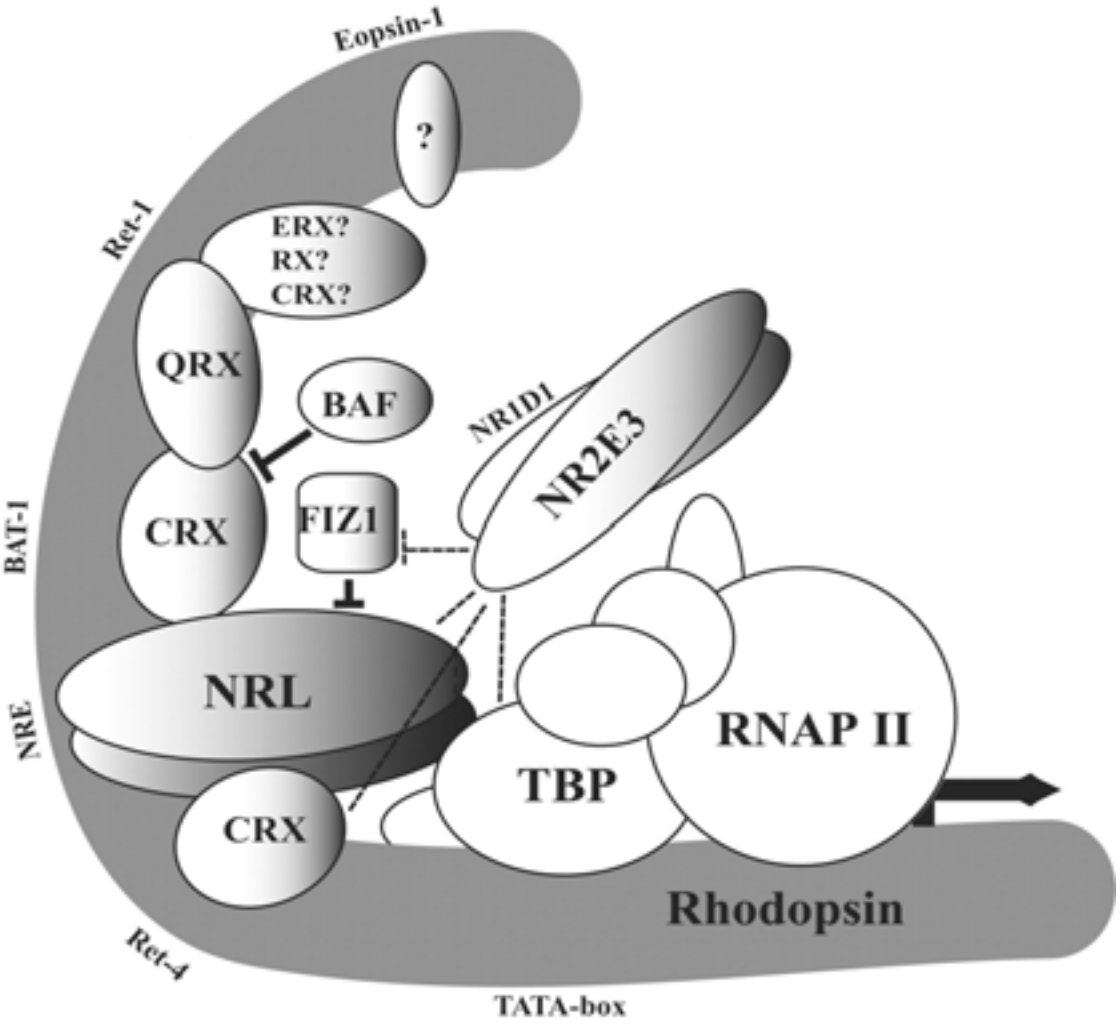

Figure 26. Model of transcription initiation complex at the rhodopsin promoter

In the dark cis-regulatory elements upstream of the transcription start site in the rhodopsin promoter include Eopsin-1, Ret-1, BAT-1, NRE, Ret-4 and TATA-box. NRL is believed to bind to NRE as a homodimer. It interacts with TATA-binding protein (TBP) (unpublished data). NRL and CRX physically interact (18). CRX can bind to Ret-4 and BAT-1 elements. BAF and FIZ-1 function as repressors of CRX and NRL, respectively. The homeodomain proteins, RX, ERX, CRX and QRX, can bind to the Ret-1/PCE-1 element in vitro. It is unclear which of these occupies this element in vivo. QRX interacts with CRX but not with NRL (57). In this report, we show that NR2E3 and NR1D1 also participate in the transcriptional activation of the rhodopsin promoter. Combinatorial and synergistic actions of various regulatory proteins recruit and stabilize the initiation complex and facilitate the transcription by RNA polymerase II (RNAP II). From Cheng *et al.*, Hum. Mol. Genet., 2004.

Beside the classical *cis*-regulators Nrl, Crx and Nr2e3 involved in *rhodopsin* promoter activation, other signaling pathways/transcription factors have been identified to modulate *rhodopsin* expression. For example, QRX in bovine (Wang, Chen et al. 2004), Fiz1 in bovine (Mitton, Swain et al. 2003) and cAMP in chicken (Voisin and Bernard 2009) were all found to regulate rhodopsin expression levels.

In summary, regulation of *Rhodopsin* expression throughout development and life requires multiple factors working in synergy for sustained transcription level (Figure 26).

1.2 Human retinal degenerations associated with NRL

As mentioned previously, inappropriate regulation of *Rhodopsin* expression, by NRL notably, is associated with retinal disease. The first study linking NRL mutation to autosomal dominant Retinitis Pigmentosa, is the work of Bessant and collaborators, who identified one nucleotide substitution in the coding region of *NRL*, a T→A change, resulting in the replacement of a Serine by a Threonine in codon 50 of the protein. This mutation was then identified as p.S50 mutation, and alteration in transactivating capacity of *Rhodopsin* promoter has been described above (Bessant, Payne et al. 1999; Kanda, Friedman et al. 2007). To date, about fifteen mutations (missense mutations or frame shift sequence variations) affecting *NRL* have been associated with retinal disease (DeAngelis, Grimsby et al. 2002; Nishiguchi, Friedman et al. 2004; Kanda, Friedman et al. 2007; Hernan, Gamundi et al. 2011). The pathogenic mechanisms triggered by NRL mutations are still under investigation; however, the approach of using biochemical evaluations gave an insight on how NRL mutants could cause the disease. Kanda and his collaborators reported changes in NRL mutants, such as a decreased phosphorylation leading to a reduced number of isoforms (as discussed earlier), decrease of protein stability, or alteration of apparent molecular weight upon immunoblotting. Moreover, another type of mutations affecting the bZIP domain of the protein caused a mislocalization into the cytoplasm (instead of an exclusive localization to the nucleus as observed in the WT). Finally, some mutations of NRL abolished or diminished the binding of the protein on *Rhodopsin* NRE (Kanda, Friedman et al. 2007).

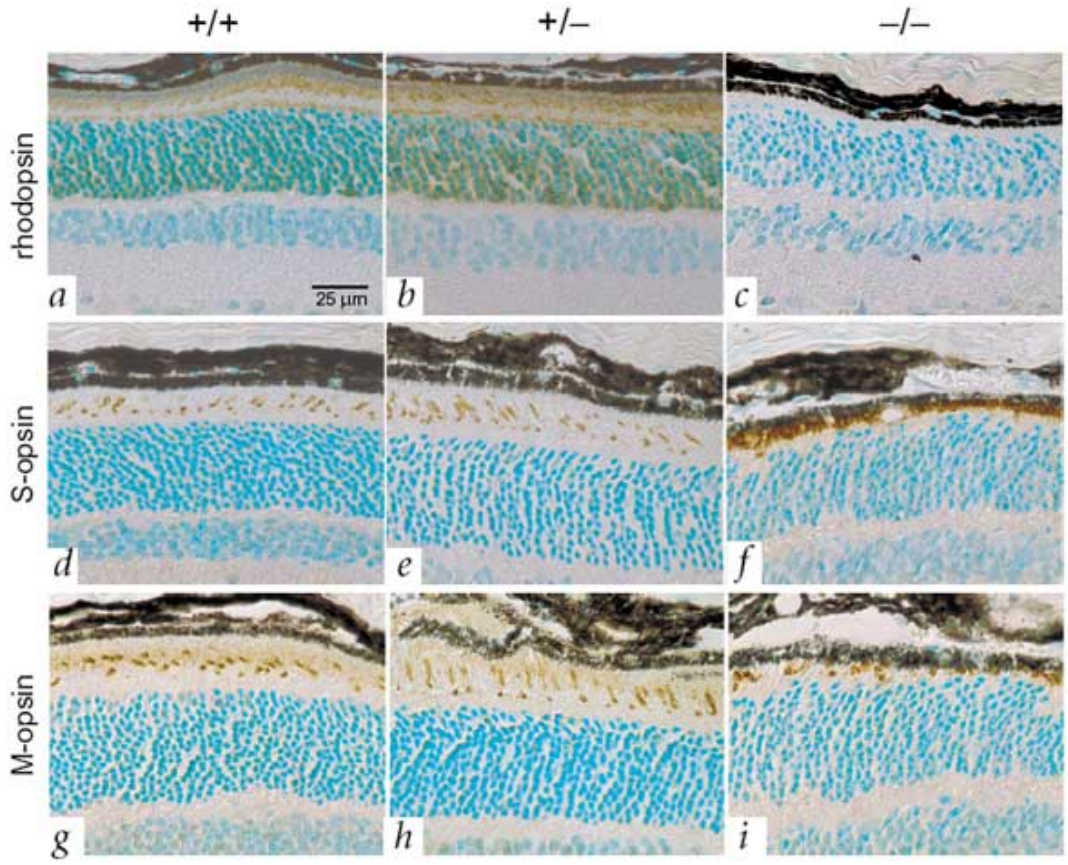

Figure 27. Opsin immunohistochemistry for Nrl KO retina

Immunostaining of retinal sections from 5-wk wildtype, *Nrl* +/- and *Nrl* -/- mice with antibodies. a,b, Wildtype and *Nrl* +/- retinae show a normal distribution of rhodopsin antibody (indicated by light brown color). c, Rhodopsin is absent in the shortened outer segment layer of the *Nrl* -/- retina. d,e, Wildtype and *Nrl* +/- retinae show normal distribution of S-opsin antibody. f, *Nrl* -/- retina shows S-opsin staining of the entire outer segment layer. g,h,i, M-opsin antibody shows normal distribution in all retinae. From Mears *et al*., Nat Genet., 2001.

According to these findings therapeutic strategies aiming at suppressing and/or replacing *NRL* mutant gene, appear valuable in the case of Retinitis Pigmentosa due to *NRL* mutations, as it has been done previously with two genes involved in this disease, *Rds-peripherin* and *Rhodopsin* (Palfi, Ader et al. 2006; O'Reilly, Palfi et al. 2007).

2. NRL is a key factor for rod-photoreceptor development

During retinogenesis, NRL plays a pivotal role in the determination of a precursor cell to become a rod. Deletion of *Nrl* in mice results in the complete loss of rods, which are converted into S cones (Mears, Kondo et al. 2001) (Figure 27). In $Nrl^{-/-}$ animals, S cone proliferation is not only explained by NRL loss but also by the failure to express NR2E3. Indeed, it was shown that NRL induces the expression of NR2E3; therefore NR2E3, which main function is to inhibit expression of cones gene, is defective in *Nrl-null* mice, resulting in the cone-only- retina observed in these animals (Oh, Cheng et al. 2008). These data suggest that NR2E3 is downstream of NRL in the transcriptional hierarchy controlling retinal development. Transgenic expression of *Nr2e3* in *Nrl-/-* animals results in the complete suppression of cone differentiation and development of rod-like cells, which are, however, non-functional (Cheng, Aleman et al. 2006). Per contra, ectopic expression of *Nrl* in the *null* background induces transformation of all photoreceptors into functional rods as attested by the scotopic ERG showing a profile similar to the WT (Oh, Khan et al. 2007). Therefore, these data suggest that contrary to NR2E3, NRL is not only essential, but is also sufficient for rod genesis. Induction of rod-specific gene expression relies on the action of multiple transcription factors. Interaction of NRL with several other transcription factors confers cell specificity in activating target genes. NRL largely contributes to the rod lineage specification and to the homeostasis of these cells by regulating genes involved in phototransduction on one hand and in cell maintenance on the other hand, such as *Lman1* and *Wisp1* (Hao, Kim et al. 2012). Therefore control of *Nrl* expression should be tightly regulated to generate a mature and functional retina.

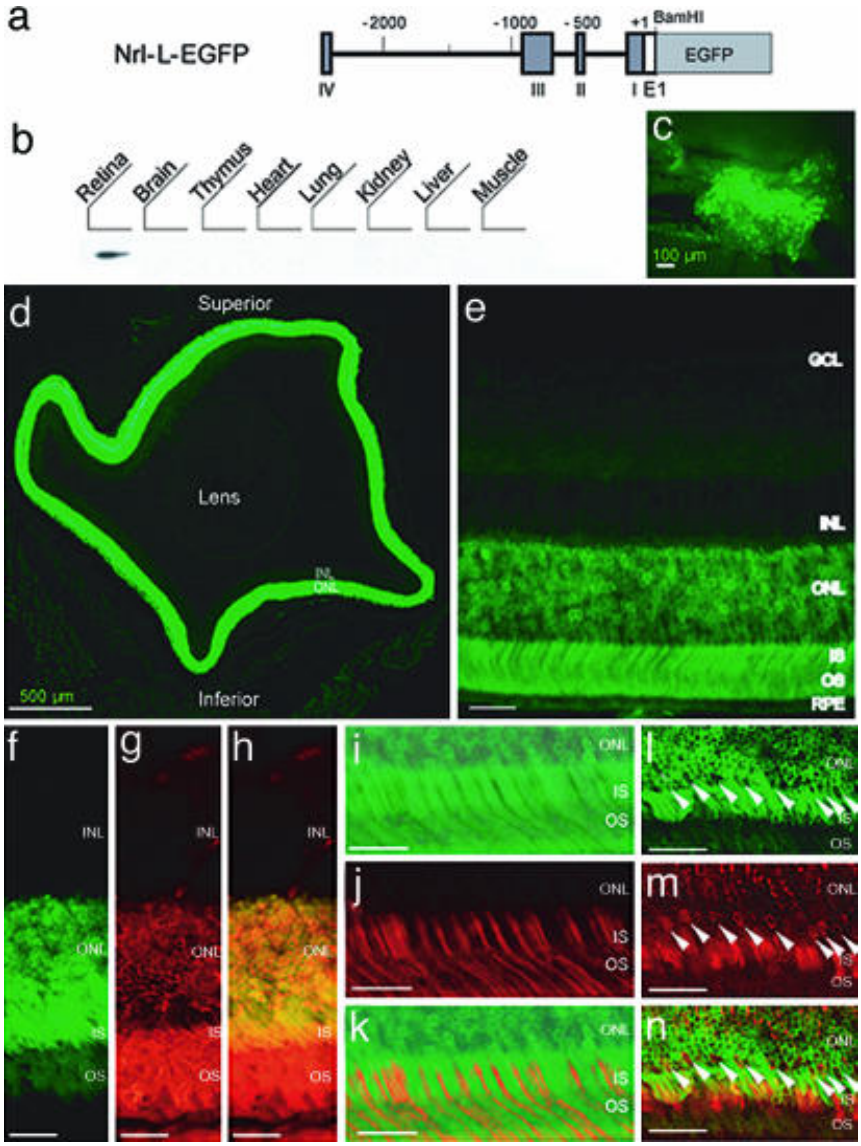

Figure 28. *Nrl* promoter directs GFP expression to rods and pineal gland in transgenic mice

(a) Nrl-L-EGFP construct. The upstream *Nrl* segment contains four sequence regions I–IV that are conserved between mouse and human. E1 represents exon 1. (b) Immunoblot of tissue extracts using anti-GFP antibody, showing retina-specific expression of GFP in the Nrl-L-EGFP mouse. (c) GFP expression in the pineal gland of Nrl-L-EGFP transgenic mice. (d) GFP expression in outer nuclear layer (ONL) of entire adult retina. (f–h) Immunostaining with rhodopsin antibody (red) showing a complete overlap with GFP (green) expression. (i–k) Cells positive for the cone-specific marker peanut agglutinin and cone arrestin (l–n) (red both) do not overlap with GFP (green)-expressing cells. Arrowheads indicate cone photoreceptor cells. RPE, retinal pigment epithelium; OS, photoreceptor outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. [Scale bar, 100 µm (c), 500 µm (d), and 25 µm (e–n)]. From Akimoto *et al.*, Proc Natl Acad Sci U S A, 2006.

3. Control of *Nrl* expression

3.1 NRL-GFP mice, a tool to study rod development

A considerable progress in the study of rod development has been made with the generation of transgenic mice expressing a reporter gene specifically in rods. Akimoto and his collaborators studied 2,5 kb of mouse *Nrl* 5'-unstranslated region (from -2408 to +115) and aligned it with its human homolog. This syntenic analysis highlighted four regions in the promoter, called clusters I-IV, which are highly conserved sequences. Cloning of this 2,5 kb *Nrl* promoter with a reporter gene, in this case EGFP, allowed to generate transgenic mice. The characteristic of these mice is to express EGFP only in rod photoreceptors and interestingly, into the pineal gland as well (Figure 28). It is then easy to follow expression of these EGFP-expressing cells during retinal development, and to determine when the reporter gene is detected (and explicitly transcribed) in these cells. EGFP positive cells are detectable in the retina as early as embryonic day E12 (Rhodopsin starts to be detected at post natal day P4) and subsequently increase in abundance over time. Expression profile of these EGFP-positive cells is central-to-peripheral, consisting with spatial and temporal rod genesis (Akimoto, Cheng et al. 2006). These data are the first to use an early-expressed factor specific to rod cells to trace their fate, from retinal precursor cell to mature photoreceptor. These mice have also proven highly useful in studies assessing the therapeutical value of transplantating more or less mature photoreceptors in mice (MacLaren, Pearson et al. 2006)

3.1 Effects of retinoic acid on *Nrl* regulation

Only a few studies looked at the mechanisms responsible for *Nrl* regulation. It was reported that serum and retinoic acid (RA) have a positive effect on *Nrl* regulation. Y79 human retinoblastoma cell cultures, shown to express NRL (Swaroop, Xu et al. 1992), were serum-deprived for 24h. Effect of deprivation resulted in the absence of *Nrl* transcript; addition of serum restored expression of the transcript, meaning that there are components in the serum activating *Nrl* gene. Among the potential candidates tested, *Nrl* promoter showed a positive activation upon retinoic acid (RA) treatment. Putative RA response elements have been identified in the 2,5 kb mouse *Nrl* promoter, and revealed a positive binding of proteins from bovine retinal nuclear extracts (Khanna,

Akimoto et al. 2006). In summary, this study was the first to investigate potential candidates for *Nrl* regulation.

Identification of factors controlling *Nrl* expression will deepen our knowledge on the transcriptional hierarchy influencing retinal precursor cells to commit to one cell fate and to maintain this cellular identity.

MATERIALS AND METHODS

A. Bioinformatic analysis

Promoter/enhancer sequences from mouse, rat, human, orangutan and dog *Nrl* genes were downloaded from the July 2007 (mm9) mouse genome assembly (Karolchik, Hinrichs et al. 2009) and submitted to syntenic alignment using CLUSTALW program (Chenna, Sugawara et al. 2003) in order to identify conserved sequence clusters. To predict the transcription factor binding sites on the promoter region, we analyzed the mouse genomic sequence with TF search program (Heinemeyer, Wingender et al. 1998), MultiTF tool and Mulan program (Matys, Kel-Margoulis et al. 2006) found in the TRANSFAC database (Matys, Fricke et al. 2003).

B. Animals

For the developmental part of our study, we purchased untimed E17-19 pregnant CD-1 mice from Charles River Laboratories (Wilmington, MA). Animals were housed in a 12 h : 12 h light/dark cycle (LD) and had access to food and water ad libitum. Neonatal animals were used for *in vivo* electroporation procedure in accordance with guidelines for animal care and experimentation established by the National Institutes of Health and approval by National Eye Institute Animal Care and Use Committee. For the circadian study, experiments were performed on Wistar rats (Charles River Laboratories, L'Arbresle, France) bred and housed in our animal facility in a 12 h : 12 h LD cycle (light intensity approx. 300 lux, Light Meter DLM2000AC; General Tools & Instruments, NY, USA). Rats had access to water and food ad libitum, and were handled according to the European Communities Council Directive of 24 November 1986 (86 / 609 / EEC) and the Animal Use and Care Committee from Strasbourg (CREMEAS). 6 to 8 week-old males (n = 24 were subjected to two lighting conditions – LD and DD cycles (dark : dark). LD animals were kept only in 12 h : 12 h LD cycle of illumination. Animals were killed with CO2 (20% in an airtight box) starting at ZT0 (light onset) every 4 h for a period of 24 h. DD (n = 32) animals were exposed to constant dark for 36 h prior to eye sampling (starting at projected ZT0, every 4 h for a 24-h period). During the dark (night in LD and the whole DD cycle), all procedures (animal handling and eye sampling) were performed by using night vision goggles (ATN NVG-7, ATN-Optics, Chorges, France).

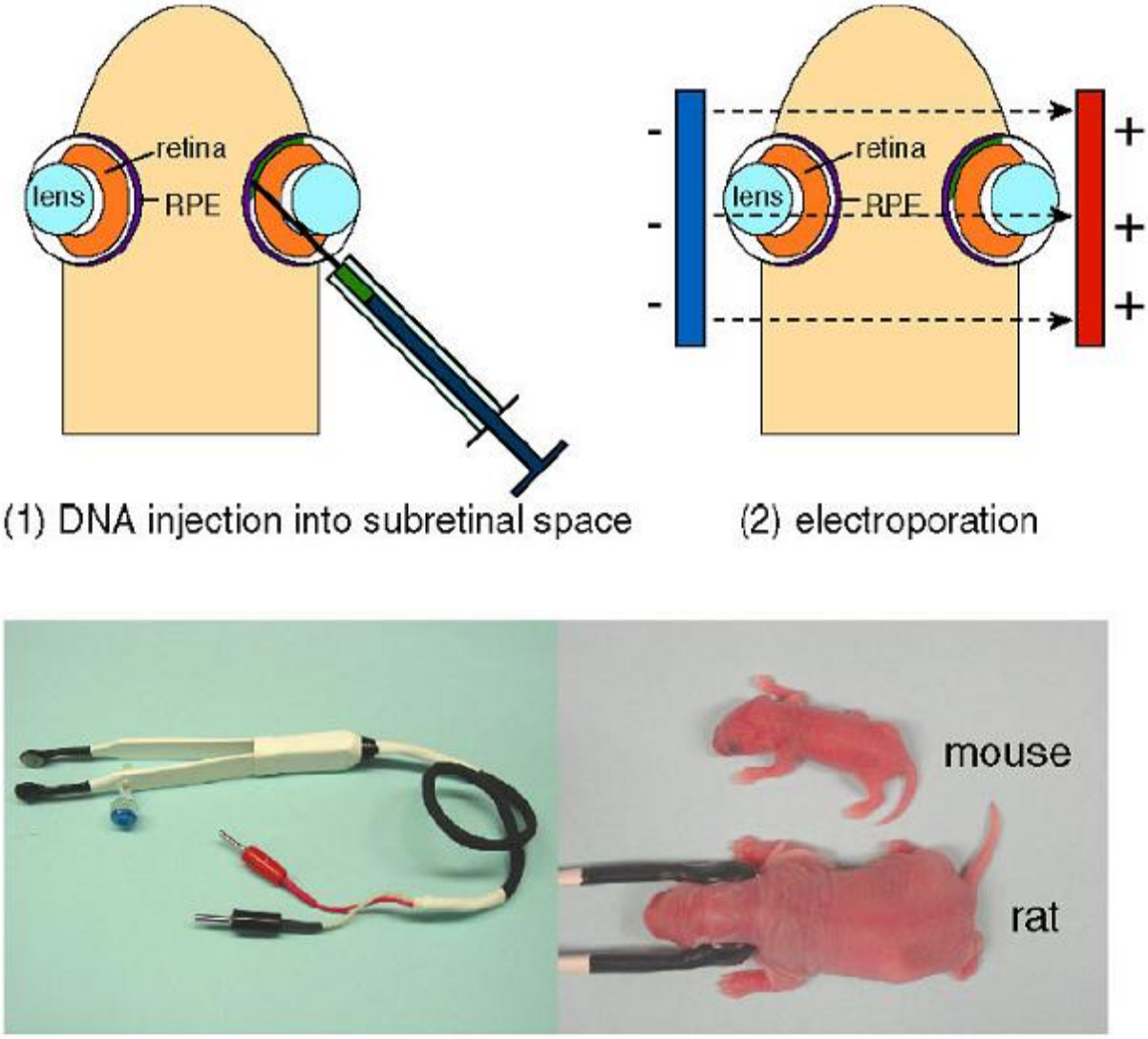

Figure 29. *In vivo* retinal transfection by electroporation

Upper panel, DNA solution is injected in the subretinal space of neonatal animals and then submitted to series of electrical pulses for DNA transfection into retinal progenitor cells. *Lower panel*, electrodes for *in vivo* electroporation. Tweezer-type electrodes are placed to hold the head of neonatal rat or mouse. From http://genepath.med.harvard.edu

C. In vivo electroporation

In vivo transfection of the retina by electroporation was performed as previously described (Matsuda and Cepko 2008). Briefly, neonatal animals were placed on ice several seconds for anaesthesia. Only the right eye was injected. At birth, the eyelid of the animal is still closed; thus, to expose the eyeball for injection, the future edge of the eyelid was cleaned with 70% ethanol and cut with the tip of a sharp 30-gauge needle. Once the eyeball was exposed, a pre-hole was made in the sclera near the cornea using the tip of a sharp 30-gauge needle. Through that same pre-hole an injection needle (Hamilton Syringe, 701SN, 10 ul, Special: Gauge 33, length 0.5 in, point style 3, blunt end) was inserted until feeling a resistance (the inserted needle is visible through the lens). DNA solution (1 µl) containing a dye (1% Fast green) was injected into the subretinal space. After the injection, the DNA was submitted to electroporation (ECM 830, BTX-Harvard Apparatus, Square Wave Electroporator). Tweezer-type electrodes were soaked in PBS and squeezed around the head of the animal. For a subretinal injection, the positive electrode was placed at the DNA-injected side, to allow the DNA to migrate towards the retinal cell progenitors (Figure 29). Five 80 volts square pulses of 50-ms duration and 950-ms intervals were applied using a pulse generator. After the procedure, the animals were placed on a heating pad until total recovery and then returned to their mother.

D. Tissue preparation and immunohistochemistry

Animals were sacrificed at post-natal day 14 (P14) and electroporated eyeballs were collected in Hank's Balanced Salt Solution (HBSS) medium. Before dissection, eyeballs positive for fluorescent reporter gene were sorted out and only positive eyeballs were kept for dissection. Two different techniques of dissection were used. First technique: under a dissection microscope, in HBSS medium, eyeballs were cut along the *ora serrata*, removing the cornea and the sclera, leaving only the retina still attached to the lens. Retina (plus lens) was immerged in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT) to allow the retina to keep its “cup-shape” for further processes. After this pre-fixation, the lens and vitreous humor were removed in HBSS. The retina was

placed back into the 4% PFA bath for an additional 20 min. After fixation, retinas were rinsed in HBSS and placed in 30% sucrose /PBS for several hours or overnight for cryoprotection. Finally, retinas were embedded in OCT (Sakura Finetek) and processed for cryosectioning (10–12 µm). Second technique of dissection: eyeballs were harvested from the animals and directly immerged in 4% PFA for 15 min at RT. After this pre-fixation, eyeballs were placed in HBSS and cut along the *ora serrata* to remove the cornea, lens and vitreous humor. An additional fixation in 4% PFA was performed for 20 min and then dissected eyeballs were rinsed in HBSS and immerged in 30% sucrose/PBS and processed as described previously.

Immunohistochemistry: Retinal sections stained only with DAPI (1 mg/ml in PBS) were rinsed several times with washing buffer (0.1 % Triton-X-100 in PBS). Then DAPI solution was applied and incubated 10 min at RT. Finally, the slide was rinsed several times in washing buffer before applying mounting medium. For antibody use in immunohistochemistry, retinal sections were first rinsed with washing buffer, then incubated for 2h in blocking buffer (DMEM, 10% FBS, Penicillin/Streptomycin, 0.1% Triton X100 and 4% goat serum). Primary antibodies were diluted in blocking buffer and applied overnight at 4°C. After rinsing the slides with washing buffer, we applied the secondary antibody diluted in blocking buffer. DAPI was applied right after the secondary antibody incubation and retinal sections were proceeded as described above for the mounting.

Table 1: List of antibodies used for immunostaining

Antigen	Host	Dilution	References
Primary Antibody			
Cone arrestin	Rabbit	1 :2000	Millipore
Rhodopsin	Mouse	1:500	(Hicks and Molday 1986)
Secondary Antibody			
Goat anti mouse-Alexa 568	Goat	1 :1000	Invitrogen
Goat anti rabbit-Alexa 568	Goat	1 :1000	Invitrogen

E. Molecular biology

1. PCR and subcloning

To generate different fragments of *Nrl* promoter/enhancer we used the technique Polymerase Chain Reaction (PCR), which allows producing several copies of target DNA flanked with specific restriction enzyme sites in 5' and 3' facilitating further subcloning into destination vector with compatible restriction sites. Briefly, synthetic forward and reverse oligonucleotides (~ 21 bases pairs, bp) were designed spanning several mouse genomic regions upstream of *Nrl* transcription start site. Specific restriction sites (SalI, MfeI) were added to these oligonucleotide sequences. PCR fragments were cloned from a Bacterial Artificial Chromosome containing *Nrl* gene that was used as template and mixed with high fidelity DNA polymerase (Pfu High Fidelity and NEB Phusion) and deoxyribonucleoside triphosphate (dNTPs) to produce amplicons. Amplified DNA fragments were purified, enzymatically digested at the new restriction sites, and then ligated into pEGFP-N1 (Clontech) and SV40-mCherry-IRES-alkaline phosphatase plasmid vectors (Kim, Matsuda et al. 2008). Individual clones were analyzed by restriction endonuclease digestion or PCR screening, and sequenced before being tested in functional assay.

2. Electrophoretic Mobility Shift Assays (EMSAs)

Based on our bioinformatic analysis, we designed oligonucleotide sequences (30 – 40 nucleotides) spanning the predicted transcription factor binding sites. Corresponding mutant oligonucleotides were obtained by substituting up to 5 nucleotides in the putative binding sites. Wild type (WT) oligonucleotides were annealed and radiolabeled as described below. Nuclear protein extracts from HEK293 cells transfected with mammalian expression constructs or from adult or neonatal (P1-2) mouse retinas were incubated with 1 µg poly (dI-dC) and 1 µg salmon sperm at 4°C for 15 minutes in binding buffer (12 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.9; 60 mM KCl; 4 mM $MgCl_2$; 1 mM EDTA [ethylenediaminetetraacetic acid]; 12% glycerol; 1 mM dithiothreitol; supplemented with protease and phosphatase inhibitors). Then 30 000 cpm of the ^{32}P-labeled double-stranded synthetic oligonucleotide probe was added

and the reaction was incubated at 4°C for 20 minutes. In competition studies, nuclear extracts were preincubated with 25 or 100 ng unlabeled probes at room temperature for 15 minutes and then incubated with labeled probe at 4°C for 20 minutes. In order to immunologically identify protein components in the protein-DNA complexes, nuclear extracts were incubated with appropriate antibody at 4°C for 15 minutes followed by the addition of labeled probe and a further incubation at room temperature for 20 minutes. The reaction mixtures were electrophoresed on 8% non-denaturating polyacrylamide gels at 80 volts for 2 hours and subjected to autoradiography.

Table 2: List of antibodies used for supershift assays

Antigen	Host	Type	References
Primary Antibody			
Otx2		Polyclonal	Chemicon
Crx		Polyclonal	(Cheng, Khanna et al. 2004)
Rorβ		Polyclonal	Diagenode
Creb		Monoclonal	Cell signaling
Nrl		Polyclonal	(Roger, Nellissery et al. 2010)
c-Fos		Polyclonal	Calbiochem
Normal Immunoglobulin G		**Polyclonal**	**Rockland**

3. Site directed mutagenesis

The DNA construct containing a mutated retinoid-related orphan nuclear receptor response element (RORE) was generated from a *Nrl* conserved region (-938 to -119) by sequential PCR using the following primers: 5'-GCTGAAAATGTATGGCACACCCCAGCC-3' and 5'-GGCTGGGGTGTGCCATACATTTTCAGC-3'. The mutations consisted in substituting two oligonucleotides of the consensus core motif AAAATGTAGGTCA. Effect of the mutation was assessed by *in vivo* tranfection by electroporation of neonatal animals.

4. Analysis of transcript level variation during 24 h cycle

4.1 Extraction of total RNA and reverse transcription

Extraction of total RNA (from whole retina and isolated photoreceptors of 6-8 week old rats) and reverse transcription were described in (Sandu, Hicks et al. 2011). 300 ng of total RNA was reverse transcribed into first strand cDNA and stored at -80ºC.

4.2 Real-time quantitative PCR

Gene expression over 24 h LD and DD cycles was analysed by real-time PCR for *Rho* and *Nr2e3* in the whole retina, and *Nrl* and *Crx* in photoreceptor layers. Real-time quantitative PCR was performed using the hydrolysed probe-based TaqMan chemistry. We used inventoried TaqMan Gene Expression Assays for *Rho*, *Nrl* and *Crx*, designed to specifically amplify mRNA (Applied Biosystems, Table1), and custom designed assays for *Nr2e3* based on the predicted RefSeq XM_002727061.1 sequence (forward 5'GGCTGCAGCGGCTTCTT3', reverse 5'CCCCTACCTGGCACCTGTAG3', probe 5'AGGAGTGTGAGACGGAG3'; amplicon size, 64bp).

Table 3: Inventoried TaqMan gene expression assays used in the study

Gene	TaqMan assay reference	RefSeq	Exon boundary	Assay location	Amplicon length (bp)
Rhodopsin	Rn00583728_m1	NM_033441.1	1-2	446	73
Nrl	Rn01481925_m1	XM_224189.2	4-5	512	103
Crx	Rn00573116_m1	NM_021855.1	1-2	99	64

Reference (Applied Biosystems), GenBank accession number (RefSeq), location of the assay in the gene (nucleotide number indicates position of the probe in the RefSeq sequence), size (bp, base pairs) of the amplicons are given for all assays. Gene expression assays used in this study are designed across the exon-exon junction indicated in the table.

Prior to qPCR total RNA was extracted from isolated photoreceptors by using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA, USA), and from whole retina using the Absolutely RNA Miniprep kit (Stratagene) according to the manufacturer's instructions. Samples were homogenized using a 1-mL syringe and a 27-gauge needle. On-column digestion with DNase was performed to ensure removal of possible genomic DNA contamination. Total RNA was eluted with elution buffer (10 mM Tris–HCl pH 7.5, 0.1 mM EDTA) using 14 and 30 µL for photoreceptor layers and whole retina,

respectively. RNA concentration and purity were measured using NanoDrop ND-1000 V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Integrity of the RNA was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity numbers were between 7.8 and 9.6. Real-time PCR was used to analyze gene expression over 24-h cycles for *Rhodopsin* and *Nr2e3* in the whole retina, and for *Nrl*, *Crx* and *Recoverin* in photoreceptor layers. qPCR was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and the hydrolysed probe-based TaqMan chemistry. We used optimized TaqMan Gene Expression Assays designed to specifically amplify mRNA (Applied Biosystems As described in (Sandu, Hicks et al. 2011),. the PCR conditions were: 1x TaqMan Universal PCR Master Mix, No AMPErase UNG (Applied Biosystems), 1x Gene Expression Assay mix (containing forward and reverse primers and cognate probe; Applied Biosystems) and 1 µL of cDNA in a total volume of 20 µL. The PCR program was as follows: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing-elongation at 60°C for 1 min. The acquisition of fluorescence data was performed at the end of the elongation step using the 7300 System Sequence Detection Software V 1.3.1 (Applied Biosystems). Each PCR reaction was done in duplicate, and the coefficient of variation among duplicates was 0.18% on average. A dilution curve of the pool of all cDNA samples was used to calculate the amplification efficiency for each assay. No-template control reactions were performed as negative controls for each assay. One 96-well plate corresponded to the analysis of one gene within one type of sample (retina or photoreceptors) and one experimental condition (LD or DD cycles). Transcript levels were normalized using *β-actin* and *Pde6b* (Kamphuis, Cailotto et al. 2005) that showed constant expression in their mRNA levels in both LD and DD during the 24-h cycle, in photoreceptor layers as well as in the whole retina (data not shown). qPCR data analysis was done using the qBase software (free v1.3.5; (Hellemans, Mortier et al. 2007)) for the management and automated analysis of qPCR data. Expression of target genes was quantified based on the ΔCq method modified to take into account gene-specific amplification efficiencies and multiple reference genes. Transcript levels were calculated relative to the sample showing lowest expression, and which was rescaled to one.

F. Cell culture

HEK293 human embryonic kidney cell line has high transfection efficiency and rapid growth in conventional cell culture environment, and therefore was used for transfection experiments. Frozen aliquot of HEK293 was rapidly thawn and transferred to a 75 cm^2 flask containing 10 ml of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Gibco, Invitrogen) pre-warmed at 37°C. Cultures were kept at 37°C in a 5% CO2 atmosphere. Medium was renewed two times a week. Cells at 80% confluence were split and seeded in 24-well plates (40000 cells/well). After 24 h cells were transiently transfected using XtremeGENE 9 (Roche Applied Science) according to the manufacturer's protocol.

HEK293T cell co-transfections were performed using 0.5 µg of the reporter plasmid expressing firefly luciferase under the control of mouse Nrl promoter (-938 to +119) (Kautzmann et al., 2011) and the following expression plasmids: i. pSV-CLOCK and CMV-BMAL1 (Travnickova-Bendova et al., 2002) (0.1 µg each); ii. FLAG-tagged CMV-RORβ (Science Applications International Corporation, Frederick, MD) (0.3 µg); iii. pcDNA4c-RevErbα(Cheng et al., 2004) (0.3 µg). Co-transfection mixtures contained also 0.001 µg of the second reporter plasmid, CMV-Renilla (Promega, Madison, WI), and the empty pcDNA4c (Life Technologies,) vector to adjust the total amount of transfected DNA to 1 µg. Cells were harvested 48 h after transfection and lysed in 100 µl of passive lysis buffer (Promega).

G. Luciferase assays

At 80% confluence, cells were split counted and seeded at 40 000 cells per well into 12-well plates. 24h after splitting, cells were transfected with Fugene6 or XtremGene9 (Roche) with 1 µg of total DNA, and further incubated 48h. Cultures were then processed with Dual-Luciferase Reporter Assay kit according to the manufacturer's instructions. 20 µl of cell lysate was loaded into each well in a 96 well-plate. Activity of *firefly* and *renilla* was measured sequentially using GloMax Luminometer (Promega) with Dual Auto-Injector.

H. Western blot

Retinas were flash-frozen on dry ice and maintained in -80°C until protein extraction. Retinas were mechanically dissociated using a sonicator (Vibra-Cell 75186, Sonics & Materials, Newtown, CT, USA) for 15 s; 30/10 pulse and 50% amplitude. Samples were prepared in an extraction buffer solution containing 20 mM Tris pH 7,6; 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 0,2% SDS. Just before use, protease inhibitor cocktail (Roche Diagnostics GmbH. Roche Applied Science, Mannheim, Germany) and 1 mM DTT were added to the buffer. Sonication products are centrifuged 20 min in a pre-chilled centrifuge and the supernatant was kept at -80°C until further use. Protein extract concentration was determined with Bradford method (Bradford 1976) by using a standard curve of Bovine Serum Albumin (BSA). Protein denaturation was done by adding one volume of 2x Laemmli buffer: 0.5 M Tris-HCl, pH 6,8, Glycerol, 10% (w/v) SDS, 0.1% (w/v) Bromophenol Blue and Beta-Mercaptoethanol (2-Mercaptoethanol) and by heating up the sample at 100°C for 5 min. Retinal proteins (3µg, denaturated in 2X Laemmli buffer) were separated by electrophoresis onto SDS-polyacrylamide gels (10-12%) at 100 V in running buffer. Composition of gels and buffers are reported in Table 2. Proteins were tranferred to a PVDF (Polyvinylidene Fluoride) membrane in a chilled tank with transfer buffer. After transfer, the membrane was rinsed with Tris Buffer Sodium Tween 20 (TBS-T) buffer, and then incubated in TBS-T supplemented with 5% fat-free milk for 1h incubation at RT. Later, the membrane was incubated in the primary antibody solution diluted in TBST overnight at 4°C under agitation. Membrane was washed thoroughly with TBS-T and then incubated with secondary antibody coupled to horseradish peroxidase diluted in TBS-T supplemented with milk. immunoreactive bands were revealed by chemoluminescence (Immobilon, Millipore) and detected by exposure to an auroradiographic film. Apparent molecular weights were estimated by comparison to pre-stained molecular size markers. Protein band densities were determined by scanning the blots on a professional scanner. Each image was subjected to quantification using ImageJ software (version 1.43u, National Institues of Health, USA).

Table 4: Composition of buffers used for Western blot analysis

	Composition
6,5% Acrylamide Stacking gel	0.5 mM Tris pH 6.8 ; 5% Sodium Dodecyl Sulfate (SDS) ; 10% Amonium persulfate (APS); 30% Acrylamide/Bisacrylamide ; 0.1% Tetramethylethylenediamine (TEMED)
10% Acrylamide Running gel	1M Tris HCl pH 8,8 ; 0.1% SDS ;10% APS ; 30% Acrylamide/Bisacrylamide ; 0.1% TEMED
Running buffer	25 mM Tris ; 1.44% Glycine ; 2.5% SDS
Transfer buffer	12 mM Tris HCl; 240 mM Glycine ; 10% SDS
Tris Buffer Sodium Tween	1.5 M NaCl ; 200 mM Tris HCl pH 7,6 ; 0,2% Tween 20

Table 5: List of antibodies used for Western blot analysis

Antigen	Host	Dilution	References
Primary Antibody			
Nrl	Rabbit	1:5000	(Roger, Nellissery et al. 2010)
Crx	Rabbit	1:2000	(La Spada, Fu et al. 2001)
Nr2e3	Rabbit	1:3000	(Peng, Ahmad et al. 2005)
β-Actin	Mouse	1:40 000	Sigma
α-Tubulin	Mouse	1:10 000	Abcam
Secondary Antibody			
Goat anti rabbit-horseradish peroxidase		1:10 000/1:80 000	Fermentas
Goat anti mouse-horseradish peroxidase		1:10 000	Fermentas

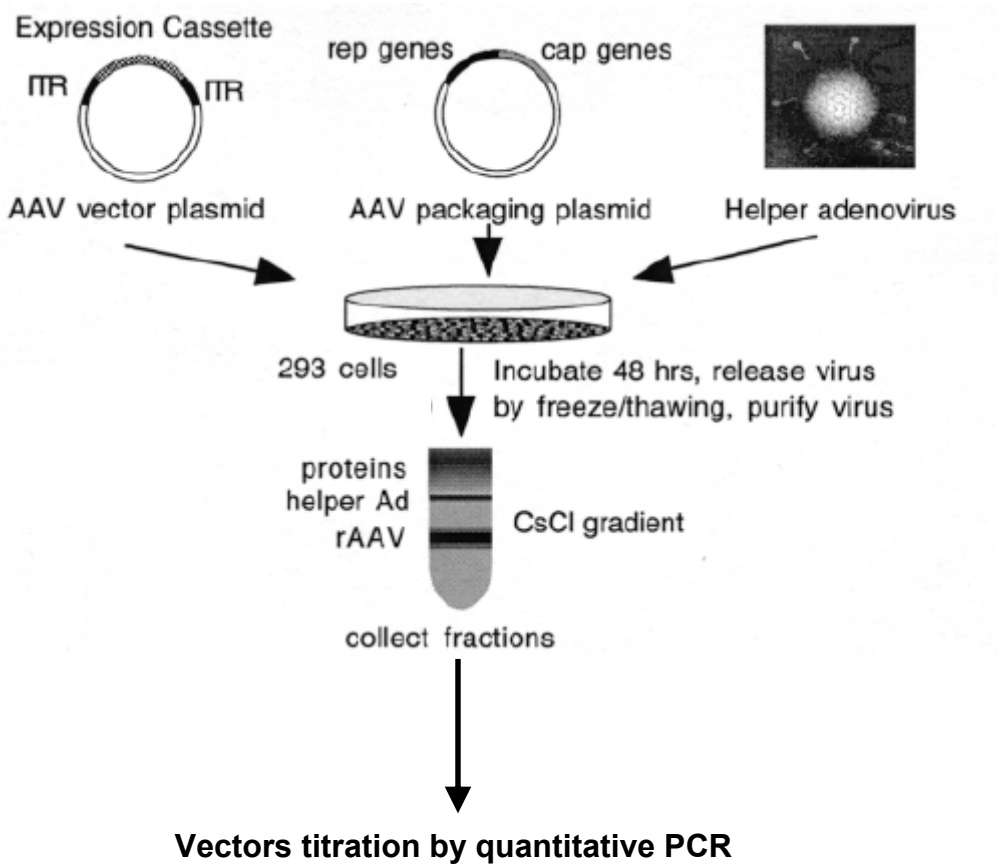

Figure 30. Recombinant Adeno-associated virus production

Co-transfection of rAAV vector and AAV packaging plasmid followed by infection with helper adenovirus, results in the generation of rAAV in 293 cells. Modified from Büeler, Biol Chem., 1999.

I. AAV production and purification.

1. Vector design

AAV2 ITRs (Inverted Terminal Repeats) were used in all AAV vector constructs. pCMV-EGFP DNA (Park, Wu et al. 2009) was digested with NotI and SacII to remove CMV promoter. 0.3 kb mouse *Nrl* promoter (composed of A1+B; -34 to +16 and -968 to -657 respectively) was PCR amplified using the following primers: A1, forward and reverse primers, 5'-ACGGGGTACCGTCCTTTAAGAGTGTC-3' and 5'-ATCCCCGCGGTCAGAACAAGGGGGC-3'; B, forward and reverse primers, 5′-ATTTGCGGCCGCGGGAATACCCTTTA-3′ and 5′-ACGGGGTACCACCACCACACTTCTGT-3′. The PCR products were digested with NotI, KpnI and SacII, and then inserted into the AAV8 vector plasmid containing enhanced green fluorescent protein. For rescue experiments, mouse *Nrl* coding sequence was inserted via BamHI and XhoI sites into AAV8-vector plasmid containing CMV promoter

2. rAAV Vector production.

rAAV particles were produced and purified as decribed in (Grimm, Zhou et al. 2003). Briefly, vented cap roller bottles (850 cm2; Corning, USA) were seeded with 3×10^7 HEK293 cells in 300 mL Dulbecco's modified Eagle medium (DMEM, Gibco) containing 9% fetal calf serum. The cultures were incubated at 37°C with 10% CO2 and rotated at 0.2 rounds per minute for 3 days before transfection. For each roller bottle, 18 mL of 300 mM CaCl2 containing 150 µg each of vector plasmid and rep/cap vector (encoding proteins required for virus replication and encapsidation) as well as adenovirus helper was rapidly mixed with 10 mL of 2 × HBS (HEPES-buffered saline; 50 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 280 mM NaCl, 1.5 mM NaH_2PO_4, pH 7.1) and was added to the roller bottle. The next morning, medium was changed and replaced with 100 mL serum-free DMEM supplemented with antibiotics. Cultures were then incubated for 72 hours. Vector-containing cells were dislodged from the sides of the roller bottles by vigorous swirling and collected by centrifugation at 1000g for 15 minutes 4°C.

3. rAAV vector purification

Virus production is summarized in Figure 30. The collected HEK293 cells were dispersed in TSM buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM $MgCl_2$, pH 8.0, 50 mL per roller bottle) before disruption by microfluidization (3 passes, model HC 2000; Microfluidics Corporation, Newton, MA). Tissue debris were removed by centrifugation (4150 rpm, 1.30 hour total, 4°C), and the supernatant was adjusted to 25 mM $CaCl_2$ by adding the appropriate amount of a 1 M stock solution. After 1-hour incubation at 4°C, the resultant precipitate was removed by centrifugation (4150 rpm, 1 hour, 4°C), and the supernatant was digested with Benzonase (250 U/mL, Novagen) for 1 hour at 37°C. Vector particles were then precipitated for 2 hours at 0°C with 8% polyethylene glycol 8000 (in 650 mM NaCl) and collected by centrifugation (4000 rpm, 30 minutes, 4°C). The pellet was completely dissolved in 50 mM HEPES, 150 mM NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 1% Sarcosyl, pH 8.0 containing 10 µg/mL RNase A (2 mL per roller bottle) and was applied to a CsCl step gradient consisting of 5 mL of 1.5 g CsCl/mL for the bottom layer, 8 mL of 1.3 g CsCl/mL for the middle layer, then a 22-25 mL sample (in 25 × 89 mm polyallomer tubes, spun in an SW32 rotor at 28 000 rpm for 18 hours at 20°C). The lower full capsid band was identified visually and collected with an 18-gauge hypodermic needle and a 10-mL syringe through the side of the tube. The vector-containing solution was again centrifuged on a linear CsCl gradient (14 × 95-mm polyallomer tubes, spun in an SW40 rotor at 38 000 rpm for 72 hours at 20°C). The full capsid band was collected with a hypodermic needle, diafiltered against Tris-buffered saline (TBS, 10 mM Tris-Cl, 180 mM NaCl, pH 7.4) and stored frozen at -80°C.

4. Titration of viral particles.

Vector genome titers were established by taking the average of 3 quantitative real-time PCR (Q-PCR) determinations. All samples were DNaseI treated before measurement and were subjected to 45 cycles of amplification (2 steps per cycle: 62°C for 60 seconds, 95°C for 15 seconds) in a 7700 Q-PCR machine (Applied Biosystems, Foster City, CA) using primer and probe sets specific for *EGFP* cDNA.

5. Animals and vector delivery

Postnatal day (P)10 wild-type mice were used for promoter expression study and P4, P10 and 1 month-old *Nrl* KO mice were used for rescue experiments (n=4 per DNA vector tested). Animals were anesthetized with intraperitoneal injection of ketamine (90 mg/kg)/xylazine (9 mg/kg). Pupils were dilated with topical application of cyclopentolate and phenylephrine hydrochloride. Under binocular microscope, a small incision was made through the cornea adjacent to the limbus with an 18-gauge needle. A 33-gauge blunt needle injection syringe (Hamilton) was inserted through the incision, with care taken to avoid the lens, and was pushed through the retina. All injections were made subretinally. P4 and P10 animals received 0.5 µL of AAV-*mNrl*p-EGFP at 0.6, 2 or 6^{e12} vector genomes per milliliter (vg/mL), 0.5 µL of AAV-*hRK*p-EGFP at 2^{e12} vg/mL for promoter characterization study, and 0.5 µL of AAV-CMV-NRL at 1^{e12} vg/mL for rescue experiments. Adult animals received 1 µL of either AAV-CMV-NRL or AAV-h*RK*p-EGFP at 0.5^{e12} vg/mL. Visualization during injection was aided by addition of fluorescein (100 mg/mL AK-Fluor; Alcon, Inc., USA) to the vector suspensions at 0.1% V/V.

6. Immunofluorescence analysis

3 weeks post-injection, eyes were enucleated, placed in 4% paraformaldehyde/PBS for 15 min, and their anterior segments and lens were removed. Fixation continued in fixative for additional 20 min. The fixed tissues were soaked in 30% sucrose/PBS for 3 hours or overnight, embedded in OCT (Sakura Finetek),shock frozen and sectioned along the superior-inferior meridian at 12 µm thickness.. For immunolabeling retinal sections were washed three times x 5 min with PBS. Non-specific binding sites were blocked with DMEM supplemented with antibiotics (penicillin and streptomycin) containing 10% FBS, 4% goat serum, and 0.1% Triton X-100 for 1 h at room temperature. Rhodopsin monoclonal antibody, Rho4D2 (Dr. R. Molday, University of British Columbia, Vancouver, British Columbia, Canada) was used as primary antibody, diluted at 1:500 in blocking solution and incubated at room temperature overnight at 4°C. The next day, sections were washed three times in PBS-T (1X PBS, 0.1 % Triton) and then were incubated for 1 h with anti-mouse antibody conjugated with Alexa Fluor 568 (Molecular Probes, Invitrogen) diluted at 1:1000 in blocking buffer and (1 mg/mL in PBS) diamidino-phenyl-indole (DAPI). After three washes with PBS-T, sections were mounted with gel mounting

medium. Immunostaining was visualized using an Olympus FluoView FV1000 confocal laser scanning unit.

RESULTS

I. Characterization of mouse *Nrl* promoter regulatory sequences.

II. Identification of a minimal *Nrl* promoter as a tool for specific photoreceptor expression using AAV.

A. Further dissection of *Nrl* promoter elements required for the transcription

B. AAV-mediated expression of *Nrl* promoter or cDNA to further decipher the developmental properties of *Nrl* gene

III. *Nrl* and co-activators of *rhodopsin* expression show daily variations in the rat retina during the 24 h cycle.

IV. A novel transcription factor, NonO, appears as a co-activator of *rhodopsin* expression and is necessary for rod-photoreceptor survival.

I. Characterization of mouse *Nrl* promoter regulatory sequences.

NRL is a key factor for rod photoreceptor differentiation and homeostasis. Deletion of *Nrl* in mice leads to a cone-only retina and mutations in this gene have been correlated in humans with Retinitis Pigmentosa (Bessant, Payne et al. 1999; Martinez-Gimeno, Maseras et al. 2001; Kanda, Friedman et al. 2007; Hernan, Gamundi et al. 2011), a hereditary disease leading progressively to blindness. Despite the developmental and clinical importance of this gene, little is known about its regulation. The main goal of my project was to decipher the mechanisms responsible for the transcriptional regulation of *Nrl* during retinal development. Previous studies had partially characterized promoter elements of *Nrl* that could play an active role in the positive regulation of this gene. A 2.5 kb promoter region containing conserved elements was shown to drive specifically reporter gene in rod photoreceptors (Akimoto, Cheng et al. 2006). Retinoic acid response elements were among the putative binding sequences identified in *Nrl* promoter; gel shift and transactivation assays demonstrated the positive role of retinoic acid for *Nrl* regulation. (Khanna, Akimoto et al. 2006). During my thesis, we aimed to extend *Nrl* promoter characterization using biochemical approaches and *in vivo* electroporation of neonatal mouse retina. We defined a minimal region of 0.3 kb that could strongly drive reporter gene expression specifically in rod photoreceptors. This fragment of promoter is composed of a proximal promoter (A1) and a promoter/enhancer element (B) that are both required for *Nrl* expression. Bioinformatic analyses of these two 5'-untranslated sequences predicted various transcription factors binding sites; further analysis of *Nrl* promoter elements evidenced the binding of RORβ, CREB, CRX and OTX2 for developing and mature retina. Discovery of these *trans*-acting factors implied in *Nrl* regulation gave insights into the transcriptional protein complexes at the origin of rod photoreceptor differentiation.

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Combinatorial Regulation of Photoreceptor Differentiation Factor, Neural Retina Leucine Zipper Gene *Nrl*, Revealed by *in Vivo* Promoter Analysis*

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Development and homeostasis require stringent spatiotemporal control of gene expression patterns that are established, to a large extent, by combinatorial action of transcription regulatory proteins. The bZIP transcription factor NRL (neural retina leucine zipper) is critical for rod *versus* cone photoreceptor cell fate choice during retinal development and acts as a molecular switch to produce rods from postmitotic precursors. Loss of *Nrl* in mouse leads to a cone-only retina, whereas ectopic expression of *Nrl* in photoreceptor precursors generates rods. To decipher the transcriptional regulatory mechanisms upstream of *Nrl*, we identified putative *cis*-control elements in the *Nrl* promoter/enhancer region by examining cross-species sequence conservation. Using *in vivo* transfection of promoter-reporter constructs into the mouse retina, we show that a 0.9-kb sequence upstream of the *Nrl* transcription initiation site is sufficient to drive reporter gene expression in photoreceptors. We further define a 0.3-kb sequence including a proximal promoter (cluster A1) and an enhancer (cluster B) that can direct rod-specific expression *in vivo*. Electrophoretic mobility shift assays using mouse retinal nuclear extracts, in combination with specific antibodies, demonstrate the binding of retinoid-related orphan nuclear receptor β (RORβ), cone rod homeobox, orthodenticle homolog 2, and cyclic AMP response element-binding protein to predicted consensus elements within clusters A and B. Our studies demonstrate *Nrl* as a direct transcriptional target of RORβ and suggest that combinatorial action of multiple regulatory factors modulates the expression of *Nrl* in developing and mature retina.

Generation of cellular diversity and homeostasis are controlled and fine tuned through regulation of gene expression. The expression of eukaryotic genes is largely modulated at *cis*-sequences of the core promoter and enhancer elements that bind to transcription initiation complex and *trans*-acting activator or repressor proteins (1, 2). In addition, protein-protein interactions, posttranslational modifications, epigenetic marks on chromatin, and microRNAs facilitate the expression of tissue or cell type-specific genes (3–5). In developmental regulatory networks, spatiotemporal expression of transcription factors primarily dictates functional specification of distinct cell types (6, 7).

The vertebrate neural retina, with its *in vivo* accessibility and a well defined cell repertoire, serves as an excellent model for investigating the origin and maintenance of cellular diversity. The retina consists of six types of neurons and one type of glia. Rod and cone photoreceptors function as specialized sensory neurons that are responsible for scotopic and photopic vision, respectively. Rod photoreceptors are highly vulnerable to genetic defects and environmental abuse (8) and are needed for cone cell viability (9). Hence, elucidation of genesis and functional maintenance of rod photoreceptors would permit better design of strategies for treatment of retinal and macular degenerative diseases.

Distinct retinal cell types originate in a conserved temporal order from multipotent retinal progenitor cells that undergo progressive changes in transcriptional states (10). Both extrinsic cues and intrinsic factors play critical roles in retinal development; however, intrinsic mechanisms largely dictate the acquisition of cell type specificity (11, 12). MASH1, NEUROD1, MATH5, and other basic helix-loop-helix transcription factors bias cells toward specific neuronal fates (13, 14). One of the key regulatory proteins that guides photoreceptor lineage from retinal progenitor cells is the homeodomain transcription factor orthodenticle homolog 2 (OTX2)[3]; its loss results in amacrine-like cells instead of photoreceptors (15). However, OTX2 is not sufficient to induce specific photoreceptor cell fate and requires interaction with other specific regulators (16, 17). BLIMP1, a zinc finger protein, appears to control the choice between photoreceptor and bipolar cell fate (18, 19). Downstream from OTX2 (and probably BLIMP1) in photoreceptor transcriptional hierarchy, retinoid-related orphan nuclear receptor β (RORβ) controls appropriate differentiation of both rod and cone photoreceptors (20, 21). The retina of *Rorb*$^{-/-}$ mice contains primitive and nonfunctional cones and no rods (17, 21).

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[3] The abbreviations used are: OTX2, orthodenticle homolog 2; bZIP, basic motif leucine zipper; CAG, CMV early enhancer/chicken β-actin promoter/rabbit β-globin intron; CREB, cAMP response element-binding protein; CRX, cone rod homeobox; NR2E3, nuclear receptor subfamily 2, group E, member 3; NRL, neural retina leucine zipper; ONL, outer nuclear layer; P, postnatal day; RORβ, RAR-related orphan receptor β; RORE, ROR response element; S-cone, short wavelength-sensitive opsin-expressing cone; IRES, Internal Ribosome Entry Site.

Cell fate specification and maturation of rod *versus* cone photoreceptors are dependent on the expression and activity of four transcription factors: cone rod homeobox (CRX), thyroid hormone receptor β2 (TRβ2), neural retina leucine zipper (NRL), and nuclear receptor subfamily 2, group E, member 3 (NR2E3) (17). The studies using knock-out mice suggest that the homeodomain protein CRX does not specify photoreceptor cell fate, yet it critically contributes to photoreceptor-specific gene activation and homeostasis (22, 23). TRβ2, together with RORβ and retinoid X receptor γ, modulates cone differentiation and patterning (24, 25). The key transcriptional regulator of photoreceptor cell fate choice is NRL (26), a basic motif leucine zipper (bZIP) protein that induces postmitotic precursors to become rods instead of cones (27). Ablation of *Nrl* in mouse leads photoreceptor precursors to acquire a "default" short wavelength-sensitive opsin-expressing cone (S-cone) state (28, 29). NR2E3 is a direct transcriptional target of NRL (30). The primary role of NR2E3 is to repress the expression of cone genes. Loss of NR2E3 results in a retina with enhanced S-cone function and many hybrid photoreceptors expressing both S-opsin and rhodopsin (17, 31–33). Together with CRX, NR2E3, and other transcription factors, NRL activates the rod differentiation pathway by inducing the expression of rod-specific genes, including rhodopsin and cGMP-phosphodiesterase (22, 34–36). Not surprisingly, mutations in *NRL* are associated with retinal degenerative diseases (37–40).

Previously we showed that a 2.5-kb genomic sequence, upstream of the *Nrl* transcription initiation site, contains four conserved regions (cluster I–IV) that might control *Nrl* expression (41, 42). Transgenic mice expressing GFP under the control of this sequence selectively express the reporter gene in developing and mature rod photoreceptors (41). Here, we report the identification of specific *cis*-control sequence elements that are required and sufficient for appropriate *Nrl* expression *in vivo*. Our studies establish RORβ as a direct transcriptional regulator of *Nrl* and implicate CRX, OTX2, and cyclic AMP response element-binding protein (CREB) in modulating *Nrl* expression.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis—Genomic sequences were analyzed using the July 2007 (mm9) mouse genome assembly (University of California Santa Cruz Genome Browser Project, Santa Cruz, CA) (43). The conserved regions upstream of *Nrl* transcription start site were aligned with CLUSTALW (44). The TFsearch program (45), MultiTF tool, and Mulan program (46) were used to find predicted transcription factor binding sites annotated in the TRANSFAC database (version 4.0) (47).

Plasmid DNA Constructs and Mutagenesis—Genomic sequences upstream of the mouse *Nrl* transcription start site were PCR-amplified and cloned into the pEGFP-N1 vector (Clontech). The SV40 basal promoter driving mCherry-IRES-alkaline phosphatase was generated by replacing GFP with mCherry sequence in SV40-GFP-IRES-alkaline phosphatase plasmid (48). Conserved sequence clusters were cloned into pEGFP-N1 and SV40-mCherry-IRES-alkaline phosphatase. Sequences contained in each cluster were as follows: cluster A (−304 to +119; relative to the transcriptional start site), A1 (−34 to +16), B (−938 to −657), B with intervening sequence before A (−938 to −305), B1 (−938 to −786), B2 (−814 to −657), C (−2734 to −2458). FLAG-tagged CMV-RORβ, CMV-CRX, and CMV-OTX2 expression constructs were generated in the pDest-515 vector (Science Applications International Corporation, Frederick, MD). A DNA construct containing a mutant ROR binding site was generated from a *Nrl* conserved region (−938 to +119) as DNA template by sequential PCRs using the following forward and reverse primers: 5′-GCTGAAAATGTATGGCACACCCCAGCC-3′ and 5′-GGCTGGGGTGTGCCATACATTTTCAGC-3′.

In Vivo Transfection Using Electroporation in Mouse Retina—Neonatal CD-1 mice (Charles River Laboratories, Wilmington, MA) were used for *in vivo* transfection by electroporation, as described (49), in accordance with guidelines for animal care and experimentation established by the National Institutes of Health and approval by National Eye Institute Animal Care and Use Committee. For co-transfection studies, equimolar amounts of plasmids were used at a concentration of 2–4 μg/μl. Injection volume was 0.2 μl.

Histology—Harvested retinas were dissected and rinsed in PBS (pH 7.4) and fixed in 4% paraformaldehyde for 15–30 min at room temperature. After a brief rinse, retinas were cryoprotected for 1 h in 30% sucrose and embedded in OCT (Sakura Finetek). Cryosections (10–12 μm) were stained for 6 min with DAPI (1 mg/ml in PBS).

Image Analysis—Confocal photomicrographs were acquired using Olympus FluoView FV1000 and Leica SP2 confocal laser-scanning microscopes. GFP and mCherry fluorescence intensities in cells were measured from maximum intensity projections (9-μm thick) of confocal images of retina sections using ImageJ software (version 1.45f).

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described (30). Briefly, nuclear extracts (5 μg) from HEK293 cells transfected with CMV-RORβ or from mouse retinas were first incubated with 1 μg each of poly(dI-dC) and salmon sperm DNA at 4 °C. After 15 min, ^{32}P-labeled oligonucleotide was added, and incubation was continued for another 10 min. To identify the components in protein-DNA complexes immunologically, samples were incubated with 1 μg of a specific antibody (anti-OTX2, anti-CRX, anti-RORβ, anti-NRL, anti-CREB, anti-c-Fos) or normal immunoglobulin G (control) for 10 min at 4 °C. The reaction mixtures were electrophoresed using 6–8% polyacrylamide gels at 80 V for 1.5 h and subjected to autoradiography.

Antibodies—The following antibodies were used: anti-OTX2 polyclonal antibody (Chemicon, Billerica, MA), anti-CRX polyclonal antibody (50), anti-RORβ polyclonal antibody (Diagenode, Denville, NJ), anti-NRL polyclonal antibody (51), anti-CREB monoclonal antibody (Cell Signaling Technology, Danvers, MA), anti c-Fos polyclonal antibody (Calbiochem), and normal immunoglobulin G (Rockland, Gilbertsville, PA).

RESULTS

Identification of Conserved Sequences Required for Rod Photoreceptor-specific Expression of Nrl in Vivo—To investigate *cis*-regulatory elements, we analyzed the mouse *Nrl* genomic sequence using bioinformatics software. Phylogenetic compar-

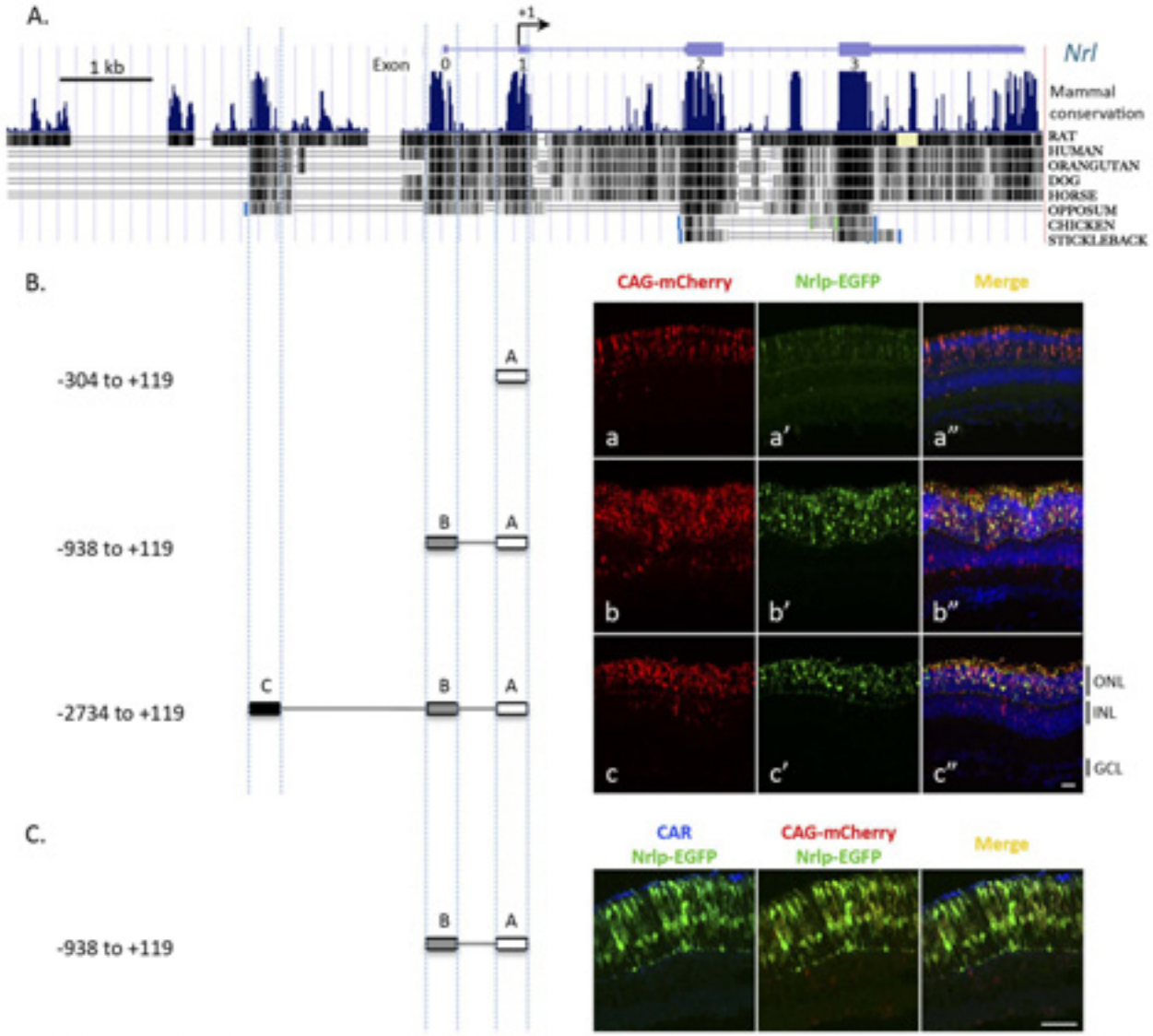

FIGURE 1. *A*, *Nrl* upstream promoter/enhancer sequence showing three major conserved regions. Conservation diagram of *Nrl* promoter sequence displays sequence homology among different vertebrates. *B*, *in vivo* dissection of promoter activity of conserved sequence clusters. One or more clusters of the mouse *Nrl* promoter sequence were used for generating the reporter constructs. Representative sections from neonatal mouse retina, transfected *in vivo* with three different *Nrl* promoter lengths −304 to +119 (*a′*), −938 to +119 (*b′*), and −2734 to +119 (*c′*), are shown. *C*, non-overlapping expression of cone arrestin (*CAR*) and EGFP expression driven by the *Nrl* promoter (−938 to +119). Retinas were harvested at P14 for examining the expression of the reporter gene. *CAG-mCherry* is used to indicate the transfected cells. *Scale bars*, 20 μm. *ONL*, outer nuclear layer; *GCL*, ganglion cell layer; *INL*, inner nuclear layer.

ison of a 4-kb sequence upstream of the transcription start site of *Nrl* revealed the presence of three noncoding sequence clusters that were highly conserved in vertebrates (Fig. 1*A*; designated as clusters A, B, and C in Fig. 1*B*). To assess their physiological relevance, we performed *in vivo* transfection of promoter-reporter constructs in neonatal mouse retinas by subretinal DNA injection followed by electroporation (Fig. 1*B*). A 2.8-kb sequence (−2734 to +119), containing all three conserved clusters A–C, was sufficient to drive EGFP reporter expression specifically in the outer nuclear layer (ONL), which contains photoreceptor cell bodies, in P14 retinas (Fig. 1*c′*). Another construct with a strong CAG promoter driving mCherry expression (referred to as CAG-mCherry) was co-transfected as control for transfection efficiency (Fig. 1, *a*–*c*). A majority of mCherry-positive ONL cells were also EGFP-positive, and no mCherry-positive cells in the inner nuclear layer (INL) expressed EGFP. These data showed that the 2.8-kb sequence was sufficient to drive photoreceptor-specific expression of *Nrl* in the retina. Transfection of a deletion construct containing clusters A and B (−938 to +119) led to robust EGFP expression (Fig. 1*b′*), whereas cluster A (−304 to +119) alone induced relatively faint reporter expression (Fig. 1*a′*). As predicted, cone arrestin immunostaining did not overlap with transfected EGFP-expressing cells in the ONL (Fig. 1*C*); hence, *Nrl*-promoter sequence (clusters A and B; −938 to +119) is indeed directing the expression to rods.

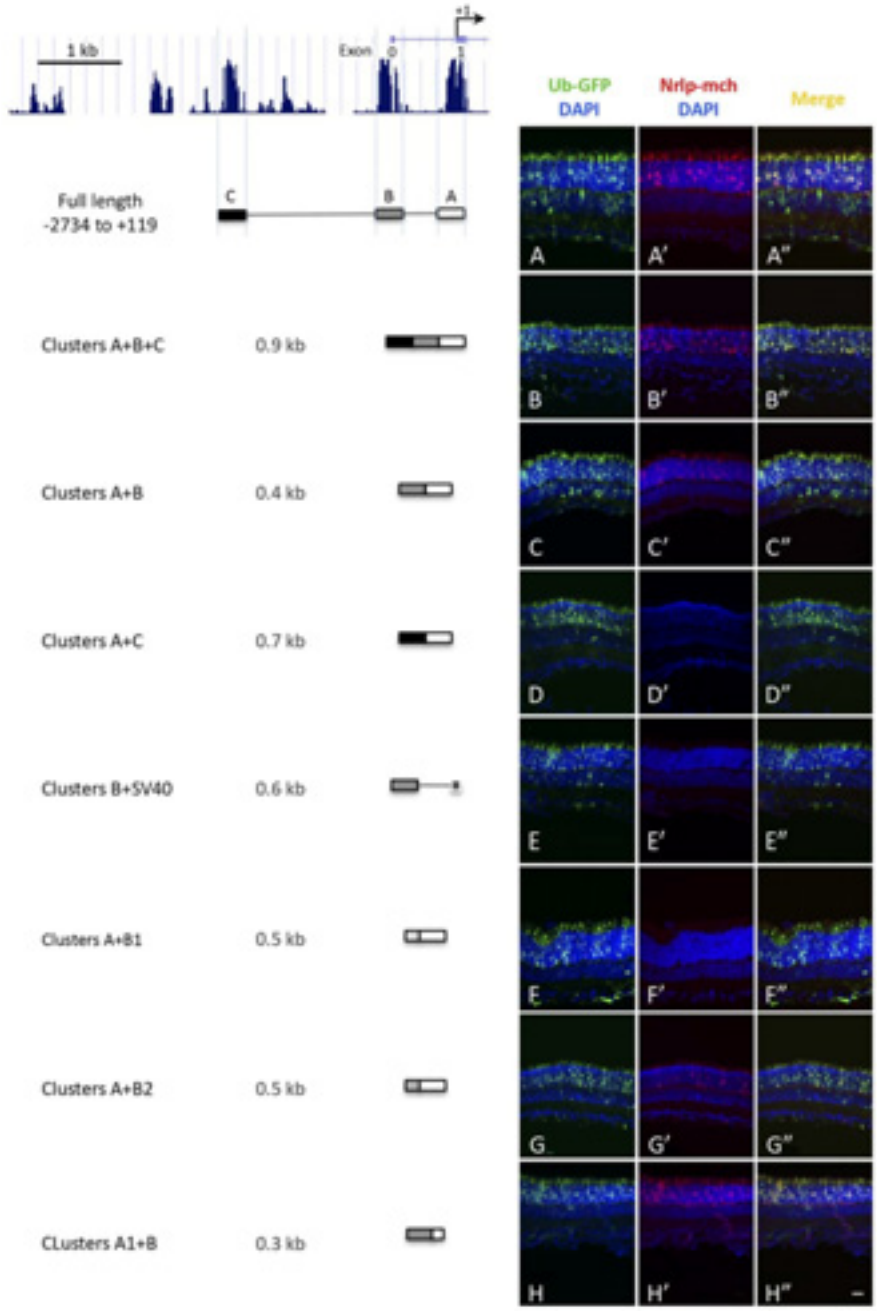

FIGURE 2. **Identification of the sequence elements within *Nrl* promoter/enhancer required for rod-specific expression *in vivo*.** Diagram of the upstream region of *Nrl* displays three main conserved clusters C, B, and A. Representative sections from mouse retina, transfected *in vivo* with the ubiquitin-GFP construct (*A–H*) and *Nrl* promoter-mCherry (*A'–H'*), are shown. Retinas were harvested at P14 for examining the expression of the reporter gene. (*A"–H"*) are merged images. *Green*, GFP fluorescence; *red*, mCherry fluorescence; *blue*, DAPI staining. *ub-GFP*, ubiquitin-GFP; *Nrlp-mCh*, Nrl promoter-mCherry. *Scale bar*, 20 μm.

Fine Mapping of cis-Regulatory Elements—To refine the *cis*-regulatory elements involved in controlling *Nrl* expression further, we tested the sequences within the three conserved clusters by *in vivo* transfection. In these experiments, mCherry reporter gene expression was controlled by *Nrl* promoter elements, and ubiquitin promoter-EGFP construct was used as control for transfections. The full-length 2.8-kb construct (−2734 to +119) and a construct with clusters A, B, and C concatenated without any intervening sequence exhibited robust and photoreceptor-specific mCherry expression (Fig. 2, *A'* and *B'*, respectively). The mCherry/EGFP fluorescence ratio (indicative of promoter strength) was higher for the full-length construct compared with the concatenated A–C construct (−2734 to +119 construct, ratio = 0.89; *versus* A + B + C,

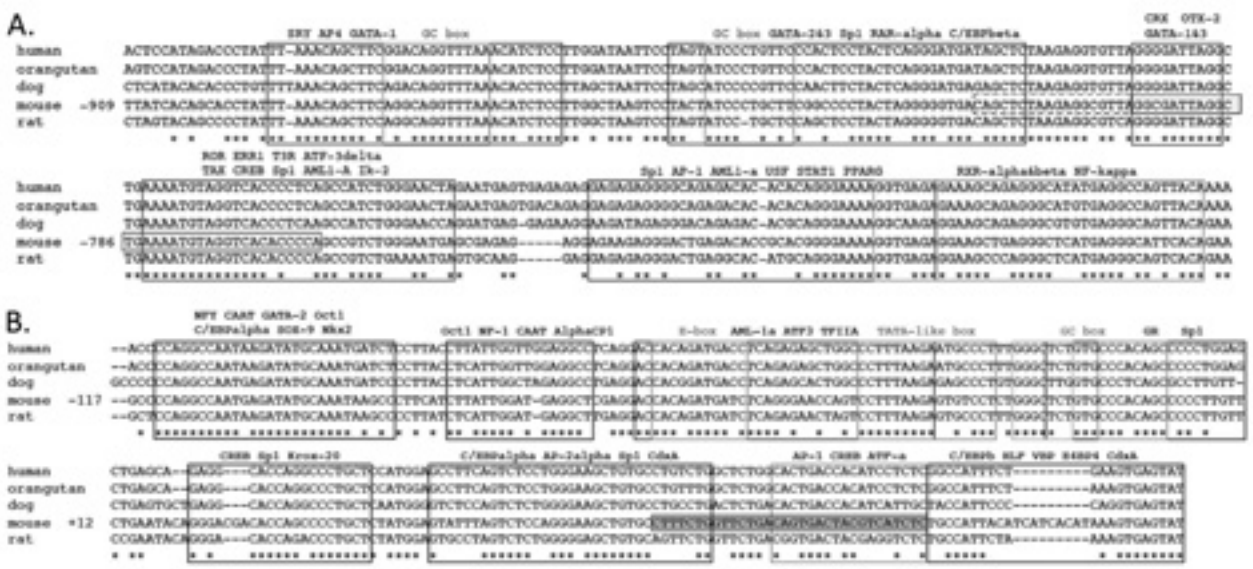

FIGURE 3. **Nrl promoter/enhancer sequence showing predicted sites for *trans*-acting regulators.** The conserved regions −909 to −667 (*A*) and −117 to +137 (*B*) in the *Nrl* promoter were aligned to syntenic sequences from human, orangutan, dog, and rat genomes. * indicates the conserved nucleotides. Sequences for putative transcription factors binding sites are *boxed*. The *gray shades* correspond to oligonucleotide sequences used for EMSA. *Light gray box, A*, shows predicted overlapping sites for many transcription factors including retinoid receptors, homeodomain proteins, ATF-3δ, CREB, and Sp1. *Dark gray box, B*, includes putative binding sites for AP-1, CREB, and ATF-α. The sequence boxed by *dashed lines* represents the extension of oligonucleotide B3 (shown in *light gray*) in oligonucleotide B4.

ratio = 0.7 ± 0.05; mean ± S.E.; n = 3 retinas per construct). Clusters A + B were also sufficient to drive strong and ONL-specific expression though somewhat lesser than the full-length or the A + B + C construct (Fig. 2*C*′; ratio = 0.56 ± 0.05). Transfection with a construct containing A + C, however, did not show reporter gene expression (Fig. 2*D*′; ratio = 0.04 ± 0.01), indicating that the critical sequences for *Nrl* expression are contained within cluster B. Transfection of a construct containing cluster B and a heterologous SV40 basal promoter resulted in little or no reporter expression (Fig. 2*E*′; ratio = 0.11 ± 0.01). Thus, sequence elements in both B and A clusters are required for appropriate reporter expression *in vivo*.

We then defined the relative importance of different sequence elements within clusters A and B in directing *Nrl* expression *in vivo*. A 129-bp sequence in 3′-region of cluster B (called B2, −814 to −657) combined with cluster A was sufficient to drive ONL-specific expression (Fig. 2*F*′; ratio = 0.57 ± 0.05), whereas the 5′-153-bp sequence in cluster B (called B1, −938 to −786) was unable *per se* to induce mCherry expression (Fig. 2*G*′; ratio = 0.07 ± 0.01). A 51-bp sequence within cluster A (called A1), together with B, induced strong reporter gene expression in ONL (Fig. 2*H*′; ratio = 1.16).

RORβ, CRX, OTX2, and CREB Bind Directly to Nrl Promoter/Enhancer Sequence Elements—In silico analysis using the TRANSFAC database (46) revealed a number of conserved putative transcription factor binding sites within B2 (transcriptionally active sequence in cluster B) and cluster A (Fig. 3, *A* and *B*, respectively). To identify the involvement of specific transcription factors, we performed EMSA using several different oligonucleotides spanning the conserved sequence elements (Fig. 4). Because of the lack of expression of NRL in *Rorb*$^{-/-}$ mouse retina (21), we specifically focused on the oligonucleotide sequence B3 (−797 to −764 in Fig. 3*A*, *light gray shaded box*) containing putative ROR, ERR1, T3R, OTX2, CRX, GATA-1, GATA-3, ATF-3δ, TAX, CREB, Sp1, AML1-A, and Ik-2 sites. EMSA demonstrated the binding of proteins in adult and neonatal (postnatal day (P) 1–P2) retinal extracts to oligonucleotide B3 (Fig. 4*A*, *lanes 2–9*, and *11*; Fig. 4*B*, *lanes 2–10*). In cluster A, we also identified the binding of retinal proteins to oligonucleotide A4 (+75 to +107; Fig. 3*B*, *dark gray shaded box*) that includes putative binding sites for bZIP transcription factors AP-1, CREB, and ATF-α (Fig. 4*D*, *lanes 2–7*; Fig. 4*E*, *lanes 2–7*). The binding of nuclear proteins to these two oligonucleotides was specific in that the addition of increasing amounts of unlabeled respective oligonucleotide reduced the EMSA signal, whereas a mutant unlabeled competitor oligonucleotide did not affect the signal intensity.

To identify the transcription factor(s) within protein complexes bound to B3 or A4 *cis*-sequence elements, we performed EMSA after the addition of specific antibodies. Addition of RORβ antibody resulted in the disappearance of the specific DNA-protein complex at B3 observed in both the adult retinal nuclear extract and extract from HEK293 cells transfected with a RORβ expression construct (Fig. 4*A*, *lanes 9* and *10*). A similar dramatic suppression of binding at B3 was observed with neonatal retinal extracts when RORβ antibody was added (Fig. 4*B*, *lane 9*). Addition of CREB antibody resulted in slight reduction of B3 EMSA signal with both adult and neonatal retinal extracts (Fig. 4, *A* and *B*, *lanes 8*). OTX2 and CRX antibodies (Fig. 4*A* and *B*, *lanes 6* and *7*) did not alter the mobility of B3 oligonucleotide. Because OTX2 and CRX are predicted to be upstream of NRL in the transcriptional hierarchy during retinal development (15, 17), we designed a longer oligonucleotide (B4, from

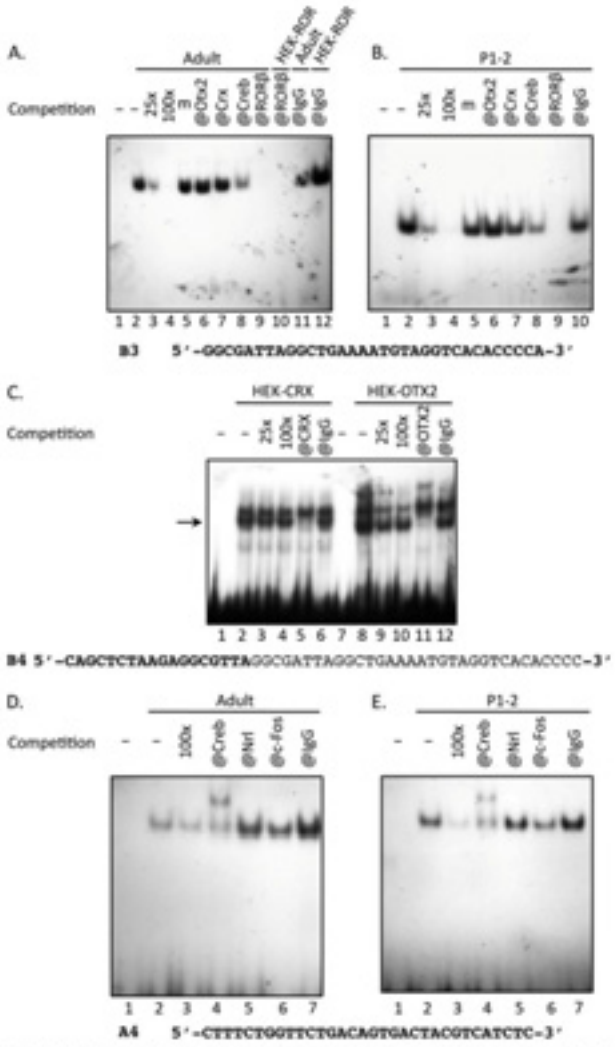

FIGURE 4. **RORβ, CRX, OTX2, and CREB bind to *Nrl* promoter/enhancer sequence elements.** Autoradiograms of EMSA using nuclear extracts from adult (*A* and *D*) and neonatal P1–2 (*B* and *E*) mouse retina and from HEK293 cells transfected with *CMV-RORβ* (*A*, *lanes 10* and *12*), CMV-CRX or *CMV-OTX2* (*C*) are shown. Addition of 25 or 100 ng of unlabeled specific oligonucleotides reduced the binding (*A* and *B*, *lanes 3* and *4*; *D* and *E*, *lane 3*), whereas unlabeled mutant (*m*) oligonucleotides (*A* and *B*, *lanes 5*) did not compete for the bound proteins. Oligonucleotide supershift assays were performed with antibodies against OTX2, CRX, CREB, RORβ, and normal IgG (*A* and *B*), with CRX and OTX2 antibodies (*C*), and with antibodies against CREB, NRL, c-Fos, and normal IgG (*D* and *E*). B3, B4 and A4 oligonucleotides are indicated below the appropriate EMSA. The mutant oligonucleotide for B3 included five substitutions in conserved binding sites. B4 contains 18 additional nucleotides at the 5′-end of B3 with better core binding sequence(s) for homeodomain proteins.

−814 to −763 containing 18 additional nucleotides at 5′-end of B3) for EMSA. Interestingly, a gel shift (indicated by an *arrow* in Fig. 4*C*) of B4, obtained by adding nuclear extracts from HEK293 cells transfected with CRX or OTX2 expression constructs, diminished by including CRX or OTX2 antibody, respectively (Fig. 4*C*, *lanes 5* and *11*). Addition of normal control immunoglobulins (IgG) (Fig. 4*A*, *lanes 11* and *12*; Fig. 4*B*, *lane 10*; Fig. 4*C*, *lanes 6* and *12*) did not affect the mobility of B3 or B4 DNA-protein complex.

With oligonucleotide A4, addition of CREB antibody to adult or neonatal nuclear extracts showed a supershift of the EMSA band (Fig. 4, *D* and *E*, *lanes 4*). Antibodies against the two other bZIP proteins, NRL and c-Fos, and normal IgG did not alter the mobility (Fig. 4, *D* and *E*, *lanes 5–7*).

Mutations in RORβ Binding Site in Nrl Promoter/Enhancer Abolish Reporter Gene Expression—To assess further the regulatory function of RORβ on the *Nrl* promoter, we performed site-directed mutagenesis by substituting two nucleotides in the consensus core motif of the ROR response element (RORE), AAAATGTAGGTCA, present in *Nrl* cluster B (−783 to −771). Whereas the wild-type construct (containing *Nrl* promoter sequence −938 to +119; see Fig. 1*B*) showed strong mCherry expression in the ONL of P14 retina (Fig. 5*A′*, ratio = 0.56), the mutant construct (carrying mutations in RORE) did not display any reporter gene expression (Fig. 5*B′*, ratio = 0.1 ± 0.01). Our data therefore establish the importance of RORE (present in cluster B) and RORβ in directly activating the expression of *Nrl* in developing retina.

DISCUSSION

Photoreceptors are highly specialized neurons that initiate visual transduction (52, 53); their death or deterioration is a major cause of untreatable vision loss (54). Because NRL is critical for determining rod *versus* cone cell fate choice (17), identification of transcription factors that induce its expression would assist in elucidation of regulatory networks that specify distinct retinal cell types. In this report, we have defined *cis*-control elements responsible for rod-specific *Nrl* expression by *in vivo* transfection methods and identified two conserved upstream sequences (cluster B, −939 to −657 and cluster A, −304 to +119) that, when combined, are sufficient to drive reporter gene expression in the rod photoreceptors *in vivo*.

The cluster A sequence includes the transcription start site, CAAT and GC boxes, and a TATA-like sequence; this cluster is likely required for the binding of transcription initiation complex. The fact that a basal SV40 promoter did not show any activity when combined with cluster B *in vivo* suggests that the cluster A sequence appears necessary for photoreceptor-specific expression of *Nrl*. We further showed that the −35 to +16 sequence harboring only the GC box and TATA-like element was sufficient for rod specificity when linked to cluster B. In addition, the conserved B2 sequence between −814 and −657 could induce highly specific ONL expression. Within this B2 sequence, a 31-bp element (B3, from −797 to −764), was highly conserved and had a large predictive score for the binding of several transcription factors. EMSA studies confirmed the binding of retinal proteins, including RORβ, to the B3 sequence.

We unambiguously show a direct role of RORβ in mediating *Nrl* promoter activity. RORβ2, a retina-specific isoform of RORβ (55), plays a key role in the differentiation of both rod and cone photoreceptors. Early expression of RORβ during initial stages of photoreceptor development is needed to induce a photoreceptor precursor to become a mature rod (21). *Rorb*$^{-/-}$ mice lack rods, overproduce immature S-cones, and exhibit the loss of *Nrl* expression, suggesting that RORβ acts upstream of NRL to promote the rod photoreceptor cell fate

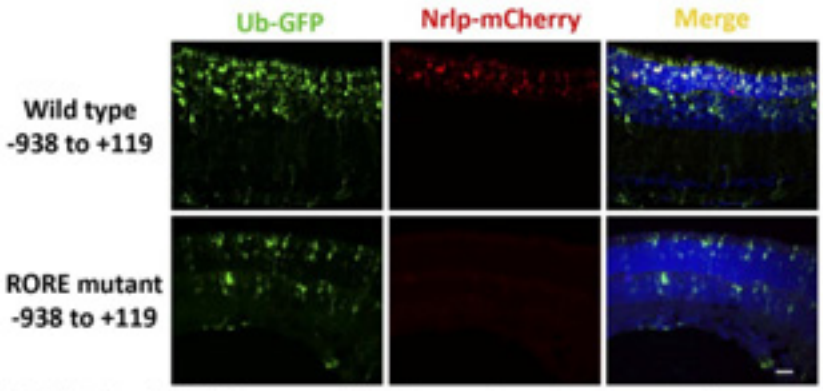

FIGURE 5. **Mutations in RORβ binding site abolish *Nrl* promoter activity.** Representative sections from neonatal mouse retina transfected *in vivo* with the ubiquitin (Ub)-GFP construct (*left panels*) and *Nrl* promoter segment −938 to +119 wild type (*upper center*) and mutant for the RORβ binding site (*lower center*, ROR site located from −783 to −771) are shown. Retinas were harvested at P14 for examining the expression of the reporter gene. *Right panels* show merged images. *Green*, GFP fluorescence; *red*, mCherry fluorescence; *blue*, DAPI staining. *Scale bar*, 20 μm.

(21). The specific binding of RORβ to *Nrl* promoter in both developing (P1–2) and adult retina indicates that RORβ, with other *trans*-acting regulators, helps to initiate the rod differentiation program and might later maintain *Nrl* expression in mature retina.

DNA-binding proteins bind to sequence elements at multiple sites within the genome, and their contribution to specific gene regulation generally depends on co-operativity and/or interaction with other regulatory factors to assemble an enhanceosome complex (56, 57). The homeodomain proteins, OTX2 and CRX, are major regulators of photoreceptor development (15, 17, 23) and hence excellent candidates for modulating *Nrl* expression. The putative target binding sites for these factors are present in the *Nrl* control element B2 (Fig. 3*A*). Although only a slight reduction in B3 gel shift signal is observed with CRX antibody when added to the neonatal retinal nuclear extract, a direct binding of CRX and OTX2 to core homeodomain binding sequences within B4 (that includes 18 additional nucleotides 5′ of B3) was clearly evident when transfected cell nuclear extracts were used (Fig. 4*C*). Because homeodomain proteins can bind promiscuously to A-T-rich sequence elements (56, 58), the specificity in gene regulation is likely accomplished by their interaction with other proteins. Further investigations are necessary to identify the role of CRX and/or OTX2 in directly controlling *Nrl* expression in early stages of photoreceptor development.

CREB mediates the effects of numerous signaling pathways during neuronal development and homeostasis (59). Notably, deregulation of cAMP in retinal degeneration mouse models (*rd1*, *rd2*, and certain rhodopsin mutants) leads to insufficient or excessive levels of cAMP and coincides with cell death (60–62). We show that CREB can bind to both B3 and A4 sequence elements in *Nrl* promoter/enhancer although binding characteristics may be different as reflected by the effect of anti-CREB antibody in EMSA. Although CREB has not been specifically associated with retinal or photoreceptor development, it may integrate spatiotemporal signals during rod differentiation and/or homeostasis via its action on *Nrl*, which may then fine tune the transcription of specific rod genes.

Expression and binding of distinct *trans*-acting regulators may be required for developmental expression *versus* homeostasis (17). In this study, we investigated differential regulation of *Nrl* using retinal extracts from two stages: P1–P2, at the time of peak rod birth when *Nrl* expression is needed to determine the rod fate in photoreceptor precursors; and adult, when NRL regulates the expression of rod-specific genes for functional maintenance. How do cells determine the appropriate time or levels of *Nrl* expression? Here we focused on defining the *cis*-control elements in the *Nrl* promoter/enhancer region. Another approach would be to examine posttranslational modifications, such as phosphorylation and SUMOylation, of transcription factors that control *Nrl* regulation. Control of *Nrl* expression may also depend on extrinsic signaling factors. Notably, FGF and retinoic acid have been implicated in inducing *Nrl* expression (42, 63). Identification of RORβ as a direct transcriptional activator of *Nrl* and potential involvement of CRX, OTX2, and CREB are consistent with combinatorial and cooperative regulation by intrinsic and extrinsic mechanisms. Our studies thus provide new insights into transcriptional control pathways and suggest how retinal progenitor cells can be directed to adopt a photoreceptor-specific identity.

Much progress has been made toward the treatment of retinal diseases using adeno-associated virus as a vector for gene therapy (64–66). As rod photoreceptors degenerate first in many retinal neurodegenerative diseases, it would be ideal to specifically target the therapy to rods by using cell-type specific promoters. Because adeno-associated virus vector can only accommodate a limited size, one critical factor in promoter choice is the length of the promoter sequence. Another factor is the promoter strength for transcriptional activation of the target gene as too high or too low expression can be counterproductive. The identification of a small *Nrl* promoter/enhancer sequence of <0.3 kb that can specifically direct the target gene to both developing and mature rod photoreceptors should be of interest in the design of adeno-associated virus-based therapies for retinal diseases.

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II. Identification of a minimal *Nrl* promoter as a tool for specific photoreceptor expression using AAV

The anatomical accessibility of the retina makes it a tissue of choice for gene therapy experiments targeting degenerating photoreceptors. Hereditary diseases such as Retinitis Pigmentosa or Leber Congenital Amaurosis primarily affect photoreceptor survival and lead ultimately to blindness. During the last decades, use of adeno-associated virus (AAV)-based vectors showed increasing promise in gene therapy due to their relative safety and long-term gene expression (High 2001). Various promoters specific for photoreceptor expression have been successfully used for foreign gene delivery in photoreceptors (Chen, Tucker et al. 1994; Glushakova, Timmers et al. 2006; Khani, Pawlyk et al. 2007). However, most promoters used for this type of study are shorter versions of opsins' promoters. Utilization of these promoters could rather be a disadvantage as opsin expression is a tightly controlled function. Opsin promoter produces abundant amounts of transcripts and may generate a potential toxicity for the cell by overproducing the protein. Identification of small promoter, for optimal expression of photoreceptor-specific genes, presents a clinical interest. Thus, primary characterization of regulatory elements in such a promoter would allow modulation of its expression.

In vivo analysis of *Nrl* promoter/enhancer emphasized the importance of evolutionary conserved regions in the transcriptional regulation of this gene. Combination of cluster sequences highlighted the significance of regions B and A1 in positive regulation of *Nrl* expression. Yet, B region shows numerous potential binding sites for known transcription factors. Thus, to further understand the role of these promoter/enhancer elements, we dissected out region B sequence and indentified a 51 bp sequence, called B4 (-814 to -763), which showed a high score for putative transcription factor binding sites by *in sillico* analysis. Gel shift assays confirmed the direct binding of RORβ, CRX and OTX2 on this sequence. Hence, we intended to test the transcriptional relevance of B4 sequence by analyzing *in vivo* regulation of a reporter gene linked to B4.

By identifying *Nrl* essential elements for its transcription, our goal was also to define a minimal *Nrl* 5'-upstream regulatory region functional and specific for rod-photoreceptor expression, and that is notably limited in size for use in Adeno-Associated

Vector (AAV)-mediated expression. This last point is a major concern in gene delivery systems using AAV; indeed, AAV vectors have a relatively small carrying capacity,

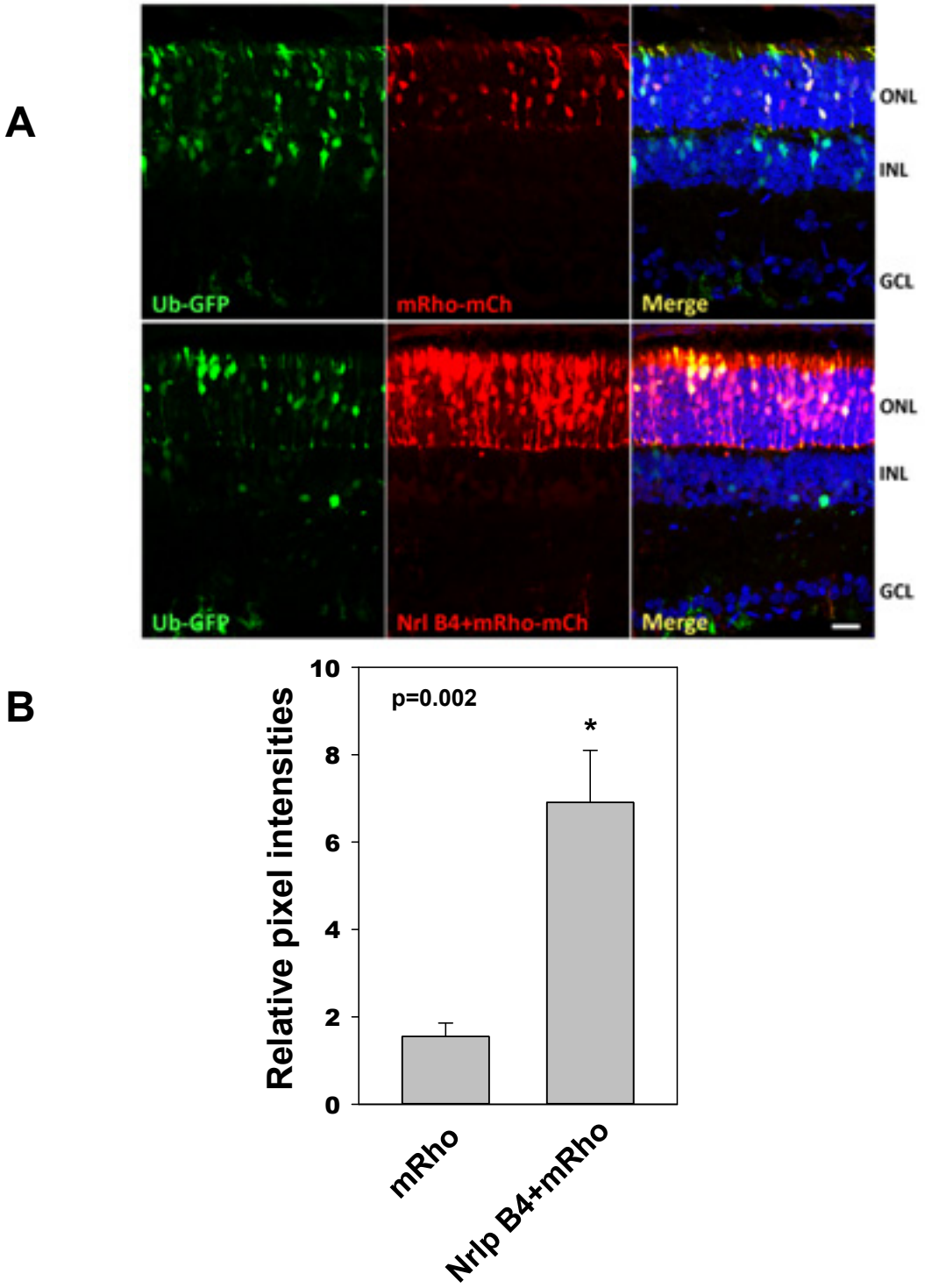

Figure 36. *Nrl* promoter B4 sequence is a transcriptional enhancer

(A) Representative sections from neonatal mouse retina transfected *in vivo* with two DNA constructs: mouse *rhodopsin* proximal promoter driving mCherry (mRho, -250 to +1 from the transcription start site) alone or coupled with mouse *Nrl* promoter B4 sequence (-814 to -763). Retinas were harvested at P14. **(B)** Chart showing the relative pixel intensity per photoreceptor double positive for the fluorescent reporter genes for each condition (n = 20). Plotted data are normalized to GFP expression. Values represent mean ± SEM. Significant promoter activity ($P < 0.05$) is indicated. Scale bars, 20 µm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

allowing the cloning of foreign DNA ≤ 4.7 kb (Wu, Yang et al. 2010). In gene therapy, the 4.7 kb cloning capacity should allow proper insertion of the cDNA sequence and it should not be impel to truncate the coding region sequence with the risk of generating a non-functional protein. To avoid any deletion in the cDNA sequence, it is more prudent to focus on the promoter region for cloning approach. In addition, bashed promoter should conserve uniform tissue-restricted transcriptional activity. A part of the activity of our laboratory (N-NRL, National Eye Institute) is devoted to the design and development of AAV vectors. The work that is described below was in part (virus production and subretinal injections) performed in collaboration with Dr. Z. Wu from the team of Dr. P Colosi.

A. Further dissection of *Nrl* promoter elements required for the transcription

We knew from previous experiments that full length cluster B (-938 to -657 bp from the transcriptional start site) cloned upstream of the minimal SV40 promoter was unable to activate transcription. Subsequently, we hypothesized that B4 would also be unable to stimulate transcription of the reporter gene. For that reason, we cloned B4 sequence upstream of a rod-specific promoter (mouse *rhodopsin* proximal promoter, -250 to +1) and compared the reporter gene expression in P0 electroporated retinas in presence or not of B4 sequence.

Addition of B4 sequence to *mRhodopsin* promoter triggered an apparent increase in *rhodopsin* promoter activity compared to the single *rhodopsin* promoter (Figure 36A). Relative pixel intensity ratio was measured using ImageJ software (National Institutes of Health, USA) to quantify the effect of B4 cloning. Differences of expression level between the two DNA constructs were checked using the one way ANOVA analysis of variance. Results showed a significant variation in mCherry expression between the two groups (n= 20; P =0.002) with the highest pixel intensity when B4 was cloned with *rhodopsin* promoter (Figure 36B). These data suggest that B4 sequence is an enhancer element for the transcription and strategies for cloning of *Nrl* upstream region should include this B4 sequence for correct gene activation.

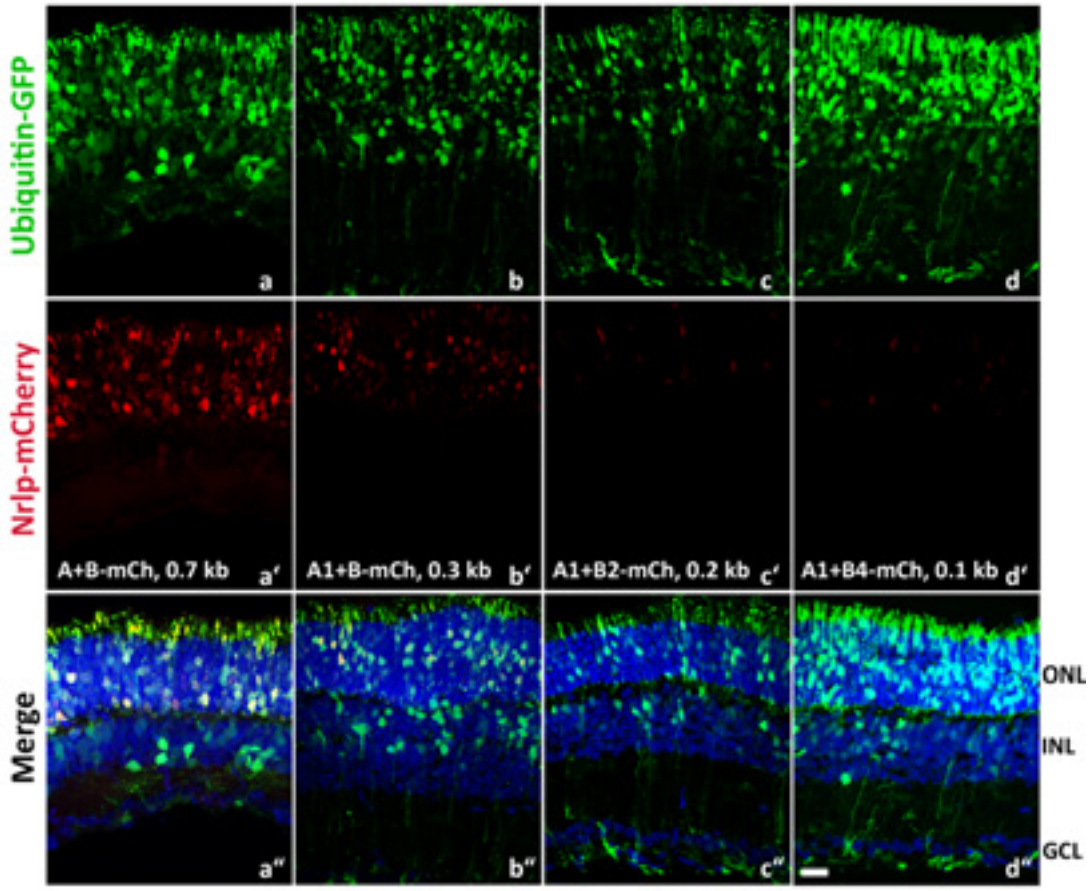

Figure 37. *Nrl* minimal and functional promoter

Representative sections from neonatal mouse retinas transfected *in vivo* with different lengths of mouse *Nrl* promoter/enhancer regions. **(a')** Conserved clusters combination A (-304 to +119 from the TSS) and B (-938 to -657) display a strong mCherry reporter gene expression specifically in the ONL. **(b')** Minimal A1 sequence (-34 to +16) in cluster A behaves as a basal promoter for *Nrl* expression when combined to cluster B. A1+B sequence shows a sustained expression of mCherry in the ONL compared to A1+B2 (B2, -814 to -657) or to A+B4 (B4, -814 to -763) sequences (**C'** and **D'** respectively). Ubiquitin-GFP was used to verify the transfection efficiency **(a-d)**. Merged images (**a"-d"**). Red, mCherry; Green, GFP; blue, DAPI. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 20 µm.

To replace B4 enhancer in the context of *Nrl* gene regulation, and based on the identification of regulatory elements previously characterized in *Nrl* promoter, we generated different lengths of promoter/enhancer by focusing on reducing as far as possible the promoter size, and performed *in vivo* retinal transfection in neonatal animals. When the animals reached P14, we harvested the retinas and confirmed the transcriptional validity of the DNA constructs tested and checked their specificity of expression in rod-cells by looking at reporter gene expression. *In vivo* electroporation analysis showed relatively strong mCherry expression for A+B and A1+B promoter fragments, as shown previously (chapter I of results), while only a few cells expressing mCherry were detected in A1+B2 and A1+B4 retinal transfections (Figure 37). Although the four *Nrl* upstream fragments showed positive expression specifically in the ONL, for cloning purposes in AAV we decided to keep the A1+B *Nrl* promoter (0.3 kb) as the best compromise between promoter size and expression level.

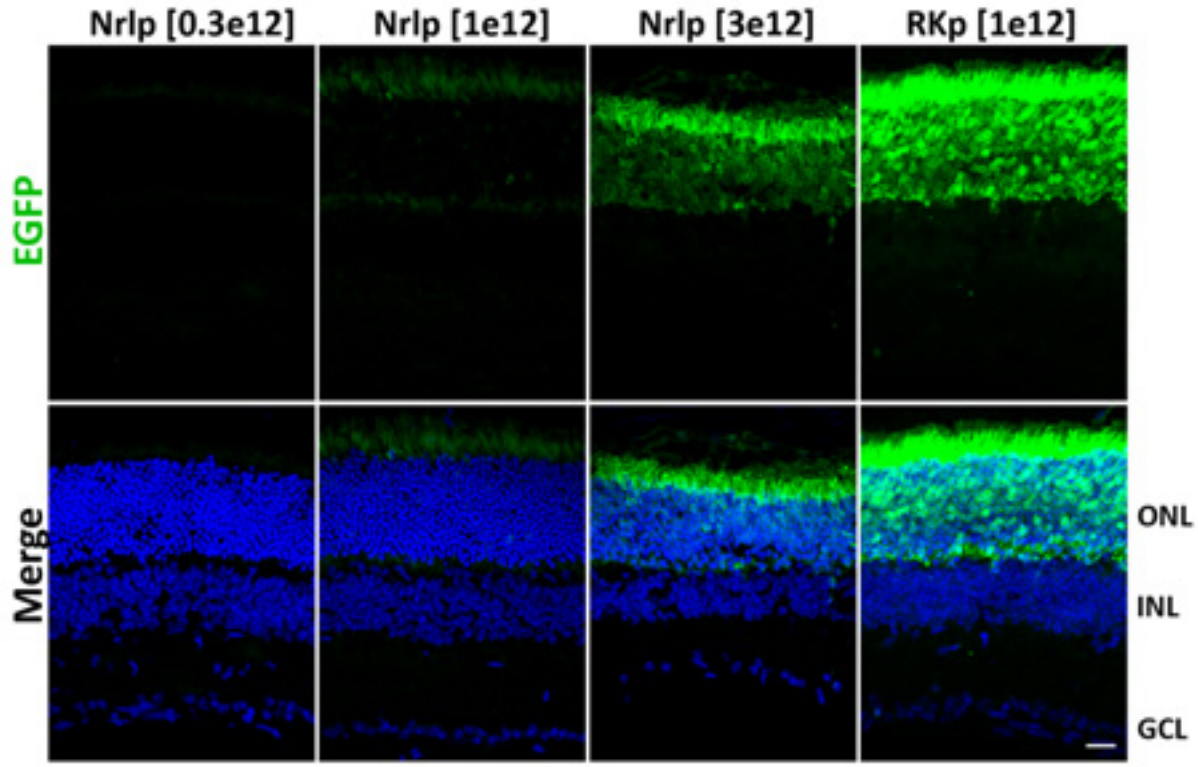

Figure 38. Determination of the working dilution for AAV-*Nrl*p-EGFP

Retinal sections from wild type (C57Bl6) P10 injected animals with AAV-*Nrl*p-EGFP and AAV-*RK*p-EGFP. Retinas were harvested 3 weeks post-injection for EGFP analysis. AAV-*RK*p-EGFP (far right column) was used as positive control. Green, EGFP; blue, DAPI. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 20 µm

B. AAV-mediated expression of *Nrl* promoter or cDNA to further decipher the developmentall properties of *Nrl* gene

Nrl promoter drives robust and specific transgene expression in retinal photoreceptors

In this study, we examined the promoter of the mouse *Neural retina leucine* zipper (*Nrl*) and tested its ability to drive gene expression in photoreceptors mediated by AAV vector. To determine the promoter activity of minimal *Nrl* promoter (A1+B, 0.3 kb) sequence *in vivo*, we placed an EGFP reporter gene under its transcriptional control and packaged the resultant expression construct into an AAV8 vector to generate AAV-*Nrl*p-EGFP. We first determined the appropriate working dilution of this construct by injecting P10 wild type (C57Bl6) retinas with a range (0.3^{e12} – 3^{e12} vector genome/milliliter) of AAV-*Nrl*p-EGFP in subretinal space. For comparison, we looked at human *Rhodopsin kinase* promoter activity in AAV (AAV-*RK*p-EGFP) previously reported to be functional and robust in both rod and cone photoreceptors (Khani, Pawlyk et al. 2007). Three weeks after injection reporter gene analysis revealed a high difference of promoter transactivation between the two promoters tested. While AAV-*RK*p-EGFP showed a strong reporter gene expression at the concentration tested (1^{e12} vg/mL), cells were only able to express strong EGFP reporter gene with the highest AAV-*Nrl*p-EGFP concentration (3^{e12} vg/mL). A barely detectable EGFP expression was seen with injection of AAV-*Nrl*p-EGFP at 1^{e12} vg/mL and no expression was detected with the lowest concentration 0.3^{e12} vg/mL (Figure 38). Neither RPE nor inner retina expressed the reporter gene, confirming the photoreceptor specificity of the two promoters. Both promoters appeared similarly effective in driving uniform expression in photoreceptors although AAV-*RK*p-EGFP showed a stronger promoter response for a lower working dilution, meaning that Rhodopsin kinase expression is actively stimulated in photoreceptors. These data prove that cloning of *Nrl* 5'-upstream sequence in AAV is a working tool for photoreceptor-specific expression and brings us some knowledge about the behavior of this promoter in AAV system as this experimental design has never been tested before.

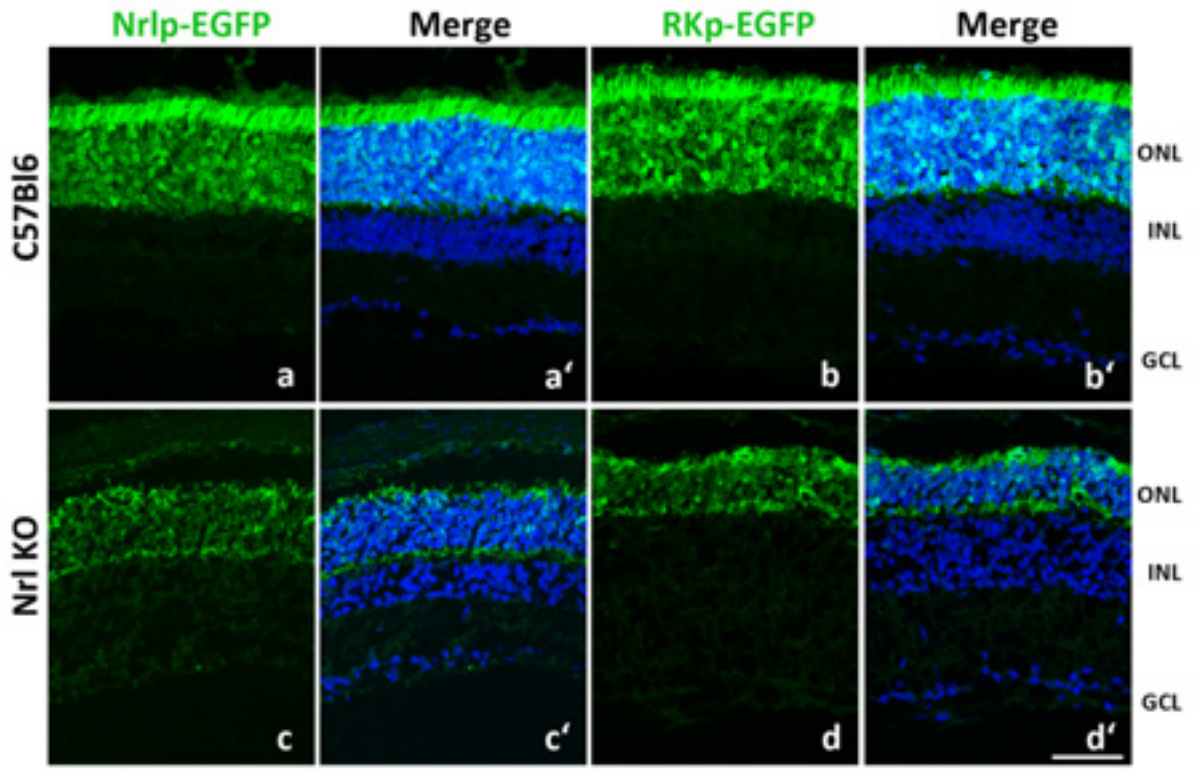

Figure 39. Analysis of GFP reporter expression in *Nrl* KO mouse retina

Representative retinal sections from wild type (C57Bl6; a-b') and Nrl KO mice (c-d'). In parallel groups, animals were injected at P10 with 1 µL of AAV-*Nrl*p-EGFP concentrated at 3^{e12} vg/mL (a, a', c, c') or with AAV-*RK*p-EGFP concentrated at 1^{e12} vg/mL. 3 weeks post-injection, retinas were harvested for EGFP analysis. Green, EGFP; blue, DAPI. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50 µm

AAV- Nrlp-EGFP is functional in cone-like cells

We next asked whether AAV -*Nrl*p-EGFP could also drive EGFP reporter expression in photoreceptors other than rods. To answer this question, we used recombinant mice deleted of *Nrl*. In these animals, the photoreceptor layer is composed of cone-like cells and does not contain rods. In *Nrl*$^{-/-}$ animals, rods are replaced by photoreceptors similar to S-cones (Mears, Kondo et al. 2001). It was previously reported that AAV-*RK*p-EGFP injection in *Nrl* KO retina showed positive expression of the reporter gene (Khani, Pawlyk et al. 2007); therefore, we used this construct as positive control. P10 *Nrl* KO or wild type retinas were injected with AAV-*Nrl*p-EGFP (3^{e12} vg/mL) or AAV-*RK*p-EGP(1^{e12} vg/mL). Retinas were harvested 3 weeks post-injection for reporter gene analysis. We found EGFP expression in the photoreceptor layer of wild type animals as reported previously (Figure 39a-b') and in the outer nuclear layer of *Nrl* KO retina (Figure 39c-d').Although EGFP was detected in the transgenic context, cells displayed an apparent diminished promoter activity as compared to the wild type background. These data suggest that *Nrl* promoter can be activated in cone-like cells.

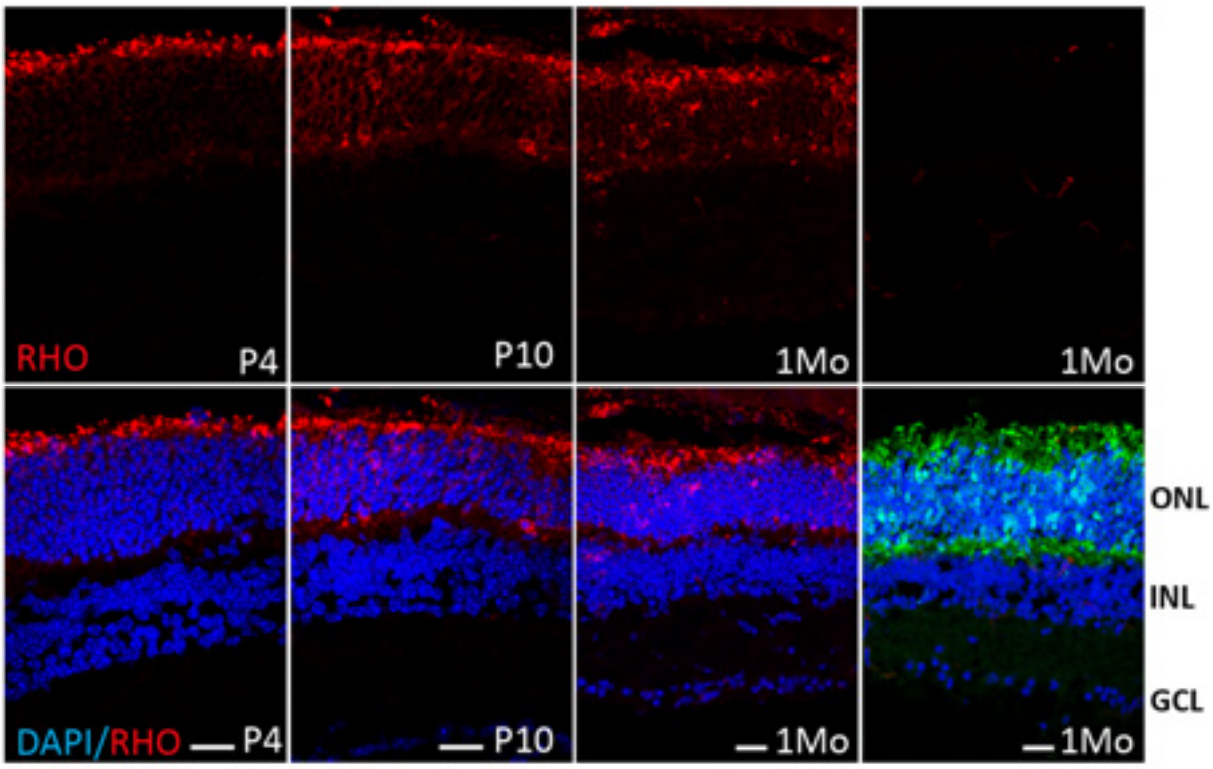

Figure 40. Expression of *Nrl* in *Nrl* KO retina restores rhodopsin expression

Representative retinal sections of *Nrl* KO retina injected at P4, P10 and 1 month-old mice with AAV-*CMV*-*Nrl* at 0.5^{e12} vg/mL or with AAV-*RK*p-EGFP at 1^{e12} vg/mL. On these retinas, assessment of rhodopsin expression was done using immunohistochemistry with anti-rhodopsin antibody (RHO). We detected specific expression of rhodopsin in the ONL for all the developmental time points tested. AAV-*RK*p-EGFP (far right column) was used as negative control, and did not show positive cells expressing rhodopsin. ONL, outer nuclear layer; INL, inner nuclear layer, GCL, ganglion cell layer. Scale bars, 20 µm.

Ectopic expression of Nrl in Nrl KO retina induces rhodopsin expression

NRL transcription factor is a major contributor to the rod cell fate specification by directing retinal progenitors to develop into rod photoreceptors. Indeed, ectopic expression of *Nrl* in immature photoreceptors leads to loss of cone genes expression and transformation into rods. Moreover, NRL activates several rod-specific genes for rod proper functioning, such as rhodopsin and cGMP-phodphodiesterase β-subunit (Rehemtulla, Warwar et al. 1996; Mitton, Swain et al. 2000; Lerner, Gribanova et al. 2001). To evaluate the cellular plasticity of post-mitotic retinal neurons, we drove the expression of *Nrl* coding sequence not only in developing (P4 and P10) and but also in adult *Nrl* KO retina. We evaluated how the action of this transcription factor modulates retinal development by ectopically expressing *Nrl* open reading frame under the control of cytomegalovirus promoter in cone-like cells. *Nrl* KO retinas were injected with AAV-*CMV-Nrl* (made by S. Hiriyanna) (0.5^{e12} vg/mL). For negative control experiment, we used AAV-*RK*p-EGFP (1^{e12} vg/mL), and as output of this experimental design, we performed immunohistochemistry with rhodopsin antibody for injected retina (Figure 40). We observed a positive rhodopsin staining for P4 and P10 *Nrl* KO retina injected with AAV-*CMV-Nrl*, and interestingly for adult retina injected with AAV-*CMV-Nrl* as well. Injection of AAV-*RK*p-EGFP did not show any rhodopsin-labeled cells. Hence, these data give additional weight to the major contributing role of Nrl in rod cell fate specification.

Discussion

Definition of a minimal, functional and yet specific promoter for photoreceptor expression is a major interest for gene therapy targeting retinal degenerative disorders.

In this study, we identified by *in vivo* electroporation *cis*-regulatory sequences of the *Neural retina leucine* zipper gene essential for its transcription. Among the identified elements, B4 enhancer showed elevated promoter activation when linked to the mouse *rhodopsin* proximal promoter. When placed in *Nrl* genomic context, B4 enhancer linked to the basal promoter A1 previously described (Kautzmann, Kim et al. 2011), could not transcribe efficiently the reporter gene, resulting in weak expression of mCherry in some cells in the ONL. In this situation, likely the combination of A1+B4 sequence (50 bp and 51 bp, respectively), similar to the B2+A1 combination, was too short for correct promoter transactivation, explaining the decline in reporter gene expression. Our results suggest that the sequences and potential interacting factors corresponding to B4 region are sufficient to drive expression specifically in the ONL, but likely other factors are missing to fully activate the promoter. Therefore, based on the information provided by *in vivo* retinal transfections, we chose *Nrl* promoter A1+B that has the characteristics to be small in size (0.3 kb) and has relatively high transactivation abilities. The specificity of expression of this promoter was partially demonstrated using *in vivo* electroporation, where we observed location of mCherry reporter gene in the ONL. The technique of *in vivo* electroporation has several advantages; one of them is the possibility to quickly evaluate expression of a gene of interest as compared to the generation of transgenic mice. Another advantage of this technique which could be perceived in some cases by a disadvantage is the limitation of cell types targeted during neonatal stages, time of the injection. Indeed, *in vivo* electroporation is performed at P0-2 in mouse retina; at this time of the development, there is a peak of rod proliferation that will thus be preferentially targeted for the ectopic gene expression. Only dividing cells will be able to express the foreign DNA as long as these cells possess appropriate transcription factors for promoter activation. Based on these data, we presumed in our study that *Nrl*p-*mCherry* expression observed in the ONL was due to rod-expression only but not to cones, as previously shown for the -938 to +119 construct (chapter I of results).

However, confirmation of this statement would require further co-labeling with cone specific markers like cone-arrestin.

Adeno-associated virus (AAV)-based vectors showed increasing promise in gene therapy because of to their relative safety and long-term gene expression (High 2001). Various genes' promoters specific for photoreceptor expression have been successfully used for foreign gene delivery in photoreceptors (Chen, Tucker et al. 1994; Glushakova, Timmers et al. 2006; Khani, Pawlyk et al. 2007). In addition, they have arisen as convenient tool in research, to deliver ectopic genes within the whole outer retina/RPE, even in the adult animal. We generated AAV vectors containing *Nrl* promoter; the cloning size limitation in AAV pushed us to preferentially use the minimal *Nrl* A1+ B promoter. We first checked the functionality of this promoter in AAV genome by generating AAV-*Nrl*p-EGFP construct and injecting it into wild type mouse retina. We tried different dilutions of the vector genomes to give us an overview of this construct expression. The best results of EGFP expression was observed with the highest concentration of AAV-*Nrl*p-EGFP (3^{e12} vg/mL), which showed a strong and specific expression of the gene reporter all over the ONL, is in the range of currently used virus dilutions and did not show any toxicity. Thus AAV-*Nrl*p-EGFP appears of potential convenient use for photoreceptor gene delivery in the retina. Once the correct AAV working dilution was determined, we assessed promoter specificity by injecting AAV-*Nrl*p-EGFP in *Nrl* KO retina, which have the specificity to contain only cone like cells in the ONL. Although EGFP expression was weaker in *Nrl* KO retina in comparison to the wild type background, we showed positive expression of GFP in the ONL. These data suggest that in this particular context, photoreceptors other than rods are able to activate *Nrl* promoter. However, we cannot precisely explain th expression in these particular cone-like cells is due to limited sequence elements found in the minimal *Nrl* promoter that may be active also in cone-like cells, or whether the characteristic molecular environment, found in the photoreceptors of the *Nrl* KO retina, is permissive for *Nrl* promoter activation. Nonetheless, we can correlate our data to previous work reporting GFP expression under the control of *Nrl* (2.5 kb) promoter in *Nrl* KO retina (Akimoto, Cheng et al. 2006). In this study, the authors crossed *Nrl*-GFP mice with *Nrl* KO mice and analyzed expression of GFP in this *Nrl* deleted background. They observed strong GFP

expression in the cone-like cells and proposed that early in development, retinal progenitors have not committed their cell fate specification yet and remain plastic. It is not until action of key transcription factors such as *Nrl* that the retinal progenitors will restrict their cellular competence to give rise to one type of neuron and restrict the panel of genes able to be expressed. Our results show that this permissive state of this cone-like cells (or maybe of cones in general) still exists in the P10 retinas and extends over 1 month, age at which retinas were harvested.

III. Nrl and co-activators of *rhodopsin* expression show daily variations in the rat retina during the 24 h cycle

Daily variation of photoreceptor specific-gene expression in the rat retina

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Paper in preparation

Abstract

Rhythmic physiology is central to visual function and allows adaptation of the retinal response to the day/night cycle. Several photoreceptor-specific processes such as melatonin synthesis and shedding of rod outer segments are controlled by a circadian clock located within the retina and self-entraining to the light/dark cycle. Rat photoreceptors harbour a functional clock machinery but the mechanisms by which it regulates their rhythmic functions has been poorly characterized. In the present study we confirm that *rhodopsin* transcript displays daily rhythmicity and show that its direct regulatory transcription factors, NRL, CRX and NR2E3 are also regulated on a daily basis. All studied mRNAs display maximal expression around the transition between night and day but don't show any significant rhythmicity in constant darkness. We provide evidence that transcription of *Nrl* is regulated by REVERBα and RORβ clock factors and suggest a novel link between the circadian clock and its outputs.

Introduction

Night/day transitions are major events to which living organisms have to adapt their physiology and behaviour. Such daily changes rely on circadian rhythmicity at cellular and molecular levels. In mammals, these rhythms are generated by a hierarchical network of oscillators, comprising a central clock located in the suprachiasmatic nuclei (SCN) which is synchronized with daily environmental light cues through the retina, and a series of peripheral oscillators receptive to synchronizing information produced by the SCN (Stratmann and Schibler 2006). Circadian rhythmicity is a cell autonomous process based on the interconnection of transcriptional/post-translational regulatory feedback loops These molecular phenomena involve clock genes such as *Bmal1*, *Clock*, *Per1-2*, *Cry1-2*, *RevErbα* and *Rorα* or *Rorβ*, which have the property to entrain the expression of "clock-controlled genes" and thereby to drive rhythmic gene expression programs (Guillaumond, Dardente et al. 2005; Ko and Takahashi 2006).

Retinas have evolved endogenous timekeeping systems ensuring the adjustment of their function to the daily changes in light intensity (Iuvone, Tosini et al. 2005). Rhythms have been described in most retina layers. For instance, phospholipid synthesis is rhythmic in avian ganglion cells (Garbarino-Pico, Carpentieri et al. 2004) as is *melanopsin* expression in rat intrinsically sensitive ganglion cells (Hannibal, Georg et al. 2005). Rhythmic synthesis and release of dopamine from the inner nuclear layer (INL) has been documented in many species (Wirz-Justice, Da Prada et al. 1984; Pozdeyev, Doroshenko et al. 2000; Doyle, McIvor et al. 2002). In photoreceptors, numerous processes, such as synthesis and release of melatonin (Tosini and Menaker 1996), rod-cone coupling (Ribelayga, Cao et al. 2008) or phagocytosis of rod outer segments (LaVail 1976; Bobu and Hicks 2009) follow a daily schedule and are regulated by a circadian clock. Expression of genes encoding elements of the phototransduction cascade is rhythmic as well, notably in rodents. In the mouse, expression of *UV-opsin* and *rhodopsin* genes were shown to cycle under constant darkness with maximum mRNA amounts found around the transition between subjective day and night (von Schantz, Lucas et al. 1999), as is also expression of *rhodopsin* in the *zebrafish* retina (Yu, Gao et al. 2007). Daily changes in elements of the phototransduction cascade were more frequently reported under light-dark cycles: in these conditions, mRNAs for the α-subunit of *transducin* (Brann and

Cohen 1987) and for *rhodopsin* (Bowes, van Veen et al. 1988), with both peaks around the night to day transition.

Rhythmic control of retina function proceeds from complex regulations involving a local internal clock in the retina, capable to be entrained by light and to maintain rhythmic synthesis of melatonin in culture (Tosini and Menaker 1996; Ruan, Allen et al. 2008). In addition, *Bmal1* gene was shown to be indispensable in the eye to generate appropriate rhythms in gene expression and to process light information (Storch, Paz et al. 2007). Interestingly, a large part of the transcriptome found cyclic under LD conditions, was also altered upon ablation of the *Bmal1* gene, confirming the central role that the circadian clock plays in the synchronization of the retina visual function with the day/night cycle (Storch, Paz et al. 2007). Photoreceptors were shown to harbor a functional circadian clock machinery (Tosini, Davidson et al. 2007; Sandu, Hicks et al. 2011). So far, clock-controlled genes directly activated by the CLOCK/BMAL1 complex have been identified in photoreceptors, such as *AC1* (*Adenylyl cyclase 1*) (Fukuhara, Liu et al. 2004) and *Aanat* which encodes the rate-limiting enzyme in melatonin synthesis (Tosini and Fukuhara 2002). It has been suggested that E-box activation through CLOCK/BMAL1 does not account for all the genes undergoing rhythmic expression. Other clock factors or tissue-specific/clock-controlled transcription factors are likely to provide activating signals at phases of the 24 h cycle that do not coincide with CLOCK/BMAL1 peak (Doherty and Kay 2010). Thus in photoreceptors, mechanisms underlying rhythmic expression of other genes remain to be identified, although the cAMP/protein kinase A (PKA)/cAMP response element binding (CREB) signaling pathway has been proposed to provide strong contribution (Iuvone, Tosini et al. 2005). In the present study, we investigated potential mechanisms for the control of *rhodopsin* rhythmic expression in the rat retina. We show that *Crx* and *Nrl*, as well as *Nr2e3* photoreceptor-specific transcription factors responsible for high level of rhodopsin expression in rod-photoreceptor, display rhythmic expression in LD, with peaks occurring around the night to day transition. We also provide evidence that *Nrl* is not a direct target of CLOCK/BMAL1 dimers but rather controlled by the REVERBα and RORβ clock factors.

Materials and methods

Animals and tissue preparation

Experiments were performed on 6-9 week old male Wistar rats (Charles River Laboratories, L'Arbresle, France) bred and housed at the Chronobiotron UMS3415 animal facility (Strasbourg) in a 12 h light/12 h dark cycle (LD). Rats had access to water and food *ad libitum* and were handled according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Animal Use and Care Committee from Strasbourg (CREMEAS). Animals were euthanized with CO_2 (20% in an air tight box). A group of animals kept in LD was euthanized starting at ZT0 (light onset; ZT12 dark onset) every 4 hours through a period of 24 h. A second group of animals was exposed to constant dark (DD, dark/dark), for 36 hours prior to eye sampling (starting at projected ZT0, every 4 hours through a 24 h period).

Whole retina sampling. After enucleation of the eye, a small incision was performed on the cornea with a sterile blade. The lens and vitreous body were discarded and the retina was directly collected with sterile forceps, immediately frozen on dry ice and stored at -80°C for further total RNA and protein extractions.

Isolation of photoreceptor layer. Photoreceptor layer was isolated from the retina by tangential sectioning using a vibratome-based procedure (Sandu, Hicks et al. 2011). Briefly, retina was dissected from the eye, flattened carefully with four radial cuts and mounted with photoreceptor surface down on a 20% gelatin block. To obtain the photoreceptor layer, a first vibratome cut of 170 µm depth from the vitreal surface was performed. The remaining photoreceptor layer was isolated with a single further slice (100 µm). The preparation was permanently maintained on ice cold medium and the photoreceptor samples were immediately frozen on dry ice and stored at -80°C.

Extraction of total RNA and reverse transcription

Extraction of total RNA (from whole retina and isolated photoreceptors of 6-8 week old rats) and reverse transcription were described in (Sandu, Hicks et al. 2011). 300 ng of total RNA was reverse transcribed into first strand cDNA and stored at -80ºC.

Real-time quantitative PCR

Gene expression over 24 h LD and DD cycles was analysed by real-time PCR for *Rho* and *Nr2e3* in the whole retina, and *Nrl* and *Crx* in photoreceptor layers. Real-time quantitative PCR was performed using the hydrolysed probe-based TaqMan chemistry. We used inventoried TaqMan Gene Expression Assays for *Rho*, *Nrl* and *Crx*, designed to specifically amplify mRNA (Applied Biosystems, Table1), and custom designed assays for *Nr2e3* based on the predicted RefSeq XM_002727061.1 sequence (forward 5'GGCTGCAGCGGCTTCTT3', reverse 5'CCCCTACCTGGCACCTGTAG3', probe 5'AGGAGTGTGAGACGGAG3'; amplicon size, 64bp).

Gene	TaqMan assay reference	RefSeq	Exon boundary	Assay location	Amplicon length (bp)
Rhodopsin	Rn00583728_m1	NM_033441.1	1-2	446	73
Nrl	Rn01481925_m1	XM_224189.2	4-5	512	103
Crx	Rn00573116_m1	NM_021855.1	1-2	99	64

Table 1

Inventoried TaqMan gene expression assays used in the study.

Reference (Applied Biosystems), GenBank accession number (RefSeq), location of the assay in the gene (nucleotide number indicates position of the probe in the RefSeq sequence), size (bp, base pairs) of the amplicons are given for all assays. Gene expression assays used in this study are designed across the exon-exon junction indicated in the table.

As described in (Sandu, Hicks et al. 2011), PCR conditions were: 1 x TaqMan Universal PCR Master Mix, No AMPErase UNG (Applied Biosystems), 1 x Gene Expression Assay mix (containing forward and reverse primers and cognate probe; Applied Biosystems) and 1 µl of cDNA in a total volume of 20 µl. The PCR program was as follows: 10 minutes at 95ºC, followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing-elongation at 60ºC for 1 minute. The acquisition of fluorescence data was performed at the end of the elongation step using the 7300 System Sequence Detection Software V 1.3.1 (Applied Biosystems). Each PCR reaction was done in duplicate and the coefficient

of variation (CV) among duplicates was 0.18% on average. A dilution curve of the pool of all cDNA samples was used to calculate the amplification efficiency for each assay (values were between 1.8 and 2 for all assays). No-template control (NTC) reactions were performed as negative controls for each assay. One 96-well plate corresponded to the analysis of one gene within one type of samples (retina or photoreceptors) and one experimental condition (LD or DD). Transcript levels were normalized using *β-actin* and *Pde6b*.

Western blot analysis

Whole retinas, sampled at ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20, were dissociated in extraction buffer (20 mM Tris pH 7.6, 150 mM NaCl, 1.5% Triton-X-100, 1 mM EDTA, 0.2% SDS supplemented with protease inhibitors (Roche Applied Science, Germany), phosphatase inhibitors (Sigma, L'Isle d'Abeau Chesnes, France) and 1 mM DTT) using a sonicator (Vibra-Cell 75186, Sonics & Materials, Newtown, CT, USA) for 15 s; 30/10 pulse and 50% amplitude. Lysates were centrifuged at 13,000 rpm (4°C, 20 min) and supernatants kept at -80°C until further use. Concentrations of total soluble proteins in the extracts were determined using the Bradford method (Bradford 1976).

Retinal proteins (3µg, denaturated in 2X Laemmli buffer) were separated by 10 % SDS-polyacrylamide gels electrophoresis and transferred on PVDF (polyvinylidene fluoride: Millipore, Molsheim, France) membranes. Membranes were blocked in TBST buffer (Tris buffered saline containing 0,2% Tween 20) supplemented with 5% fat-free milk and then incubated overnight at 4°C in primary antibodies diluted in blocking buffer. Membranes were washed in TBST buffer and incubated with secondary antibody coupled to horseradish peroxidase diluted in TBST supplemented with 5% fat-free milk. Membranes were further processes using the chemoluminescence Immobilion reagent (Millipore), exposed to autoradiographic films. For loading control analysis, immunoblots were stripped and probed with α-tubulin or β-actin primary antibody solution.

Protein band densities were determined by scanning the blots on a professional scanner (Epson 4990). Each image was subjected to quantification using ImageJ software (version 1.45s, National Institutes of Health, USA). Plotted data represent the ratio of normalized

pixel band values obtained for the studied protein with respect to those measured for the loading control protein.

Antibodies

The following antibodies were used: anti-NRL polyclonal antibody, a gift from Dr A. Swaroop (Swain, Hicks et al. 2001); anti-CRX polyclonal antibody (La Spada, Fu et al. 2001) and anti-NR2E3-p183 polyclonal antibody (Peng, Ahmad et al. 2005), gifts from Dr S. Cheng; anti-β-actin (Sigma, L'Isle d'Abeau Chesnes, France); anti-α-tubulin (Abcam, Paris, France); anti-rabbit and anti-mouse light chain specific horseradish peroxidase-conjugated anti-IgG antibodies (Beckmann-Coulter, Nyon, Switzerland).

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Saint Aubin, France) containing 10% fetal bovine serum, 100 units/ml penicillin G and 100 µg/ml streptomycin and 2 mM L-glutamine. Cells at 80% confluence were split and seeded in 24-well plates (40000 cells/well). After 24 h cells were transiently transfected using XtremeGENE 9 (Roche Applied Science) according to the manufacturer's protocol.

HEK293T cell co-transfections were performed using 0.5 µg of the reporter plasmid expressing firefly luciferase under the control of mouse *Nrl* promoter (-938 to +119) (Kautzmann, Kim et al. 2011) and the following expression plasmids: i. pSV-CLOCK and CMV-BMAL1 (Travnickova-Bendova, Cermakian et al. 2002) (0.1 µg each); ii. FLAG-tagged CMV-RORβ (Science Applications International Corporation, Frederick, MD) (0.3 µg); iii. pcDNA4c-RevErbα (Cheng, Khanna et al. 2004) (0.3 µg). Co-transfection mixtures contained also 0.001 µg of the second reporter plasmid, CMV-*Renilla* (Promega, Madison, WI), and the empty pcDNA4c (Life Technologies,) vector to adjust the total amount of transfected DNA to 1 µg. Cells were harvested 48 h after transfection and lysed in 100 µl of passive lysis buffer (Promega).

Dual luciferase assays

Firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega) and measured with Glomax luminometer (Turner

BioSystems, Sunnyvale, CA). *Renilla* luciferase activity was used as internal control for transfection efficiency. All the transfections were done in triplicate.

Statistical analysis

Results are presented as mean ± SEM.

Differences among groups were analysed using the one-way ANOVA analysis of variance *(P* < 0.05 was considered significant) (STATISTICA 8.0, StatSoft Inc., Tulsa, OK, USA) and post-hoc analysis (Tukey's HSD test).

Data were further analysed by the cosinor method (Sigmaplot V 10.0, Systat Software Inc., San Jose, CA, USA): rhythmicity in gene expression was assessed by fitting the 24 h data to a cosine curve $f=a+(b*\cos(2*3.1416*(x-c)/24))$ (Nelson, Tong et al. 1979), with *x* indicating the time (h), *a* indicating the mean value of the cosine curve (mesor), *b* indicating the amplitude of the curve (half of the sinusoid) and *c* indicating the acrophase (h).

Results

Rhodopsin gene expression in whole rat retina

Profiles of *rhodopsin* were analysed over 24 h in whole rat retinas under LD and DD conditions. In LD (n = 3-5 per time point, total n = 24) a statistically significant temporal variation was found for *rhodopsin* transcript by one-way ANOVA analysis (Figure 1) (*rhodopsin* $F_{5,18}$ = 8.9419, P = 0.0002). Cosinor analysis confirmed a daily rhythmic pattern for *rhodopsin* (P < 0.01, Table 2), with a peak at ZT23.

In DD conditions (n = 3-6 per time point, total n= 32), *rhodopsin* transcript (Figure 1) did not show any significant temporal variation (Table 2).

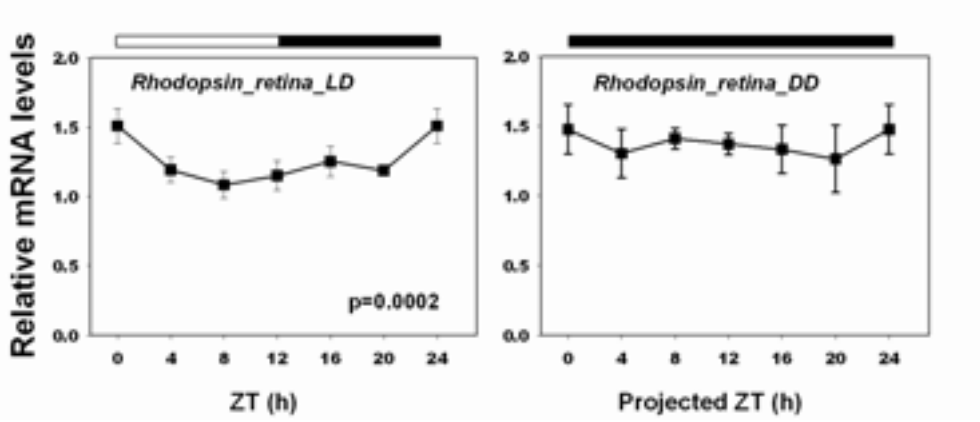

Figure 1

Expression profiles of *rhodopsin* transcript in rat retina under LD and DD conditions during 24 h (white bar, light period; black bars, dark period). Values represent mean ± SEM. Significant temporal variations (P < 0.05) are indicated. Data for ZT0 are double-plotted at ZT24.

Nrl and Crx expression in rat photoreceptor layers

Nrl and *Crx* are photoreceptor-specific genes encoding the major transcriptional regulators of *rhodopsin* promoter in the adult retina by acting on neighbouring enhancer sequences (Mitton, Swain et al. 2000; Cheng, Khanna et al. 2004). 24 h expression kinetics of *Nrl* and *Crx* transcripts in LD conditions (n = 5 per time point, total n = 30) showed strong daily variations (Figure 2A) by one-way ANOVA analysis: *Nrl* $F_{5,24}$ = 2.78, *P* = 0.04; *Crx* $F_{5,24}$ = 12.07, *P* = 0.0001. Expression of these genes showed circadian rhythmicity with acrophases around ZT1 for *Nrl* ZT20 for *Crx* (Table 2, cosinor analysis $P < 0.05$). In DD conditions (n = 3-8 per time point, total n = 33) the amplitude of *Nrl* and *Crx* profiles was strongly reduced with respect to LD condition and no significant temporal variation could be revealed by one-way ANOVA analysis (Figure 2A) (*Nrl* $F_{5,27}$ = 1.14, *P* = 0.37; *Crx* $F_{5,27}$ = 2.14, *P* = 0.09).

To correlate gene transcript variation with protein expression, we harvested retinas of animals sacrificed every 4 hours over a 24 h period (ZT0-20) (n = 3-4 animals per time point, total n = 22). We extracted whole retina proteins and performed immunoblotting. Figure 2B shows the daily variations of NRL and CRX with their respective immunoblots. NRL shows rhythmic protein expression, according to one-way ANOVA analysis (*F(5,16)* = 2.8618, *P* = 0.04948), with acrophase around ZT7-8 (Table 3, cosinor analysis $P < 0.05$). CRX expression also revealed significant changes over 24 h (*F(5,16)* = 10.911, *P* = 0.0001) but no rhythmic profile (Table 3).

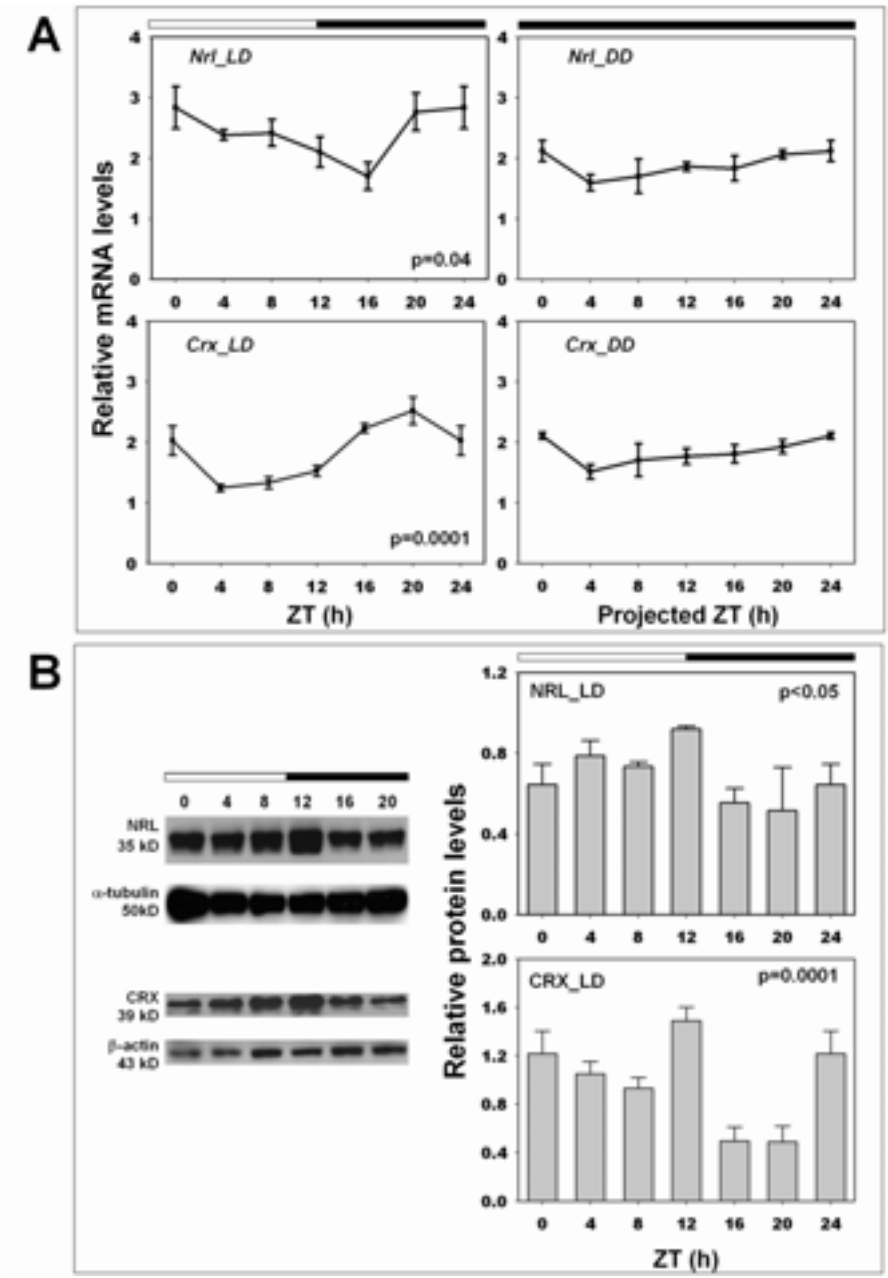

Figure 2

Expression kinetics of *Nrl* and *Crx* transcripts and protein products.
A. 24 h expression profiles of *Nrl* and *Crx* transcripts in rat photoreceptors under LD and DD conditions were analysed by qPCR; B. Daily profiles of NRL and CRX protein levels in the retina of rats maintained in LD. Protein levels were analyzed in total extracts and quantified by Western blots. *Left panel*, representative immunoblots of LD samples pooled per time point (ZT). Respective molecular weights (kDa) are reported *Right panel,* values are shown as relative amount of protein after normalization with the indicated reference protein.
Data are expressed as mean ± SEM. Significant temporal variations ($P < 0.05$) are indicated. (White bar, light period, black bar, dark period). Data for ZT0 are double-plotted at ZT24.

	a (mesor)	b (amplitude)	c (acrophase) (h)	*F*-value	*P*-value
Retina LD					
Rhodopsin	1.26 ± 0.03	0.13 ± 0.04	22.83 ± 1.21	*F(2,21)* = 5.7967	0.0099
Nr2e3	0.68 ± 0.03	0.18 ±0.05	23.84 ± 1.05	*F(2,17)* = 8.1446	0.0033
Retina DD					
Rhodopsin	1.37 ± 0.03	0.02 ± 0.04	3.82 ± 6.57	*F(2,29)* = 0.1801	0.8362
Photoreceptors LD					
Nrl	2.36 ± 0.10	0.43 ± 0.15	0.87 ± 1.37	*F(2,27)* = 3.9153	0.0321
Crx	1.81 ± 0.06	-0.66 ± 0.09	19.20 ± 0.50	*F(2,27)* = 29.6640	<0.0001
Photoreceptors DD					
Nrl	1.89 ± ∞	0.12 ± ∞	0.00 ± 0.00	*F(2,30)* = 0.7083	0.5005
Crx	1.84 ± ∞	0.13 ± ∞	0.00 ± 0.00	*F(2,30)* = 1.3029	0.2867

Table 2

Cosinor analysis of mRNA levels of clock output genes in whole retina and isolated photoreceptors.

Nr2e3 expression in whole rat retina

Photoreceptor specific transcription factor NR2E3 was also reported to regulate *rhodopsin* expression (Cheng, Khanna et al. 2004; Cheng, Aleman et al. 2006). *Nr2e3* transcript levels show a strong tendency to vary with time (*F(5,14)* = 2.8780, *P* = 0.054) (Figure 3A). These data were confirmed by cosinor analysis (Table 2, *P* < 0.05). Figure 3B shows the daily variation of NR2E3 protein with its respective immunoblot. NR2E3 protein expression profile shows strong variation over 24 h as evidenced by one-way ANOVA analysis (*F(5,16)* = 22.454, *P* < 0.0001) and peaks around ZT7 (Table 3, cosinor analysis *P* < 0.05).

	a (mesor)	b (amplitude)	c (acrophase) (h)	*F-value*	*P-value*
Retina LD					
NRL	0.69 ±0.03	0.15 ±0.05	8.38 ±1.31	*F(2,19)* = 3.9596	0.0365
CRX	0.93 ±0.07	0.29 ±0.11	7.27 ±1.39	*F(2,19)* = 3.1559	0.0656
NR2E3	0.70 ±0.02	0.24 ±0.03	6.88 ±0.49	*F(2,19)* = 26.3104	<0.0001

Table 3

Cosinor analysis of clock output protein levels in rat whole retina.

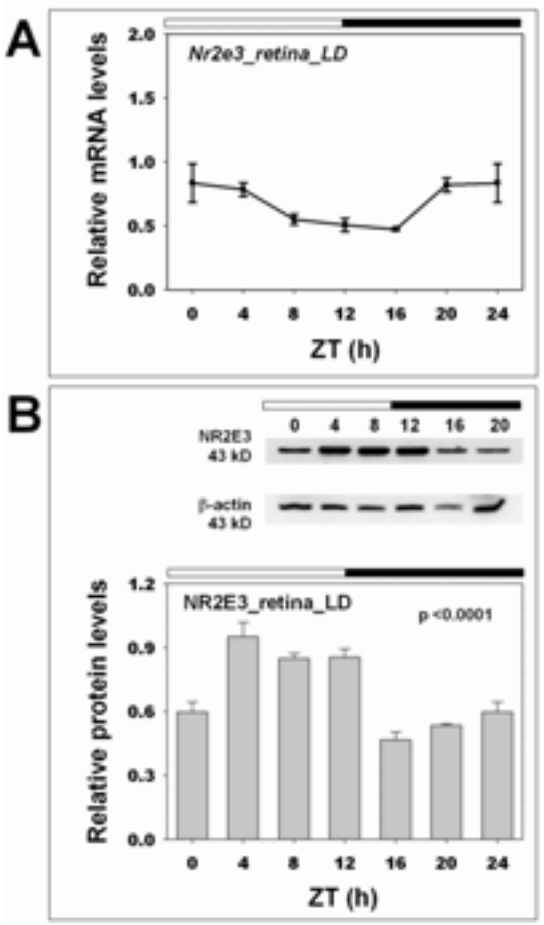

Figure 3

Expression kinetics of *Nr2e3* transcript and protein product.

A. 24 h expression profile of *Nr2e3* transcript in the rat retina under LD condition was obtained by qPCR; B. Daily profiles of NR2E3 protein levels in the retina of rats maintained in LD. Protein levels were analyzed in total extracts and quantified by Western blots. *Left panel*, representative immunoblots of LD samples pooled per time point (ZT). Respective molecular weights (kDa) are reported *Right panel,* values are shown as relative amount of protein after normalization with β-actin.

Data are expressed as mean ± SEM. Significant temporal variations ($P < 0.05$) are indicated. (White bar, light period, black bar, dark period). Data for ZT0 are double-plotted at ZT24. Here *Nr2e3* transcript levels were normalized with respect to *Pde6b* only.

Effects of clock transcription factors on mNrl promoter

We tested the abilities of clock factors to transactivate luciferase reporter activity driven by mouse *Nrl* promoter fragment (-938 to +119), previously reported to be sufficient and necessary for rod-specific expression (Kautzmann, Kim et al. 2011) and to carry E-box (-74) and RORE (Retinoic acid-related Orphan Receptor binding Element; -783) sequences . We performed luciferase assays to compare the transactivation abilities of different factors reported to be involved in the molecular clock machinery. Co-expressed CLOCK and BMAL1 did not lead to any increase in *Nrl* promoter activity (Figure 4), although they strongly stimulated the promoter of the *Per1* clock gene in parallel assays (data not shown). In contrast, REVERBα and RORβ triggered statistically significant increase of *Nrl* promoter activity as compared to its basal level. These data suggest that the daily regulation of *Nrl* may be controlled by secondary clock factors rather than by the direct action of the core clock elements CLOCK and BMAL1.

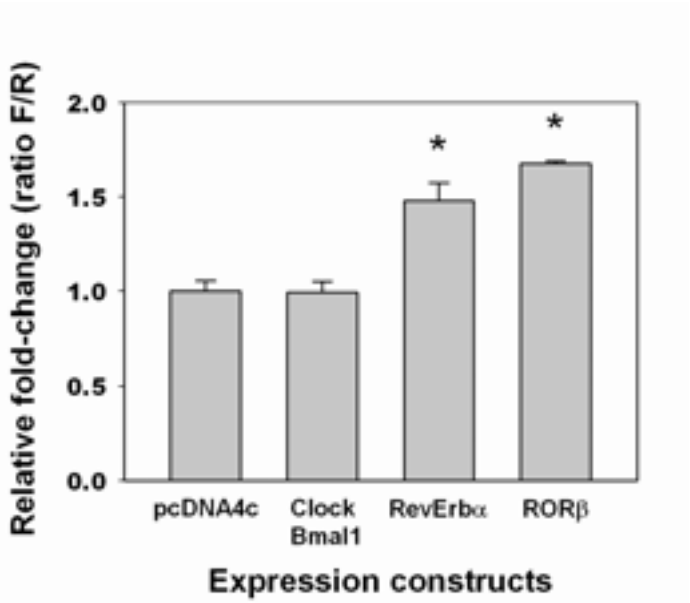

Figure 4

Transcriptional effect of clock factors on *Nrl* promoter. HEK293T cells were co-transfected with a construct of m*Nrl* (-938 to +119) promoter region linked to firefly luciferase reporter gene and a combination of CLOCK plus BMAL1 expression vectors (0.1 µg each), or with of RevErbα alone (0.3 µg), or RORβ alone (0.3 µg). Fold-changes are relative to the mock expression of pcDNA4c control vector. Data (ratio of firefly to *Renilla* luciferase activity) are expressed as relative to the mock expression. Each bar represents the mean ± SEM (n = 3). Asterisks indicate $P < 0.05$.

Discussion

Photoreceptor properties are synchronized to the light/dark cycle allowing organisms to optimize visual function to each photic situation. Although molecular mechanisms regulating outputs have not been yet characterized, it is clear that a circadian clock plays a central role in the temporal distribution of retina specific functions over the 24 h cycle (Storch, Paz et al. 2007). Altered expression of *rhodopsin* gene induces photoreceptor degeneration, suggesting that a strict control of its synthesis is required for retina homeostasis (Humphries, Rancourt et al. 1997; Tan, Wang et al. 2001). In the present study, we investigated 24 h expression profiles of *rhodopsin* and of its principal regulators NRL, CRX and NR2E3 and show that all of them display rhythmic expression in LD, with peaks occurring by the end of the night or beginning of the day. None of these genes was found rhythmic in DD, although mRNA profiles look rather similar to the ones in LD, probably owing to decreased amplitude of circadian oscillations previously observed in photoreceptors in DD, for clock genes as well as for outputs (Sandu, Hicks et al. 2011). We also provide evidence that *Nrl* transcription might be controlled by clock factors REVERBα and RORβ.

By performing qPCR analysis with whole retina, we show that in Wistar rats, *rhodopsin* expression is rhythmic in LD with maximal transcript amount by the end of the night. This result is in agreement with what was reported in mice (Bowes, van Veen et al. 1988) but was found different in regard to data obtained previously in Fisher rats (Sakamoto, Liu et al. 2006) and likely reflects strain differences. Interestingly, levels of *α-transducin* mRNA also peak by the night/day transition, indicating that expression of some phototransduction cascade elements cluster around that moment (Brann and Cohen 1987). To assess this hypothesis we analyzed 24 h expression profiles of *recoverin*, another gene associated with phototransduction which product inhibits rhodopsin kinase and sustains light effects on rhodopsin. Since recoverin was shown to be expressed in the inner retina (McGinnis, Stepanik et al. 1999), we performed the qPCR analysis on vibratome isolated photoreceptor layers and found that this gene also displays sustained rhythmicity in LD with peak expression around ZT21 and a large dampening in DD (Supplemental data, Figure S1). Thus, several key players of the phototransducing

function appear to be maximally co-expressed around the transition between night and day in the Wistar rat. While we do not have any clear explanation for the occurrence of these peak expressions around that time of the cycle, it is possible that it reflects similar regulatory mechanisms allowing coincidence between synthetic pathways and catabolic processes such as outer segment disk phagocytosis which takes place just after lights on (Bobu and Hicks 2009).

Our results show that expression of three photoreceptor-specific transcription factors varies along the day/night cycle, both at the level of their mRNA and gene products which levels increase from the beginning of the light phase and peak 7 to 11 hours after their respective transcript. Interestingly, mRNA profiles also display maxima by the end of the night/beginning of the day, as was also reported for *Crx* and *Nr2e3* upon microarray analysis of the circadian eye transcriptome: peak expression was found at ZT20.5 and ZT4.5 respectively (Storch, Paz et al. 2007). However, *Crx* rhythmicity was not observed in a previous Northern blot study performed in whole retinas from Wistar rats (Sakamoto, Oishi et al. 1999) possibly because of the lower dynamic range of the technique. Because *Nrl* has been reported to be a “master gene” for rod differentiation and the principal activator of *rhodopsin* promoter, we further investigated its daily regulation. Transcriptional activity of NRL, notably toward *rhodopsin* promoter, is regulated by post-translational mechanisms involving phosphorylation (Bessant, Payne et al. 1999; Kanda, Friedman et al. 2007) and sumoylation (Roger, Nellissery et al. 2010). We did not detect any variation in NRL apparent mobility in western blot, which remained at the highest apparent molecular weights (3 bands around 30-35kDa) (Figure 2B) described to correspond to phosphorylated proteins (Swain, Hicks et al. 2001) indicating that the daily regulation of *Nrl* principally takes place at the transcriptional level. We recently mapped the minimal promoter of *Nrl* required for rod-specific expression in the postnatal retina and described the presence of both RORE and E-box sequences therein (Kautzmann, Kim et al. 2011). Here we investigated whether *Nrl* promoter could be a target for clock factors binding to these elements and found that it is not transactivated by the CLOCK/BMAL1 dimer but shows significant stimulation by two clock components that are also involved in photoreceptor development (Swaroop,

Kim et al. 2010; Mollema, Yuan et al. 2011; Forrest and Swaroop 2012): RORβ and, to a lesser extent but still significant, by REVERBα (Figure 4). We previously reported that RORE site is bound by RORβ and that this factor is required for expression of *Nrl* in rods (Kautzmann, Kim et al. 2011). In addition, *Rorβ* expression shows robust rhythmicity in photoreceptors in LD, with maximum occurring at ZT20 (Sandu, Hicks et al. 2011), a few hours before the peak of *Nrl* transcript. Thus, it is possible that RORβ takes part in the rhythmic regulation of *Nrl* gene. While REVERBα was essentially described as a transcriptional inhibitor, we found a mild activating effect on *Nrl* promoter. Similar positive effect was reported on the promoter of *rhodopsin*, yet in the context of a transcriptional complex containing other transcription factors and in which REVERBα might play an unusual role. Since *Reverbα* also cycles in photoreceptors with peak expression around ZT22, further analysis is required to definitely understand the role of this factor in *Nrl* regulation and notably the competitive effect it might display towards RORβ effect.

Taken together, our results suggest that synchronization of rhythmic expression patterns by the LD cycle in photoreceptors likely proceeds from a complex interplay of transcription factors. We provide evidence that NRL expression is regulated by the clock factor RORβ. One could hypothesize that NRL, together with CRX and NR2E3 which likewise peak around the middle of the day, contribute to rhythmic expression of *rhodopsin* gene. Interestingly, REVERBα which is also part of the photoreceptor clock is recruited by direct interaction with NR2E3 into this complex and also provides activating effect (Cheng, Khanna et al. 2004). Respective contribution of each of these factors will need additional studies. While our study, together with results from other groups, put forward the expression of *rhodopsin*, *recoverin*, *Nrl*, *Crx* and *Nr2e3* around the end of the night, it did not explore the possible contribution of the cAMP/PKA/CREB pathway which is maximally activated by the end of the dark phase as well and was shown to regulate other clock outputs in photoreceptors Effects of cyclic AMP regarding the studied genes will require further experiments. Finally, alternative post-transcriptional regulatory mechanisms involving mRNA decay, deadenylation of polyA tails or microRNAs (Tan, Wang et al. 2001; Staiger and Koster 2010) should not be forgotten in order to get a

complete picture of the molecular pathways linking the photoreceptor clock to its outputs.

Supplemental data

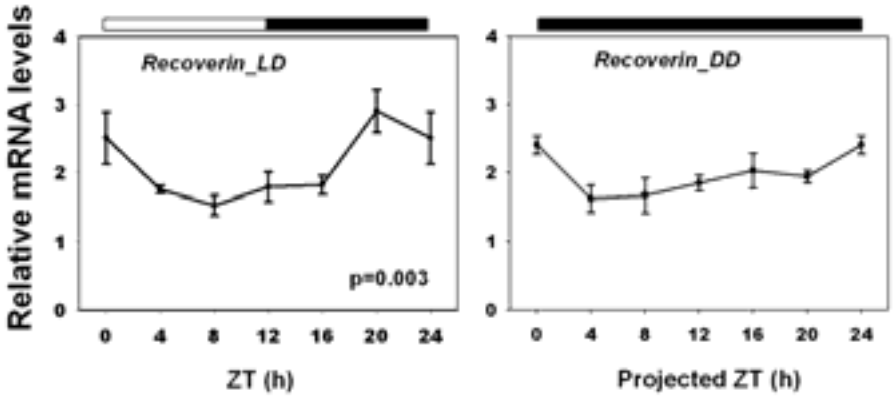

Figure S1

Expression profiles of *recoverin* transcript in isolated photoreceptors under LD and DD conditions during 24 h analysed by qPCR (inventoried TaqMan assays Rn00590194_m1, Applied Biosystems) (white bar, light period; black bars, dark period). Significant temporal variation ($P < 0.05$) is indicated: LD, $F_{5,24} = 5.08$, $P = 0.003$; DD, $F_{5,27} = 2.41$, $P = 0.06$. Rhythmicity in LD conditions was confirmed by cosinor analysis (mesor, 2.05 ± 0.1; amplitude 0.62 ± 0.14; acrophase 21.18 ± 0.87 h, $F_{2.,27} = 9.6181$, $P = 0.0007$) and the tendency to be rhythmic in DD conditions was shown (mesor, 1.98 ± 0.08; amplitude 0.31 ± 0.12; acrophase 21.09 ± 0.87 h, $F_{2.,30} = 3.2393$, $P = 0.0532$). Data for ZT0 are double-plotted at ZT24. Values represent mean ± SEM.

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IV. A novel transcription factor, NonO, appears as a co-activator of *rhodopsin* expression and is necessary for rod-photoreceptor survival.

BIOLOGICAL SCIENCES **Biochemistry**

The non-POU domain containing octamer binding protein NonO/p54^{nrb} augments *rhodopsin* transcription synergistically with NRL and CRX

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Abstract

Rhodopsin is the visual pigment in outer segments of rod photoreceptors that constitute around 70% of cells in the retina of most mammals. The daily renewal of almost 10% of outer segment discs requires a stringent homeostatic control to maintain high levels of rhodopsin expression. The expression of *rhodopsin* is controlled by two *cis*-regulatory sequences upstream of the transcription start site – a proximal promoter region (RPPR) and an enhancer region (RER). RPPR contains binding sites for the two key transcription regulatory factors – neural retina leucine zipper (NRL) and cone rod homeobox (CRX). However, a larger genomic region, including RER, is essential for precise high-level *rhodopsin* expression *in vivo*. Here, we report the identification of RER binding proteins from bovine retinal nuclear extract by mass-spectrometric analysis. We detected the largest number of unique peptides for the non-POU domain containing octamer-binding protein (NonO/p54^{nrb}). We show that NonO activates the expression from a *rhodopsin* promoter in HEK293 cells and acts synergistically with NRL and CRX. Furthermore, shRNA knockdown of NonO by *in vivo* electroporation in the mouse retina induced rod cell death. Our studies suggest that NonO functions at the level of the RER to produce high-level expression of rhodopsin and might contribute to coupling of *rhodopsin* transcription with splicing in retinal rod photoreceptors.

Introduction

Complex biological processes, including development and homeostasis, require quantitatively precise expression of genes in specific spatiotemporal patterns (Swaroop, Kim et al. 2010). Regulatory information necessary for the transcription of a gene is generally confined to the proximal promoter region, upstream of the transcription start site (TSS); however, distal sequence elements (called "enhancers") are frequently required for cell type-specific expression (Bulger and Groudine 2010). Enhancer elements are not directionally selective and can exert their influence over long distances (Banerji, Rusconi et al. 1981). Most enhancers contain binding sites for multiple proteins; some of these can associate with transcriptional co-activators, relocate into physical proximity of TSS through a looping mechanism, and enhance RNA polymerase II-mediated gene expression (Visel, Rubin et al. 2009). The combinatorial interaction of specific factors that bind to promoter and/or enhancer elements together determines the activation or repression of a gene (Ravasi, Suzuki et al. 2010). The evolution of enhancers in developmentally regulated genes is believed to exert a major drive for animal morphology (Boffelli, Nobrega et al. 2004). Recent genome wide studies have revealed that a majority of variations associated with complex multifactorial human diseases are present in non-coding regions, and many of these may be within potential enhancer sequence elements (Manolio, Collins et al. 2009).

Retinal and macular neurodegenerative diseases are a major cause of incurable blindness (Neitz and Neitz 2011), with photoreceptor death being the primary cause of visual dysfunction (Malanson and Lem 2009). Rod photoreceptors constitute ~70% of the retinal cells in most mammals and contain the visual pigment, rhodopsin (Curcio, Sloan et al. 1990). Rhodopsin represents as much as 90% of the total protein in rod outer segments (ROS) that capture photons to initiate visual transduction (Deretic 2006). The renewal of almost 10% of ROS discs, subsequent to their daily circadian clock-regulated shedding requires quantitatively precise expression and transport of rhodopsin and other phototransduction proteins (Young 1967; Winkler 2008). Indeed, abnormal expression and/or trafficking of rhodopsin are associated with dysfunction or death of rod photoreceptors (Malanson and Lem 2009)**.**

The expression of *rhodopsin* is primarily regulated at the level of transcription. Two distinct sequence elements have been defined upstream of rhodopsin TSS: Rhodopsin Proximal Promoter Region (RPPR) and Rhodopsin Enhancer Region (RER) (Kumar, Chen et al. 1996; Nie, Chen et al. 1996). RPPR includes about 200 bp sequence, immediately upstream of TSS, that can direct rod-specific expression of a reporter gene in the transgenic mice (Zack, Bennett et al. 1991). Electrophoretic mobility shift assays and promoter activity analyses have delineated the binding sites for multiple transcription factors in RPPR; these include basic motif leucine zipper transcription factor NRL, cone rod homeobox CRX, and orphan nuclear receptor NR2E3 (Chen and Zack 1996; Rehemtulla, Warwar et al. 1996). Loss of *Nrl* results in a cone-only retina with no rhodopsin expression (Mears, Kondo et al. 2001), whereas loss of *Crx* leads to rods but no outer segments and minimal rhodopsin expression (Furukawa, Morrow et al. 1999), establishing their synergistic and pivotal role in regulating rhodopsin expression (Mitton, Swain et al. 2000; Hao, Kim et al. 2012).

Transgenic mouse studies with murine or bovine RPPR revealed somewhat leaky and lower level of reporter gene expression (Zack, Bennett et al. 1991); however, a larger approximately 5 kb upstream fragment demonstrated more precise and high expression levels (Zack, Bennett et al. 1991), suggesting the importance of sequences upstream of RPPR in determining *rhodopsin* expression *in vivo*. Footprinting of bovine *rhodopsin* promoter identified a highly conserved sequence RER, approximately 2 kb upstream of TSS (Nie, Chen et al. 1996). More recently, RER was shown to contact physically with RPPR and within the rhodopsin coding region by intrachromosomal loop formation in rod photoreceptors while maintaining a linear configuration in other cell types (Peng and Chen 2011). Despite its demonstrated significance, RER binding proteins have not been identified as yet.

In this study, we took a proteomic approach to identify protein, which bind to and might be involved in bringing RER in close proximity to RPPR in order to achieve quantitatively precise expression of rhodopsin in rod photoreceptors. We hypothesize that being a highly transcribed gene in rod photoreceptors, the splicing of *rhodopsin* pre-messenger RNA might be coupled to transcription of this gene by looping of RER on RPPR. By mass spectrum analysis we identified several candidate proteins bound to RER and selected

the non-POU domain containing octamer-binding protein (NonO/p54^{nrb}) for downstream analysis since it showed the maximum number of peptides. Here we report that NonO can activate NRL and CRX transcriptional activity on the *rhodopsin* promoter. Finally, we looked at the effect of the NonO gene transcript knockdown in neonatal murine retina and evaluated the survival of rod photoreceptors.

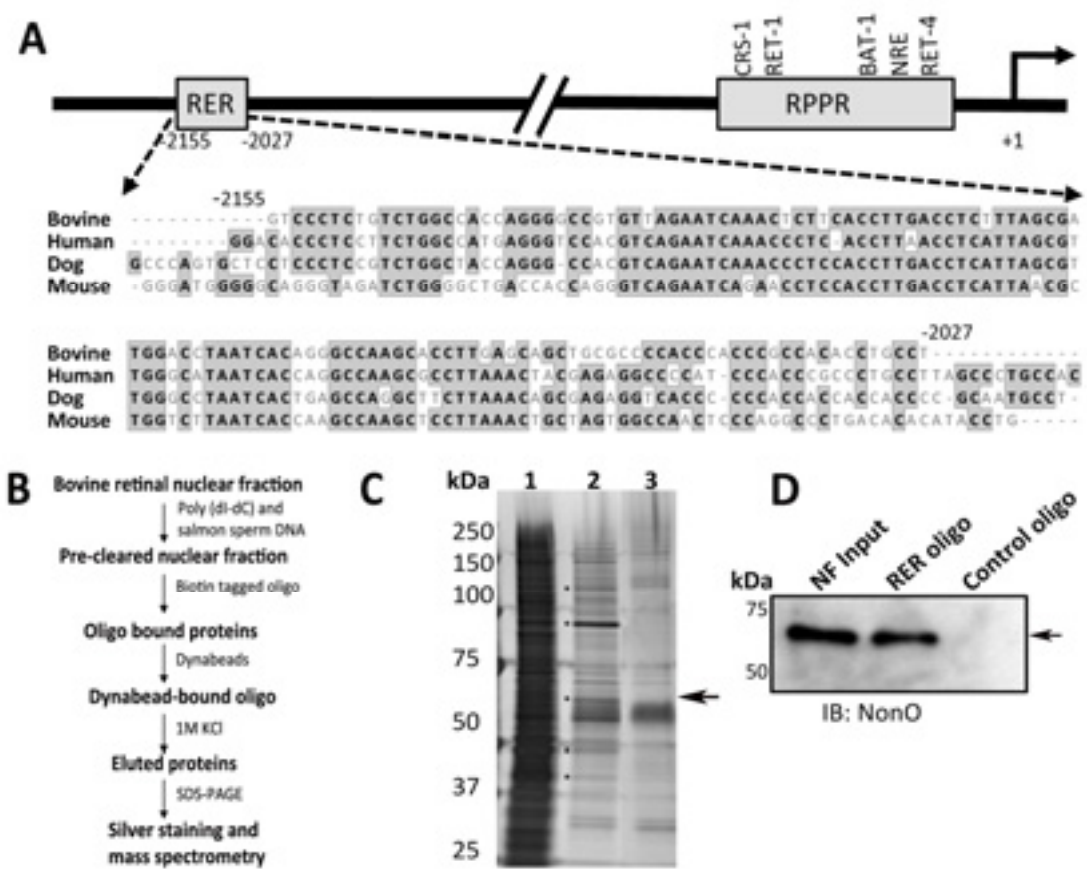

Figure 1

Schematic representation showing upstream regulatory elements in *rhodopsin* promoter, sequence alignment of RER and scheme of the isolation of bovine retina proteins binding to it . Panel (A) shows the proximal and distal elements in bovine *rhodopsin* promoter and sequence alignment of distal enhancer region (RER) in different mammalian species. Shaded regions represent the conserved sequences. Panel (B) shows the flow chart of the isolation scheme for the control and RER oligonucleotide-binding proteins from the bovine retinal nuclear extract. Panel (C) silver stained SDS PAGE gel showing the different eluted proteins bound to *rhodopsin* RER and control oligonucleotide from bovine retina. Lanes: 1- Input nuclear fraction, 2-RER- and 3-Control oligonucleotide eluted proteins. Asterisks indicate the protein bands used for mass spectra analysis. Panel (D) shows the western immunoblot with NonO antibody of the RER and control oligonucleotide eluted fractions. Arrow indicates the NonO band.

Results

Identification of proteins binding to RER of bovine rhodopsin promoter.

To identify factors binding to RER, we first analyzed the bovine *rhodopsin* RER genomic sequence using MacVector (version 11.11.1). Phylogenetic comparison of the -2155 to -2027 region relative to TSS revealed the presence of several evolutionarily conserved elements (Figure 1A). RER contains predicted binding sites (Transfac software AliBaba2.1) for transcription factors and co-activators such as ERα, RXRβ, RevErbα, RARα1, MyoD, NF-1, SP-1, ATF, COUP, GATA-1, E1, ACE2 and ARP-1 however their functional relevance in *rhodopsin* regulation is not yet reported. To identify cis-regulatory factors binding to *rhodopsin* RER, we generated a biotin tagged 129 bp RER oligonucleotide based on the bovine sequence and a scrambled oligonucleotide as control. Bovine retinal nuclear fraction was prepared by differential centrifugation and incubated with biotin tagged oligonucleotides (Figure 1B). Streptavidin tagged Daynabeads were used to isolate RER- and scrambled oligonucleotide-bound proteins. After several washings, RER and control oligonucleotide bound protein complexes were eluted in high salt buffer, further resolved on SDS-PAGE and visualized by silver staining (Figure 1C). Five protein bands present in the RER but absent in the scrambled oligonucleotide elute lane (Figure 1C, asterisks) were selected and excised out from the gel for subsequent mass spectrometry analysis. Proteins for which two or more unique peptides were obtained from the mass spectra analysis, were considered significant (Table 1). Gene ontology analysis of the identified proteins revealed that they were mostly involved in splicing, transcription, cell cycle regulation and signal transduction. A few were protein kinases and phosphatases modulating the activity of a wide range of regulatory proteins. Several candidate co-activators bound to RER were identified, such as NonO, PPP1CA, BUB3, CCAR1, 14-3-3γ, SAP180, HNRPM and P. Some of these proteins are known to be part of transcriptional complexes involved in the regulation of nuclear receptor gene promoters. We selected for further investigation the non-POU domain containing octamer-binding protein (NonO/p54^{nrb}) that returned the highest number of unique peptides in our mass spectrometry analysis. NonO protein binding specificity to RER oligonucleotide as compared to scrambled oligonucleotide was confirmed by Western blot analysis of eluates (Figure 1D).

In summary, we identified several candidate proteins that bind to *rhodopsin* RER in rod photoreceptors and identified a candidate protein, NonO, which may be involved in rhodopsin regulation.

NonO physically interacts with RER and acts in synergy with NRL and CRX to activate rhodopsin promoter.

To investigate the functional correlation between NRL, CRX and NonO, we performed reporter gene assays using a 2.2 kb bovine *rhodopsin* promoter-*firefly luciferase* reporter containing both RPPR and RER *cis* regulatory regions (Figure 2A). A plasmid containing *CMV* promoter-driving *renilla luciferase* was used as control. HEK 293 cells were used to examine promoter activity. Transfection of NonO alone in HEK 293 cells resulted in minimal dosage-dependent increase of *rhodopsin* promoter- *luciferase* reporter activity. Either NRL or CRX alone transactivated the *Rhodopsin* promoter two to three times more than NonO. However, co-transfection of NonO with NRL and CRX resulted in ~ 80-fold increase over background in *rhodopsin*-luciferase reporter activity. In contrast, only minimal effect was observed when the luciferase reporter driven by the 130 bp bovine rhodopsin promoter lacking RER, was co-transfected with NonO, NRL and CRX (Figure 2B). This suggests that NonO specifically acts on RER and enhances the transactivation activities of NRL and CRX on *rhodopsin* promoter. To test whether bovine NonO interacts directly with NRL or CRX, we performed immunoprecipitation from bovine retinal extract with anti-NonO, anti-NRL, anti-CRX anti-bodies. Immunoprecipitation with anti-RNA Polymerase II (Pol II) antibody was used as positive control and anti-Brn3b was used as negative control. Blots were immunoreacted with anti-NonO antibody. Upon cross-linking, NonO was immunoprecipitated from bovine retinal extract using NRL, CRX and Pol II antibodies, but not with anti-Brn3b and control rabbit IgG (Figure 2C). However, immunoprecipitation of NonO with NRL and CRX was not observed in the absence of cross-linking from bovine retina. These results suggest that NonO-containing transcriptional complexes are in close proximity to the NRL- and CRX-containing complexes on the *rhodopsin* promoter. However, we did not get a direct interaction of these proteins in bovine retina.

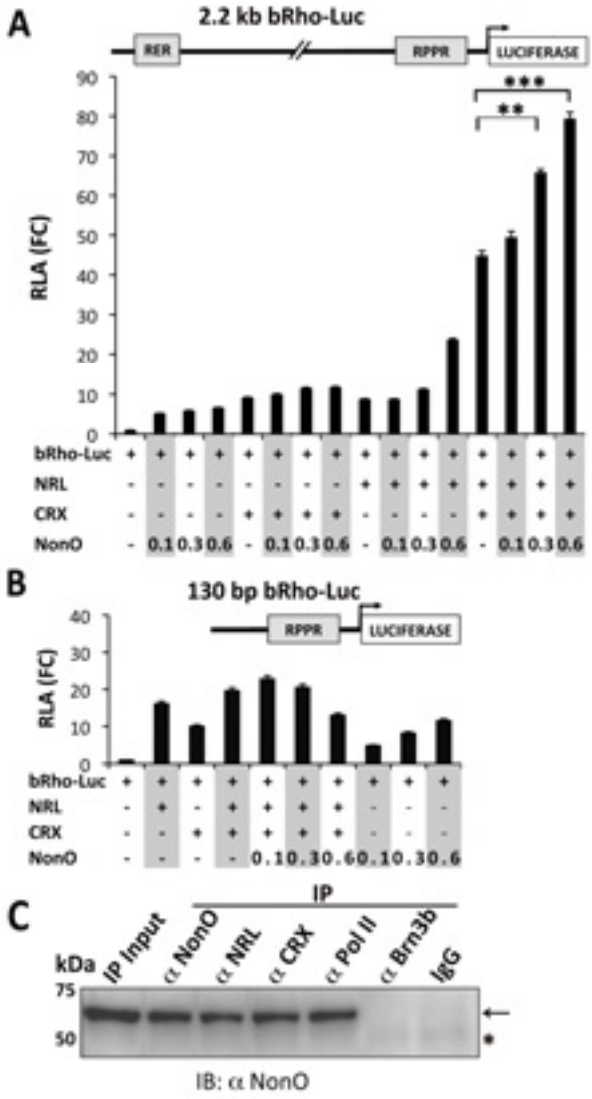

Figure 2

(A) **Luciferase reporter assay showing transactivation of the 2.2 kb bovine *rhodopsin* promoter by NonO**. HEK293 cells were co-transfected with 2.2 kb bovine *rhodopsin* promoter driving *firefly luciferase* reporter (0.2 µg) and increasing concentrations (0.1–0.6 µg) of NonO alone or in association with 0.1 µg of either NRL or CRX or both. Fold change is relative to the mock expression vector control. All the experiments were done in triplicate. T test was performed using Prism software version 5 and P values <0.05 were considered significant. P values <0.05 to 0.01 were given two asterisks and <0.01 were given three asterisks. (B) HEK293 cells were co-transfected with 130 bp bovine *rhodopsin* promoter driving *firefly luciferase* reporter (0.2 µg) and increasing concentrations (0.1–0.6 µg) of NonO alone or in association with, 0.1 µg of NRL and CRX together. Fold change is relative to the mock expression vector control. All the experiments were done in triplicate. T test was performed using Prism software version 5 and P values <0.05 were considered significant.

This may be due to transient interaction of these proteins on RPPR due to post-translation modifications. Furthermore immunoprecipitation of the NRL and CRX with NonO was also performed from transfected HEK293 cells. Indeed NonO can pull down NRL and CRX from the transfected HEK293 cell extracts. This suggests that NonO might be involved in looping of RER on RPPR by interacting with NRL and CRX. In summary NonO specifically binds to RER and enhances the quantitatively precise expression of *rhodopsin* by transiently interacting with the NRL and CRX containing complexes present on the RPPR and modulating the activity of the RNA Pol II present on the core promoter.

NonO is required for rod photoreceptor survival.

To investigate the role of *NonO* in maintaining rod photoreceptor homeostasis, we knocked down *NonO in vivo* in neonatal mouse retina using shRNA. We first generated a shRNA targeting exon 2 of the mouse *NonO* transcript and a control shRNA of the same nucleotide composition but scrambled. To visualize the transfected cells, both shRNA constructs contained GFP driven by *signal recognition particle alpha* promoter and shRNA hairpins driven by *histone H1* promoter. We then assessed the effects of the knockdown in the developing mouse retina. Neonatal mouse retinas were co-electroporated with 2.2 kb bovine *rhodopsin* promoter driving DsRed (b*Rho*DsRed) and either control or *NonO*-shRNA constructs. Fluorescent reporter gene expression was analyzed at P7, P10 and P21 (Figure 3). Our results show that almost all the cells expressing DsRed reporter in the outer nuclear layer (ONL) were co-labeled with GFP reporter in P10 control, however in *NonO*-shRNA transfected retinas, we observed a diminution of the cells co-expressing both DsRed and GFP (Figure 3a and b, respectively) . At P21, only a few co-labeled cells were found in the ONL (Figure 3c, d) in *NonO*-shRNA treated retina but not in control retina. To further investigate the causes of rod cells reduction in *NonO*-shRNA electroporated retina, we immunostainned P7 retinal sections with caspase-3, a marker for apoptosis (Figure 3g, h). Immunoreactivity for caspas 3 was stronger in the ONL of *NonO*-shRNA treated retina compared to control. These results suggest that knockdown of *NonO in vivo* in mouse retina reduces the expression of *rhodopsin* and leads to death of rod photoreceptors by apoptosis during early stages of retinal development.

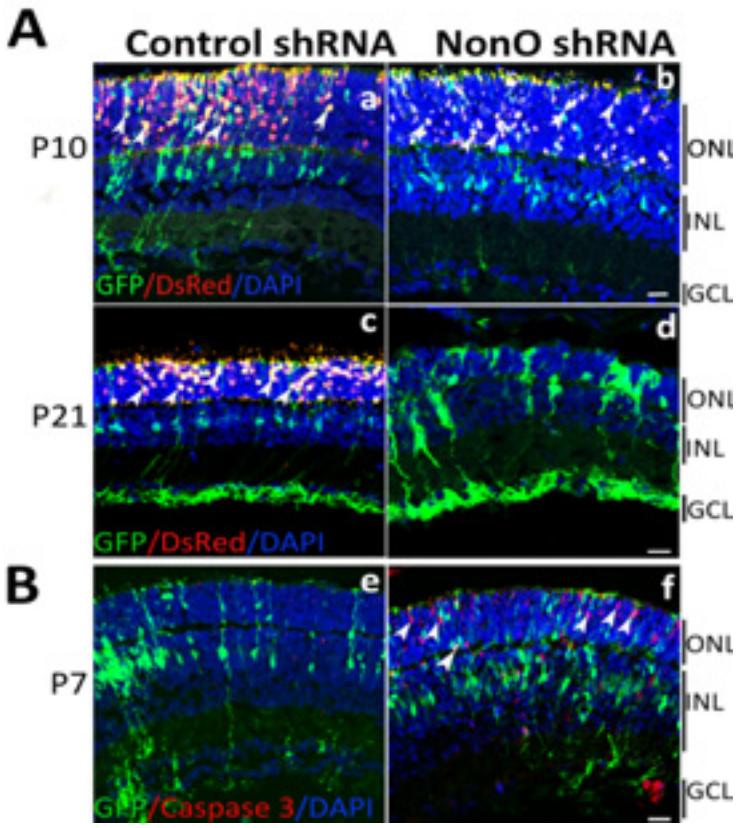

Figure 3

NonO is required for rod photoreceptor survival. (A) Neonatal mouse retinas were co-electroporated with 2.2 kb bovine rhodopsin promoter driving DsRed (b*Rho*DsRed) and either control or *NonO*-shRNA constructs. Fluorescent reporter gene expression was analyzed at P10 and P21. Cells expressing either control or *NonO*-shRNA are green, cells expressing bovine *rhodopsin* promoter-driven DsRed are Red. Nuclei were stained with DAPI. Left panel (a and c) represents control shRNA and right panel (b and d) represents *NonO*-shRNA electroporated retina at P0 and harvested at P10 and P21. Green GFP, red 2.2 kb *rhodopsin* promoter driven DsRed. White Arrow heads indicate the GFP and DsRed double positive cells. White line indicates the 20 µm length of the section. (B) **Depletion of *NonO* by shRNA leads to rod cell death by apoptosis**. Neonatal mouse retinas were electroporated with either control or *NonO*-shRNA constructs. Caspase-3 staining was performed at P7 to find out the rod cell death in *NonO*-shRNA electroporated retina as compared to control. (e) *NonO*-shRNA, (f) Control shRNA. Green GFP, Red caspase-3 and nuclei were stained with DAPI.

Discussion

Regulation of transcription is mainly accomplished through activity of gene promoter and enhancer regions. Although proximal regions of promoters are sufficient for minimal expression of the majority of the genes,those genes which are highly transcribed need additional regulation, which is largely accomplished by enhancers. Enhancers contain multiple binding sites for a variety of transcription factors and act independently of their location, distance or orientation with respect to the gene (Banerji, Rusconi et al. 1981). In some cases, they can even activate transcription of genes located in a different chromosome (Geyer, Green et al. 1990; Lomvardas, Barnea et al. 2006). Clusters of DNA sequences in enhancers can bind combinations of transcription factors and then interact with components of the mediator complex or transcription factor II D (TFIID) to help recruit RNA polymerase II (RNAPII) (Maston, Evans et al. 2006) by looping out the intervening sequences on gene promoters (Schoenfelder, Clay et al. 2010). Non-coding intergenic sequences and most likely enhancers contain the majority of the genetic variations associated with complex human diseases.

Rhodopsin is the major structural protein present in the ROS and required for the outer segment integrity and phototransduction. Mutations in its coding region are associated with rod photoreceptor degeneration. Information about the genetic variations in *rhodopsin* regulatory region is not well characterized but there are evidences that mutations in the proteins binding to RPPR region cause underexpression of *rhodopsin*, which ultimately leads to death of rod cells by apoptosis. Indeed *rhodopsin* null mice have no ROS and undergo degeneration after a month. Due to daily circadian-clock driven shedding of ROS discs a high level of rhodopsin expression is required to maintain the rod photoreceptor homeostasis.

Rhodopsin promoter contains two *cis-regulatory* elements: a RPPR which binds the transcription factors NRL, CRX, NR2E3 and Fiz-1 and recruits RNA Pol II, gives basal level of expression and a second RER region located ~2 kb upstream from the TSS that is required for restricted expression in rods and is bound and regulated by yet unknown factors. Here we show that several *cis*-regulatory proteins bind to distal enhancer region and may be required for quantitative and precise expression of *rhodopsin* in rod cells.

Gene ontology analysis of these identified proteins revealed that they were mostly involved in splicing, transcription, cell cycle regulation and signal transduction. We propose that expression of the *rhodopsin* in rod photoreceptors might be co-transcriptionally coupled to splicing to cope with the timing of ROS biogenesis during daily rhythmic shedding as well as during terminal maturation of the rod cells during retinal development.

It is well established that ROS undergo disc shedding during early morning and need more membrane lipids and proteins particularly rhodopsin which constitutes about 90% of total ROS protein. Presence of several splicing proteins on the rhodopsin RER suggests that its splicing may be coupled with transcription to facilitate high expression in rod photoreceptors. Finding of several splicing proteins such as NonO, PPP1Ca and HNRPM on RER further supports the hypothesis of *rhodopsin* co-transcriptional coupling. Our data appear to be consistent with the ‘‘recruitment’’ model involved in co-transcriptional coupling of splicing to transcription (Munoz, de la Mata et al.), in the sense of a recruitment of splicing regulators at the promoter, or of their interaction with transcriptional regulators associated with the nascent mRNA. However, it is also possible that splicing factors like NonO may be playing a dual role in the expression of the highly transcribed genes such as *rhodopsin* in the retinal rod photoreceptors by altering the kinetics of transcription along with splicing.

We showed that NonO activates rhodopsin expression in *in vitro* cell cultures by interacting with NRL and CRX and increases the recruitment of RNA Pol II on *rhodopsin* promoter. In depth analyses using *rhodopsin* promoter *luciferase* reporter analysis and knockdown of *NonO* by *in vivo* electroporation of the shRNA against it revealed a functional significance of its role in *rhodopsin* expression in rod photoreceptors. Remarkably, *NonO* shRNA knockdown displayed increased death of rod photoreceptors in the retina very early. Therefore these genetic and biochemical lines of evidence suggest that *Nono* is involved in the co-transcriptional coupling of the *rhodopsin* transcription with its splicing.

Notably, two proteins identified by our RER binding/mass spectrometry screening, serine/threonine-protein phosphatase 1 alpha catalytic subunit (Ppp1ca) and

heterogeneous nuclear ribonucleoprotein M (Hnrpm), are known to interact with NonO (Marko, Leichter et al. 2010; Liu, Xie et al. 2011). Furthermore, Ppp1ca modulates the transcriptional activity of several transcription factors including NonO and PSF as well RNA Pol II by changing their phosphorylation status. We hypothesize that NonO forms a regulatory complex with Ppp1ca and Hnrnp on *rhodopsin* RER that in turn loops on RPPR and interacts with the transcriptional complexes containing NRL and CRX in order to enhance the recruitment of RNA Pol II on *rhodopsin* promoter and increase its expression in rod photoreceptors particularly in early morning when its higher amount of synthesis needed (Figure 4). Similarly, recent reports have shown that NonO is involved in regulating transcription of several genes such as steroid hormones estrogen, androgen as well as progesterone receptors, RXR and Cyp17 (Yang, Hanke et al. 1993; Sewer, Nguyen et al. 2002).

Among other proteins identified in our screening are several co-activators such as BUB3, CCAR1, 14-3-3γ, CREAP1, Sap180 and ERBP. Presence of these proteins on *rhodopsin* RER was not reported earlier and based on their known function it is possible that they might be playing an important role in *rhodopsin* regulation. Recently, yeast two hybrid screening studies have identified retinoic X receptor (RXR) chain of the nuclear hormone receptor superfamily as a NonO interacting protein (Yang, Hanke et al. 1993). RXR is a nuclear receptor activated by 9-cis retinoic acid (RA). The role of RA in eye development is well established and interaction of NONO with the RA receptor (RAR) may have indirect effect on the transactivation abilities of NRL and CRX on *rhodopsin* promoter. To evaluate the effect of *NonO* knockdown on rhodopsin level we used P7 retina electroporated with either *NonO* shRNA or control shRNA and looked for the endogenous rhodopsin expression in GFP positive dissociated retinal cells by staining with anti-Rho.. Role of the retinoic acid in activation of NRL expression in Y79 cells has been recently reported (Khanna, Akimoto et al. 2006). Since NonO interacts with RXR receptor and RER has a predicted binding site for RXR, it may be possible that this interaction might be helping in increasing the *rhodopsin* promoter-*luciferase* reporter activity. The recent evidence that ERR beta is involved in rod differentiation and photoreceptor survival (Onishi, Peng et al. 2010) might be another line of evidence that

orphan nuclear hormone receptors such as ERR beta and RXR play an important role in *rhodopsin* gene regulation.

In conclusion, we identified several candidate proteins binding to RER and studied the role of NonO/p54^{nrb} in *rhodopsin* gene regulation. NonO enhances *rhodopsin* gene transcription in the presence of NRL and CRX by a mechanism that may involve looping of RER on to RPPR on *rhodopsin* promoter and might be co-transcriptionally coupled to splicing (Figure 4). Finally, knockdown of *NonO* leads to rod photoreceptor cell death by apoptosis in the post-natal mouse retina.

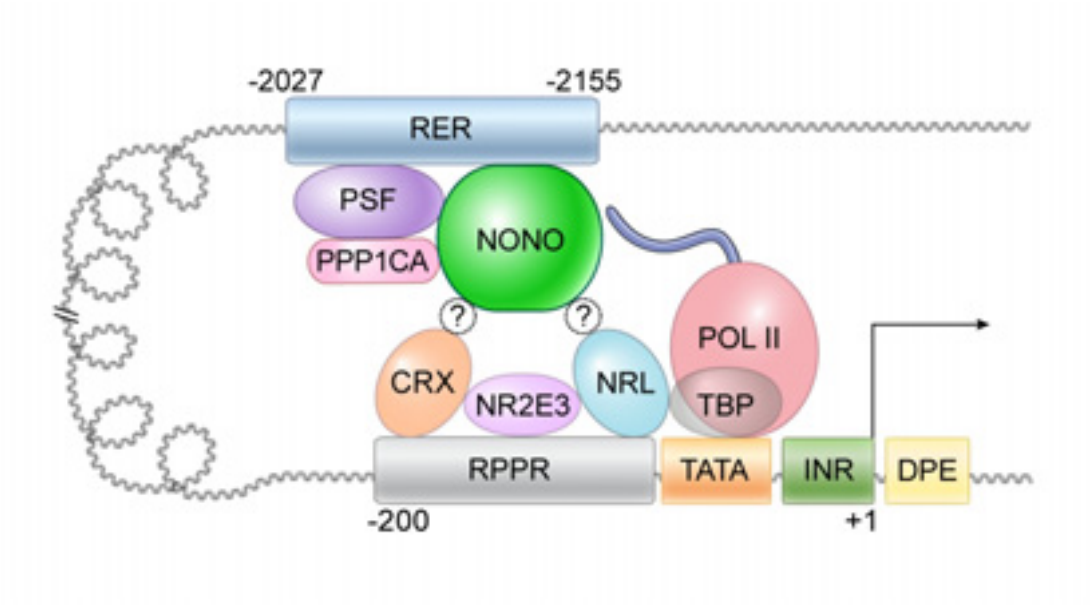

Figure 4

Schematic representation of the RPPR and RER bound transcriptional complexes on *rhodopsin* promoter. A schematic representation of *rhodopsin* promoter showing the RPPR and RER with bound known representative proteins. Location and binding to *rhodopsin* promoter is shown for different proteins. Based on our data and previous studies we propose a model in which RER bound transcriptional complexes containing NonO along with other proteins identified in this study loops to RPPR to further enhance the recruitment of the general transcription machinery and RNA Pol II to the *rhodopsin* promoter in order to regulate its quantitative precise expression in rod photoreceptors.

Materials and Methods

RER oligonucleotide synthesis. All oligonucleotides were custom designed and provided by IDT (USA). Biotin was covalently attached to forward primers to facilitate the isolation of complexes from bovine nuclear extracts. Bovine genomic RER fragment (-2155 to -2027) was PCR amplified followed by gel extraction and sequencing to confirm the integrity of the sequence. A control oligonucleotide was designed after scrambling the RER sequence. Oligonucleotides were annealed in 20mM Tris HCl pH-7.5, 100mM NaCl and 20mM $MgCl_2$, by heating complementary stranded oligonucleotides at 94 ^{0}C for 5 min followed by a cooling at RT. Excess single stranded oligonucleotides were removed by standard gel purification method.

Isolation of RER bound protein complexes from bovine retina. Bovine retinal nuclear fraction was isolated by differential centrifugation. Nuclear extract was pre-mixed with 2X binding buffer (12 mM HEPES pH-7.9, 60 mM KCl, 4mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 12% glycerol and protease inhibitor cocktail) for equilibration, then poly dI-dC and salmon sperm DNA were used to remove non-specific DNA binding proteins. Equimolar amount of oligonucleotides were incubated overnight at 4 ^{0}C with 300 mg of nuclear extract. Next day, 30 ml of the 50% mixture of Dynabeads® MyOne TM Streptavidin C1 (Invitrogen) and Dynabeads®MyOne TM Streptavidin T1 (Invitrogen) was added to the above mixture to allow the binding of oligonucleotides with nuclear proteins. Beads were washed several times and bound proteins were eluted in 12 mM HEPES pH-7.9, 1 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 12 % glycerol and protease inhibitor cocktail. Eluted proteins were separated on SDS-PAGE, visualized by silver staining and differential bands were excised out and subjected to mass spectra analysis.

Plasmid construction. Total RNA was isolated from one month-old C57BL6 mice retina using QIAGEN kit (Qiagen, Valencia, CA) and cDNA was prepared as described in the protocol Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Appropriate restriction enzyme sites were incorporated in the forward and reverse primers. Coding regions of *NonO* were PCR amplified using the newly synthesized cDNA. Amplified product was gel purified and cloned into pGemT-Easy vector (Promega, Madison, WI). *NonO* cDNA was excise out from the pGemT-Easy vector and sub cloned

into pcDNA4c (*NonO*-pcDNA4C) mammalian expression vector (Invitrogen) between BamHI and NotI sites. Sense and anti-sense primers used for the cloning of *mNono* are as follows- (BamHI)-NonOF: 5'-ATGCGGATCCATGCAGAGCAATAAAGC-CTT-3' and (NotI)-NonOR: 5'-ATGCGCGGCCGCCTAATATCGGCGGCGTTTATTT-3'. Bovine rhodopsin promoter was excised out from pRho-DsRed using Sal I and Hind III and cloned into PGL3 basic vector between XhoI and HindIII sites. GFP expressing shRNA vectors were made using pGSuper vector (Kojima, Vignjevic et al. 2004). In brief, *NonO*-shRNA (pGS-NonOshRNA) was generated by annealing the sense and antisense oligonucleotides and cloned between Bgl II and Hind III sites of the pG-Super vector. The *NonO* shRNA target sequence (GACCTTTACACAGCGTAGC) was from the exon 2. Sense and antisense oligonucleotides were designed in such a way that they will make a hairpin after transcription. Bgl II half site was added at 3' end and Hind III half site was added on 5' end to facilitate the ligation of the annealed double stranded oligonucleotide to the pG-Super vector. Sense and antisense used oligonucleotides are as follow GATCCCCGACCTTTACACAGCGTAGCTTCAAGAGAGCTACGCTGTGTAAAGGTCTTTTTGGAAA and AGCTTTTCCAAAAAGACCTTTACACAGCGTAGCTCTCTTGAAGCTACGCTGTGTAAAGGTCGGG. Corresponding control vector (pGS-Control shRNA) was prepared by scrambling the target sequence (AGATAACTGCGCCGTACTC). Sense and antisense used oligonucleotides are as follow GATCCCCAGATAACTGCGCCGTACTCTTCAAGAGAGAGTACGGCGCAGTTATCTTTTTTGGAAA and AGCTTTTCCAAAAAAGATAACTGCGCCGTACTCTCTCTTGAAGAGTACGGCGCAGTTATCTGGG. Sequences of the all constructs were verified by sequencing.

Immunobloting. Expression of Xpress-tagged fusion protein was confirmed by transfection of *NonO*-pcDNA4c plasmid in HEK293 cells using FuGENE (Roche Applied Science, Indianapolis, IN). Cells were harvested after 48hrs, lysed with SDS loading buffer; 20mg of total protein from each samples were separated on 12% SDS-PAGE gels and transferred to PVDF membrane. Immunoblots were incubated with mouse anti-Xpress (1:5000 dilution in TBST buffer), for 2hrs and after 4 washes with TBST, anti-

mouse HRP secondary antibody was incubated for another 1hour. Proteins were visualized by enhanced chemiluminescence plus (Thermo Scientific, Rockford, IL).

Luciferase reporter assays. Dual luciferase reporter assays (Promega, Madison, WI) were performed using HEK-293 cells co-transfected with 0.001 µg of CMV-Renilla, 0.2 µg of bovine *rhodopsin* promoter driving firefly *luciferase* (2294b*Rho*P-Luc), 0.1 µg of NRL-pcDNA4C, 0.1 µg of Flag-CRX, and 0.1 to 0.6 µg of m*NonO*-pcDNA4c. Empty pcDNA4c was used to adjust the total amount of transfected DNA. Cells were harvested after 48hrs and washed with chilled PBS and lysed in 100 ml of the passive lysis buffer provided with kit. Firefly and renilla Luciferase activities were determined using Dual Luciferase Reporter System (Promega, Madison, WI) and measured with a Modulus Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). *Renilla* Luciferase activity was used as an internal control for the transfection efficiency. All experiments were repeated three times. T-test was performed for statistical analysis, and p value < 0.05 was considered significant.

Immunoprecipitation. Bovine retinas were isolated and cross-linked with 5 mM Dimethyl 3, 3'-dithiobis propionimidate *2HC (DTBP) (Thermo Scientific Pierce) on ice for 1 hour and reaction was stopped by adding 1 M Tris pH-7.5 for 20 minutes. Retinas were washed and further homogenized and nuclear fraction was prepared by differential centrifugation. All the buffers used contain both protease as well as phophatase inhibitors (Roche) in all the steps. For immunoprecipitation, nuclear fraction was incubated with anti-NonO, anti-NRL, anti-CRX, anti-Pol II, and anti-Brn3b antibody overnight at 4°C. Immunoprecipitation with IgG was used as control. Immunoprecipitated fractions were incubated with Dynabeads® Protein A (Invitrogen) for 2 hours. Bound proteins complexes were eluted in 0.2 mM glycine pH-2.5 and neutralized by 1M Tris pH-9.0, samples were prepared by adding 5X SDS-PAGE loading buffer in equal volume of the eluted immunoprecipitates. Proteins were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. After 1 hour of blocking with 5% skimmed milk in TBST (Tris-buffered saline, 0.1% Tween 20) membranes were incubated overnight at 4 °C with primary antibody. After three washes

with TBST, membranes were incubated with secondary antibody coupled to horseradish peroxidase for 1 h in TBST. Blots were washed twice in TBST and protein bands were visualized by enhanced chemiluminescence plus (Thermo Scientific, Rockford, IL).

***In vivo* electroporation.** Neonatal CD-1 mice (Charles River Laboratories, Wilmington, MA) were used for *in vivo* transfection by electroporation, as described (Matsuda and Cepko 2004). For cotransfection studies, equimolar amounts of plasmids were used at a concentration of 10 µg/µL. Injection volume was 0.2 µL. Transfected eyeballs were harvested at P5, P10 and P21 for immunohistochemical analysis.

Immunohistochemistry. Cryosections were probed with specific antibodies as described (Matsuda and Cepko 2004) and visualized using Leica SP5 confocal laser scanning unit (Leica Microsystem Inc., Buffalo Grove, IL).

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GENERAL DICUSSION

In this thesis work we focused on the transcriptional regulation of the major rod differentiation factor, *Neural retina leucine zipper*, *Nrl*. Our first goal, which constituted the main part of the thesis, was to understand the regulation of *Nrl* expression during development. To do so, we focused on three main evolutionarily conserved elements found in *Nrl* 5'-upstream sequence, A, B and C. We made different promoter bashings and cloned these different promoter fragments upstream of fluorescent reporter genes. The technique of *in vivo* electroporation of neonatal mice retina, which allows investigating expression of DNA plasmidic vectors under the control of particular genes, led to the demonstration that the -938 to +119 fragment of the promoter (containing A+B but not intermediate sequences) was sufficient for driving strong expression in the ONL, whereas A fragment alone was not. Although we did not further focus on the whole promoter fragment (-2734 to +119 containing A, B and C) it should be underlined that this full-length 2.8 kb fragment was also efficient in driving reporter expression in the ONL, a result that is in agreement with the observation of Akimoto et al. by using 2,5kb *Nrl* promoter to drive GFP expression in transgenic mice. Our results (data not shown) even suggested that 2.8 kb fragment expression was more restricted compared to the shorter fragment (-938 to +119). This result indicates that there are likely additional regulatory elements upstream of region B, to support other functions such as expression level regulation in rods and/or inhibition of expression in non-photoreceptor cell types. We then focused on cluster B (-938 to -657) and cluster A (-304 to +119) and the observation that A+B but not A alone was working in ONL upon electroporation led to the conclusion that B is the required fragment for ONL expression. B sequence contains numerous putative binding elements and by performing gel shift experiments we indeed showed specific binding of RORβ, CRX, OTX2 and CREB. In addition, site directed mutagenesis against ROR response element abolished expression of the reporter gene upon *in vivo* electroporation of mouse pup retinas, demonstrating the relevance of RORβ in *Nrl* regulation. These data support the previous work of (Jia, Oh et al. 2009) showing the lack of *Nrl* expression in *Rorb* -/- animals. These data, together with the transactivation of *Nrl* promoter-reporter construct by RORβ (Results, chapter III) bring further insight into the transcriptional networks leading to rod development and

maturation, by showing that in the sequential intervention of specific transcription factors leading to retinogenesis, *Nrl* probably lies downstream of RORβ. However, since OTX2 and CRX also interact with *Nrl* promoter, we can hypothesize that these factors (and likely others) which are expressed earlier in photoreceptor development, also contribute to activate *Nrl* expression at the right place and moment.

To further characterize the conserved *Nrl* promoter elements, we used different cluster combinations and tested them by *in vivo* electroporation; we dissected out clusters B and A to reveal regions within these fragments essential for promoter activation in the ONL. We identified a minimal *Nrl* promoter sequence constituted of cluster B and a sub-part of cluster A (-34 to +16) that we called basal promoter A1. A1 roughly contains the TATA-like box sequence and a GC rich potential binding site for Sp1 factor that probably contributes to basal constitutive expression (Tan and Khachigian 2009). Combination of B and A1 yielded relatively strong reporter expression in the ONL by *in vivo* retinal transfection. Based on the main transcription factor binding region defined by gel shift experiments within cluster B, we identified the 51 bp B4 sequence as a potential crucial regulator of *Nrl* expression. Indeed, when this sequence was combined with *rhodopsin* promoter in retinal *in vivo* electroporation experiments, reporter labeling was increased four-fold, demonstrating its enhancer properties. However, this element triggered very limited expression in the ONL when combined with A1. The same observation was done with B2 sequence-containing B4. Thus, 0.3 kb A1+B is so far the promoter sequence that most truly reflects *Nrl* promoter properties and should be used as a tool to further dissect the developmental mechanisms inducing *Nrl* expression.

One of the main advantages of the *in vivo* electroporation technique is to rapidly evaluate gene expression as compared to the generation of transgenic mice. However, this technique does not allow investigating early developmental points in the retina. Knowing the histological order of the retinal development, we hypothesize that cone photoreceptors (peak of postmitotic cells at E16 in the mouse) would not be transfected in our neonatal electroporations. Therefore, the specificity of the tested promoter was not fully proven by using only this technique.

To tentatively answer this question, we produced EGFP constructs under the control of *Nrl* promoter A1+B sequence cloned in recombinant adeno-associated virus (AAV). Generation of such viral vectors represent several advantages. First the specificity of promoter can be tested as mentioned above, since viruses are infecting cells independently of their cell type (although some AAV serotypes were proven to display higher affinity and better gene delivery for certain cell types (Pang, Lauramore et al. 2008)). Second, use of a minimal promoter is a clear benefit for AAV use, since the cloning size limitation is < 4.7 kb and should include the coding sequence (CDS) to test, as well as the regulatory untranslated regions plus the two inverted terminal repeat sequences essential for AAV transcription. Hence, the importance of reducing as much as possible the size of foreign DNA to integrate into AAV genome is evident. In gene therapy targeting retinal disease, the idea is to replace the deficient gene by its wild type version carried by AAV. Uses of AAV vectors show a biological interest since they are non-pathogenic and provoke a mild immune response. In some cases, replacement genes can be particularly long and it is necessary to diminish the CDS with a risk of translating truncated protein that will have an unrewarding effect for gene therapy. To avoid this problem, it is better to focus on the promoter region by minimizing as much as possible its size. Defining a minimal *Nrl* promoter region for specific photoreceptor expression was among our goals. We showed expression of AAV-*Nrl*p-EGFP in wild type retina specifically in the photoreceptor layer; however complementary immunostaining with cone or rod-markers would have informed us on the precise identity of photoreceptor-cell types that are expressing EGFP. We took a different approach to answer this question; by expressing AAV-*Nrl*p-EGFP in *Nrl*-/- animals we could evaluate whether *Nrl* promoter could be activated or not in cones. We found EGFP expression in the ONL of injected *Nrl*-/- retinas, showing that *Nrl* promoter can indeed be activated in the default S-cones typical of *Nrl* KO. These data corroborate the previous finding of our laboratory showing expression of the GFP transgene placed under the control of the 2.5 kb *Nrl* promoter in *Nrl* -/- retina (Akimoto, Cheng et al. 2006). In our case, we showed the promoter activation from P10, while Akimoto and his collaborators assessed the expression earlier in retinal development by generating transgenic mice. Nonetheless, it is complicated based only on these observations to affirm

that cones are able to activate *Nrl* since the biological outcome of this activation would be transformation of cones in rods as proven by Oh and collaborators (Oh, Cheng et al. 2008). One piece of element we can bring forward is the fact that there are more likely further 5'-upstream regulatory elements in *Nrl* promoter (even above 2.5 kb) that are not taken in consideration with artificial *Nrl* promoter constructions. Therefore, further promoter characterization is required for better understanding of the regulatory mechanisms implicated in the control of *Nrl* expression that will give rise to mature and functional retina.

Nrl regulation found its interest also in the investigation of rhythmic function of the retina. Previous studies showed a daily rhythm in rhodopsin transcription (Bowes, van Veen et al. 1988), as is also the case for other genes involved in the phototransduction cascade (Iuvone 2005). Given the central role played by rhodopsin in photoreceptor physiology and pathology, we wanted to understand the underlying mechanisms responsible for this rhythmicity. We looked at well-characterized *rhodopsin* transcription factors NRL, CRX and NR2E3. We used either whole retina or isolated photoreceptor layers, and found a daily variation of rhodopsin *transcript*, as well as daily variations of Nrl, Crx and Nr2e3 transcript and protein. While *rhodopsin* transcript as well as its transcription factors' mRNA showed a peak of expression at the dark/light transition, the factors' protein were found maximally expressed at the middle of the light phase, differences of expression probably reflecting the delay between transcription of the gene and translation of the protein. It will be interesting to analyze rhodopsin protein daily variation as well. Interestingly, none of the tested photoreceptor-genes was shown to be significantly rhythmic in constant conditions, suggesting the dependency of the retina to the daily light/dark cycle entrainment for certain rhythmic functions. Indeed, rhythmic functions in the retina are known to strongly depend on exposure to the light/dark cycle which synchronizes the component oscillators. Thus, in complete darkness, individual oscillators present within the retina likely lose their phase coherence leading to an overall flattening of the oscillations, including those of their target genes. To further investigate how the rhythms in photoreceptors are orchestrated, we looked at potential upstream transcriptional mechanisms. Functional circadian clockwork exists in photoreceptors (Tosini, Davidson et al. 2007; Sandu, Hicks et al. 2011) and this

probably drive rhythmic expression of rod and cone functions by entraining more or less directly expression of key transcription factors and their target genes. Based on our partial *Nrl* promoter characterization, we can relate the control of its regulation with the action of RORβ as transcriptional regulator. RORβ is a molecular component of the circadian clock and was shown to display robust rhythmic expression in photoreceptors (Sandu, Hicks et al. 2011). Control of *Nrl* promoter by RORβ transcription factor may explain in part the daily variations of *Nrl*; these data are corroborated by *luciferase* expression showing *Nrl* promoter activation by RORβ. The overall picture we can draw from this study is that *rhodopsin* expression proceeds from complex mechanisms, which ensure expression of this gene during rod photoreceptor development and throughout adulthood, by making sure that *rhodopsin* is expressed at the right time of the day. Our data suggest that *rhodopsin* is highly expressed at the end of the night, in phase with expression of other elements of the phototransduction cascade and also with the rhythmic shedding of photopigment-filled outer segments. The concerted regulation of these processes probably contributes to the fine regulation of an over wise highly active outer segment renewal that is fundamental for photoreceptor survival.

Finally, we investigated the transcriptional relevance of a novel *trans*-acting factor NonO in *rhodopsin* regulation. NonO was identified on *rhodopsin* proximal promoter and was shown to highly regulate reporter gene expression in combination with NRL and CRX. To characterize NonO function *in vivo*, we knocked-down this factor in the developing retina using shRNA targeted against NONO transcript. We showed early degeneration of rod-photoreceptors mediated by apoptosis probably due to *rhodopsin* down-regulation, a data which puts further emphasis on the fact that abnormal expression levels of rhodopsin strongly impair photoreceptor homeostasis. These data present NonO as an original transcription factor required for *rhodopsin* activation. From this observation, it is legitimate to wonder whether NonO could play a role in *rhodopsin* daily variation. It was previously reported that NonO interacts with Period1 protein in mammalian cells and this interaction was suggested to be essential for normal circadian rhythmicity in mammals and drosophila (Kyriacou and Hall 1980). It would be interesting

to investigate the profile of expression of *Nono* to determine whether this gene is also rhythmic in the retina.

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PROMOTER DISSECTION OF THE ROD-SPECIFIC *NRL* TRANSCRIPTION FACTOR: DEVELOPMENTAL AND DAILY REGULATION

The vertebrate retina consists of seven major cell types generated from multipotent progenitors by a hierarchical and stepwise process controlled by both extrinsic factors and intrinsic genetic programs. Photoreceptors (PRs) account for up to 60% of all cells in the adult neural retina and contain generally a majority of rods responsible for night vision. PRs are highly metabolically active cells undergoing intensive outer segments renewal on a daily rhythmic basis. Mutations in rod photoreceptor-genes are linked to retinal dystrophy such as Retinitis Pigmentosa (RP), leading ultimately to blindness. Cell transplantation and gene therapy present feasible strategies to restore visual function; given the central role played by rods in these pathologies, it is meaningful to understand molecular mechanisms controlling their development and maintenance.

The Neural Retina Leucine zipper transcription factor (Nrl) plays a central role in rod photoreceptor development and homeostasis. Nrl, combined with other photoreceptor-specific transcription factors activates expression of rod-specific genes such as the visual photopigment, Rhodopsin. Moreover, Nrl is both essential and sufficient for rod cell fate specification as shown by using transgenesis. Finally, mutations in *Nrl* have been associated with RP. Thus, *Nrl* gene is an interesting model for understanding genetic programs controlling PRs development and homeostasis.

This thesis work aimed at characterizing regulatory mechanisms of *Nrl* expression. It mainly focused on transcriptional control during retinal development. By using *in vivo* electroporation of reporter vectors carrying distinct portions of *Nrl* promoter into neonatal mouse retina, we identified the minimal sequences necessary to drive reporter gene expression specifically in PR layer. We identified RORβ as being required for this expression and showed that OTX2, CRX and CREB transcription factors also directly bind to the defined regulatory regions.

Based on these results we designed a novel adeno-associated virus (AAV) vector containing a minimal *Nrl* promoter fragment of 0.3 kb, and showed that it is well-suited for gene delivery specifically into PRs.

Nrl promoter analysis also proved to be useful to get new insights into the mechanisms controlling daily physiological rhythms in PRs. We showed that NRL, CRX, and NR2E3, the main transcriptional regulators of *Rhodopsin* gene, display rhythmic expression over 24 h. and that *Nrl* might undergo cyclic activation by RORβ which is part of the PR circadian clock. Finally, we investigated the role of a novel *Rhodopsin* transcriptional regulator, NonO, identified in the *Rhodopsin* proximal promoter region. We demonstrated that NonO co-activates *Rhodopsin* promoter along with NRL and CRX. By knocking down this gene during retinal development we provided evidence for its role in rod development and homeostasis.

Key words: Retina, photoreceptors, Neural retina leucine zipper, retinogenesis, transcription, gene regulation, circadian clock, Rhodopsin