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Molecular determinants of *Xotx2* and *Xotx5b* action in retinal cell fate specification

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"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Isaac Newton

Index

Introduction

1.1 The Vertebrate retina

"No one ever have the courage to start a historical overview of any topic in neuroscience without mentioning Cajal's contribution to that given field. It is particularly true for retinal research, where he has been instrumental in defining retinal connection pattern and possible function of the main neuron classes (Cajal, 1892)."

- Robert Gabriel -

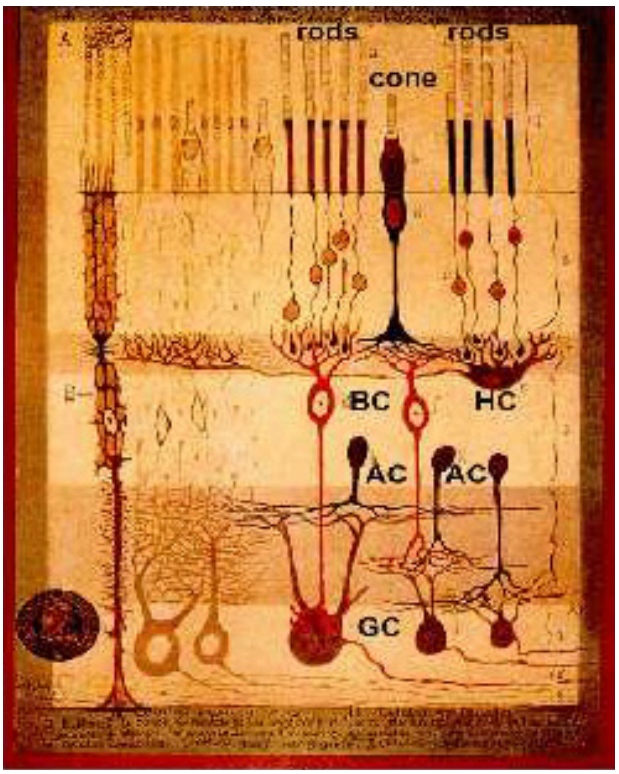


Figure 1.1. Structure of the Mammalian Retina.

Cross-sectional microscopic drawing of the nerve cells in the retina made by Santiago Ramon y Cajal (1900). http://hubel.med.harvard.edu/12.jpg

The retina has arguably the most intricate and aesthetically pleasing cytoarchitecture of any sensory system (Fig. 1.1). The combination of highly specialized cell types in a well-organized wiring and complex modulatory activity, results in an amazing and flexible sensory processing system.

Although our knowledge of how the retina is organized and functions is absolutely essential, understanding how it is assembled during development is a big challenge in Neurobiology. Indeed, understanding how the retina arises is attractive not only to developmental neuroscientists interested in vision, but to all neuroscientists interested in neural development, because the retina is "an approachable part of the brain" and developmental processes required to build up this exquisitely organized system are basically relevant to all other parts of the Central Nervous System.

The vertebrate retina comprises five major classes of nerve cells (see Wässle, 2004, for review). Rod and cone photoreceptors convert light information to chemical and electrical signals that are relayed to interneurons in the outer retina. Bipolar interneurons are contacted by photoreceptors and convey signals from the outer retina to the inner retina. Transmission from photoreceptors is modulated by horizontal cells that also contact the bipolar cell. In the inner retina, bipolar cells form chemical synapses with two classes of neurons, amacrine interneurons and retinal ganglion cells. Amacrine cells not only modulate signals from the bipolar cells by providing inhibition directly onto ganglion cells, but also modulate transmitter release from the bipolar cells. Light information leaves the retina and reaches the other stations in the brain via axons of the ganglion cells that collectively form the optic nerve (Wong, 2006).

Beside these five major classes of neurons, in the retina a type of macroglia exists: the Müller glia cells. Müller cells span the depth of the retina and provide important, structural and functional support for the retinal neurons.

The cell bodies and connections of retinal neurons are arranged in layers and this laminar organization of the retina is stereotypic across species.

Connections are restricted to two major laminae, the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The nuclei of nerve and glial cells are organized in three nuclear layers. Photoreceptor cell bodies form the outer nuclear layer (ONL); horizontal, bipolar, amacrine and Müller glia cell bodies are located in the inner nuclear layer (INL) and, finally, ganglion cells form the ganglion cell layer (GCL) (Fig. 1.2).

Embedded within this basic organization of the vertebrate retina many specialized subcircuits are present, working together in parallel to process different features of the visual image. For example, rods are sensitive to low-light levels and rod-driven circuit exists for visualizing objects under dim light conditions. In most vertebrates, this circuit involves connections among rod photoreceptors, rod bipolar cells and a specialized type of amacrine cells, the AII amacrine cells connecting to ganglion cells (Strettoi et al., 1990). On the other hand, under high light level conditions, cones work involving two vertical pathways, giving also a chromatic information of the visual stimulus. Cones contact a variety of cone bipolar cells, some of which are depolarized (ON) and others hyperpolarized (OFF) by increased illumination. ON and OFF-cone bipolar cells contact ganglion cells, which respond to changes in illumination according to their bipolar input. Together, the ON and OFF pathways provide contrast information. In addition to these basic features, the retina has also specialized circuits that can compute other features of the visual scene, such as the direction of motion and orientation of edges (Wong, 2006)

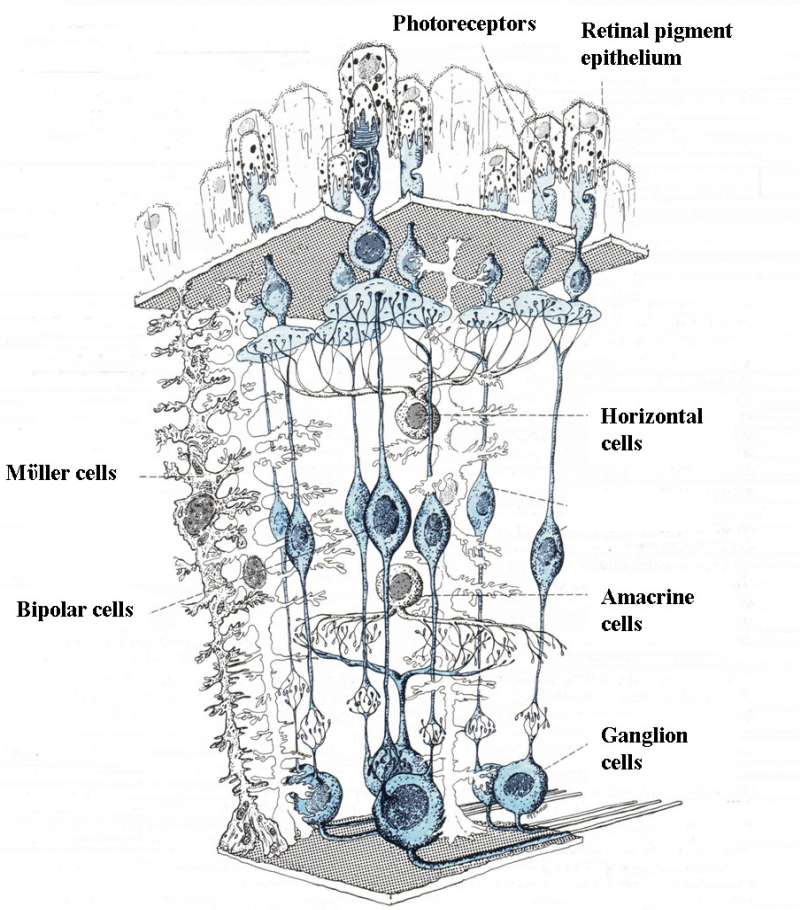


Figure 1.2. Tri-dimensional representation of the retinal structure.

The retinal structure and the principal retinal cell types are shown (modified from Balboni et al., 1993).

1.1.a The *Xenopus* retina

In this work great relevance has been given to *Xenopus laevis* as model system. The clawed frog, *Xenopus laevis,* is an ancient Anuran amphibian, exploited in developmental studies because of its well-characterized larval stages (Nieuwkoop and Faber, 1956). In relation to experimentation on the visual system, *Xenopus* has been utilized as a model system for studies of retinotectal

projection (Gaze and Keating, 1970) and also for biochemical studies of visual pigment, thanks to its large rod photoreceptors (Dartnall, 1954; Wald, 1955; Moritz et al., 1999). In the recent era of molecular biology, the *Xenopus* embryo is a favoured system for studies of gene function during neural development.

Frogs usually have a good vision and Wilhelm and Gabriel suggested that the potential spatial resolution power of the *Xenopus* retina is approximately as good as that of the central retina of mammals (Wilhelm and Gabriel, 1999). The basic structural and functional organization of the amphibian retina is fundamentally similar to the mammalian/primate one (Krizaj, 2000).

However, it is generally accepted that the relative simplicity of the brain visual system in lower vertebrates may require a higher degree of visual information processing at the retinal level (Vigh et al., 2000). For this reason, the retina of non-mammalian vertebrates seemed to posses more complex receptive field properties than those of mammals. Edge, dimming and convexity detectors, directional selectivity and processing of moving stimuli, all the neural circuits that analyze these properties seemed to be present in the *Xenopus* retina (Gabriel et al., 2000).

On account of this, the *Xenopus* retina shows some peculiarities in respect to the mammalian retina.

Photoreceptors. Rod photoreceptors represent 53% of total photoreceptors (Wilhelm and Gabriel, 1999). Rods are rather uniform regarding their morphology, although a minor population (2-3%) of blue-sensitive rods, with a thinner outer segment, has been described on the basis of its visual pigment content (Witkovsky et al., 1981). Cones have also been classified on the basis of their visual pigment content and can be divided into three types: miniature, ultraviolet-wavelength-sensitive (UWS) (4% of all cones); large, short-wavelength-sensitive (SWS) (10%); and large, long-wavelength-sensitive (LWS) cones (86%) (Rohlich et al., 1989).

Horizontal cells. The *Xenopus* retina differs from the pattern in mammals, in which horizontal cell dendrites contact only cones, while the axon terminal contacts rods (Steinberg, 1969; reviewed in Wassle and Boycott, 1991). There are two types of horizontal cells in *Xenopus* retina (Stephan and Weiler, 1981). In the axon-bearing cell of the luminosity type (i.e. lights of any spectral composition elicit a hyperpolarization), both dendrites arise in the cell body and axonal branches contact both rods and cones. The other horizontal cell type lacks an axon and is of the chromatic type (depolarized by red light, hyperpolarized by blue light) (Stone et al., 1990). Its dendrites contact what are short-wavelength-sensitive cones and rods (Witkovsky et al., 1995).

Bipolar cells. The main point in the structure of the lower vertebrates is that retinal circuits did not evolve separate rod-dedicated and cone-dedicated pathways. So, bipolar cells receive direct rod and cone inputs and specific rod bipolar cells do not exist. In intermediate light condition (mesopic state), when both cones and rods are active, it is probably disadvantageous for an animal to receive double information concerning the same object (due to, for instance, the different latency of the two kinds of photoreceptors). However, the mutual antagonism between rod and cone signals decreases the magnitude of this problem; this mechanism of the mutual inhibition involves the neuromodulator dopamine, synthesized by the dopaminergic amacrine cells (reviewed in Krizaj, 2000).

Amacrine cells. Sporadic attempts to note down the cell types by their morphology have been made by early researchers, particularly by Cajal (1892). He distinguished 13 amacrine cell types, mostly on the basis of ramification pattern in the IPL. In the Anuran retina, the majority of the amacrine cells takes up GABA and glycine (Voaden, 1974). Moreover, dopaminergic cells are present at low density, representing about 0.5% of the total amacrine cell number. In addition, in the *Xenopus* retina other amacrine cell types are present, identified by

different markers: serotonin-immunoreactive, nitric-oxide producing cells, neuropeptide Y, substance P, somatostatin and colecystokinin immunoreactive (reviewed in Vigh et al., 2000). The importance of diverse amacrine populations in retinal information processing can be explained by the relative simplicity of the brain visual system in lower vertebrates (Vigh et al., 2000).

Ganglion cells. It can be cautiously considered the presence of 12 types of ganglion cells in the frog retina. There has been a long-standing debate on the existence of direct bipolar to ganglion cell contacts in the lower vertebrate retina. Definite bipolar to ganglion cell synapses have been identified in *Xenopus laevis* (Buzas et al., 1996), representing about 10% of all ganglion cell inputs in the IPL. This fact can be explained by considering that there is a higher divergence of bipolar cell output to amacrine cells in frog than in mammals (reviewed in Gabriel et al., 2000).

Ciliary marginal zone. A peculiarity of the amphibian and fish retina is that its peripheral portion, termed ciliary marginal zone (CMZ), is a pseudo-stratified neuroepithelium from which retinal precursors differentiate, allowing growth of the retina throughout the whole life of the animal. In fact, after the embryonic phase of retinogenesis, new cells are added to the central retina from this peculiar proliferative region (Wetts et al., 1989). CMZ progenitors are multipotent and can give rise to all retinal cell types, including pigmented epithelial cells (Wetts and Fraser, 1988). The main feature of this region is that the retinoblasts are ordered along the CMZ, from its peripheral edge towards the centre, according to their grade of commitment.

Therefore, stem cells are located in the most peripheral region of the ciliary margin, post-mitotic precursors are adjacent to the central retina, and proliferating neuroblasts are distributed between these two regions. Because each set of precursors, in their state of commitment, is characterized by the expression of an unique combination of genes, the consequence of such a defined spatial

distribution of the precursors is that the CMZ recapitulates spatially the temporal sequence of gene expression during retinogenesis (Wetts and Fraser, 1988; Perron et al., 1998).

Such a spatially arrayed ciliary margin does not exist in the mammalian and bird retina, even if both types of retina contain stem cells. The mouse retinal stem cells are located in the pigmented ciliary margin (PCM) (Tropepe et al., 2000). These cells may be the evolutionarily homologs of the amphibian and fish CMZ precursors, but their location is completely different; in fact, the non-pigmented iris margin of the mammalian retina (corresponding to the amphibian and fish CMZ) is devoid of stem cells. The PCM stem cells can differentiate into various retinal neuronal types including photoreceptors, bipolar neurons and Müller glia (Tropepe et al., 2000).

More recently, retinal stem cells have been isolated from the human retina. These cells display self-renewal properties and, above all, when transplanted into mouse post-natal or embryonic chick eyes, are able to survive and differentiate in the host retina (Coles et al., 2004). Thus, the adult mammalian eye harbours stem cells, which can be induced to re-enter the cell cycle and initiate neuronal differentiation.

1.2 Retinal development

Vertebrate retina is a complex neural structure that comprises highly organized, laminated networks of nerve and glial cells. The biological question we ask is how this complexity arises during development.

The vertebrate retina shares a common origin with the rest of the central nervous system. Retinal development begins with specification of the eye primordia during early stages of embryonic life, highlighted by the appearance of a bilateral evagination of the ventro-lateral diencephalon. Upon continuous evagination of the optic primordia, two optic vesicles are generated which extend

towards the overlying, non-neural ectoderm which will ultimately originate the lens and the cornea. The optic vesicle invaginates and gives rise to a double-layered optic cup: the inner layer (facing the lens placode) will give rise to the neural retina, while the outer layer will differentiate into the retinal pigmented epithelium (reviewed in Chow and Lang, 2001).

The retinal progenitors in the inner layer of the optic cup are proliferating and share an uniform morphology; they are initially arranged as a pseudostratified neuroepithelium, whereby cells contact both surfaces of the layer (Fig. 1.3). This layer is apposed at its outer (scleral) surface to the retinal pigmented epithelium (RPE), but remains separated by the potential space of the obliterated neural tube lumen (optic ventricle). The nuclei of progenitors undergo S-phase distal to the neural tube lumen but enter M-phase at the luminal surface. During mitosis retinal progenitors likely retain their basal process, divide and subsequently re-establish contact with both sides of the layer (Cayouette and Raff 2003).

It is interesting to note that during retinal progenitor proliferation there is a change of cell cycle length and all studies of cell cycle timing in the retina are in accordance with the fact that it slows during development (Rapaport, 2006; Decembrini et al., 2006).

Another mechanism affecting the production of cells is the mode of division, of which three can be described. Early progenitors go through a period of symmetrical divisions, each daughter returning the cell cycle. This mode allows the pool of progenitors to expand exponentially. Later, retinal progenitors divide asymmetrically, one daughter returning the cell cycle, the other exiting, migrating and differentiating. At some late stage, progenitors go through a terminal symmetrical division in which both daughters become post-mitotic and differentiate in the correct retinal cell types (Rapaport, 2006).

In this regard, it is noteworthy that the sequence of cell genesis in the vertebrate retina is highly conserved.

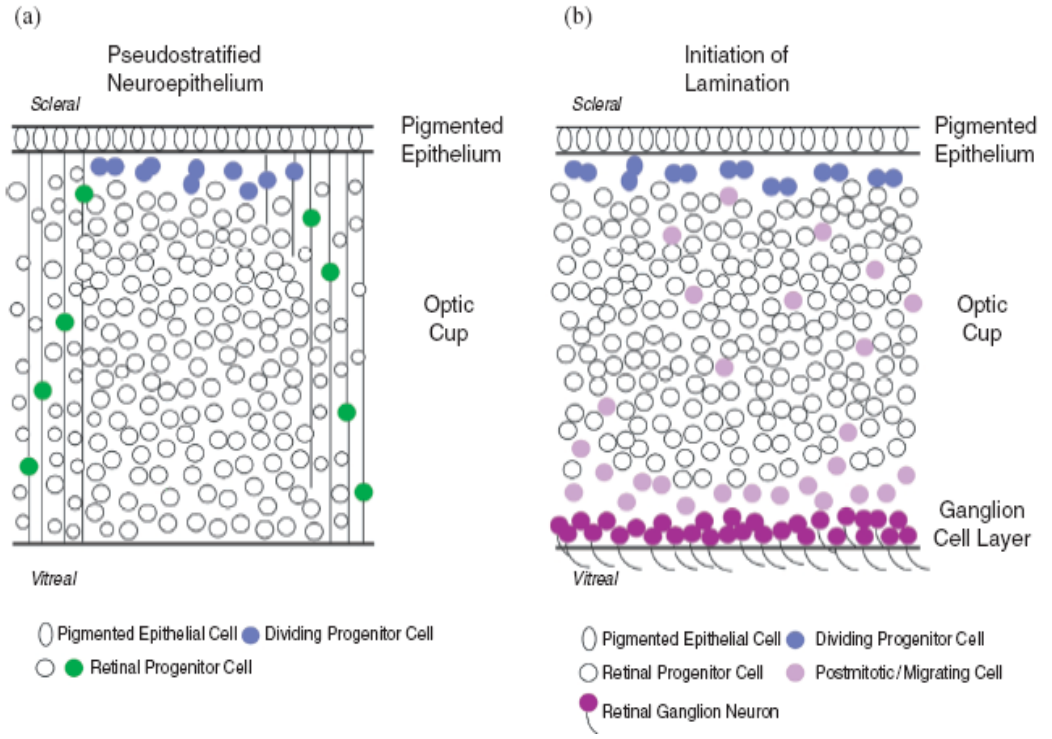


Figure 1.3. Cellular arrangement during retina development.

(A) The initial neural retina is arranged as a pseudostratified neuroepithelium. Prior to neuron formation, uncommitted progenitor cells contact both surfaces of the optic cup. At the onset of mitosis, cells move their nuclei to the scleral side, lose a contact with the optic cup and round up. This process is reversed after division is complete.

(B) When neurogenesis begins, proliferation still occurs at the outer side of the optic cup, but the migration of committed neuroblasts and the accumulation of mature ganglion cells at the vitreal side is now apparent (from Vetter and Brown, 2001).

1.2.a Retinal neurogenesis

The characteristics of the retina make it an ideal tissue to study neurogenesis. Its development proceeds through three overlapping steps starting with retinal progenitor cell proliferation, followed by birth of post-mitotic retinal

transition cells (also referred to as precursors), and ending with terminal differentiation of the seven major cell types (Chen et al., 2007).

Despite the differences among each vertebrate class, two common features are shared by the newly generated neurons during development of the retina. First, as shown by birthdating analyses performed in chick (Prada et al., 1991), monkey (LaVail et al., 1991), rat (Rapaport et al., 2004) and *Xenopus* (Stiemke et al., 1994; Decembrini et al., 2006), the seven major retinal cell types are generated in an extremely conserved histogenetic order, with ganglion cells born first and Müller cells born last (Cepko et al., 1996). In particular, in all species studied cell birth proceeds in the following retinogenetic timing: ganglion cells, horizontal cells, cones, amacrine cells, rods and bipolar cells. Finally, Müller glial cells differentiate. Moreover, the first cells to be generated, ganglion and horizontal cells, are the largest in the retina, supporting the hypothesis that large neurons are generated before small ones. Likewise, the last cells to be born are the Müller cells, supporting a trend in the CNS for glia to be generated late (Rapaport, 2006).

The second important fact concerning the retinal neurogenesis is that, as demonstrated by means of lineage tracing analyses and cell ablation studies, retinal progenitors are multipotent at the different developmental stages and a single progenitor is able to produce all the different retinal cell types (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). So, retinal neurogenesis follows a precise and evolutionarily conserved order which suggests the conservation of the underlying molecular mechanisms among the Vertebrates.

An attractive hypothesis to accommodate these findings was that, once specified as retinal progenitors, the various cell fates of postmitotic neurons are determined by environmental signals. Alternatively, these inducing signals might be present at many stages, but an autonomous clock could regulate the competence of cells to respond to them. To differentiate between these mechanisms, *in vitro* heterochronic transplant experiments had been performed in both chick and rodents, in which progenitors from different stages of development

were placed in an environment of a different age (Livesey and Cepko, 2001). For example, early chick progenitors, which normally generate ganglion cells *in vivo*, originate ganglion cells regardless of the age of the environment that they are placed in (Austin et al., 1995). Another strong evidence, in this regard, derives from experiment of heterochronic transplantation. Cells from young embryonic retinae were dissociated and grown together with those from older embryos, and the timing of rod determination assayed. Young cells appeared uninfluenced by older cells, expressing photoreceptor markers on the same time schedule as when cultured alone, even if there is a change in the percentage of differentiated rods (Watanabe and Raff, 1990). A similar result was obtained when the heterochronic mixing was done *in vivo* by grafting a small plug of optic vesicle from younger embryos into older hosts. Even the graft cells at the immediate margin of the transplant failed to express photoreceptor markers earlier than normal, despite their being in contact with older cells (Rapaport et al., 2001).

1.2.b The competence model of the retinal cell fate determination

The above-mentioned and other observations led Connie Cepko and co-workers to the elaboration of the "competence state" model (Cepko et al., 1996). The competence model states that progenitors pass through a series of competence states, during each of which the retinal progenitors are competent to produce one or a subset of retinal cell types. Within a given competence state, the generation of a particular type of cell is regulated by positive and negative extrinsic signals (Livesey and Cepko, 2001) (Fig. 1.4).

Although the competence model was formulated to explain cell fate choice in the vertebrate retina, it is clear that cell specification in many other regions of the developing nervous system - including neural crest (Selleck and Bronner-Fraser, 1996), spinal cord (Ericson et al., 1996), and cerebral cortex (McConnell, 1988; Qian et al., 2000) - involves changes in progenitor competence over time,

frequently resulting in altered sensitivity to extrinsic factors (Blackshaw et al., 2001). Moreover, the model of temporal changes in competence is strongly

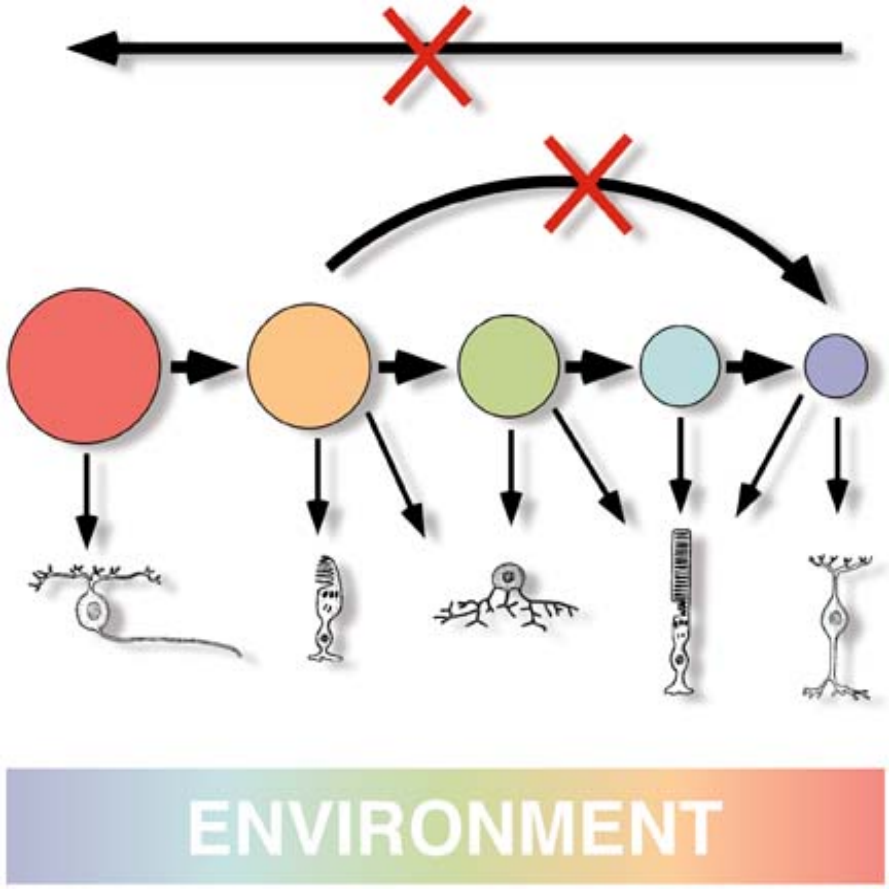


Figure 1.4. The competence model of retinal cell fate determination.

Retinal progenitors comprise a dynamic mixture of mitotic cell types that interact with the environment to make the different postmitotic cell types. Each progenitor cell is thought to be controlled by a complex of transcription factors that define its competence state. Retinal progenitors are modulated to progress from one state of competence to another in only one direction. The environment is shown to be changing over time (from Cepko et al., 1999).

supported by elegant studies of *Drosophila* CNS development (Isshiki et al., 2001; Pearson and Doe, 2003), where the sequential expression of the transcription factors Hunchback, Krüppel, Pdm and Castor was found to change cell fate competence (Isshiki et al., 2001). Noteworthy, *cdc25* mutants, whose cell cycle is arrested at the G2-M transition, fail to progress through the normal sequence of gene expression (Isshiki et al., 2001), suggesting the importance of a cell cycle-dependent clock (Cremisi et al., 2003).

It is interesting to note that the situation in the retina, where early progenitor cells cannot be induced to adopt late fates and *vice versa*, is distinct from the progressive developmental restriction that is seen in the cerebral cortex, where early cortical progenitor cells are competent to generate cells of upper (late-born) and lower (early-born) layers of the cortex, but become restricted to generate only late-born fates as development proceeds (Desai and McConnell, 2000).

The obvious questions are what defines the cellular differences among progenitors at different times, how those differences define different competences and how passage between one state and the next is regulated. The main mechanisms for control of a competence state are transcriptional program and post-transcriptional regulation, such as protein expression, modification, accumulation and degradation (Livesey and Cepko, 2001).

In order to identify genes that might regulate retinal development, Cepko and coworkers recently performed a gene expression study in the mouse developing retina using serial analysis of gene expression (SAGE). SAGE is a technique that provides a comprehensive profiling of gene expression; moreover, genes that show dynamic expression via SAGE have been analyzed by *in situ* hybridization. In this way, a molecular atlas of the expression patterns of 1051 genes in the developing and mature retina was thereby constructed (Blackshaw et al., 2001). The laminar structure of the retina makes it relatively simple to assign an identity to cells expressing a given gene. During early stages of retinal development, mitotic progenitors form the outer neuroblastic layer (ONBL), while

newborn neurons reside in the inner neuroblastic layer (INBL). As we have seen, the position of mitotic progenitors within the ONBL varies depending upon their progress through the cell cycle, with S phase cells being found on the vitreal side of the ONBL near the border with the INBL and M-phase cells being found on the scleral side of the ONBL, near the retinal pigment epithelium. Blackshaw *et al.* analyzed the gene expression in the scleral and vitreal portions of both the ONBL and INBL separately. Virtually every gene previously reported to regulate retinal development was detected in this analysis and showed dynamic expression during development. For instance, *NeuroD1,* which regulates rod photoreceptor survival, as well as possibly rod differentiation (Morrow et al. 1999; Wang et al. 2001), is overexpressed at P4.5.

In the case of genes previously shown to be required for production of certain cell types in the developing retina, such as *Ath5* and *Chx10* - which are required for ganglion cell and bipolar neurons, respectively (Burmeister et al., 1996; Brown et al., 2001; Wang et al., 2001) - peak expression typically occurred around or just after the peak time of exit from mitosis for that cell type (Blackshaw et al., 2001).

On the other hand, the authors have identified a number of genes that show temporally restricted expression in early ONBL. By analyzing the expression of a large number of genes that were highly expressed early in development they found that are expressed in broad but temporally restricted subsets of mitotic progenitor cells. For example, *sFrp2* (*secreted Frizzled related protein 2,* a modulator of the Wnt signalling pathway) RNA was found to be broadly expressed in the ONBL until E16, after which it rapidly decreased. Expression of *Fgf15* (*Fibroblast growth factor 15*) was seen to persist longer, but never was easily detected after P0. *Lhx2,* by contrast, was weakly expressed in subsets of cells in the ONBL until P0, when it was dramatically and transiently upregulated throughout the ONBL.

From this study it has been confirmed that the population of progenitors is complex at any time, as there is evidence for progenitor heterogeneity at several

points during development (Livesey and Cepko, 2001). In fact, a limited number of genes have previously been reported as expressed in subsets of mitotic retinal progenitor cells, including genes such as *Ath5,* and have been shown to be required for retinal ganglion cell development (Brown et al. 2001; Wang et al. 2001. They identified a large number of genes that showed selective expression at certain times during development in relatively small subsets of cells in the ONBL. These include a large number of known and putative transcription factors, such as *Sox2, Sox4, Tbx2, Eya2* and *Mbtd1* (a novel polycomb family member), along with many genes of other functional classes.

Particularly intriguing is the early and transient expression of *Pum1,* a mammalian homolog of the *pumilio* gene, which has been shown to mediate asymmetric mRNA distribution in *Drosophila* (Micklem, 1995). Many of these genes showed highly dynamic expression during development - rapidly shifting their cellular expression patterns in the course of a few days. In some cases, these genes were scattered throughout the ONBL, such as *Eya2* at E14, while for other genes, such as *Pum1* and *Pgrmc2* (a surface membrane progesterone receptors), expression was in only the scleral portion of the ONBL, suggesting that these genes may show strongest expression near M-phase in retinal progenitor cells.

When retinal neuron differentiation begins, several genes involved in cell fate specification and/or in the early steps of the differentiation process are expressed only in newly postmitotic cells and cells actively undergoing differentiation. Some of these genes, namely *Crx, Nrl,* and *Nr2e3* (Furukawa et al. 1997; Chen et al. 1997; Haider et al. 2001; Mears et al. 2001) have been shown to play an active role in regulating photoreceptor differentiation (Blackshaw et al. 2001).

Finally, in this work the authors found that genes selectively expressed in Müller glia share a great degree of transcriptional overlap with mitotic retinal progenitor cells. Among the genes identified as being specifically expressed in Müller glia after the first post-natal week, 68% was found to be enriched in mitotic progenitor cells based on their *in situ* hybridization pattern. This extensive

overlap raises the question of how closely these two cell types are at the functional level. Müller glia morphologically resembles mitotic progenitor cells in having apical and basal processes that span the radial dimension of the retina - a feature that is shared with retinal progenitor cells as well as radial glia of the developing brain, a cell type known to be the cortical progenitor cell (Doetsch, 2003). Müller glia is the last cell type to exit mitosis and represents the only cells in the mature retina that can reenter mitosis following retinal injury (Dyer and Cepko 2000; Vetter and Moore, 2001; Bernardos et al., 2007).

Finally, data from chicken suggest that Müller glia can be induced to divide and give rise to some types of retinal neurons for a short period of time near the end of retinal development (Fischer and Reh, 2001).

Recently, it has been shown that Müller glia-derived progenitors express *Crx* and are late retinal progenitors that generate the rod photoreceptor lineage in the post-embryonic zebrafish retina (Bernardos et al., 2007). Moreover, Müller glial cells also are competent to produce earlier neuronal lineages, in that they respond to injury-induced loss of photoreceptors by specifically regenerating missing cones and rods (Bernardos et al., 2007).

The question arises whether Müller glia cells of mammalian retina are fundamentally multipotent progenitor cells that are quiescent regarding cell division and the production of neurons (Morest and Silver, 2003; Walcott and Provis, 2003), conversely to the fish retina. Indeed, if they are progenitor cells, they have acquired the specialized properties needed for a support role in the mature retina, e.g., neurotransmitter reuptake and structural roles (Blackshaw et al., 2001).

Beside mRNA expressed during retina development, a number of RNA transcripts that do not appear to encode proteins were strongly expressed in the developing retina (Blackshaw et al., 2001). These transcripts are typically spliced and polyadenylated, but do not encode evolutionarily conserved open reading frames (ORFs), or any ORFs encoding proteins longer than 100 amino acids, while often showing high similarity at the nucleotide level between mouse and

human (Numata et al., 2003). Putative non-coding transcripts that showed developmentally dynamic expression include *retinal non-coding RNA 1 (RNCR1),* which was expressed throughout the ONBL during early development and which was later restricted to Müller glia. It was transcribed in a head-to-head fashion, and largely coexpressed, with *Six3*. *RNCR2,* on the other hand, was expressed in a large subset of cells in both the ONBL and INBL prenatally, with expression restricted to the INL and GCL postnatally (Blackshaw et al., 2001).

Large-scale EST sequencing efforts from mouse have uncovered up to several thousand putative spliced transcripts that do not appear to encode for proteins (Numata et al., 2003). The functional role of these transcripts is obscure, although non-coding spliced RNAs such as *Xist* and *H19* in mammals and *Rox1* and *Rox2* in *Drosophila* have been implicated in a variety of epigenetic processes (Mattick, 2003).

1.2.c Regulators of competence state: extrinsic versus intrinsic signals

There is a long-standing debate on the relative importance of the extrinsic signals versus intrinsic regulators on retinal cell fate during retinal development. One idea in favour of the extrinsic possibility is that the addition of new differentiated cells could feed signals back to the dividing progenitors and influence the fate of their daughters (Agathocleos and Harris, 2006).

Experimental evidence supports a feedback inhibition mechanism: by cell-mixing experiments, using amacrine-enriched or amacrine depleted cellular environments, Belliveau and Cepko demonstrated that the postnatal environment had at least two signals that affected the cell fate; one signals inhibited the production of amacrine cells and the second affected the production of cones. In particular, previously generated amacrine cells produce a feedback signal that inhibits the production of the amacrine cell themselves. At the same time, this inhibition is compensated by the production of cones and no changes in other cell

type frequency are observed (Belliveau and Cepko, 1999).

The authors suggested that extrinsic signals can influence progenitor decision, in order to control the number of differentiated cells, but the choice of the cell fate is restricted by the intrinsic biases of progenitor cells (Belliveau and Cepko, 1999). On the other hand, signals in the embryonic retinae inhibit rod and favour bipolar cell generation from postnatal progenitors (Belliveau et al., 2000). This alteration in cell fates appeared to be caused by a secreted factor released by embryonic cells that requires the LIFR, a receptor for LIF (Leukaemia Inhibitory Factor), CNTF (Ciliary Neurotrophic Factor) and other cytokines (Belliveau et al., 2000). Another molecule, Sonic Hedgehog (Shh), has been identified as a factor having the potential to be both a feedback inhibitory signal for the production of ganglion cells (Zhang and Yang, 2001) and a positive factor for the differentiation of other retinal cell types (Stenkamp et al., 2002; Shkumatava et al., 2004).

Taurine, an unusual amino acid, is an extrinsic factor produced from P0 rat retinal cultures and its addition to retinal explants promotes rod differentiation (Altshuler et al., 1993), acting via glycine receptor and $(GABA)_A$ receptor (Young and Cepko, 2004). It has also been shown that isolated progenitors differentiate to rods or cones according to the relative amounts of retinoic acid and thyroid hormone (Kelley et al., 1995 and 1999).

Among the extrinsic factors important for retinal development there are neurotrophins, a family of growth factors consisting of NGF (Nerve Growth Factor), BDNF (Brain-Derived Neurotrophic Factor), NT-3 (Neurotrophin-3) and NT-4/5. Besides their critical importance for correct specification and survival of a number of classes of neurons in the central and peripheral nervous system (Lewin and Barde, 1996), neurotrophins have an important role in earlier stages of development (Pearson, 2006).

For example, NT-3 is expressed in retinal pigmented epithelium and then in neural retina (Rodriguez-Tebar et al., 1993). It has been demonstrated that NT-3 stimulates the birth of new neurons. By inhibiting the NT-3 action using specific antibodies to neutralize endogenous NT-3 (Bovolenta et al., 1996) there is a

marked decrease in retinal neuron differentiation, ganglion cells being most affected. Additionally, the impairment of NT-3 signalling causes a decrease in clonal expansion of cells derived from a single retinal progenitor (Das et al., 2000). In contrast, NGF and BDNF have a role during programmed cell death occurring during retinogenesis (Frade et al., 1999).

Ultimately, extrinsic signals need to be translated into an internal code that will drive a cell towards one fate or another, by switching on a precise transcriptional program. The Notch-Delta pathway is a paradigmatic example of how an extracellular signal can do so (Agathocleos and Harris, 2006).

Notch is a transmembrane receptor that transduces an extrinsic cue, that of the binding of its ligand Delta or Serrate, to directly regulate the transcription of several target genes, in particular repressing proneural genes coding for basic helix-loop-helix (bHLH) transcription factors (Artavanis-Tsakonas et al., 1999) (Fig. 1.5). Studies in frog, rat, chick and mouse have shown that *Notch1* is expressed by proliferating and undifferentiated cells (Dorsky et al., 1995; Bao and Cepko, 1997; Lindsell et al., 1996) and its expression is retained by Müller glial cells (Furukawa et al., 2000; Dorsky et al., 1995).

It has been demonstrated that constitutive activation of Notch pathway in fish, frog, chick and rat retina inhibits neurogenesis (Dorsky et al., 1995; Austin et al., 1995; Bao and Cepko, 1997; Scheer et al., 2001) and promotes gliogenesis (Furukawa et al., 2000; Scheer et al., 2001).

A good progress in this regard comes from a recent work where Jadhav *et al*. (2006) demonstrated, by means of a comprehensive molecular characterization, that activation of Notch in early progenitors allowed them to retain appropriate early progenitor gene expression. When examined at later stages of development, however, the cells exhibited expression of an inappropriate mixture of progenitor genes (like *fgf15* and *cyclin D1*) and glial genes (Jadhav et al., 2006). Moreover, a functional assay showed that these cells could form neurospheres, similar to stem cells derived from the retinal pigmented epithelium of the mammalian peripheral retina (Jadhav et al., 2006).

Furthermore, selective reactivation of Notch pathway in newly generated postmitotic cells that had previously released Notch activation during development, led to their differentiation in proper Müller glial cells (Jadhav et al., 2006). In conclusion, prolonged Notch activity in progenitors permits them to progress through multiple states without perturbing temporal identity, promoting early progenitor characteristics early in development and late characteristics later in development. Remarkably, constitutive Notch activation led these cells to acquire both glial and stem cells characteristics (Jadhav et al., 2006).

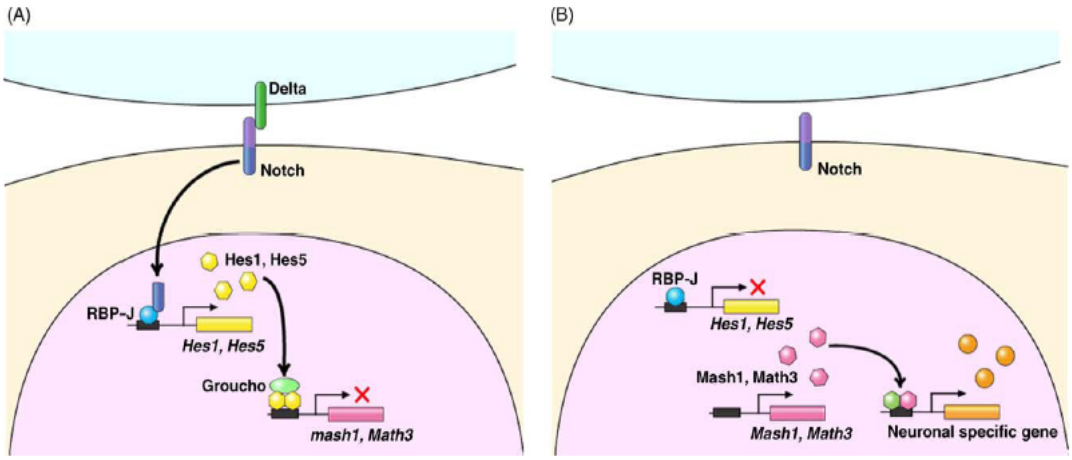


Figure 1.5. Molecular mechanism of Notch signalling.

(A) Upon activation by Notch ligands from surrounding cells, the intracellular domain of Notch (ICN) is cleaved off from the transmembrane region and translocated into the nucleus. In the nucleus, the ICN forms a complex with the DNA-binding protein RBP-J. This complex induces expression of bHLH repressors such as *Hes1* and *Hes5*. *Hes1* and *Hes5* repress transcription of bHLH activators and inhibit neuronal differentiation. Thus, the Notch pathway links the extrinsic signals (Notch ligands from neighbouring cells) to the intrinsic factors (*Hes1*/*Hes5*) for regulation of cell differentiation.

(B) When Notch is not activated, *Hes1*/*Hes5* expression is off, allowing bHLH activators to trigger neuronal-specific gene expression (from Hatakeyama and Kageyama, 2004).

On the other hand, the relationship between lineage and histogenesis is certainly consistent with the idea of an intrinsic developmental clock (Cayouette et al., 2006). Beside the studies of extrinsic regulation of cell fate, we have several evidences on the role of intrinsic factors during retinal neuron determination. bHLH proneural genes, mentioned above as target of Notch signalling, and homeobox genes are basically the best characterized transcription factors involved in generating the diversity of retinal cell fates (Fig. 1.6).

A prime example of a bHLH gene is *Ath5*. The *Xenopus* homologue, *Xath5*, promotes retinal ganglion cell genesis when overexpressed *in vivo* (Kanekar et al., 1997). It can induce the expression of *Xbh1,* a homeodomain transcription factor involved in ganglion cell differentiation (Hutcheson and Vetter, 2001; Liu et al., 2001; Poggi et al., 2004). When *ath5* gene is non functional, such as in zebrafish *lakritz* mutants (Kay et al., 2001) or in *Math5* mutant mice, there is a depletion of ganglion cells (Brown et al., 2001; Wang et al., 2001). *Ath5* has an interesting effect on cell cycle because cells that express *Xath5* tend to exit the cell cycle early, at the appropriate time for ganglion cell genesis (Ohnuma et al., 2002a).

Other bHLH have different profiles of activity with respect to cell determination in the retina. NeuroD, for example, promotes amacrine over bipolar cell fate and favour photoreceptor survival (Morrow et al., 1999). *Mash1* and *Math3* are both expressed in bipolar cells and in their double mutation virtually all bipolar cells are abolished (Tomita et al., 2000).

The loss of *Chx10* gene*,* coding for a homeodomain transcription factor, results in the complete loss of bipolar cells in mice, too (Burmeister et al., 1996).

Recently, it has been identified a bHLH transcription factor, Bhlhb4, that is required for rod bipolar cell maturation. *Bhlhb4*$^{-/-}$ mice lack specifically rod bipolar cells, while the other retinal neurons are unaffected (Bramblett et al., 2004).

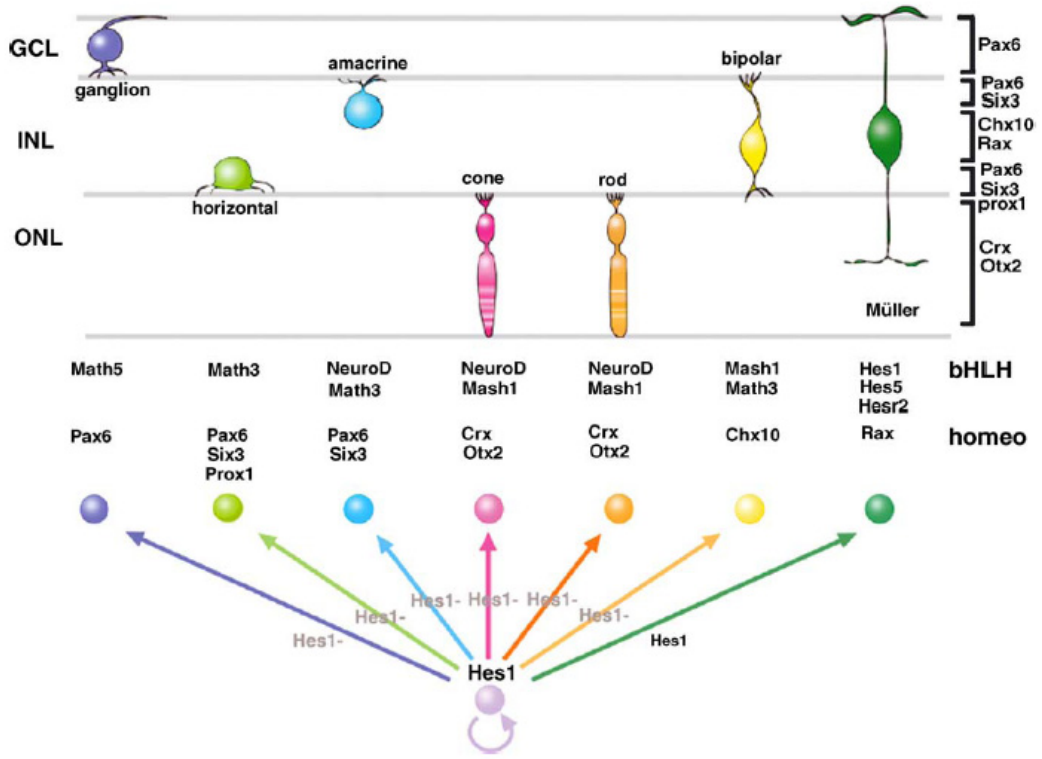


Figure 1.6. Cooperation of bHLH and homeodomain transcription factors for retinal cell type specification.

Hes1 inhibits neuronal differentiation and maintains progenitors. Differentiating neurons lose Hes1 expression. Homeodomain and bHLH factors determine the neural fate of retinal cell type. The cells that do not lose Hes1/Hes5 expression during neurogenesis stages adopt the Müller glial fate (from Hatakeyama and Kageyama, 2004).

Instead, *Prox1* is involved in horizontal cell differentiation and null mice lack horizontal cells (Dyer et al., 2003).

Another gene, *Foxn4*, coding for a winged/helix forkhead transcription factor, has been isolated and controls the competence state of amacrine and horizontal cells. Mutation in *Foxn4* result in the elimination of horizontal cells and a great reduction of amacrine cells (Li et al., 2004).

Finally, *Crx/otx5* is involved in photoreceptor differentiation in mouse and *Xenopus* (Furukawa et al., 1997; Viczian et al., 2003). It is also demonstrated that CRX/OTX5 works in synergism with NRL, a leucine zipper transcription factor, in order to promote rod differentiation (Mitton et al., 2000; Mears et al., 2001).

1.2.d Towards an integrated model

On the basis of the above-mentioned observations, the competence model can explain the different roles of the extrinsic and intrinsic factors. Environmental signal can alter the relative proportion of each cell type generated at a given time but it cannot influence progenitors to make temporally inappropriate cell types. Competence states seem to be intrinsically defined and within a given competence state, the generation of a particular type of cell is regulated by positive and negative extrinsic signals (Livesey and Cepko, 2001).

Retinal histogenesis offers a good example of a complex phenomenon where multiple players are involved in establishing the generation of a specific type of neuron. The emerging scenario is that the presence of these players must be tightly controlled in space and time to generate the different retinal cell types. In other words, the molecular players must be active in the right cellular type at the appropriate time. This regulation is a multi-step process which is carried out at different levels by means of transcriptional, translational and post-translational mechanisms.

In this point of view, the debate on the major importance of the extrinsic or intrinsic factors on cell fate determination should be downsized. For example, most of the extrinsic factors, like growth factors, neurotrophins and secreted peptides, are molecules produced by specific cells at a specific time and are the result of a strong-intrinsic-programmed cell activity.

On the other hand, these extrinsic factors do usually act on specific receptors whose expression, localization and function is highly controlled by

transcriptional and post-transcriptional mechanisms.

Moreover, some extrinsic factors can regulate the expression of several transcription factors. For example, it has been demonstrated that CNTF/LIF, extrinsic factors able to block rod differentiation, as we have seen, specifically activate a molecular cascade that acts on *Crx* promoter, inhibiting its transcription (Ozawa et al., 2004). This observation represents an evidence of the strong interplay between extrinsic signals and intrinsic factor activity during neuroretinal histogenesis.

Transcription factor activity is well-controlled at different levels. For instance, Moore and co-workers have shown that, in *Xenopus*, the activity of bHLH factors can be regulated post-translationally in a temporally tight specific manner, so that each factor is active at the right time during retinogenesis (Moore et al., 2002). This is the case of *NeuroD* which is regulated through phosphorylation by glycogen synthase kinase-3β (GSK 3β). GSK 3β prevents XNeuroD to promote frog retinal neurogenesis at early stages, by phosphorylation at a specific site; at later stages, GSK 3β inhibition is released, allowing XNeuroD to promote later cell type differentiation. Interestingly, a mutated form of XNeuroD which cannot be phosphorylated by GSK 3β promotes ganglion cell fate like *Xath5* does (Kanekar et al., 1997; Moore et al., 2002).

Importantly, during retinal development, it seems that several different transcription factors may be expressed in the same progenitor cells, a fact that suggests the possibility of a combinatorial mode of action. There are several findings that support such an idea. Double mutants of *Math3* and *NeuroD* have no amacrine cells, whereas single mutants of either gene exhibit normal amacrine cell number. Overexpression of either *Math3* or *NeuroD* in murine retinal explants results in an increase in rods, however both produce amacrine cells when coexpressed with either the homeodomain transcription factors *Pax6* or *Six3* (Inoue et al., 2002).

Finally, although the function of the transcription factors controlling retinal cell development has been revealed, little is known regarding the

regulation of their transcription. Chen and Cepko (2007) have investigated this aspect by analysing histone acetylation, a post-translational modification that leads to changes in chromatin structure and transcription. The acetylation level of histones is governed by opposing effects of two enzymes, histone acetyltransferase (HATs) and histone deacetylases (HDACs). HDACs lead to transcription repression by packaging chromatin structure, while HATs relax it increasing transcriptional activity.

Interestingly, the authors found that inhibition of HDACs on P2 mouse retinal explants produces a significant reduction of RNA level for genes that regulate retinal development, as well as cell cycle regulators (Chen and Cepko, 2007).

Surprisingly, several of these genes encode transcription factors essential for photoreceptor differentiation, like *Otx2*, *Nrl, Crx, NeuroD1 and NeuroD4/Math3*. Moreover, using luciferase reporter assays, the promoter activity of both *Nrl* and *Crx* was found to be compromised by HDAC inhibition, suggesting that they may directly regulate their transcription (Chen and Cepko, 2007).

The effects of HDAC inhibition was essayed on retinal development, too. Beside the reduced proliferation and increased apoptosis, the block of HDAC activity produces a complete loss of rods (and Müller glia), and an increase of bipolar cells (Chen and Cepko, 2007), suggesting that HDACs are involved in regulating key transcription factors involved in rod differentiation and that loss of their activity can drive bipolar cell differentiation at the expense of other cell types, mainly rods.

1.2.e Cell cycle progression and cell fate determination

An important question for understanding histogenesis is how cell cycle exit is coordinated with cellular determination (Cayouette et al., 2006). Several

observations show that cell fate determination events are linked to specific phases of the cell cycle.

As we have seen before, *Xath5* overexpression in the retina not only biases progenitors to give rise to ganglion cells, but also induces these cells to exit the cell cycle at the appropriate histogenetic window for ganglion cell genesis, indicating that this cell fate determinant modulates the cell cycle machinery (Ohnuma et al., 2002a).

Prox1 represents another paradigmatic example of a transcription factor coupling cell fate determination and cell cycle control. Dyer *et al.* (2003) demonstrated that *Prox1*, necessary and sufficient for horizontal cell differentiation, regulates at the same time the exit of progenitor cells from the cell cycle in the embryonic mouse retina (Dyer et al., 2003).

If cell determination factors affect the cell cycle, it might not seem surprising that cell cycle factors can affect determination. Indeed, when the cyclin-dependent kinase (CDK) inhibitor p27 (Xic1) is overexpressed, it strengthens the ganglion cell-promoting activity of *Xath5*. In contrast, when *Xath5* and *cyclinE1* are cotransfected, progenitors are kept in the cell cycle and the effect of *Xath5* is largely abolished (Ohnuma et al., 2002a).

In this regard, the action of *Xrx1,* a homeobox gene promoting proliferation of retinal progenitors, is remarkable (Casarosa et al., 2003). By comparing the effects of *Xrx1* with those of *cyclin-dependent kinase 2* (*cdk2*), a strong mitotic promoter, the authors demonstrated that despite the similar increase in clonal proliferation, the two factors act differently on retinal cell determination. Indeed, while *cdk2* promotes the differentiation of late-born retinal cell types (such as bipolar cells) at the expense of a decrease in early-born cell types, *Xrx1* does not produce any change in the proportions of the different cell types, suggesting a role in supporting proliferation and multipotency of retinal progenitors (Casarosa et al., 2003).

Another important aspect concerns the observation that formation of a cell lineage involves multiple rounds of cell division and that symmetric versus

asymmetric mode can influence progenitors in generating neural cell diversity.

In invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans,* asymmetric segregation of cell fate determining proteins and mRNAs to daughter cells makes an important contribution to cell diversification (Knoblich, 2001; Lu et al., 2000; Rose and Kemphues, 1998).

In *Drosophila,* for example, asymmetric segregation of the cell fate determinant Numb (an inhibitor of Notch signalling) to only one daughter of the sensory organ precursor cell is essential to confer distinct fates (Rhyu et al., 1994). Evidence that this mechanism may operate in mammalian central nervous system came from a pioneering study by Chenn and McConnell (1995) on the developing ferret cortex.

In the developing retina, the process of symmetric and asymmetric division has been studied in terminal divisions. A mammalian homologue of Numb (mNumb), is asymmetrically localized at the apical pole of the dividing progenitor (Cayouette et al., 2001; Dooley et al., 2003) and is asymmetrically inherited by the apical daughter cell in vertical divisions, whereas it is symmetrically inherited by both daughter cells in horizontal divisions (Cayouette et al., 2006).

Imaging of labelled retinal progenitors has demonstrated that the two daughter cells in a horizontal terminal division tend to become the same cell type, whereas the two daughter cells in a vertical division tend to produce daughters that become different cell types (Cayouette and Raff, 2003).

Moreover, overexpression of mNumb in progenitors resulted in more daughter cells of the same cell type (rods, at the expense of interneurons and Müller glia). This finding indicates that the plane of division influences cell fate choice in the retina and that asymmetric segregation of mNumb normally influences some of this choice (Cayouette and Raff, 2003).

1.2.f Concluding remarks: wiring cell components of the retina

Following proliferation, differentiation and migration of the retinal neurons, the major sequence of developmental events in the retina pertains the formation of connections between its cellular components and between the retina and its brain targets. Within the retina, organization of its networks occurs progressively and with precision.

First, the various cell types need to express their appropriate neurotransmitters for intercellular communication. Second, retinal neurons need to extend processes (Wong, 2006).

One important requirement for dendritic outgrowth of retinal neurons is that their arbors overlap, leading to a complete coverage of the retinal surface, in order to avoid any perceptual blind spot in the visual field (Eglen and Galli-Resta, 2006). Different cell types show different amount of overlap. The mechanism by which these mosaics of cell territories arise is fascinating and important because they relate to spatial processing by each cell population. In fact, ganglion cells that can sample at high acuity have small dendritic arbors that hardly overlap, whereas those that detect motion show great overlap (Wong, 2006).

It is surprisingly that early circuits are functional and able to generate electrical activity before the retina is sensitive to light. Amacrine and ganglion cells form the first synaptic circuit in the retina (Wong, 2006).

In particular, it has been demonstrated that spontaneous discharges of neighbouring ganglion cells are correlated during prenatal life and this activity is believed a process to refine retinotopic maps in the brain (Maffei and Galli-Resta, 1990).

Photoreceptors develop later and bipolar cells connect the outer retina to the inner retina after the eye is wired to the visual stations in the brain. Then, light responses emerge shortly before eye opening in mammals, preparing the retina to perform its important function in visual processing (Wong, 2006).

1.3 *Otx* genes and retinogenesis

Otx/otd genes are a class of homeobox genes related to the *orthodenticle (otd)* gene of *Drosophila*, required for normal development of anterior nervous system, eye and antenna of the fly (Cohen and Jurgens, 1990; Finkelstein et al., 1990; Boncinelli et al., 1994) and for regulating the expression of rhodopsin in photoreceptors (Tahayato et al., 2003).

Two *otx* genes, *Otx1* and *Otx2*, were initially isolated in mouse (Simeone et al., 1992) and shown to be essential for correct development of the rostral brain and sensory structures, including ear, nose and eye (Simeone et al., 1993; Martinez-Morales et al., 2001).

Ectopic expression studies in *Xenopus* suggest that OTX2 operates early in eye development, interacting with a network of eye-field transcription factors, including RX1, PAX6 and SIX3 (Zuber et al., 2003).

During eye morphogenesis, initial expression in the entire optic vesicle becomes restricted to the presumptive retinal pigmented epithelium (Simeone et al., 1993; Bovolenta et al., 1997; Martinez-Morales et al., 2003), where OTX2 protein interacts with the transcription factors MITF, leading to the activation of target genes, including *tyrosinase*, coding for a melanogenic enzyme (Martinez-Morales et al., 2003). Later, *Otx2* is increasingly expressed in neural retinal cells, including postmitotic precursors in the ONL and INL (Bovolenta et al., 1997; Nishida et al., 2003). In mouse and rat adult retina, OTX2 is present in the cytoplasm of photoreceptors and nuclei of bipolar cells (Baas et al., 2000; Rath et al., 2007).

Homozygous *Otx2* knock-out phenotype is severe, the embryos have gastrulation defects, die early in embryogenesis and lack anterior neuroectoderm fated to become forebrain, midbrain and rostral hindbrain (Acampora et al., 1995 and 1996; Ang et al., 1996; Matsuo et al., 1995). Heterozygotes show highly variable phenotypes - ranging from acephaly, micrognathia, microphthalmia, anophthalmia, to normal - depending on genetic background (Acampora et al.,

1995; Matsuo et al., 1995; Ang et al., 1996).

Recently, a wide spectrum of mutations in human *OTX2* has been analyzed. The expression pattern of *OTX2* in human embryos is consistent with the eye phenotypes observed in the patients, which range from bilateral anophthalmia, microphthalmia to retinal defects resembling Leber congenital amaurosis and pigmentary retinopathy (Ragge et al., 2005), thus confirming its importance in retinal development.

On the other hand, *Otx1*$^{-/-}$ phenotype is less severe, causing a reduction of cerebral cortex, loss of the lateral semicircular canal in the inner ear and ciliary process in the eye (Acampora et al., 1996; Morsli et al., 1999).

Several evidences demonstrated an extensive functional conservation among *otx/otd* genes. For instance, *Otx1*$^{-/-}$ and *Otx2*$^{-/-}$ phenotypes can be rescued by the *Drosophila otd* gene (Acampora et al., 1998 and 2001).

Conversely, the effects of *otd* mutation in *Drosophila* are rescued by either human *OTX1* or *OTX2* (Leuzinger et al., 1998; Nagao et al., 1998). Finally, *Otx1* and *Otx2* seem interchangeable with respect to many aspects of mouse anterior development (Acampora et al., 2003).

Crx (*Cone-rod homeobox*) is an *otx* gene important for the differentiation and maintenance of photoreceptors and pinealocytes, where it is specifically expressed (Freund et al., 1997; Furukawa et al., 1997). Phylogenetic analysis confirmed the relationship between the *otx5/5b* genes characterised in amphibians and condrichthyans and the *crx* member of mammals and fishes (Plouhinec et al., 2003). However, in contrast to *otx5/5b* genes, *crx* underwent a relaxation of the constraints during evolution, leading to a loss of its early expression and of its role in pineal photoreceptor specification (Plouhinec et al., 2003). Probably the loss of a direct sensitivity to light in the epiphysis of adult mammals could account for this divergence of *Crx* in mammals (Plouhinec et al., 2003).

The CRX protein is able to bind and activate photoreceptor specific genes such as interphotoreceptor retinoid-binding protein (IRBP), β-phosphodiesterase, arrestin and opsin (Chen et al., 1997; Furukawa et al., 1997).

CRX biological activity greatly depends on molecular interactions with partners such as NRL, an essential cofactor for vertebrate rod development (Mears et al., 2001).

Mutations in *CRX* are associated to diverse human retinal diseases: dominant cone-rod dystrophy (CORD2) (Freund et al., 1997, Swain et al., 1997), Leber congenital amaurosis (Freund et al., 1998; Swaroop et al., 1999; Rivolta et al., 2001; Tzekov et al., 2001) and late-onset dominant retinitis pigmentosa (Sohocki et al., 1998).

In mouse, *Crx* function seems essential for terminal differentiation: in $Crx^{-/-}$ mice, outer segment morphogenesis of photoreceptors is blocked at the elongation stage, leading to the failure in production of the phototransduction apparatus (Furukawa et al, 1999; Morrow et al., 2005). Further, photoreceptors demonstrated severely abnormal synaptic endings in the outer plexiform layer (Morrow et al., 2005).

However, though defective, photoreceptors do initially develop in $Crx^{-/-}$ mice, suggesting that their commitment rely on other players. In particular, results of conditional *Otx2* loss-of-function in the mouse retina suggest that *Otx2* controls photoreceptor (and pinealocyte) initial specification by activating *Crx* expression in committed precursors (Nishida et al., 2003).

In *Xenopus*, *Xotx2* and *Xotx5b* (the homolog of *Crx*) are expressed in different patterns during retinal histogenesis: transcription of both genes starts at tailbud stage in a diffused fashion throughout the retina, but then their expression is progressively restricted and, in the mature retina, *Xotx2* mRNA is found only in bipolar cells, while *Xotx5b* is transcribed in both photoreceptors and a subset of bipolar cells (Viczian et al., 2003). Even more dramatic is the difference in the protein expression pattern: XOTX2 protein is detected only in bipolar cells, while XOTX5b is produced only in photoreceptors at larval stage, due to precise translational control through the 3'UTR regions of their mRNAs (Decembrini et al., 2006).

Consistent with the pattern of protein distribution, lipofection of progenitors with constitutively expressed *Xotx2* and *Xotx5b* cDNAs of their coding sequences showed dramatic differences of effects, with *Xotx2* driving cells toward bipolar cell fate, and *Xotx5b* toward photoreceptor cell fate (Viczian et al., 2003; Wang and Harris, 2005; Decembrini et al., 2006).

Interestingly, swapping domain experiments showed that the differential activities of XOTX2 and XOTX5b are due to their carboxy-terminal (C-terminal) parts (Viczian et al., 2003). Significantly, lipofection of chimeric constructs (*Xotx2engR* and *Xotx5bengR*), in which the transactivation domain of either XOTX2 or XOTX5b is replaced with the repressor domain of the *Drosophila* Engrailed protein, had specific effects on either bipolar cells or photoreceptor cells, respectively, this time leading to a decrease, instead of an increase, in their frequency (Viczian et al., 2003).

This suggested that these XOTX-EngR chimeric proteins retain a region of the XOTX2 or XOTX5b proteins crucial for their differential activities. Interestingly, swap-experiments performed by replacing the C-terminal of XOTX5b with the one of XOTX2 (and *vice versa*) showed that the obtained chimeric construct XOTX5b/2 (or XOTX2/5b) is capable of producing the same phenotype as XOTX2 (or XOTX5b, respectively), according to the C-terminal fused to (Viczian et al., 2003).

1.3.a Molecular characteristics of OTX proteins

Otx genes code for transcription factors with a homeodomain of the K_{50} Paired-like class, characterised by a lysine residue at position 50 of the homeodomain. The homeodomain is a 60 amino acid module representing a variation on a helix-turn-helix motif of prokaryotic repressor. Three α-helical regions are separated by turns in the protein backbone. Helix 3 (recognition helix) of the homeodomain binds to the major groove of DNA, while helices 1 and 2 lie

outside the double helix. Helix 3 contacts both the phosphate backbone and specific bases. An N-terminal arm lies in the minor groove, and makes additional contacts (Lewin, 2003).

The homeodomain is followed by a glutamine-rich region, a basic region (rich in lysine and arginin) and a WSP domain, a highly conserved region of unknown function (Fig. 1.7). The OTX proteins have an OTX-tail, at first identified in CRX (Furukawa et al., 1997) but usually present in tandem repetition. By deletion analysis it has been demonstrated that multiple regions in the C-terminal portion of CRX contribute to its transactivating activity. AD-1 region (formed by two subregions, a and b) plays a major role in transactivation. In contrast, AD-2 region (that comprises the basic region and the WSP domain) plays a minor role in transactivation (Chau et al., 2000a; Chen et al., 2002).

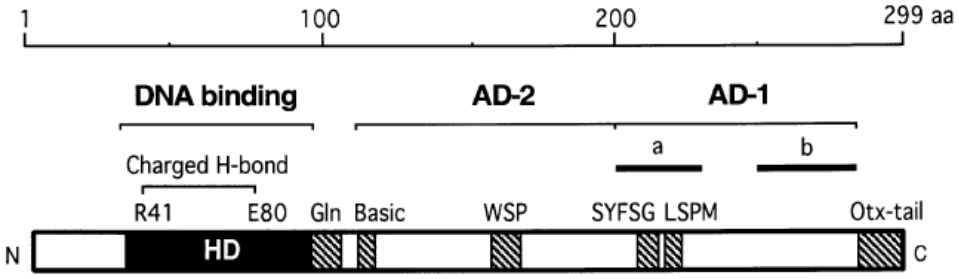


Figure 1.7. Schematic representation of the CRX structure.

The drawing shows the main regions of the primary structure of the CRX protein (from Chen et al., 2002).

OTX nuclear trafficking is highly regulated. The pathway of transport to the nucleus is mediated by nuclear localization signal (NLS) sequences that are characterized by one or more clusters of basic amino acids (Fei and Hughes, 2000). By deletion analysis it has been demonstrated that CRX NLS resides in the

C-terminal of the homeodomain, between residue 88 and 107 (Fei and Hughes, 2000). Moreover, nuclear translocation of CRX is mediated by Karyopherin 13 (also referred as Importin 13), that directly binds to the CRX homeodomain and to its flanking regions, mediating the nuclear translocation (Ploski et al., 2004).

Recently, a structural characterization of OTX2 was carried out. As for CRX, OTX2 nuclear localization is controlled by a nuclear localization sequence located within the homeodomain. Moreover, it works in conjunction with a novel nuclear retention domain, located downstream of the homeodomain (Chatelain et al., 2006).

In the context of protein trafficking, it is interesting to mention the presence in homeoproteins of a peptide, called penetratin, firstly identified in *Drosophila* Antennapedia (Dom et al., 2003). Penetratin is a 16-amino acid long peptide corresponding to the third α-helix of the homeodomain. Penetratin allows a translocation through biological membrane by means of a receptor-, endocytotis- and energy-independent mechanism, in which a tryptophan residue has an instrumental role (Christiaens et al., 2004). OTX2 protein also contains a penetratin sequence that allows it to translocate transynaptically from bipolar cells to target visual stations in the brain (Prochiantz, unpublished). This finding may explain the presence of OTX2 protein in the cytoplasm of ganglion cells in mouse and rat retina, although there is no *Otx2* transcript in those cells (Baas et al., 2000; Rath et al., 2007).

Besides DNA binding and protein trafficking, the homeodomain is involved in protein-protein interactions. Several cofactors have been identified that interact with OTX proteins. For example, it has been demonstrated that CRX binds to NRL (Mitton et al., 2000) and to NR2E3, forming a trimeric complex able to induce photoreceptor differentiation (Peng and Chen, 2005).

CRX interacts and synergizes also with SP4 (Lerner et al., 2005); p300/CBP (Yanagi et al., 2000); HMGA1 (Arlotta et al., 1997; Chau et al., 2000b) and QRX (Wang et al., 2004).

By contrast, several CRX interactors have been characterized that repress

CRX transactivation ability. For example, Phosducin (Phd) and Phd-like orphan protein1 (PhLOP1) - two G protein interactors - directly bind to CRX and inhibit its transactivation ability (Zhu and Craft, 2000); interestingly, the authors speculate that light-activated phototransduction events produce a Phd peptide that interacts with CRX preventing CRX-regulated gene expression in a light-dark dependent manner (Zhu and Craft, 2000).

Other transcriptional corepressors have been isolated, such as BAF (Barrier to Autointegration Factor) (Wang et al., 2002) and Ataxin-7, in which polyQ expansion, responsible for spinocerebellar ataxia 7, antagonized CRX function producing retinal degeneration (La Spada et al., 2001; Chen et al., 2004). Moreover, another mechanism of CRX inhibition is performed by MOK2, that represses its transcription by competing for DNA-binding (Arranz et al., 2001).

1.3.b Insights on OTX interaction: molecular network underlying photoreceptor differentiation

During retinal development in mouse, cells of the photoreceptor lineage turn on the expression of *Otx2,* which is essential but not sufficient for the photoreceptor differentiation. This has been established by means of an *Otx2* conditional knock-out (CKO), in which *Otx2* was inactivated under control of the *Crx* promoter (Nishida et al., 2003): CKO mice showed a complete loss of retinal photoreceptors. Moreover, it was found that *Otx2* is a direct upstream regulator of *Crx* (Nishida et al., 2003). On the other hand, *Crx* is able to regulate its own expression and its promoter contains four CRX-binding sites (Furukawa et al., 2002). So, the upregulation of *Crx* may be a necessary step for the expression of both rod and cone genes (Chen et al., 1997).

Several evidences have demonstrated that some extrinsic factors are able to influence photoreceptor differentiation (see Paragraph 1.2.c). In particular, two related cytokines, CNTF and LIF, inhibit the function of photoreceptors by

suppressing the expression of photoreceptor genes, including *opsin* (Kirsch et al., 1998; Neophytou et al., 1997; Ezzeddine et al., 1997; Schulz-Key et al., 2002). CNTF acts via CNTF/gp130 receptor and consequent STAT3 phosphorylation, inhibiting *Crx* expression (Ozawa et al., 2004). On the other hand, to induce signal transduction, LIF binds to LIF receptor β/gp130. In particular, it has been demonstrated that LIF neither prevents nor alter the timing of outer and inner nuclear layer separation, but it inhibits phototransduction gene expression in both rods and cones, thereby blocking functional maturation of photoreceptors. In particular, LIF reduces the expression of *Crx*, *Nrl* and *Nr2e3* and upregulates the expression of the transcription inhibitors *Baf* and *Fiz1* (a NRL corepressor) (Graham et al., 2005).

So, a complex interplay between extrinsic factors on transcription factors and among transcription factors themselves, regulates photoreceptor cell fate and terminal differentiation.

A number of experiments has been performed in order to clarify the regulatory targets of CRX and its cofactors, in particular NRL and NR2E3.

Nrl (neural retina leucine zipper) was identified as a Maf-family protein (Swaroop et al., 1992) specifically expressed in rods and required for their differentiation. Missense mutations in human *NRL* have been associated with autosomal dominant retinitis pigmentosa (Bessant et al., 1999; Martinez-Gimeno et al., 2001). *Nrl*$^{-/-}$ mice show a complete loss of rod function and supernormal cone function, mediated by S-cones, similar to the clinical phenotype of enhanced S-cone syndrome (ESCS) in humans, characterized by night-blindness and increased S-cone sensitivity (Mears et al., 2001).

In a recent work, Oh *et al.* (2007) investigated, by means of an elegant genetic tool, the pivotal role of *Nrl* in rod differentiation. They generated transgenic mice that express *Nrl* under the control of *Crx* promoter, obtaining functional retinas with only rod photoreceptors, thus demonstrating that *Nrl* is not only essential, but also sufficient for rod differentiation, able to transform cone precursors to functional rods (Oh et al., 2007). In addition, the authors found that

NRL is associated with specific promoter sequences in *Thrb* (encoding TRβ2, a transcription factor required for M-cone differentiation) and *S-opsin,* thus directly participating in transcriptional suppression of cone development (Oh et al., 2007).

The same results have been confirmed in *Xenopus*, in which *Xnrl* lipofection is able to increase rods at the expense of cones (McIlvain and Knox, 2007). Moreover, the authors found that *Xnrl* (but not *hNRL*) is at the same time able to promote lens fiber cell differentiation, thus demonstrating an additional role of *nrl* in *Xenopus* (McIlvain and Knox, 2007).

One of the downstream targets of NRL is the photoreceptor nuclear receptor *Nr2e3,* which was identified as an orphan nuclear receptor specifically expressed by rods (Bumsted O'Brien et al., 2004). Noteworthy, loss-of-function mutations in the human *NR2E3* have been identified in patient with ESCS (Wright et al., 2004). NR2E3 was found to activate rod genes and suppress cone genes (Cheng et al., 2006), and it appears to have overlapping functions with NRL during photoreceptor differentiation. *rd7* mice, which harbor mutations of *Nr2e3,* exhibit similarities to $Nrl^{-/-}$ retinae, however the majority of photoreceptors in the *rd7* retinas represent a morphologically hybrid cell type that express both rod- and cone- specific genes (Corbo and Cepko, 2005).

Moreover, expression of *Nr2e3* in $Nrl^{-/-}$ retina completely suppresses cone differentiation and results in morphologically rod-like photoreceptors, that are however not functional (Cheng et al., 2006). Also in *Xenopus*, by lipofecting *hNR2E3* there is an increase of differentiated rods and the effect is more evident when *hNRL* and *hNR2E3* are colipofected, thus supporting the functional synergy between themselves (McIlvain and Knox, 2007).

In order to study the genetic network regulated by these key transcription factors, Qian *et al.* (2005) have performed a bioinformatic prediction, confirmed by biochemical analysis, identifying 169, 166 and 97 putative targets of CRX, NRL and NR2E3, respectively (Fig. 1.8). They also examined the combinatorial regulation of these transcription factors, demonstrating that they form a complex that co-regulates photoreceptors genes (Qian et al., 2005).

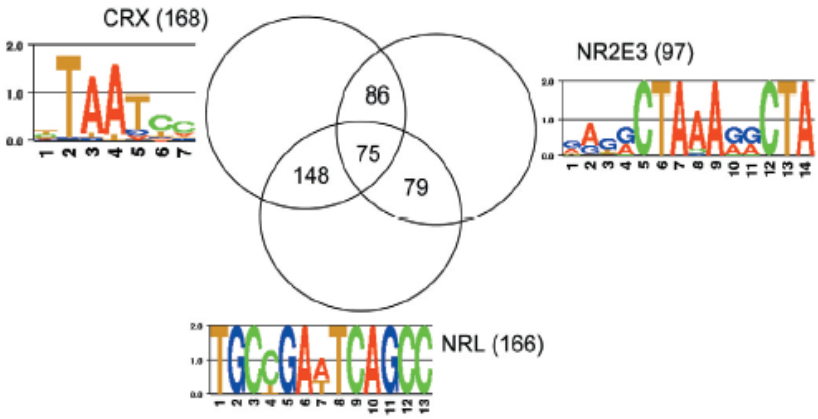


Figure 1.8. Diagram for the target genes of CRX, NRL and NR2E3.

The Venn diagram shows the target genes of CRX, NRL and NR2E3. The binding motive logos for each transcription factor are shown. The numbers in the parentheses represent the total number of predicted targets for each factor (from Qian et al., 2005).

By means of chromatin immunoprecipitation assay, Peng and Chen (2005) have demonstrated that CRX, OTX2, NRL and NR2E3 co-occupy the promoter/enhancer of several retinal genes. Moreover, by examining promoter occupancy using *Crx*$^{-/-}$ mice, they identified CRX-dependent (NR2E3) and CRX-independent (OTX2 and NRL) target binding. In particular, while OTX2 and NRL have identical distribution patterns on promoter in wild-type or *Crx*$^{-/-}$ mice, no NR2E3 gene targets are present in immunoprecipitates from *Crx*$^{-/-}$ mice (e.g.: *M-cone opsin, S-cone opsin, Arrestin3, Rhodopsin, etc.*), even if two CRX-independent genes were found (*Rhodopsin kinase* and *Rbp3*) (Peng and Chen, 2005).

Based on these observations and on the time of expression during development, availability, target genes and loss/gain of functions analysis of the key transcription factors, it is possible to propose a model of photoreceptor commitment and differentiation (Fig. 1.9). *Crx* is expressed in all postmitotic photoreceptor precursors and CRX-expressing cells are committed to

photoreceptor lineage, but not to a specific fate. Successive expression of *Nrl* dictates the rod fate, versus an otherwise default cone-fate. NRL and CRX synergize then with NR2E3, by activating rod genes and turning off cone ones (Oh et al., 2007).

Cone subtypes are then specified by other players. Ng *et al.* (2001) demonstrated that thyroid hormone receptor β 2 (TRβ2), a ligand-activated transcription factor, is important for M-cone formation, since the deletion of TRβ2 results in a selective loss of M-cones and an increase in S-cones (Ng et al., 2001; Yanagi et al., 2002).

On the other hand, S-cones are specified by retinoid-related orphan receptor β (RORβ), able to activate *Opsin1* promoter alone and in synergy with CRX. Moreover, RORβ-deficient mice fail to induce S-opsin appropriately during development (Srinivas et al., 2006).

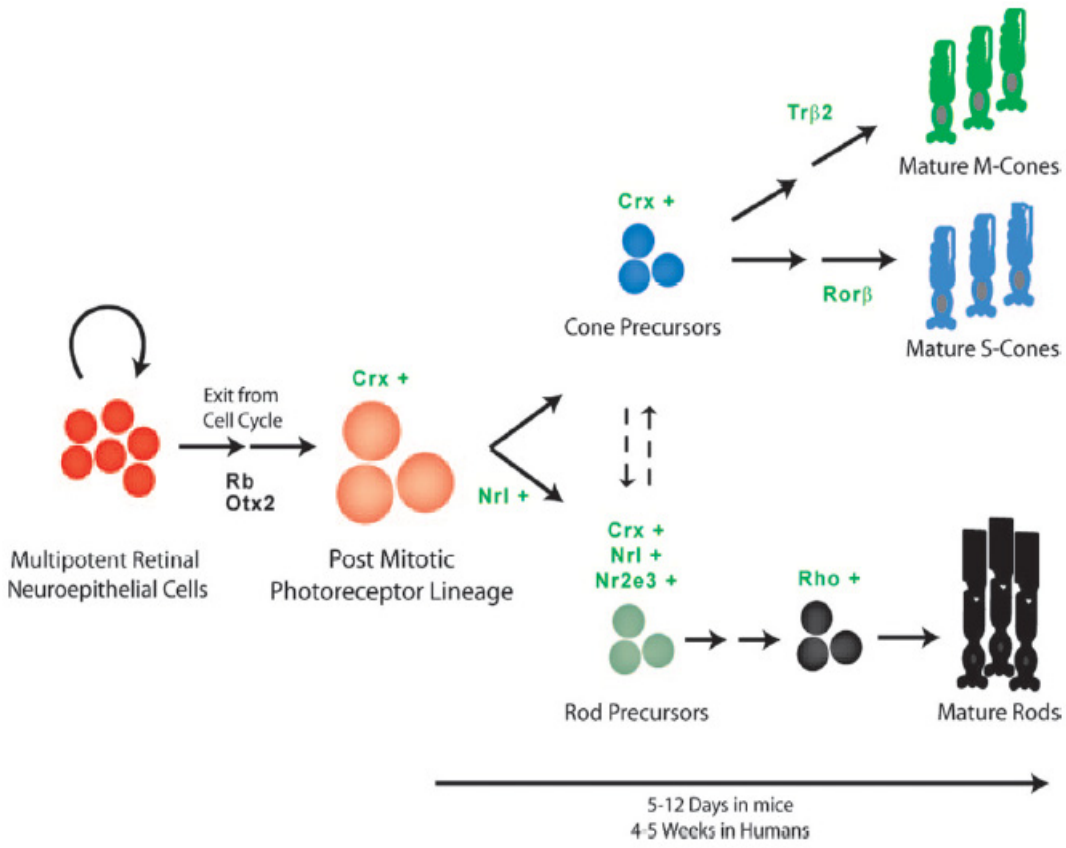


Figure 1.9. Model of photoreceptor specification.

Retinoblastoma (RB) influences multipotent retinal neuroepithelial cells to exit cell cycle. *Crx* (activated by OTX2) is the competence factor in postmitotic photoreceptor precursors. The cells that express *Nrl* are committed to rod fate, with subsequent expression of *Nr2e3*. The cells expressing only *Crx* are cone precursors. Additional transcription factors, such as *TRβ2* and *RORβ* are important for specification of M-cones and S-cones, respectively (from Oh et al., 2007).

1.3.c Phylogenetic considerations on photoreceptor lineage

The above-mentioned observations of the choice of rod versus cone fate arises the question on the relationship between these two types of photoreceptors. If we consider the timing of generation of cones and rods (cones appear at first,

then rods at the end of retinogenesis, see Paragraph 1.2.a) we can speculate that photoreceptor precursors appear that give rise to a default-cone fate early during retinogenesis. Then, when some factors are expressed, such as *Nrl*, they act as a "molecular switch" that represses cone fate and activates rod fate.

This ontogenetic pattern can be evaluated in the context of the phylogenetic finding that the first Vertebrates did possess cone photoreceptors, but not rods (Collin and Trezise, 2004). Generally, it is agreed that ancestral vertebrate visual system was based on cone-like photoreceptors, functioning under photopic condition (Collin and Trezise, 2004).

Molecular investigation has discovered five visual pigments in Agnathans (Lamprey) and that Agnathan lineage probably does not possess a true rod photoreceptor and true rhodopsin that, conversely, can be found in Gnathostomes (Collin et al., 2003). A phylogenetic analysis demonstrated that five kinds of opsins exist. It is interesting to remark that at first the all cone opsins appear, and then the rhodopsin, which derives from the S-opsin (Carleton et al., 2005).

Interestingly, the phylogenetic analysis of the Maf-family proteins revealed the NRL seems to not be present in Agnates, but appears in Gnatostomes (Coolen et al., 2005), according to the rod appearance.

The second aspect to consider is retinal circuitry. Basically, cones connect to bipolar cells, which in turn connect to ganglion cells. On the other hand, rods do not possess a dedicated retinal pathway. During evolution an attempt has been made in order to separate the two information and the mammalian retina possesses a rod pathway that "piggyback" the cone pathway (rods connect to rod bipolar cells that connect via AII amacrine cells to cone bipolars connecting to ganglion cells - see Paragraph 1.1) (Fig. 1.10).

A clear support for the cell diversity between photoreceptors comes from a recent microarray analysis in which cone gene expression, obtained using all-cone retinae derived from *Nrl*$^{-/-}$ mice, has been compared to that of rod-dominated wild type retinae (Corbo et al., 2007). Surprisingly, the authors found that photoreceptor genes can range from entirely rod- or cone-specific, showing

varying degrees of rod/cone co-expression.

In particular, 1934 transcripts were found upregulated and 991 downregulated in *Nrl*$^{-/-}$ versus wild type retinae, thus supporting the type-specific gene expression pattern of photoreceptors (Corbo et al., 2007).

Finally, it is interesting to note that typical retinopathies, such as retinitis pigmentosa, usually start with rod degeneration and then cause a second wave of degeneration involving cones, leading to total blindness.

Recently, Leveillard *et al.* (2004) have identified a rod-derived cone viability factor (RdCVF), a truncated thioredoxin-like protein, that is secreted by rods and maintains the cones alive (Leveillard et al., 2004). So, we can speculate that a kind of symbiosis exists between rods and cones. When rods appear during the evolution, they exploit the pre-existing cone pathway and in their turn produce a survival factor for cones.

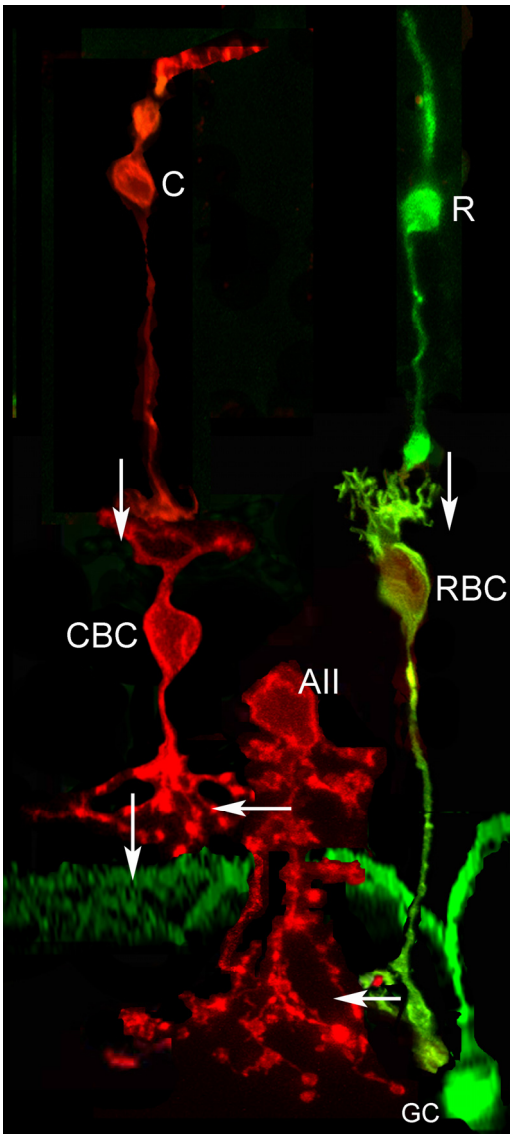


Figure 1.10. Rod pathway.

This is a five-neuron chain, typical of mammalian retinae. Rod photoreceptors (R) converge upon a single type of rod bipolar cell (RBC). These neurons have a bushy dendritic arborization with long axons running throughout the entire thickness of the inner plexiform layer. Rod bipolar cells connect via AII amacrine cells to cone bipolar cells (CBC), connecting to ganglion cells (GC) (from Strettoi, 2007).

Thesis scope and design

The general aim of this experimental work is to investigate the molecular mechanisms involved in determining neural cell fate during retinal neurogenesis.

The vertebrate retina is made of six main types of neurons, plus the Müller glia cells. All these different cell types are generated from a common pool of multipotent retinal progenitor cells, according to a precise time schedule that is largely conserved among different vertebrates (see Paragraph 1.2.a). The molecular players driving the progenitors towards specific cell fates are under intense scrutiny and several lines of evidence have shown that a crucial role in retinal cell differentiation is played by transcription factors.

In particular, this work focuses on the role of *Otx* genes, the orthologues of the *Drosophila orthodenticle* gene (*otd*), coding for homeodomain transcription factors, that play crucial roles in vertebrate brain and sensory organs development.

In the *Xenopus laevis* retina*, Xotx2* and *Xotx5b* respectively promote bipolar and photoreceptor cell fates (Viczian et al., 2003). Interestingly, swapping domain experiments showed that the differential activities of XOTX2 and XOTX5b are due to their carboxy-terminal regions, downstream of the homeodomain (Viczian et al., 2003).

To understand the mechanisms of the different effects of XOTX2 and XOTX5b on retinal cell fate, we performed an *in vivo* molecular dissection by transfecting retinal precursors with several constructs of *Xotx2*, *Xotx5b* and *otd*. We identified a 10 amino acid divergent region that is crucial for the specific retinal activity of XOTX2 and XOTX5b; therefore we called it "Retinal Specific box" (RS box). In particular, by means of *in vitro* mutagenesis, we demonstrated that when RS box of XOTX5b is converted into the corresponding one of XOTX2, the biological activity of XOTX5b is switched to that of XOTX2, and

vice versa. Moreover, deletion of both XOTX2 and XOTX5b RS box leads to complete loss of function of the two proteins in retinal cell fate specification.

Furthermore, because of previous work demonstrating extensive functional conservation of OTX/OTD proteins in early development of anterior region of fly and mouse embryos (see Paragraph 1.3), we asked whether similar functional conservation was also true for retinal cell fate specification. We therefore tested if *Drosophila otd* was able to direct progenitors toward any specific cell fate in lipofection experiments in the *Xenopus* retina. These experiments showed no OTD activity in this context. Then, we replaced the OTD region C-terminal to the homeodomain with either that of XOTX2 or XOTX5b, and we found that the C-terminal is able to "rescue" the OTD function. In particular, OTD/XOTX2 drove a bipolar cell fate, while OTD/XOTX5b promoted photoreceptor differentiation.

Finally, we tested whether the RS box could be sufficient to provide the biological activity of either XOTX2 or XOTX5b to *Drosophila* OTD. To test this we generated chimeric *otd/box2* and *otd/box5b* constructs, and we found that the RS box enabled OTD/box2 or OTD/box5b proteins to drive retinal progenitors toward bipolar or photoreceptor fates, respectively.

In order to clarify the molecular mechanisms of action of the OTX/OTD transcription factors, we performed a transactivation assay in order to test the ability of several *otx/otd* constructs (alone or together with *Xenopus* NRL, XNRL, a specific rod transcription factor) to activate a *rhodopsin* promoter. Interestingly, we demonstrated that RS box is able to switch the ability of the different constructs to transactivate one of the key photoreceptor specific genes.

One possible way to explain the different abilities of XOTX2 and XOTX5b is that the two proteins differentially interact with other key molecular players involved in retinal differentiation, such as XNRL itself. We performed a GST-pull down assays to monitor the ability of several OTX/OTD constructs to interact with XNRL and we found that XOTX2 and XOTX5b differentially interact with XNRL, though this property is not strictly dependent on the RS box.

Moreover, in the light of previous observations showing a functional dominant effect of XOTX2 on XOTX5b (Viczian et al., 2003), we performed a GST-pulldown assay between XOTX proteins, demonstrating that they are able to directly interact each other and that XOTX2 can impair the functional synergy between XOTX5b and XNRL in transactivating the *rhodopsin* promoter.

These experiments provide *in vivo* and *in vitro* molecular evidences on how homeodomain transcription factors can differentiate their functions. Moreover, the results we obtained highlight the role and unravel the mechanisms of action of two key homeodomain transcription factors in generating neural diversity in the vertebrate retina.

Materials and Methods

2.1 DNA constructs

pCS2$^+$-GFP (a kind gift of Dr. Federico Cremisi). The cDNA encoding the green fluorescent protein was cloned into EcoRI/HincII sites of pCS2$^+$ plasmid.

pCS2$^+$-RFP (a kind gift of Dr. Federico Cremisi). The cDNA encoding the red fluorescent protein was cloned into the pCS2$^+$ plasmid.

pCS2$^+$-Xotx2 (Viczian et al., 2003). This construct was generated by Prof. Robert Vignali (University of Pisa). The coding region (plus small flanking regions) was amplified by means of PCR and inserted into EcoRI site of pCS2$^+$.

pCS2$^+$-Xotx5b (Vignali et al., 2000; Viczian et al., 2003). This construct was generated by Dr. Sara Colombetti. The coding region (plus small flanking regions) was amplified by means of PCR and inserted into StuI site of pCS2$^+$.

pCS2$^+$-otd. (Lunardi and Vignali, 2006). This construct was generated by Dr. Yang Liu. The coding region (plus 50 nt of the 5'UTR and 24 nt of the 3'UTR) was amplified by PCR and inserted into EcoRI site of pCS2$^+$; fragments were amplified from an *otd* plasmid (kindly provided by Dr. Antonio Simeone).

Xop-GFP. (a kind gift of Barry Knox; Whitaker and Knox, 2004). This construct contains the *Xenopus rhodopsin* promoter -508 / +41 (*Xop*) driving expression of GFP.

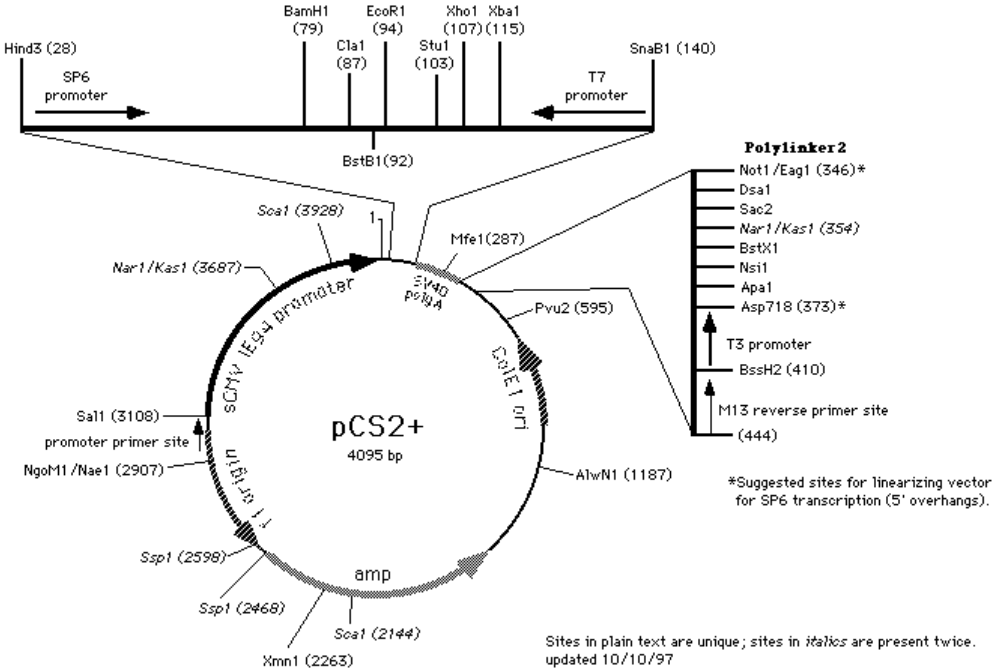


Figure 2.1. Map of the pCS2$^+$ plasmid.

pCS2$^+$ is a multipurpose expression vector. Although originally designed for expressing proteins in *Xenopus* embryos from either injected RNA or DNA, pCS2$^+$ is also useful for high-level transient expression in a wide variety of eukaryotic cells. pCS2$^+$ contains a strong enhancer/promoter (simian CMV IE94) followed by a polylinker and the SV40 late polyadenylation site. A SP6 promoter is present, allowing *in vitro* RNA synthesis of sequences cloned into the polylinker. A T7 promoter in reverse orientation is present between the polylinker and the SV40 polyA site for probe synthesis, as well as a second polylinker after the SV40 polyA site to provide several possible sites to linearize the vector for SP6 RNA transcription. The vector backbone is from pBluescript II KS+ and includes the amp resistance gene and an f1 origin for producing single stranded DNA. A number of derivatives of pCS2 have been constructed that allow fusions to epitope tags (i.e.: Myc-tag) and other marker proteins, as well as nuclear localization signals (http://sitemaker.umich.edu/dlturner.vectors/home).

p3xFLAG-Xnrl. This construct was generated for the present work. *Xnrl* full-length cDNA was cloned by RT-PCR from stage 42 *Xenopus* embryo RNA into the EcoRI/BamHI site of p3XFLAG-CMV10 (Sigma).

Mutant constructs

pCS2$^+$-Xotx5bMut1. This construct was generated by Dr. Yang Liu. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), 2 amino acid residues of pCS2$^+$-Xotx5b were converted: S100N and T101G.

pCS2$^+$-Xotx5bMut2. This construct was generated by Dr. Yang Liu. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), 2 amino acid residues of pCS2$^+$-Xotx5bMut1 were converted: A104N and P106V.

pCS2$^+$-Xotx5bMut3. This construct was generated by Dr. Yang Liu. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), 2 amino acid residues were inserted in pCS2$^+$-Xotx5bMut2: QQ99-100ins.

pCS2$^+$-Xotx2Mut3. This construct was generated for the present work. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), the region AA 100-109 QQNGGQNKVR of pCS2$^+$-Xotx2 was converted into STGQAKPR.

Deletion constructs

pCS2$^+$-Xotx2Δ. This construct was generated for the present work. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), the region AA 100-109 QQNGGQNKVR of pCS2$^+$-Xotx2 was deleted.

pCS2$^+$-Xotx5bΔ. This construct was generated for the present work. By using QuikChange^{TM} Site-Directed Mutagenesis kit (Stratagene), the region AA 100-107 STGQAKPR of pCS2$^+$-Xotx5b was deleted.

Chimeric constructs

pCS2$^+$-otd/Xotx2. This construct was generated for the present work. This is an in frame fusion encoding AA 1-96 of OTD and AA 62-288 of XOTX2, plus 50 nt of 5'UTR of *otd* and 4 nt of 3'UTR of *Xotx2*.

pCS2$^+$-otd/Xotx2. This construct was generated for the present work. This is an in frame fusion encoding AA 1-96 of OTD and AA 62-290 of XOTX5b, plus 50 nt of 5'UTR of *otd* and 25 nt of 3'UTR of *Xotx5b.*

pCS2$^+$-otd/box2. This construct was generated for the present work. This is an in frame fusion correspond to pCS2$^+$-otd, but have an insertion encoding AA 100-109 of XOTX2 replacing AA 132-137 of OTD.

pCS2$^+$-otd/box5b. This construct was generated for the present work. This is an in frame fusion correspond to pCS2$^+$-otd, but have an insertion encoding AA 100-107 of XOTX5b replacing AA 132-137 of OTD.

Myc-tagged constructs

pCS2$^+$-Myc-Xotx2, pCS2$^+$-Myc-Xotx5b, pCS2$^+$-Myc-Xotx2Mut3, pCS2$^+$-Myc-Xotx5bMut3, pCS2$^+$-Myc-Xotx2Δ, pCS2$^+$-Myc-Xotx5bΔ, pCS2$^+$-Myc-otd, pCS2$^+$-Myc-otd/box2, pCS2$^+$-Myc-otd/box5b, pCS2$^+$-NLS-Myc-otd were prepared by PCR cloning from the parental *Xotx2*, *Xotx5b*, *otd* or chimeric plasmids into pCS2$^+$-Myc and pCS2$^+$-NLS-Myc vectors.

GST-fusion constructs

GST-Xotx2. This construct was generated for the present work by in frame PCR cloning of fragments encoding XOTX2 AA 32-165 into the pYEX vector (a modified pGEX 2TK, kind gift of Dr. Luciana Dente).

GST-Xotx5b. This construct was generated for the present work by in frame PCR cloning of fragments encoding XOTX5b AA 32-164 into the pYEX vector.

GST-Xnrl. This construct was generated for the present work by in frame PCR cloning of *Xnrl* coding region into the pYEX vector.

GST-PDZ (a kind gift of Dr. Elena Landi). The cDNA encoding for the PDZ domain of PTP-BL was cloned into pYEX plasmid.

GST-Xsix3.2 (a kind gift of Dr. Massimiliano Andreazzoli). The cDNA encoding for *Xsix3.2* was cloned into pGEX plasmid.

2.1.a Purification of plasmid DNA

Plasmid DNA was extracted from bacterial cells (*Escherichia coli*, DH5α) by alkaline lysis and purified by chromatography over Nucleobond columns (Macherey- Nagel). The plasmids were used as templates for antisense probe or lipofected or transfected or transformed.

2.1.b Antisense labelled riboprobes synthesis

Standard RNA synthesis from linearized plasmids using SP6 or T7 RNA polymerases were carried out incorporating a digoxigenin conjugated

ribonucleotide. In particular, transcription reactions were carried out in the presence of 1mM each of ATP, CTP and GTP, 350 µM UTP and 650 µM DIG-11 UTP for 2 hours at 37 °C. After DNase digestion to remove template DNA, the RNA was precipitated with ammonium acetate and ethanol. The concentration of the RNA was estimated by agarose gel electrophoresis and spectrophotometry. Probes were then diluted in hybridisation mix at the final stock concentration of 10 µg/ml and stored at -20 °C for several months.

2.2 *Xenopus laevis* embryos

In order to obtain embryos, *Xenopus* females were pre-injected with 800 units of human chorionic gonadotropin (Gonasi HP 5000, Serono) the night before eggs collection. The next day, eggs are obtained by gently squeezing the frogs and then fertilized with testis homogenates. The embryos are cultured in 0.1X MMR (10X MMR: 10 M NaCl, 200 mM KCl, 100 mM $MgSO_4$, 200 mM $CaCl_2$, 500 mM HEPES, 10 mM EDTA). After half a hour from fertilization, jelly coats are removed keeping the embryos for some minutes in dejelling solution (0.2 mM Tris- HCl pH 8.8, 3.2 mM DTT). Embryos were staged according to Nieuwkoop and Faber (1956).

2.2.a Lipofections

By means of this technique, single retinal progenitors can be transfected *in vivo* at different stages of retinal differentiation (Fig. 2.2). It has been estimated that translation of the lipofected plasmids occurs after 6/8 hours after transfection (Dorsky et al., 1997), thus a certain control of timing of expression is possible. DNA isolated by Nucleobond midi preps was diluted in nuclease-free water to a concentration of 2 µg/µl. These stocks were spun down for at least 10 minutes at

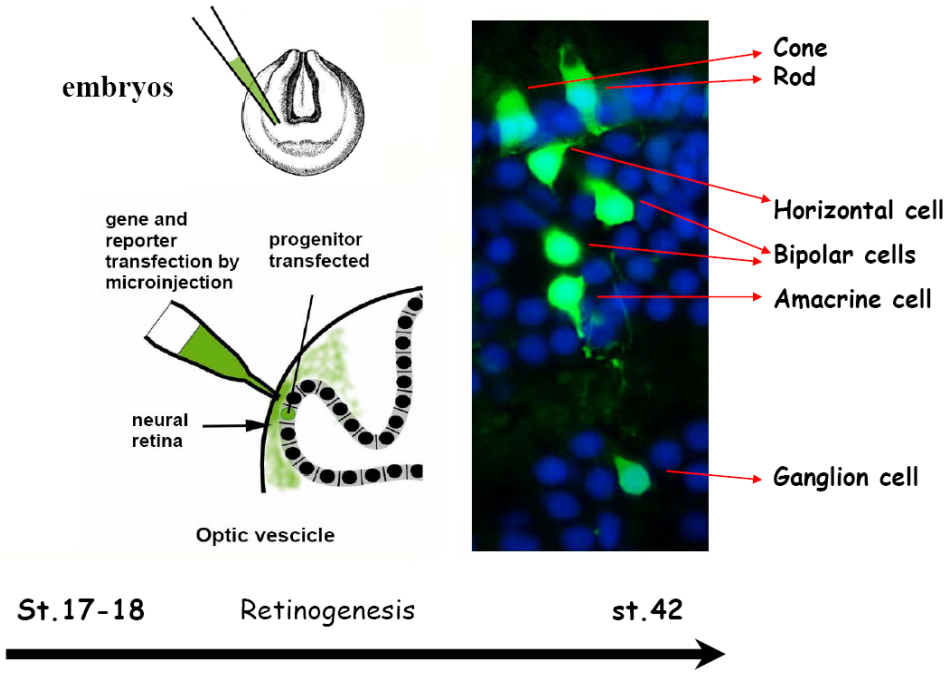


Figure 2.2. Schematic representation of the lipofection technique.

The scheme illustrates the lipofection technique performed on *Xenopus* embryos (st. 18). Single progenitors are transfected with a DNA construct + GFP. At the end of retinogenesis (st. 42), the clones derived from lipofected progenitors are analysed.

4°C before use. 1 µl of each construct was mixed with 1 µl pCS2^{+}-GFP (Green Fluorescent Protein) DNA to label the progeny of transfected cells. pCS2^{+}-GFP alone was used as the control. DOTAP (Roche) was added to DNA in a ratio of 1µg of DNA to 3 µl (Holt et al., 1990; Ohnuma et al., 2002b). The mixture was then injected into the presumptive eye region of stage 17-18 embryos using a Nanoject apparatus (Drummond). At stage 42, embryos were fixed in 4% paraformaldehyde for 1 hour at room temperature and sunk in 20% sucrose overnight at 4°C. Embryos were then embedded in O.C.T.^{TM} compound (Sakura), frozen and cryostat-sectioned (12 µm). Samples were rehydrated with two washes of 1X PBS for 5 minutes, mounted in Aqua Polymount (Celbio) and dried overnight at room temperature before microscope analysis.

Lipofected cells were scored by GFP fluorescence and assigned to the different cell types on the basis of their position within layers and their morphology; their identity was confirmed by molecular marker analysis. Statistical analysis on cell frequencies was performed by means of one-way ANOVA and Tukey-Kramer multiple comparison test.

2.2.b *In situ* hybridization

Embryos were cryostat-sectioned to be further processed for *in situ* hybridisation. Sections were unfrozen and dried at room temperature. *In situ* hybridisation was performed as follows. On the first day, sections were incubated overnight in probe mix (1 µg/ml hybridisation mix: 50% formamide, 10% dextran-sulphate, 1 mg/ml Torula RNA, 1X Denhart's, 1X salts - 10X salts**:** 114 g NaCl, 14.04 g Tris HCl, 1.34 g Tris base, 7.8 g NaH_2PO_4. $2H_2O$, 7.1 g NaH_2PO_4) at 65 °C in a humidified chamber (50% formamide, 1X salts).

The next day, sections were washed 30 minutes for 3 times in washing solution to eliminate the unbound probe which may lead to background. After two washes in MABT, 30 minutes each, the sections were incubated for two hours at RT in blocking solution. After this time, they can be incubated in antibody solution overnight at room temperature.

The unbound antibody must be removed with 5 washes, 30 minutes each, in MABT. To reveal the hybridized probe, sections were washed twice for 10 minutes in Alkaline Phosphatase Buffer (APB: 100mM Tris- HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 5mM Tetramisole (Sigma), 0,1% Tween-20), For the detection reaction, sections are incubated with Fast Red (Roche) until the staining reaches the desired intensity.

Probes used for *in situ* hybridizations were: *Xirbp* for photoreceptors (Gonzalez-Fernandez et al., 1993), *Xhermes* for ganglion cells (Gerber et al.,

1999), *Xprox1* for horizontal cells (Dyer et al., 2003), *Xvsx1* for bipolar cells (D'Autilia et al., 2006).

2.2.c Immunostaining and immunofluorescence

Cryostat-sections were unfrozen and dried at room temperature, then rehydrated with two washes of 1X PBS for 5 minutes. The primary antibody was added and incubated at room temperature for 2 hours (or at 4°C over night). After three washes of PBSX (PBS + Triton X-100 0,01%), secondary antibody (with 1 µg/ml Hoechst solution, to visualize nuclei) was added and incubated for 2 hours. The samples were washed three times with PBSX and mounted in Aqua Polymount (Polysciences). To identify lipofected cells after *in situ* hybridisation an anti-GFP antibody (Invitrogen) was used. To identify amacrine cells we used anti-5-hydroxitryptamine (5-HT), anti-γ-amino-butiryc acid (GABA) and anti-tyrosine hydroxylase (TH), all purchased from DiaSorin. The anti-XOTX2 and anti-XOTX5b antibodies were described in Decembrini *et al.* (2006).

2.3 GST-pull down assay

2.3.a GST-fusion protein production and purification

GST-fusion proteins were expressed in *Escherichia coli* BL21 upon transformation with appropriate constructs. Cultures were grown to mid-log phase (A_{600}=0.7) in Luria-Bertani medium at 37° C, induced with 1.0 mM isopropyl thio-β-D-galactopiranoside, and grown for an additional 3-4 hours. 50 ml of culture were centrifuged at 4000 rpm for 15 minutes, resuspended in ice-cold PBS and lysed on ice. After addition of lysozyme (200 µg/mL), 10 mM DTT (in AcONa 10 mM pH 5.2), protease inhibitor mix (AEBSF 2 mM, EDTA 1 mM, bestatin 130 µM, E-64 14 µM, leupeptin 1 µM, aprotinin 0.3

μM) (Sigma) - final concentrations - mixture was left on ice for 30 minutes. Then the Triton X-100 1% (v/v); $MgCl_2$ 10 mM, DNase 100 μg/ml (final concentrations) were added; the mixture was left on ice for further 30 minutes and then centrifuged at 4°C, 14.000 rpm for 20 minutes.

100 μl of Glutathione Sepharose 4B resin (Amersham) were used for each experiment and control. Resin was washed three times with ice-cold PBS and centrifuged at 2500 rpm for 1 minute, following each wash. BL21 extract was then incubated with the resin for 1 hour at 4°C on a shaker. Then three washings were carried out as above. A small aliquot of the functionalized resins (10 μl) was denatured with loading buffer (Tris HCl pH 6.8 125 mM, 2 mercaptoethanol 10% SDS 4%, glycerol 20%, Bromophenol blue 0.1%) at 95°C for 5 minutes. Different dilutions of this aliquot were subjected to SDS-PAGE, to estimate the relative quantities of each functionalized resin in the experimental and control resins. Finally, resins were soaked in a solution of 3% BSA (w/v) in PBS to achieve blocking, and left at 4°C overnight.

2.3.b Cell transfection and pull-down assay

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% (v/v) Foetal Bovine Serum (GIBCO). Transfections were performed using Lipofectamine 2000 (Invitrogen). Following a 48 hours incubation at 37° C and 5% CO_2, cells were washed with ice-cold PBS and lysed with 100 μl ice-cold lysis buffer [1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, HEPES pH 7.5 50 mM, NaCl 150 mM, glycerol 1%, $MgCl_2$ 1.5 mM, EGTA 5mM, Na_3VO_4 1 mM and protease inhibitor cocktail (Sigma)]. After 30 minutes incubation on ice, lysates were cleared by centrifugation for 40 minutes at 14000 g and 4°C. After protein quantification of the extracts (Bradford assay), Myc-fusion proteins were purified on anti-cMyc Antibody agarose beads (Clontech n.

631208), quantified again and subsequently incubated with Glutathione–Sepharose-bound GST-XNRL or GST alone in the binding buffer (0.1% Triton X-100, HEPES pH 7.5 50 mM, NaCl 150 mM, glycerol 1%, $MgCl_2$ 1.5 mM, EGTA 5mM) overnight at 4°C on a shaker; then washed three times and finally denatured with loading buffer for 5 minutes at 95° C.

FLAG-XNRL protein extract was directly incubated with GST-XOTX2, GST-XOTX5b or GST alone in the binding buffer for 2 hours at 4°C on a shaker; then washed three times and finally denatured with loading buffer for 5 minutes at 95° C.

2.3.c Western blotting

Protein samples were loaded onto a 12% polyacrylamide gel for size separation. Subsequently, proteins were transferred to Immobilon-P Tranfer membrane (Millipore) by electroblotting for 1-2 hours. Blots were blocked for 1 hour using 5% nonfat dry milk in TBS-T [10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% (v/v) Tween-20 (Sigma)]. Monoclonal primary anti-MYC antibody (Sigma) (dilution 1:500) and secondary anti-mouse IgG (peroxidase conjugate) were used to detect MYC-tagged proteins. An anti-FLAG M2 Monoclonal-peroxidase conjugate antibody (Sigma) (dilution 1:1000) was used to detect FLAG-tagged proteins. Filters were incubated for 1 hour at room temperature for each antibody, and then washed three times with TBS-T to remove excess antibody. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to visualize immunoreactive bands by exposure to Amersham Hyperfilm. Samples from at least two independent experiments were analyzed.

2.4 Transactivation assays

HEK 293T cells were co-transfected in 24-well plates with a total of 600 ng of DNA using Lipofectamine. 400 ng of *Xop*-GFP construct (Whitaker and Knox, 2004) were added to each well, along with various combinations of 100 ng of *pCS2$^+$-Xotx2, pCS2$^+$-Xotx2Mut3, pCS2$^+$-Xotx2Δ, pCS2$^+$-Xotx5b, pCS2$^+$-Xotx5bMut3, pCS2$^+$-Xotx5bΔ, pCS2$^+$-otd, pCS2$^+$-otd/Xotx2, pCS2$^+$-otd/Xotx5b, pCS2$^+$-otdbox2, pCS2$^+$-otdbox5b, p3xFLAG-Xnrl*, or empty pYEX expression constructs. RFP (Red Fluorescent Protein) has been as a reporter of the quality of the transfection. GFP fluorescence was analyzed using flow cytometry. FACS analyzes 10000 cells, measuring intensity of fluorescence, percentage of fluorescent cells and mean of intensity (Fig. 2.3).

In order to link together the percentage of fluorescent (gated) cells and the intensity of fluorescence we chose the following parameter, called Volume of fluorescence (Soboleski et al., 2005):

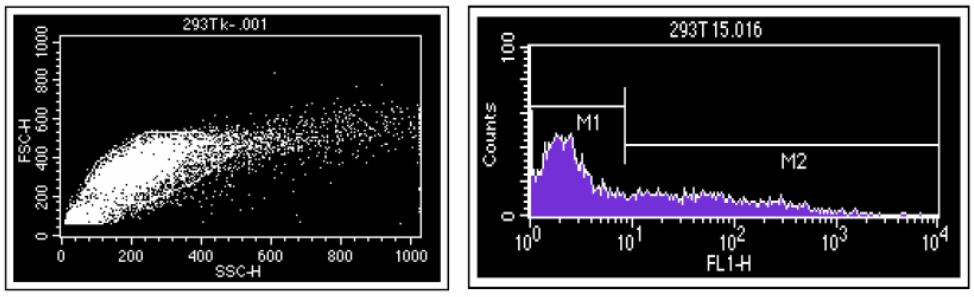
$$\text{Volume of Fluorescence} = \%\ \text{of gated GFP} \times \text{mean of intensity}$$

After that, we calculated the folds of promoter activation, as the ratio between the Volume of Fluorescence of a sample and the Volume of Fluorescence of basal activation (obtained from the activation of *Xop*-GFP alone):

$$\text{Fold activation} = \frac{\text{Volume of sample n}}{\text{Volume of basal activation (}Xop\text{)}}$$

Samples from at least three independent experiments were analyzed.

For confocal microscopy images, transfected cell dishes were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed two times with 1X PBS for 5 minutes and mounted in Aqua Polymount (Celbio).



Histogram Statistics

File: 293T 15.016 — Log Data Units: Linear Values
Sample ID: 293T 15 — Patient ID:
Tube: Untitled — Panel: Untitled Acquisition Tube List
Acquisition Date: 24-Jul-06 — Gate: G1
Gated Events: 8871 — Total Events: 14215
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8871	100.00	62.41	46.29	6.93	370.74	3.31	1
M1	1, 9	5695	64.20	40.06	2.59	2.23	61.55	2.09	1
M2	9, 9910	3182	35.87	22.38	124.43	53.00	216.54	46.14	17

Figure 2. Results of the flow cytometry analysis.

The diagrams show the results of the FACS analysis for *Xotx5b* + *Xnrl* transactivation of *Xop*-GFP. The FACS analyses a population of 10000 HEK 293T cells according to cell size (FSC-H: forward scatter) and granulosity (SSC-H: side scatter). Then, it analyzes the background fluorescence from a dark reference (range intensity: 10^0-10^1) to be subtracted to the measurements. M1 population represents the cells with background fluorescence while M2 population represents GFP fluorescent (gated) cells (with the help of Dr. Elisa Zabogli).

Results

3.1 Molecular dissection of XOTX2 and XOTX5b during *Xenopus* retinogenesis: a 10 AA box switches XOTX2 and XOTX5b cell fate choice activities.

Previous lipofection experiments of retinal progenitors with *Xotx2* and *Xotx5b* showed dramatic differences of effects, with *Xotx2* driving cells toward bipolar cell fate and *Xotx5b* toward photoreceptor cell fate (Viczian et al., 2003; Wang and Harris, 2005; Decembrini et al., 2006; see Paragraph 1.3).

Interestingly, swapping domain experiments showed that the differential activities of XOTX2 and XOTX5b reside in their carboxy-terminal (C-terminal) regions (Viczian et al., 2003): the replacement of the C-terminal of XOTX5b with that one of XOTX2 showed that the obtained chimeric XOTX5b/2 construct is capable of producing the same phenotype as XOTX2, driving progenitors toward bipolar cells, according to the C-terminal fused to (Viczian et al., 2003) (Fig.3.1). The same results were obtained with the XOTX2/5b construct, able this time to promotes photoreceptor differentiation.

These observations strongly suggested that the C-terminal regions of XOTX proteins possess the information sufficient to drive precursors toward a specific cell fate. Moreover, lipofection of chimeric constructs (*Xotx2engR* and *Xotx5bengR*), in which the transactivation domain of either XOTX2 or XOTX5b was replaced with the repressor domain of the *Drosophila* Engrailed protein, had specific effects on either bipolar cells or photoreceptor cells, respectively, this time leading to a decrease, instead of an increase, in their frequency (Viczian et al., 2003). This suggested that these XOTX-EngR chimeric proteins retain a region of the XOTX2 or XOTX5b proteins crucial for their differential activities. This region spans amino acids 100-109 (for XOTX2) or amino acid 100-107 (XOTX5b), where the two proteins differ in six residues (Fig. 3.1).

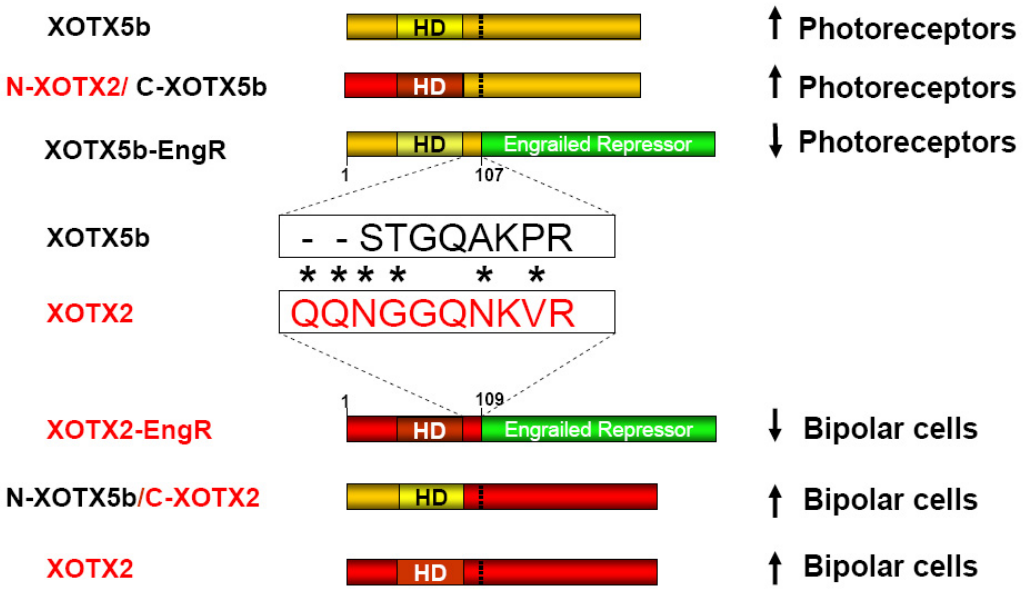


Figure 3.1. Schematic representation of the XOTX constructs and their effects on retinal cell fate.

The schemes of the different wild type and chimeric XOTX constructs, used in Viczian *et al.* (2003), are shown. On the right are indicated their effects on retinal cell fate. The magnified boxes show the potential crucial regions important for XOTX2 and XOTX5b cell fate determining activity. The divergent amino acid residues are indicated with an asterisk. Different colours identify the parental sequences of XOTX5b (yellow), XOTX2 (red) and Engrailed (green).

Based on these observations, we asked whether these residues were crucial for the respective activities of the two factors. We changed the XOTX5b amino acid sequence of this region into the corresponding one of XOTX2. By means of *in vitro* site directed mutagenesis, we first generated three sequential constructs encoding mutant forms of XOTX5b, in which two (construct *Xotx5bMut1*), four (*Xotx5bMut2*), or six amino acid residues (*Xotx5bMut3*) of the relevant region were changed to those of XOTX2, thereby switching this region of XOTX5b into that of XOTX2 (Fig. 3.2).

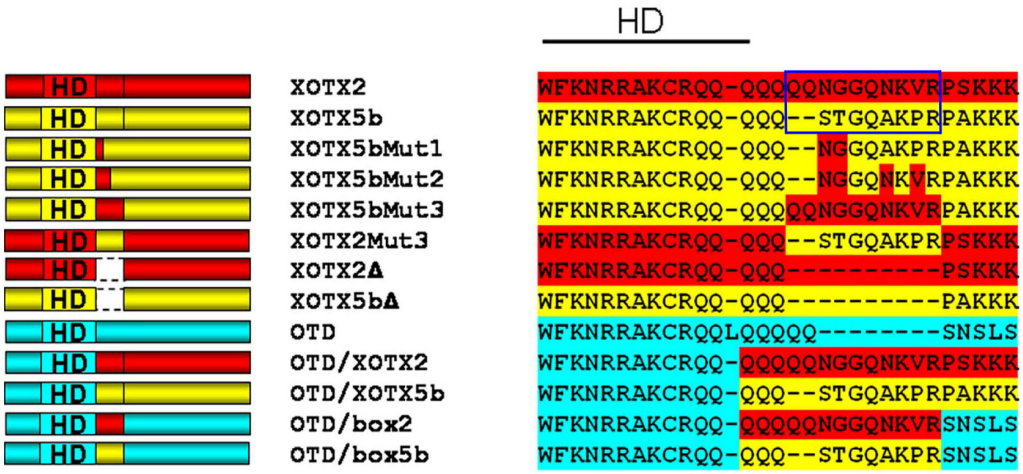


Figure 3.2. Scheme of the XOTX constructs used in this work.

On the left are schematics of the different constructs; on the right are their sequences in the final region of the homeodomain (HD) and directly downstream of it, with different colours shading the parental sequences of XOTX5b (yellow), XOTX2 (red) and OTD (blue). Lines are introduced for sequence alignment. The divergent region responsible for the different retinal activities of XOTX2 and XOTX5b (RS box) is shown in the blue box.

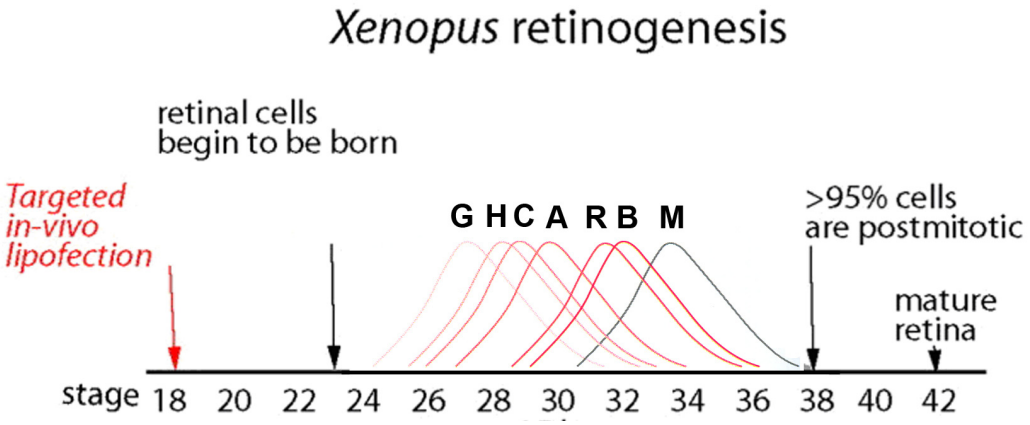


Figure 3.3. Developmental timing of *Xenopus* retinogenesis and lipofection assay.

The diagram illustrates the developmental times of the *Xenopus* retinogenesis. The different cell types are generated with a scheduled order. Lipofection experiments are performed and the beginning of retina development. (G: ganglion cells, H: horizontal cells, C: cones, A: amacrine cells, R: rods, B: bipolar cells, M: Müller glia) (modified from McIlvain and Knox, 2007; Sanes et al., 2006).

We tested the activity of the generated constructs by means of *in vivo* lipofection of retinal progenitors of the optic cup of *Xenopus* embryos at neurula stage (st. 18). All the DNA constructs were lipofected using GPF as reporter, in order to identify the clonal progeny of the lipofected retinoblast. At tadpole stage (st. 42) the retinae were analysed to check for the frequency of the differentiated lipofected retinal cells (Fig. 3.3 and see Paragraph 2.2). We performed a characterization of all retinal cell neurons using molecular markers specific for the *Xenopus* retina (Fig. 3.4).

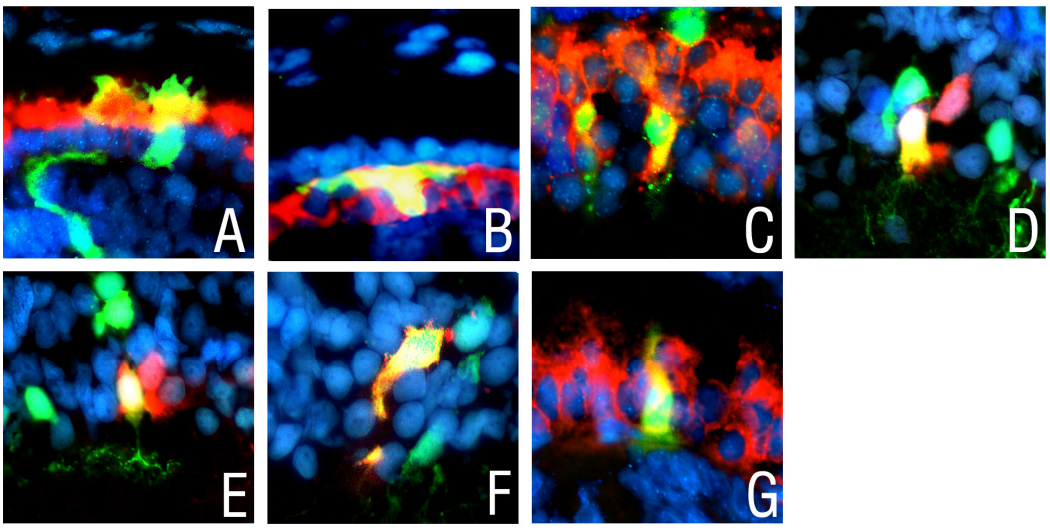


Figure 3.4. Expression of retinal markers in *Xenopus laevis*.

Lipofected GFP-positive cells (green) can be identified by using specific markers: (A) an *Xirbp* probe identifies photoreceptors by *in situ* hybridization (red); (B) *in situ* hybridization with *Xprox1* identifies horizontal cells (red); (C) *in situ* hybridization with *Xvsx1* identifies bipolar cells (red); (D) a specific antibody identifies GABAergic amacrine cells (red); (E) a specific antibody identifies 5-HT positive amacrine cells (red); (F) a specific antibody identifies TH-positive amacrine cells (red); (G) *in situ* hybridization with *Xhermes* identifies ganglion cells. Hoechst staining (in blue) identifies cell nuclei.

By means of *in situ* hybridisation a *Xirbp* (*Xenopus interphotoreceptor retinoid-binding protein*) probe identifies photoreceptors (Gonzalez-Fernandez et al., 1993) (Fig. 3.4A); a *Xprox1* probe recognizes horizontal cells (Dyer et al., 2003) (Fig. 3.4B); a *Xvsx1* (*Xenopus visual homeobox 1*) probe labels bipolar cells (D'Autilia et al., 2006) (Fig. 3.4C); a *Xhermes* probe identifies ganglion cells (Gerber et al., 1999) (Fig. 3.4G). A specific anti- γ-amino-butiryc acid (GABA) antibody identifies GABAergic amacrine cells (Fig. 3.4D); an anti 5-hydroxitryptamnine (5-HT) antibody recognizes serotonergic amacrine cells (Fig. 3.4E) and anti-tyrosine hydroxylase antibody identifies dopaminergic amacrine cells (Fig. 3.4F) (Decembrini et al., 2006).

The *Xotx5b* mutant constructs were lipofected and their activities compared to those of wild type *Xotx2, Xotx5b* and of the negative control (only GFP). As expected, lipofection with wild type *Xotx5b* or *Xotx2* constructs respectively promoted photoreceptor or bipolar cell fate (Figs. 3.5A-C; 3.6).

On the other hand, unlike wild type *Xotx5b*, the *Xotx5bMut3* construct yielded the same effect as wild type *Xotx2*, increasing bipolar cell ($p < 0.001$, ANOVA test and Tukey-Kramer post-test) and decreasing photoreceptor frequency ($p < 0.01$) (Figs. 3.5D; 3.6). Interestingly, lipofections with the *Xotx5bMut2* construct (4 AA change) increased bipolar cells ($p < 0.001$), but did not decrease photoreceptors; even more interestingly, the *Xotx5bMut1* construct (2 AA change) increased bipolar cells ($p < 0.001$) as well as photoreceptor cells ($p < 0.05$), therefore showing the joint effects of both parental proteins (Fig. 3.6).

Molecular markers confirmed the identity of cells lipofected with these different constructs: in particular, cells lipofected with *Xotx5b* and scored as photoreceptors, expressed *Xirbp,* thus showing to be *bona fide* photoreceptors (Fig. 3.5E); on the other hand, cells transfected with *Xotx5bMut3* and scored as bipolar cells expressed the bipolar cell marker *Xvsx1* (Fig. 3.5F).

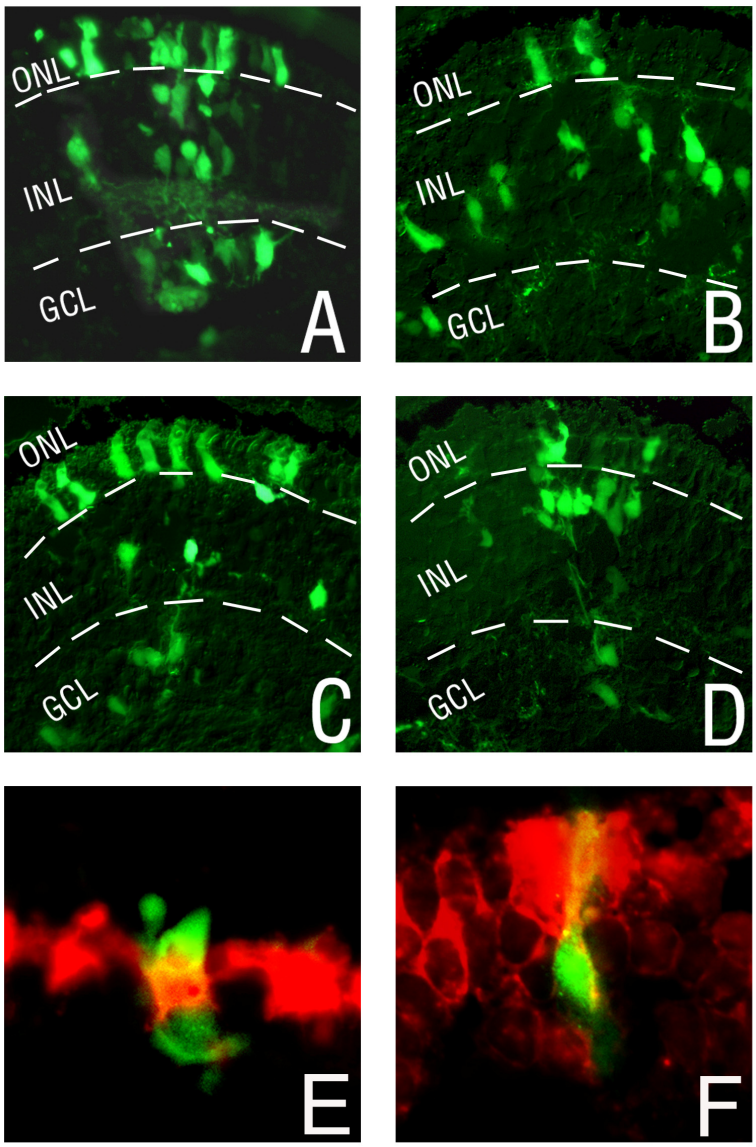


Figure 3.5. Cross-sections of *Xenopus* retinae lipofected with wild-type *Xotx2, Xotx5b* and *Xotx5bMut3* constructs.

(A-D) Sample sections are shown for control retinae lipofected with *GFP* alone (A); *GFP+Xotx2* lipofection increases bipolar cells and decreases photoreceptors (B), *GFP+Xotx5b* lipofection increases photoreceptors (C); *GFP+Xotx5bMut3* increases bipolar cells and decreases photoreceptors (D).

(E, F) *In situ* hybridization analyses showing examples of GFP-positive (green), *Xotx5b*-lipofected photoreceptor cell positive for *Xirbp* probe (Fast Red detection) (E), and a *Xotx5bMut3*-lipofected bipolar cell expressing *Xvsx1* (Fast Red detection) (F). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

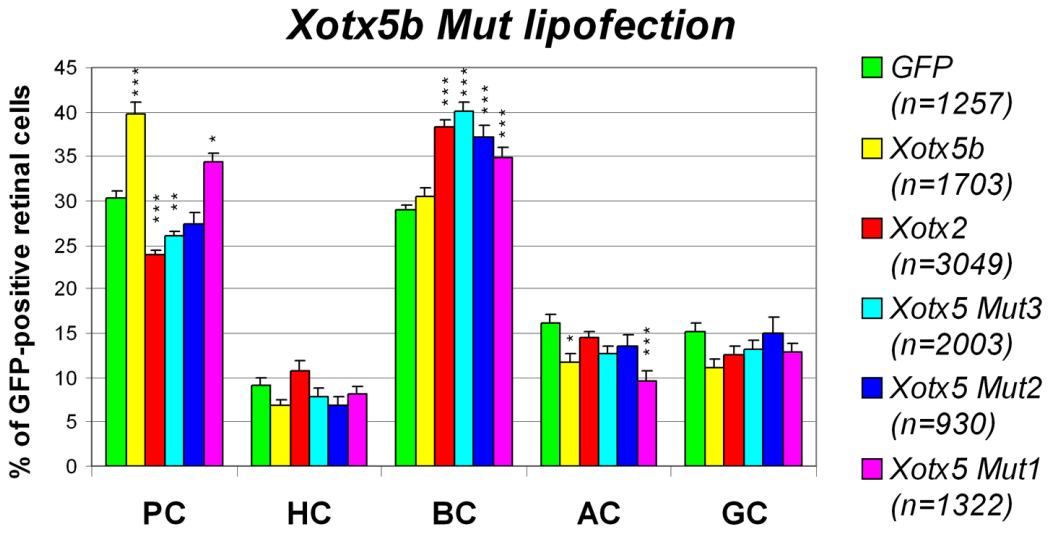


Figure 3.6. Results of *in vivo* lipofection of *Xenopus* retinae with wild-type *Xotx2, Xotx5b* and mutant *Xotx5b* constructs.

The histogram shows the overall distribution of retinal cell types in clones lipofected with the different constructs; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the standard error of the mean (S.E.M.). Counted cells are as indicated in the histogram (*n*), from 15 retinae for *GFP*, 15 retinae for *Xotx5b*, 18 retinae for *Xotx2*, 16 retinae for *Xotx5bMut3*, 10 retinae for *Xotx5bMut2*, and 13 retinae for *Xotx5bMut1*. Asterisks represent significant differences between *Xotx* constructs and *GFP*, as calculated by ANOVA analysis and Tukey-Kramer post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

In order to better characterize the importance of this small region, we performed the mirror experiment, by switching *Xotx2* into *Xotx5b*. For this, we generated a mutant *Xotx2* construct (*Xotx2Mut3*) in which the crucial region of XOTX2 was converted to that of XOTX5b. The activity of *Xotx2Mut3* in lipofections was essentially identical to that of *Xotx5b*: instead of promoting bipolar cell fate like *Xotx2*, the mutant *Xotx2Mut3* construct promoted photoreceptor fate ($p < 0.001$) (Figs. 3.7A, B; 3.8).

The identity of photoreceptors generated by progenitors lipofected with *Xotx2Mut3* was confirmed by testing the expression of *Xirbp* marker (Fig. 3.7C, D).

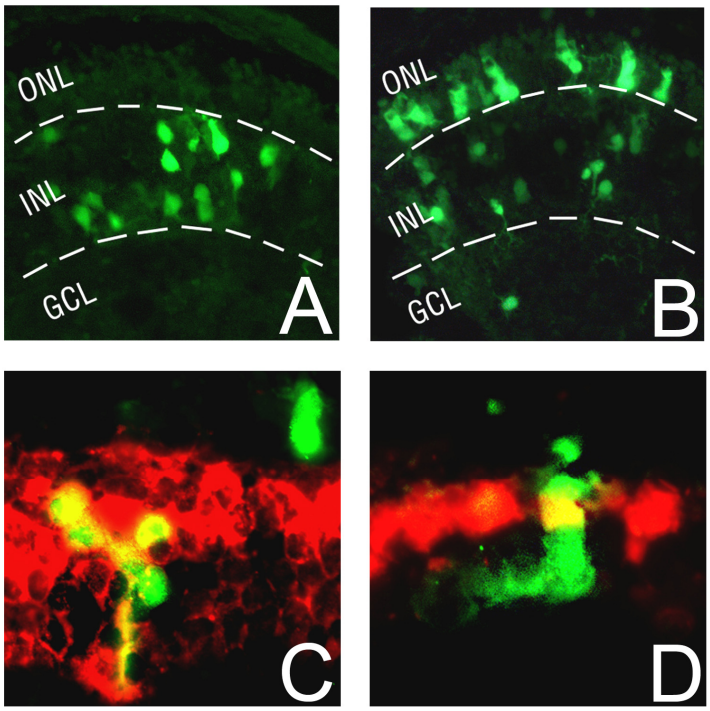


Figure 3.7. Cross-sections of *Xenopus* retinae lipofected with wild-type *Xotx2* and *Xotx2Mut3* constructs.

(A, B) Sample sections are shown for retinae lipofected with *GFP+Xotx2,* increasing bipolar cells (A) and *GFP+Xotx2Mut3,* promoting photoreceptor increase (B).

(C, D) *In situ* hybridization analyses showing examples of GFP-positive (green), *Xotx2*-lipofected bipolar cell positive for *Xvsx1* probe (Fast Red detection) (C), and a *Xotx2Mut3*-lipofected photoreceptor expressing *Xirbp* (Fast Red detection) (D). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Based on these evidences, we conclude that this small region works as a "Retinal Specificity box" (RS box) that is sufficient to confer to XOTX2 and XOTX5b proteins their respective ability to drive retinal progenitor cells toward specific fates, bipolar cells and photoreceptors, respectively.

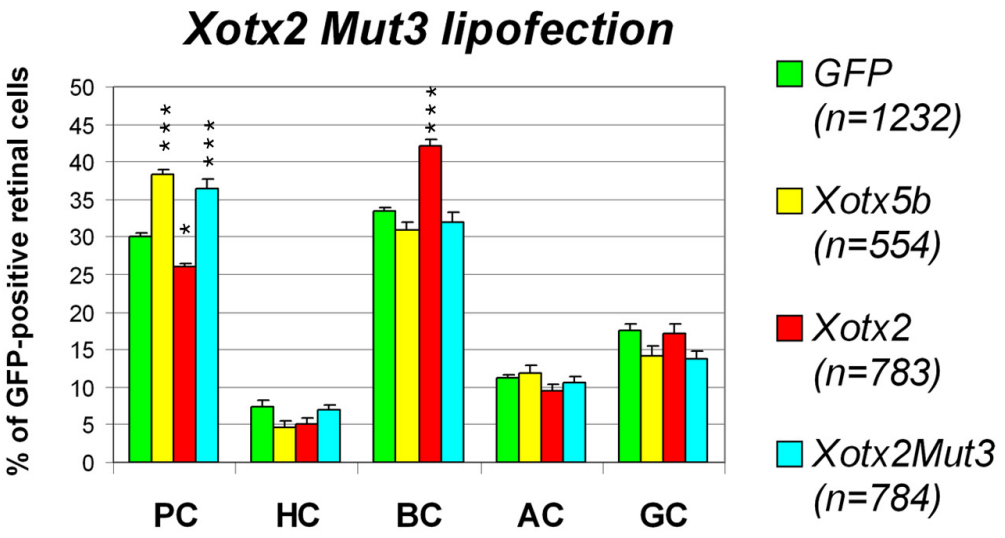


Figure 3.8. Results of *in vivo* lipofection of *Xenopus* retinae with wild-type *Xotx2, Xotx5b* and *Xotx2Mut3* constructs.

The histogram shows the overall distribution of retinal cell types in clones lipofected with the different constructs; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M. Counted cells are indicated in the histogram (*n*), from 9 retinae for *GFP*, 10 retinae for *Xotx5b*, 9 retinae for *Xotx2*, and 14 retinae for *Xotx2Mut3*. Asterisks represent significant differences between *Xotx* constructs and *GFP*, as calculated by ANOVA analysis and Tukey-Kramer post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

We next asked whether the RS box is also required for XOTX proteins activity in retinal cell fate specification, or if without it XOTX proteins still possess a retinal "default" activity. To test this, we generated deletion constructs (*Xotx2*Δ and *Xotx5b*Δ) by removing the RS box and compared their activities to that of wild type constructs.

We lipofected *Xotx2*Δ and *Xotx5b*Δ and we found that these constructs do not drive retinal progenitors toward a specific cell fate. So, the deletion of the RS box completely abrogates any biological effect of either XOTX2 or XOTX5b, showing that this small region is necessary and sufficient for their activity in the frog retina (Fig. 3.9).

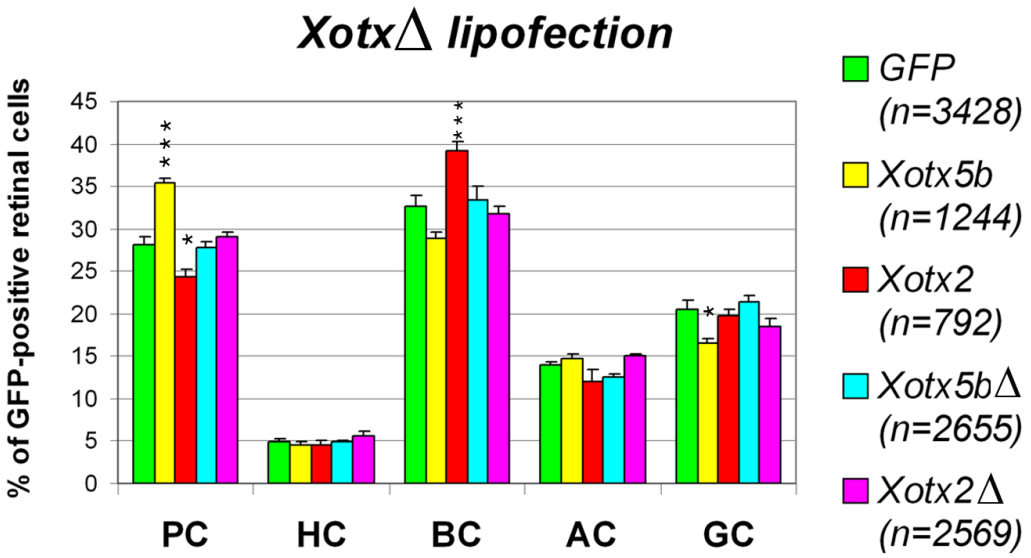


Figure 3.9. The RS box is required for the biological action of either XOTX2 or XOTX5b proteins.

The histogram reports the overall distribution of retinal cell types in clones lipofected with the different constructs, as indicated; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M Counted cells are as indicated in the histogram (*n*), from 11 retinae for *GFP*, 9 retinae for *Xotx5b*, 6 retinae for *Xotx2*, 8 retinae for *Xotx2Δ*, and 7 retinae for *Xotx5bΔ*. Asterisks represent significant differences between *Xotx* constructs and *GFP*, as calculated by ANOVA analysis and Tukey-Kramer post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.2 The RS box confers specific activities to *Drosophila* OTD

Previous works have demonstrated extensive functional conservation of OTX/OTD proteins in early development of anterior central nervous system of Vertebrates and fly (see Paragraph 1.3).

In order to investigate a possible conservation also in later stages of neural development, and in particular in the context of retinal neurogenesis, we tested whether *Drosophila otd* was able to direct retinal progenitors to any specific cell fate in lipofection experiments in the *Xenopus* retina. However, as shown in Fig. 3.10, no difference was observed in the frequency of the different retinal cell types between *otd* lipofected and control clones, suggesting that more specific activities in the vertebrate retina may be novel evolutionary acquisition of OTD/OTX class of proteins.

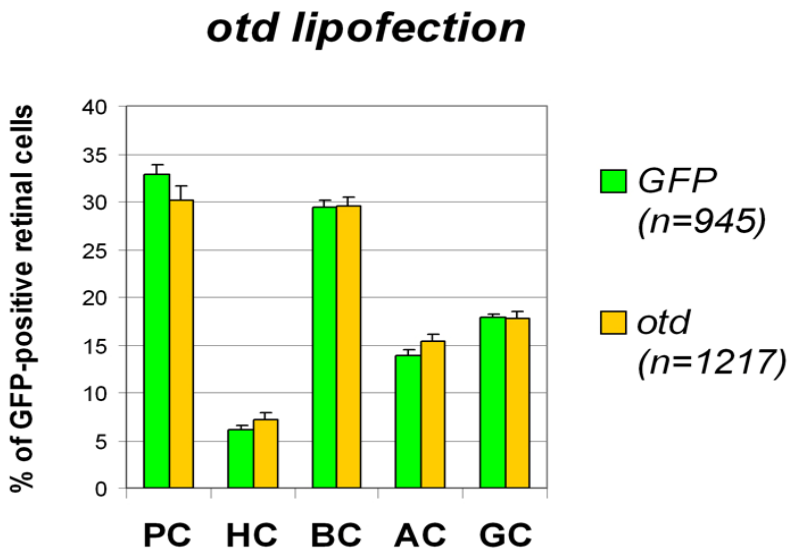


Figure 3.10. *otd* doesn't show any activity in *Xenopus* retinal cell fate determination.

The histogram reports the results of lipofection of retinal progenitors with *GFP* alone or with *GFP+otd*; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M. Counted cells are as indicated in the histogram (*n*), from 17 retinae for *GFP*, and 11 retinae for *otd*.

Then, we performed an analysis of homology among OTD and XOTX proteins. Outside the homeodomain, which results very conserved (about 97% at amino acid level), the other regions do not show high conservation. Indeed, the C-terminal of OTD is quite divergent compared to XOTX2 or XOTX5b (only 11.4% identities to XOTX2 and 8% to XOTX5b); instead, XOTX2 and XOTX5b show 75% identity, suggesting that a possible reason for the lack of OTD activity could be due to such a strong divergence. To test this hypothesis, we replaced the OTD region C-terminal to the homeodomain with either that of XOTX2 or XOTX5b, and compared the activity of chimeric OTD/XOTX2 and OTD/XOTX5b with that of wild type OTD in lipofection experiments.

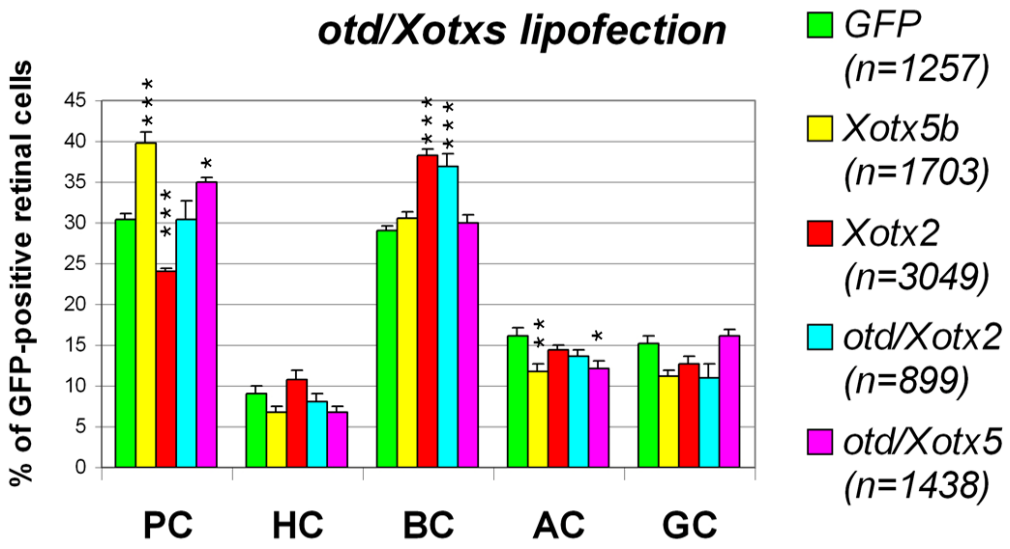


Figure 3.11. *otd/Xotx* chimeric constructs specifically act on retinal cell fate determination.

The histogram reports the results of lipofection with *GFP* alone, with *GFP+Xotx* wild-type constructs or *GFP+otd/Xotx* chimeric constructs, as indicated; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M. Counted cells are as indicated in the histogram (*n*), from 15 retinae for *GFP*, 18 retinae for *Xotx2*, 15 retinae for *Xotx5b*, 9 retinae for *otd/Xotx2*, and 11 retinae for *otd/Xotx5b*. Asterisks represent significant differences between *Xotx* constructs and *GFP*, as calculated by ANOVA analysis and Tukey-Kramer post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Significantly, we found that OTD/XOTX2 drives progenitors toward the bipolar cell fate ($p < 0.001$), while OTD/XOTX5b increased photoreceptors ($p < 0.05$) (Fig. 3.11). However, the decrease in photoreceptor cell frequency observed with wild type *Xotx2* was not detected with the *otd/Xotx2* chimeric construct. These data support the importance of the C-terminal regions of XOTX proteins described in previous experiments and demonstrated that they are able to confer to OTD a good ability to drive retinal differentiation.

Then, we asked whether the RS box, that in OTD seems not present, could be sufficient to provide the biological activity of either XOTX2 or XOTX5b to *Drosophila* OTD. To verify this idea we generated chimeric *otd/box2* and *otd/box5b* constructs in which we inserted the RS boxes of XOTX2 and XOTX5b immediately C-terminal to OTD homeodomain and transfected them into *Xenopus* retina. Although the C-terminal of OTD is strongly divergent from those of either XOTX proteins, the specificity box enabled OTD/box2 or OTD/box5b proteins to drive retinal progenitors toward bipolar ($p < 0.01$) or photoreceptor fates ($p < 0.01$), respectively (Fig. 3.12 A-C). Similar to OTD/XOTX2, OTD/box2 did not lead to the reduction in photoreceptor cells observed with wild type XOTX2.

Molecular markers confirmed the identity of retinal cells lipofected with these different constructs: cells lipofected with *otd/box5b* and scored as photoreceptors expressed *Xirbp,* (Fig. 3.12D); on the other hand, cells transfected with *otd/box2* and scored as bipolar cells expressed *Xvsx1* (Fig. 3.12E).

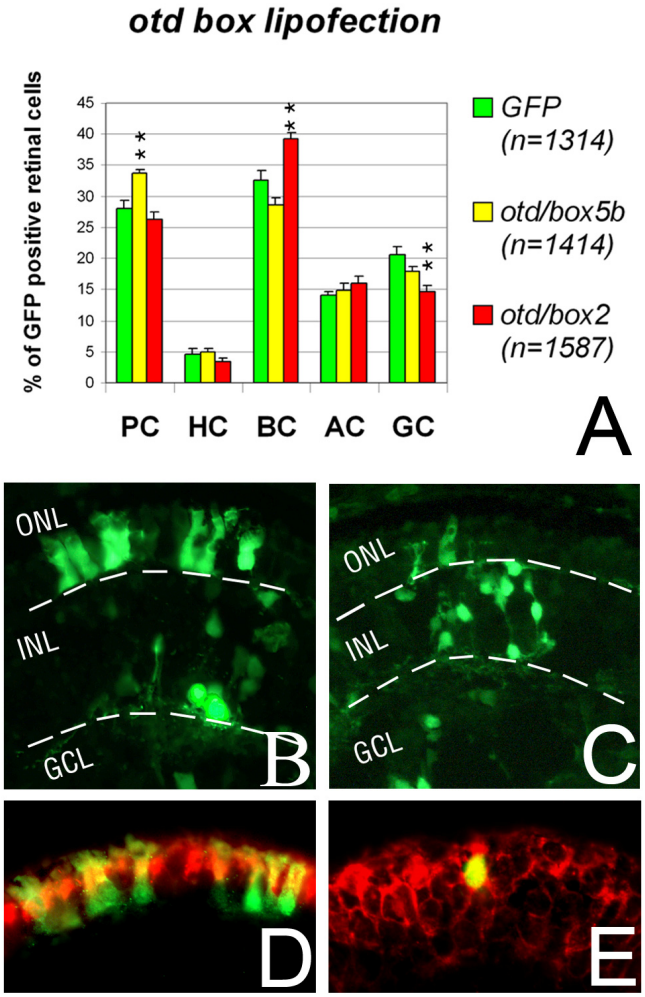


Figure 3.12. The RS box is sufficient to confer a specific retinal action on *Drosophila* OTD protein.

(A) The histogram shows the results of lipofection with *GFP* alone, with *GFP+otd/box2 or GFP+otd/box5b* chimeric constructs, as indicated; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M. Counted cells are indicated in the histogram (*n*), from 6 retinae for *GFP*, 9 retinae for *otd/box2*, and 9 retinae for *otd/box5b*. Asterisks represent significant differences between *Xotx* constructs and *GFP*, as calculated by ANOVA analysis and Tukey-Kramer post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(B, C) Lipofected retinae with *otd/box5b* are enriched in photoreceptors (B), those with *otd/box2* are enriched in bipolar cells (C); GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(D) An example of *GFP+otd/box5b*-lipofected photoreceptor cell positive for *Xirbp* probe after *in situ* hybridization (Fast Red detection). (E) A *GFP+otd/box2*-lipofected bipolar cell expressing *Xvsx1* is shown following *in situ* hybridization (Fast Red detection).

3.3 The RS box is involved in the correct nuclear localization of XOTX/OTD proteins in retinal neurons

Previous works on CRX have shown that a nuclear localization signal (NLS) is present at the C-terminal of the homeodomain (see Paragraph 1.3.a); moreover, several CRX mutations are associated with human retinal pathologies and have effect on CRX nuclear localization (Fei and Hughes, 2000). One of such mutations leads to CRX mislocalization and replaces R98 with a L residue; interestingly, a L residue is present in *Drosophila* OTD at the same position.

We therefore asked whether the inability of OTD on retinal specification was due to insufficient translocation to the nucleus, rather than to the absence of the RS box. To test this, we first compared the distributions of MYC-XOTX2, MYC-XOTX5b and MYC-OTD proteins in lipofected retinal cells, with those of endogenous XOTX2 and XOTX5b. We also examined the distribution of MYC-OTD/box2 and MYC-OTD/box5b in similarly lipofected retinal cells (Fig. 3.13).

Endogenous XOTX2 and XOTX5b are detected only in the nuclei of bipolar and photoreceptor cells, and were not detectable in the cytoplasmic compartment (Fig. 3.13B, not shown for XOTX5b). A nuclear distribution was found for MYC-XOTX2 and MYC-XOTX5b in all lipofected cells. Interestingly, while MYC-OTD showed a diffuse distribution in both the nuclei and the cytoplasmic compartments of lipofected cell, MYC-OTD/box2 and MYC-OTD/box5b had a clear nuclear localization.

While this suggested that the specificity box may also be important for correct nuclear targeting of XOTX proteins, it left opened the possibility that OTD might have potential effects on retinal cell fate that were not expressed due to insufficient nuclear translocation. To rule out this possibility, we prepared a *NLS-myc-otd* construct (containing a nuclear localization signal) and tested it by means of lipofection.

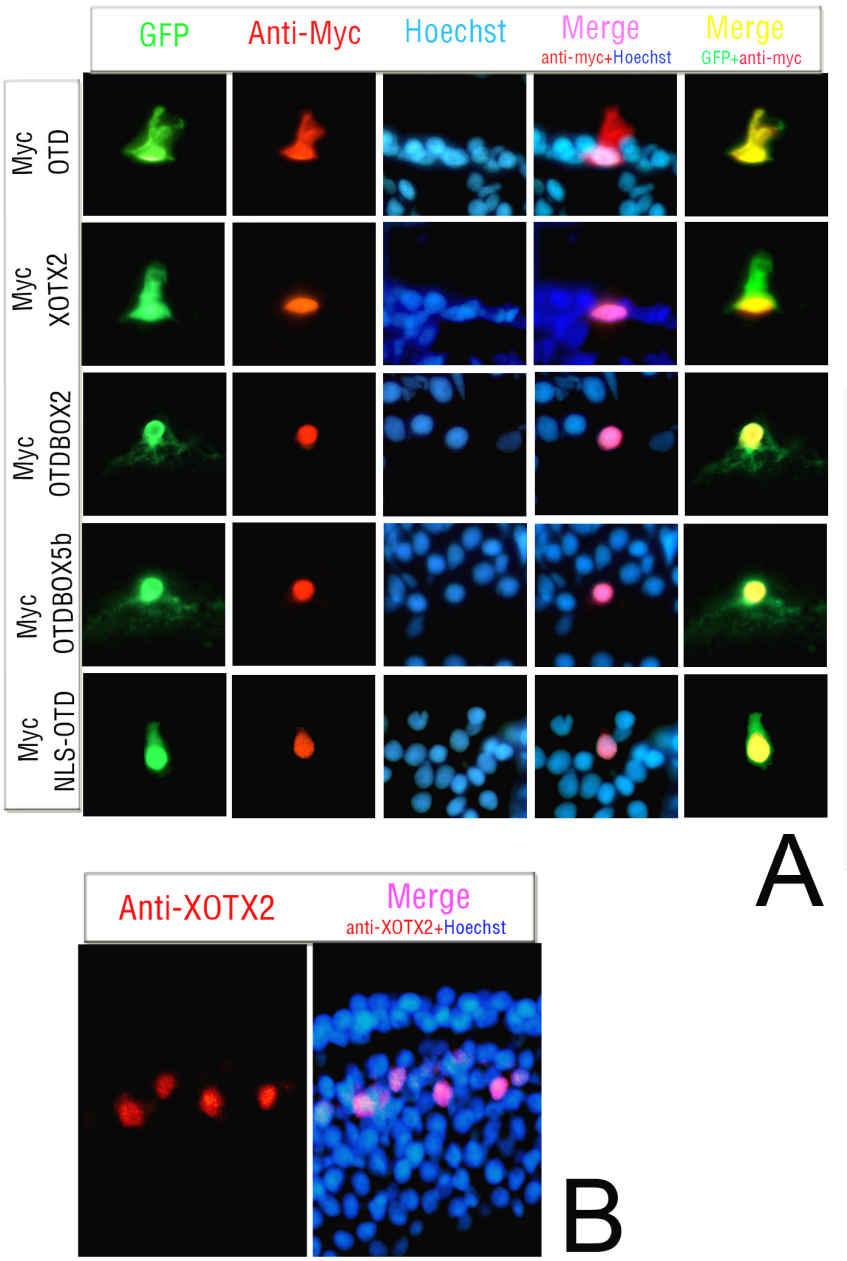


Figure 3.13. Subcellular localization of XOTX and OTD constructs.

(A) The nuclear/cytoplasmic distribution of MYC-XOTX2 and MYC-OTD is shown in lipofected retinal cells and is compared to cytoplasmic GFP fluorescence; while MYC-XOTX2 shows an exclusively nuclear localization, MYC-OTD is partly cytoplasmic; on the contrary, MYC-OTD/box2 and MYC-OTD/box5b are targeted to the nucleus; a NLS-MYC-OTD fusion protein is forced into the nucleus (bottom row).

(B) Endogenous XOTX2 protein distribution is detected by a specific antibody (red fluorescence) in the *Xenopus* retinal nuclei. Nuclei are counterstained with Hoechst.

As expected, the NLS-MYC-OTD protein was correctly localized to the nucleus (Fig. 3.13A); however, forcing OTD to the nucleus did not significantly affect cell fate (Fig. 3.14). Therefore, we conclude that efficient translocation to the nuclei of retinal cells did not provide OTD with the ability to drive frog retinal progenitors toward a precise neuronal fate; instead, OTD gained this ability when its homeodomain was directly followed by a RS box of the XOTX2 or XOTX5b type.

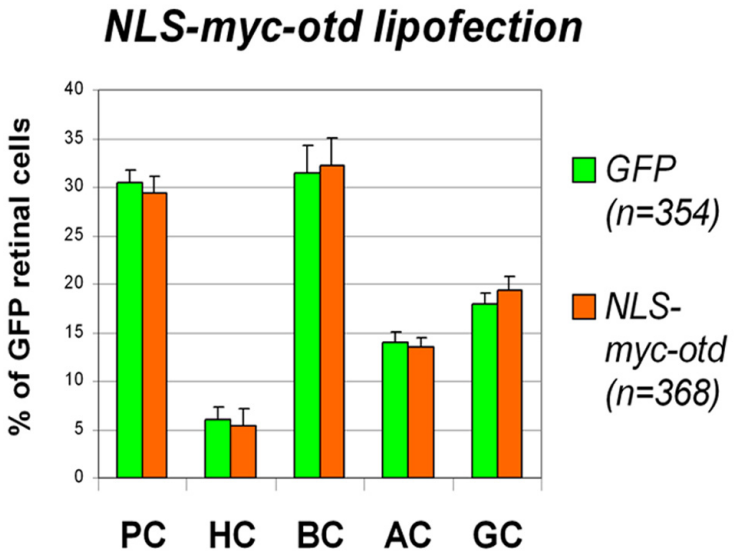


Figure 3.14. Results of *NLS-myc-otd* lipofection.

Although it forces OTD to the nucleus, a *NLS-Myc-otd* fusion construct does not have any effect on cell fate of retinal progenitors; PC, photoreceptor cells; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GC, ganglion cells. The proportion of each cell type is represented as an average. Error bars indicate the S.E.M. Counted cells are indicated in the histogram (*n*), from 6 retinae for *GFP* and 4 retinae for *NLS-Myc-otd*.

3.4 XOTX2 and XOTX5b differentially synergize with XNRL to transactivate the *Xenopus rhodopsin* promoter

In order to investigate the molecular bases of the action of the OTD/OTX transcription factors and the importance of the RS box, we performed a promoter transactivation assay.

Previous work showed that CRX/XOTX5b interacts with NRL to activate the *rhodopsin* promoter (Mitton et al., 2000; Whitaker and Knox, 2004) and that a lower level of activation is instead obtained when *Otx2* and *Nrl* are co-transfected in cultured cells (Peng and Chen, 2005).

We therefore asked whether the *Xotx* mutant constructs also switched their activity in similar transactivation assays. We thus monitored the ability of XOTX2 and XOTX5b (alone or together with XNRL) to activate a *Xenopus rhodopsin* promoter (-508 to +41 bp) driving GFP expression (*Xop*-GFP) in HEK 293T cells, and compared it with the activities of XOTX5bMut3, XOTX2Mut3, OTD, OTD/XOTX2, OTD/XOTX5b, OTD/box2, OTD/box5b, XOTX2Δ and XOTX5bΔ. Each of these constructs was co-transfected with the *Xop*-GFP reporter in the absence or presence of XNRL; we cotransfected RFP (Red Fluorescent Protein) as a reporter of the quality of the transfection (Fig. 3.15).

After the transfection, we measured quantitatively the ability of transactivation (that is the amount of GFP fluorescence) of the different constructs by means of the flow cytometry analysis. FACS analyzes 10000 cells, measuring intensity of fluorescence, percentage of fluorescent cells, mean of intensity.

Fold activation was assumed as the ratio of the volume of fluorescence between each sample and the basal activation sample (*Xop*-GFP transfection alone), where the fluorescent volume is the fraction of GFP positive (gated) cells in the population multiplied for the mean fluorescence intensity (Soboleski et al., 2005; see Paragraph 2.4).

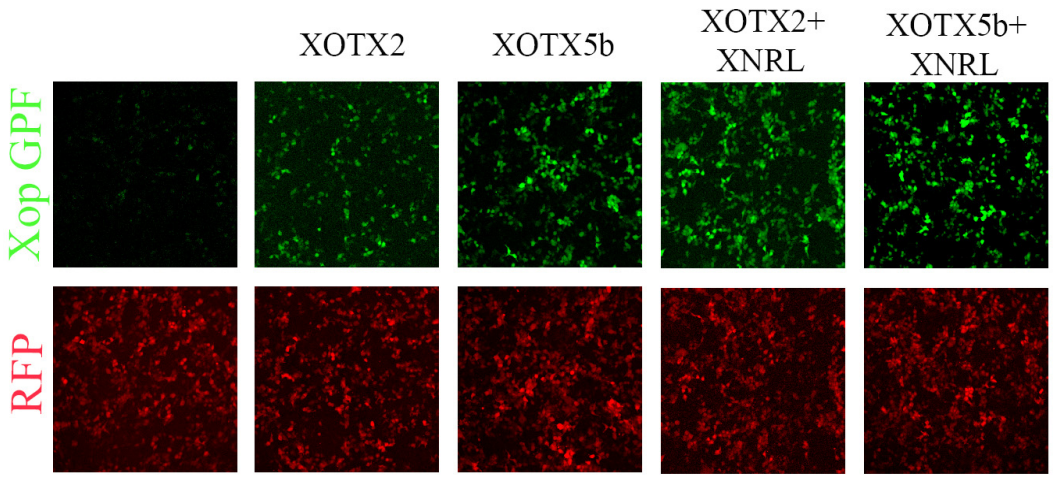


Figure 3.15. GFP and RFP fluorescence in transactivation assay.

Confocal microscopy images of transfected HEK 293T cells. The transfection of the *Xop*-GFP alone represents the control for its basal activation. The cotransfection of *Xotx2* or *Xotx5b* (with or without *Xnrl*) elicits different level of GFP fluorescence. RFP represents the control for the transfection: red fluorescence is uniform among the different samples (with the help of Dr. Riccardo Parra).

When each of the constructs was transfected alone, scarce activation of the reporter gene was detected (5-13-fold activation compared to the ground level given by transfection of *Xop*-GFP alone); the same was observed with transfection of *Xnrl* alone (Fig. 3.16). A significant difference ($p < 0.01$; bilateral *Student's t*-test) was observed between *Xotx2*+*Xnrl* and *Xotx5b*+*Xnrl* transfections, that respectively elicited activation of the reporter 43- or 105-fold over the ground level; these results are consistent with those of Peng and Chen (2005). More significantly, *Xotx2Mut3*+*Xnrl* transfection gave similar results to *Xotx5b*+*Xnrl* (101-fold reporter activation; $p = 0.035$ *Xotx2Mut3*+*Xnrl* vs. *Xotx2*+*Xnrl*; $p = 0.89$ *Xotx2Mut3*+*Xnrl* vs. *Xotx5b*+*Xnrl*), while *Xotx5bMut3*+*Xnrl* (32-fold activation) was similar to *Xotx2*+*Xnrl* (*Xotx5bMut3*+*Xnrl* vs. *Xotx2*+*Xnrl*, $p = 0.55$; *Xotx5bMut3*+*Xnrl* vs. *Xotx5b*+*Xnrl*, $p = 0.02$).

Therefore, exchanging the RS box in XOTX2 or XOTX5b leads to a switch in their ability to transactivate, together with XNRL, one of the key photoreceptor specific genes. In addition, *otd, otd/Xotx2* or *otd/box2,* when combined with *Xnrl,* all gave results similar to *Xotx2* (with 48-, 47- and 56-fold activation respectively); a slightly stronger effect was observed with *otd/Xotx5b+Xnrl* (*otd/Xotx5b+Xnrl* vs. *otd+Xnrl*, $p = 0.003$) and *otd/box5b+Xnrl* (74- and 72-fold activation respectively). Surprisingly, a rather strong effect was obtained by *Xotx2*Δ*+Xnrl* (82-fold activation, *Xotx2*Δ*+Xnrl* vs. *Xotx2+Xnrl, p* = 0.047; *Xotx2*Δ*+Xnrl* vs. *Xotx5b+Xnrl*, $p = 0.22$; *Xotx2*Δ*+Xnrl* vs. *Xotx5b*Δ*+Xnrl*, $p = 0.029$), but not by *Xotx5b*Δ*+Xnrl* (28-fold activation; $p = 0.014$ vs. *Xotx5b+Xnrl*). Significant differences were also found between *Xotx5b+Xnrl* and *otd+Xnrl* ($p = 0.002$) (Fig. 3.16).

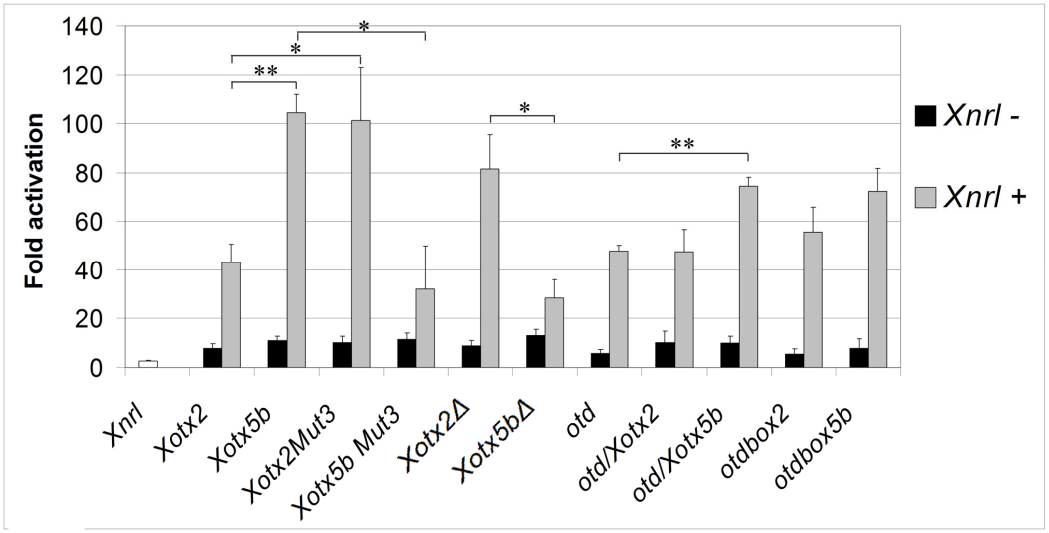


Figure 3.16. XOTX2 and XOTX5b differentially synergize with XNRL to activate the *rhodopsin* promoter.

Results of *rhodopsin* promoter transactivation assays with several *Xotx/otd* constructs (with or without *Xnrl*). Error bars indicate the S.E.M. The *p*-value was calculated by bilateral Student's *t*-test. Asterisks in histograms show statistically significant differences only for more relevant comparisons (*$p < 0.05$, ** $p < 0.01$). Samples from at least three independent experiments were analyzed.

3.4 *In vitro* interactions of XOTX/OTD proteins with XNRL

Different hypothesises may explain the differential biological abilities of XOTX2, XOTX5b, OTD and mutant constructs in cell fate specification. One possible way is that they bind differential DNA consensus, thus selectively activating different sets of instrumental genes for a program of neural differentiation. However, OTX proteins basically all recognize the same DNA-consensus motive TAATCC/T (Wilson et al., 1996; Furukawa et al., 1997; Briata et al., 1999). Moreover, Peng and Chen (2005), by means of chromatin immunoprecipitation, demonstrated that CRX and OTX2 occupy and bind the same promoter/enhancer regions of the target retinal genes (Peng and Chen, 2005).

Another hypothesis is that XOTX2 and XOTX5b differentially interact with other key molecular players involved in retinal differentiation, such as XNRL itself. Full length CRX, or truncated CRX forms containing the homeodomain, the Q-rich region and the basic region were shown to strongly interact with NRL, whereas the sole CRX homeodomain showed a much lower interaction (Mitton et al., 2000). Because the "specificity box" spans between the Q-rich and part of the basic region (Chau et al., 2000a), we decided to test whether XOTX2 and XOTX5b showed differential abilities to interact with XNRL. We therefore prepared *GST-Xotx2* and *GST-Xotx5b* fusion constructs spanning the region that in CRX is relevant for interaction with NRL, to produce the corresponding fusion proteins (AA 32-165 for XOTX2; AA 32-164 for XOTX5b). These were used in GST-pull down assay to monitor the respective ability of GST-XOTX2 and GST-XOTX5b to interact with a full length FLAG-tagged form of XNRL (XNRL-FLAG). The results of these experiments are shown in Fig. 3.17. While no binding of XNRL-FLAG was detected by GST alone (negative control), we were able to detect, as expected, strong interaction of GST-XOTX5b with XNRL-FLAG. Instead, the interaction of GST-XOTX2 with XNRL-FLAG was much lower. The films of the pull down experiments were

scanned and the intensity of the pixels analysed by Image J. We found that XOTX5b binds XNRL 2.59 folds better than XOTX2 ($p < 0.01$) (Fig. 3.17B).

The differential physical interaction we found between XOTX2 and XOTX5b with a proteic partner supports the hypothesis that these transcription factors may distinguish their activity *in vivo* through a different affinity with several transcriptional cofactors.

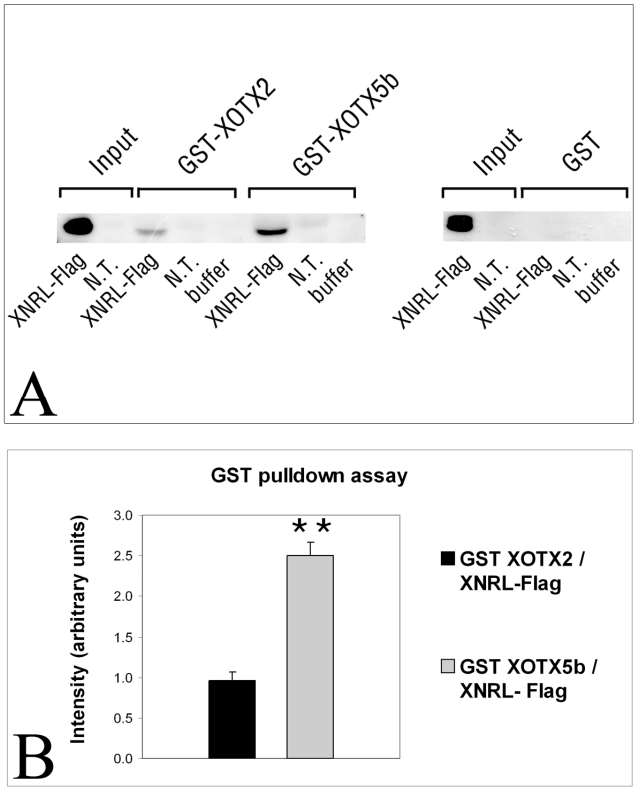


Figure 3.17. XOTX2 and XOTX5b differentially interact *in vitro* with XNRL.

(A) GST-pull down assays compare the interaction of either GST-XOTX5b or GST-XOTX2 fusion proteins or GST (control) with XNRL-FLAG; protein extracts from untransfected cells (N.T.), or buffer alone were used as negative controls in these assays.

(B) Results of three of such experiments, analysed by Image J, were statistically analyzed, and showed a significant difference in the efficiency of GST-XOTX5b or GST-XOTX2 in binding XNRL-FLAG. Error bars indicate the S.E.M. The *p*-value was calculated by bilateral Student's *t*-test. Samples from three independent experiments were analyzed

Then, we decided to perform the reciprocal experiment, preparing a GST-XNRL fusion construct to test in a pull down assay against the full-length XOTX proteins fused to a MYC epitope. Moreover, we decided to extend our analysis to all the mutant constructs used in the *in vivo* experiments: MYC-XOTX2, MYC-XOTX2Δ, MYC-XOTX2Mut3, MYC-XOTX5b, MYC-XOTX5bΔ, MYC-XOTX5bMut3, MYC-OTD, MYC-OTD/box2 and MYC-OTD/box5b.

After the production of the MYC-proteins, we purified them with anti-MYC agarose beads (see Paragraph 2.3) and quantified them before the pull down assay, in order to use the same amount of the starting samples.

The results of the pull down experiments are shown in Fig. 3.18A; the films of the pull down results were scanned and analysed by Image J, and the resulting data were plotted (Fig. 3.18B). We found that MYC-XOTX5b interacts with GST-XNRL about 2.4 times compared to MYC-XOTX2 ($p < 0.01$, *Student's t*-test); significant differences were also observed compared to MYC-XOTX2Δ, MYC-XOTX5bΔ, MYC-XOTX5bMut3, MYC-OTD and MYC-OTD/box2, but not compared to MYC-XOTX2Mut3, or MYC-OTD/box5b. Therefore, while XOTX5b interacts more strongly with XNRL, also other XOTX/OTD proteins interact *in vitro* with XNRL, even without the box.

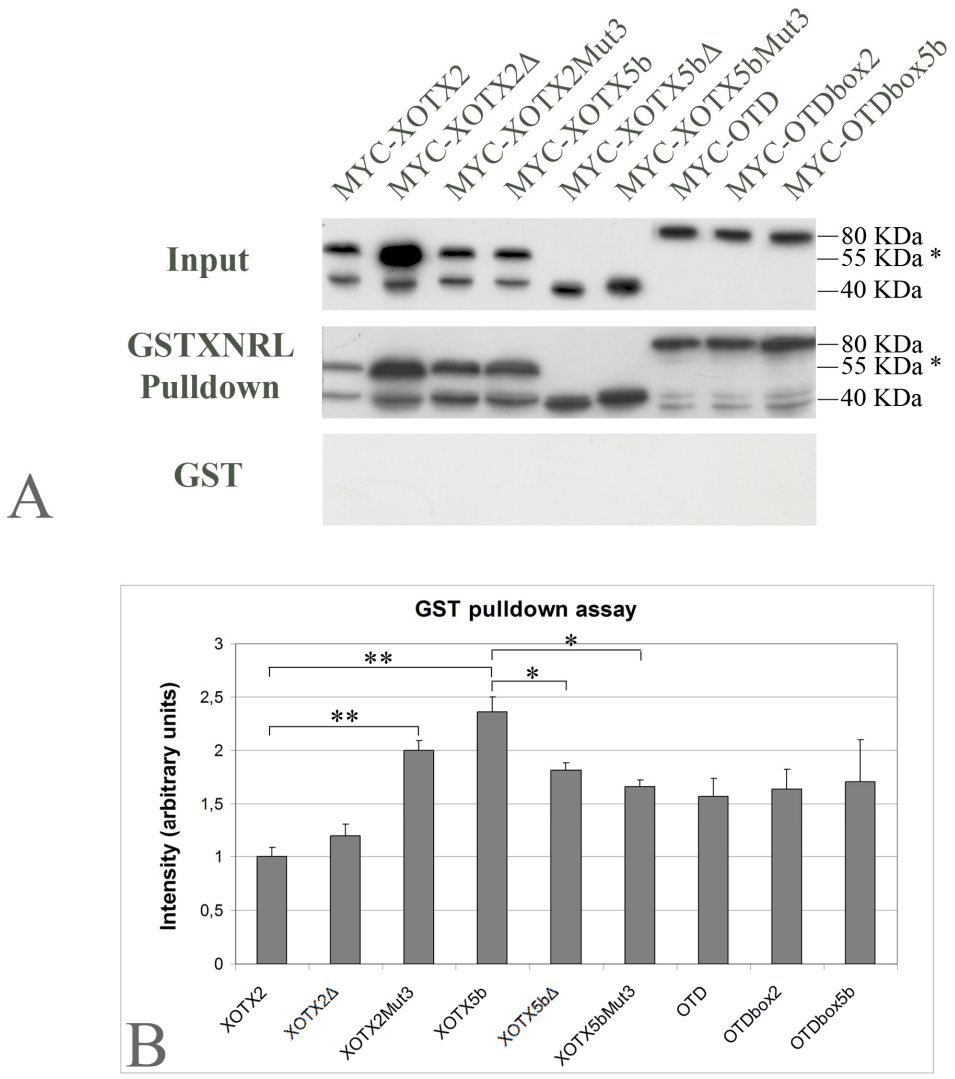


Figure 3.18. XOTX2 and XOTX5b differentially interact *in vitro* with XNRL.

(A) GST-pull down assays compare the interaction of MYC-XOTX/OTD fusion proteins to GST-XNRL or GST alone. The band indicated by an asterisk corresponds to a higher molecular weight (55 kDa) than the one expected for XOTX proteins (41 kDa) and may result from post-translational modification.

(B) Results of two of these experiments, analysed by Image J, were statistically processed; columns show the ratio of the retained MYC-tagged proteins relative to their respective input, normalized with respect to the MYC-XOTX2 retained/input ratio. Error bars indicate the standard deviation. The *p*-value was calculated by bilateral Student's *t*-test. Asterisks in histograms show statistically significant differences only for more relevant comparisons (*$p <$ 0.05, ** $p <$0.01). Samples from two independent experiments were analyzed

3.5 XOTX2 and XOTX5b can form homo/heterodimers that influence their activity

After considering the instrumental role of RS box in modulating the biological functions of the XOTX2 and XOTX5b, we investigated the possibility that XOTX protein interact each others (see Paragraph 4.5).

Indeed, previous work by Briata *et al.* (1999) showed that the homeodomain of the human OTX2 initially binds to target promoters as a monomer, an then a conformational change in the protein allows interaction with, and recruitment of, a second OTX2 monomer.

So, we investigated if XOTX2 and XOTX5b can form dimers. We therefore tested GST-XOTX2 and GST-XOTX5b proteins for their ability to bind MYC-tagged full length XOTX2 and XOTX5b. Results of these experiments show that GST-XOTX2 and GST-XOTX5b can physically interact each other. Dimerization seems specific and may not occur with a PDZ domain or any type of homeoprotein, since we failed to detect any interaction of GST-XOTX fusion proteins with a MYC-tagged fusion to XSIX3 homeodomain (Fig. 3.19).

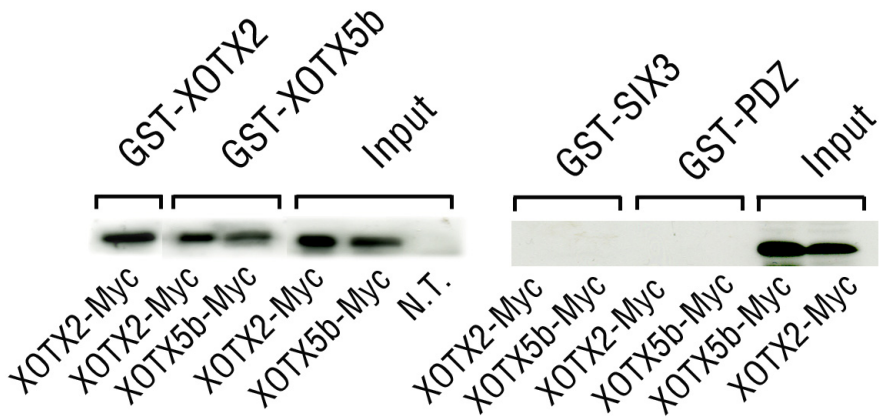


Figure 3.19. XOTX2 and XOTX5b interact *in vitro* each other.

A GST-pull down assay shows the ability of XOTX2 and XOTX5b to interact each other. This interaction is not present between SIX3 or PDZ and XOTX proteins.

Then we investigated the effect of XOTX2 and XOTX5b interaction in a *Xop*-GFP transactivation assay. As previously showed, we found that *Xotx2* and *Xotx5b* alone are able to transactivate the *rhodopsin* promoter and that they respectively elicit an activation of 7,8- and 11-fold over the basal level. Interestingly, when the same amount of *Xotx2* and *Xotx5b* are co-transfected, we observed a decrease of promoter transactivation to 5,9 folds, that is about one half of the *Xotx5b* transactivation power (although the difference is not statistically significant).

Then we asked if the physical and functional interaction between XOTX proteins could, in some degree, affect the functional synergy with other cofactors. In particular, we wondered if the dominant negative effect of XOTX2 on XOTX5b activity could impair also the synergy with XNRL. We tested this hypothesis by co-transfecting *Xotx2, Xotx5b* and *Xnrl* and analysed the *Xop*-GFP transactivation, in comparison with *Xotx2*+*Xnrl* and *Xotx5b*+*Xnrl.*

The results of this experiment are shown in Fig. 3.20: while *Xotx2*+*Xnrl* transactivate *Xop*-GFP about 43- folds and *Xotx5b*+*Xnrl 105*- folds, the combination *Xotx2*+*Xotx5b*+*Xnrl* elicits a response of 69- folds of transactivation, that is significantly different from *Xotx5b*+*Xnrl* ($p < 0.05$, bilateral Student's *t*-test), but does not differ from *Xotx2*+*Xnrl* ($p > 0.087$).

This kind of competition experiment demonstrates that XOTX2 suppresses XOTX5b synergy with XNRL in transactivating a key promoter photoreceptor gene.

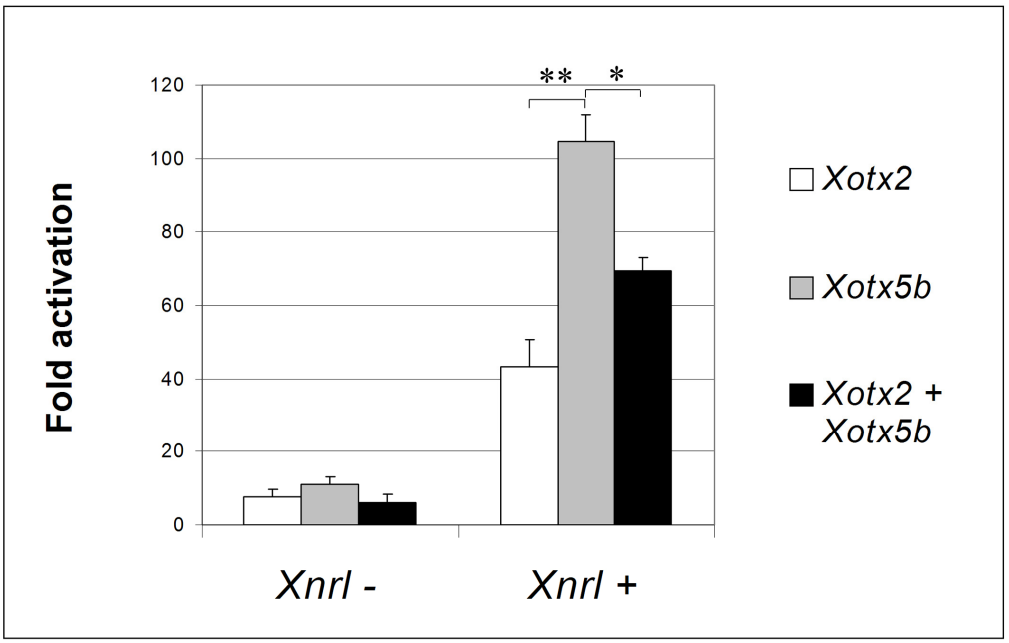


Figure 3.20. XOTX2/XOTX5b interaction impairs their transactivation ability on *rhodopsin* promoter.

Results of *rhodopsin* promoter transactivation assay with *Xotx2* and *Xotx5b* constructs (with or without *Xnrl*). Error bars indicate the S.E.M. The *p*-value was calculated by bilateral Student's *t*-test. Asterisks in histograms show statistically significant differences (*$p < 0.05$, ** $p < 0.01$). Samples from three independent experiments were analyzed.

Discussion

4.1 Molecular dissection of XOTX2 and XOTX5b during *Xenopus* retinogenesis: a 10 AA box switches XOTX2 and XOTX5b cell fate choice activities.

The nervous system is composed by an impressive array of different neural cells. The question the developmental neurobiologists have been trying to answer for many years is how such diversity arises.

In the present thesis we approached this topic by investigating the development of the retina, as a good model of a complex neural structure. As already described in the Introduction, the Vertebrate retina is composed of seven major cell types, which derive from a common group of multipotent and heterogeneous progenitors, according to a conserved histogenetic order (Cepko et al., 1996; Livesey and Cepko, 2001).

Cepko and co-workers have proposed that, during retinogenesis, the progenitors pass through a series of competence states, each defined by a combinatorial code of intrinsic factors. For each competence state, precursors would be competent to generate only certain cell types rather than others, according also to the extrinsic cues present in the extracellular environment, itself changing over time (Cepko et al., 1996; Livesey and Cepko, 2001).

Transcription factors have a pivotal role during neural differentiation of retinal cell types, but the precise mechanisms of their actions remain still unknown. We focused our attention on *Xotx2* and *Xotx5b* genes, coding for homeodomain transcription factors, with *Xotx2* driving cells toward bipolar cell fate and *Xotx5b* toward photoreceptor cell fate (Viczian et al., 2003).

Several findings have showed that a number of proteins, and in particular OTX transcription factors, are formed by independent domain able to perform

specific functions. For instance, the homeodomain is a DNA-binding domain that mediates also protein-protein interactions; the basic region and the WSP domain contribute to the transactivating activity, together with the OTX-tail. In OTX proteins a nuclear localization signal (NLS) sequence resides C-terminal to the homeodomain, between residue 88 and 107 (Fei and Hughes, 2000). This sequence works in conjunction with a recently-discovered nuclear retention domain, located downstream of the homeodomain (AA 117-146) (Chatelain et al., 2006). It is interesting to mention the presence in homeoproteins of penetratin, a 16-amino acid long peptide corresponding to the third α-helix of the homeodomain. Penetratin allows a translocation through biological membrane by means of a receptor-, endocytotis- and energy-independent mechanism (Christiaens et al., 2004).

These observations support the idea that OTX transcription factors are made up of a modular structure where single modules can cooperate to allow the biological function of the entire protein.

During our investigation we have identified a small, divergent region that confers the specific activities to either XOTX2 or XOTX5b in retinal cell fate determination and we called it "Retinal Specificity box" (RS box). This box lies directly C-terminal to the homeodomain, extending for 8-10 amino acid residues. In this box, six amino acid residues differ between XOTX2 and XOTX5b.

Remarkably, this region is necessary and sufficient to confer to these XOTX proteins their specific cell fate specification activities in the frog retina. Indeed, deletion of the box completely abrogates any cell fate activity of either XOTX2 or XOTX5b. Furthermore, when the sequence of the XOTX5b RS box was turned into that of XOTX2 box (construct *Xotx5bMut3*), the *in vivo* biological activity of XOTX5b was completely switched to that of XOTX2.

Conversely, the mutant *Xotx2* construct (*Xotx2Mut3*), where the XOTX2 RS box was changed into that of XOTX5b, had the same biological effects as wild type *Xotx5b*. We tested also the activity of other two *Xotx5b* mutant constructs: *Xotx5bMut2* (4 amino acid change) was able to push progenitors toward a bipolar

cell fate (like *Xotx2*), but had no significant effect in decreasing photoreceptor cell frequency (unlike *Xotx2*); finally, *Xotx5bMut1* (2 amino acid change) showed activities of both *Xotx2* and *Xotx5b*, since it was able to increase both bipolar and photoreceptor cells (though in this latter aspect with significantly lower efficiency than wild type *Xotx5b*).

These data show that the first two changes in the XOTX5b sequence (S100N, T101G) are sufficient to endow XOTX5bMut1 with a great part of XOTX2 ability to promote bipolar cell fate; in fact, there is no statistical difference between *Xotx2* and *Xotx5bMut1* ($p > 0.05$) (or *Xotx5bMut2* or *Xotx5bMut3;* $p > 0.05$) in their efficiency to promote bipolar cells. This suggests that N102 and/or G103 are particularly important residues for the ability of XOTX2 to promote bipolar cell fate.

On the other hand, mutant and wild type *Xotx5b* constructs showed graded effects on photoreceptor commitment: *Xotx5bMut1* promotes, rather then represses, photoreceptors, similar to *Xotx5b*; *Xotx5bMut2* shows no effect on photoreceptor frequency; *Xotx5bMut3* produces significantly fewer photoreceptors compared to GFP controls. In particular, *Xotx5bMut1* photoreceptor promoting activity is significantly lower than that of *Xotx5b*; furthermore, constructs *Xotx5bMut1* and *Xotx5bMut2* yield significantly different effects ($p < 0.001$), whereas no significant difference occurs between *Xotx5bMut2* and *Xotx5bMut3* ($p > 0.05$) (or these two and *Xotx2;* $p > 0.05$).

These results suggest that the first mutational step (2 AA change) may not completely compromise the photoreceptor promoting activity of *Xotx5b*, while the two successive steps (4 AA or 6 AA change) lead to its abrogation and reversal of effect. These data may suggest additive roles of the six residues in determining XOTX2 repressive effect on photoreceptors.

The function of the RS box sequence may be considered in the light of the presence of several serine residues that might undergo specific post-translational modification, such as phosphorylation, as predicted by a bioinformatic analysis performed using Netphos 2.0 software.

4.2 The RS box confers specific activities to *Drosophila* OTD

Several works have investigated the homology among *otd/otx* gene family and basically have demonstrated an extensive functional conservation during early development (see Introduction). For instance, *Otx2*$^{-/-}$ phenotype is severe, leading to complete lack of anterior neural structures in mouse (Acampora et al., 1995 and 1996; Ang et al., 1996; Matsuo et al., 1995). These anterior phenotypes can be rescued by the *Drosophila otd* gene with a good efficiency, although 5' and 3'UTR of *Otx2* gene are important (Acampora et al., 1998 and 2001).

Conversely, in *Drosophila*, *otd* mutations cause deletion in protocerebral *anlage* and central nervous system differentiation defects in midline neurons and glia (Hirth et al., 1995; Finkelstein et al., 1990; Klambt et al., 1991). Surprisingly, these defects are rescued by either human *OTX1* or *OTX2* (Leuzinger et al., 1998; Nagao et al., 1998).

Recently, a genome-wide microarray analysis has been performed in *Drosophila* in which *otd* or *OTX2* genes were overexpressed (Montalta-He et al., 2002). Noteworthy, the authors found that approximately one-third (93) of the *otd*-regulated transcripts also respond to overexpression of the human *OTX2* gene in fly (Montalta-He et al., 2002). These common downstream genes are likely to represent the molecular basis of the functional equivalence of *otd* and *OTX2* gene action in *Drosophila*, even if the evolutionary conservation is not absolute.

In the light of the extensive functional conservation of *otd/otx* genes we have studied if the fly *otd* shared with either *Xotx* genes any specification activity in the *Xenopus* retina. We performed an experiment of *otd* mix-expression in retinal progenitors cells, in order to investigate whether *otd* could promote a differentiation program toward a specific retinal fate. However, we observed that *otd* has no effect in lipofected retinoblasts, suggesting that it is not able to mimic *Xotx2* or *Xotx5b* in the *Xenopus* retina.

This observation can be explained by a recent work in which Tahayato *et al.* (2003) investigated the role of *otd* in *Drosophila* eye photoreceptors (Tahayato

et al., 2003).

The adult fly compound eye is composed of approximately 800 ommatidia, each formed by 8 photoreceptors, R1-R8. Photoreceptors fall into two classes based on their position within the ommatidia: outer (R1-R6) and inner (R7 and R8) (Cook and Desplan, 2001). Six Rhodopsins (Rh) are expressed in the adult fly visual system: R1-R6 contain the wide-spectrum Rh1, while three main classes of ommatidia can be distinguished based on the Rh content in inner photoreceptors. In the dorsal rim area, both R7 and R8 contain UV-Rh3, to detect polarized ultraviolet light.

The other two classes are distributed stochastically in the rest of the eye: yellow (y) photoreceptors (70% of ommatidia) and pale (p) photoreceptors (30%) (Kirschfeld and Franceschini, 1977; Franceschini et al., 1981; Pichaud and Desplan, 2001). The y ommatidia express UV-Rh4 in R7 and green-Rh6 in R8, whereas p ommatidia express UV-Rh3 in R7 and blue-Rh5 in R8 (see Cook and Desplan, 2001 for a review). In order to investigate the transcriptional control of *rhodopsin*s expression, the authors performed a molecular analysis and found that OTD promotes *rh3* and *rh5* expression, while represses *rh6* (Tahayato et al., 2003). Moreover, in *otd* mutant flies Rh3 and Rh5 are lost, while Rh6 is expanded to outer photoreceptors and a subset of R8 photoreceptors. However, the authors demonstrated that p and y inner photoreceptors remain specified in *otd* mutants, thus supporting the idea that *otd* acts downstream of the p/y decision pathway, controlling, at this stage of development, only the *rhodopsin* expression (Tahayato et al., 2003).

Therefore, this finding strongly supports our observation that *otd* is not able to drive retinal progenitor cells toward a specific cell fate in *Xenopus*.

In order to investigate the divergence between OTD and XOTX proteins we performed a homology research analysis. Outside the homeodomain, that results very conserved, the other regions do not show high conservation and, in particular, the C-terminal of OTD is quite divergent compared to XOTX2 or XOTX5b.

Based on the important role of C-terminals in XOTX action, we asked what could be the effect of OTD C-terminal replacement with that of XOTX2 and XOTX5b. Interestingly, we found that the chimeric OTD/XOTX2 and OTD/XOTX5b constructs "rescue" the ability to drive progenitors toward specific cell fates, in a direction consistent with the substitute C-terminal. Even more significantly, the simple insertion of either the XOTX2 or the XOTX5b specificity box into the OTD protein is able to provide OTD with the ability to promote bipolar or photoreceptor cell fate, respectively. This is remarkable since OTD lacks some of the functional domains, such as the OTX tail and the WSP domain, considered important for the transactivating ability of CRX/OTX proteins. Therefore, the RS box is sufficient to promote specific cell fates also in a rather divergent context than that of vertebrate OTX proteins.

However, not all XOTX2 or XOTX5b retinal functions depend on the RS box. While OTD/XOTX2 is able to promote bipolar cells with the same efficiency of wild type XOTX2, its efficiency in repressing photoreceptors is null, differently from XOTX2. This is at variance with the effect shown by the *Xotx5b/Xotx2* chimeric construct, which retains *Xotx2* anti-photoreceptor activity (Viczian et al., 2003) and suggests that some features of XOTX retinal activity may also depend on the amino-terminal (N-terminal).

The XOTX5b and XOTX2 N-terminal regions (excluding the HD) are about 73% identical, while OTD N-terminal is only about 15% identical to that of XOTX2. It is possible that the N-terminal of XOTX5b may better match the C-terminal of XOTX2 (and *vice versa*) than the N-terminal of OTD, thus allowing the exploitation of the full spectrum of protein activities. Such requirements on the N-terminal could be due to possible interactions with other parts of the XOTX proteins at the intramolecular level, for example to allow proper folding of the protein; as well as at the intermolecular level, for example with other XOTX monomers (Briata et al., 1999 and present data), or other molecular partners.

4.3 The RS box is involved in the correct nuclear localization of XOTX/OTD proteins in retinal neurons

Another subtle difference between OTD and OTX proteins exists just in a stretch of amino acids important for the nuclear localization of this transcription factors.

Interestingly, mutations in CRX NLS are associated with human retinal pathologies and have effect on CRX nuclear localization (Fei and Hughes, 2000). In particular, R98L mutation leads to CRX mislocalization; interestingly, a L residue is present in *Drosophila* OTD at corresponding position (Fig. 4.1).

In fact, we observed that OTD, when misexpressed in *Xenopus* retinae, has a nuclear-cytoplasmic localization.

We therefore asked whether the inability of OTD on retinal specification could be due to insufficient translocation to the nucleus. However, our results show that it is not the mere deficiency in nuclear localization that explains why OTD does not influence retinal cell fate choices: first, because forcing OTD to the nucleus by a additional NLS does not have any effect on retinal cell fate; second, because the effect of OTD/box2 and OTD/box5b is specifically depending on the type of RS box inserted in OTD.

Therefore, we suggest that while the C-terminal domain of OTD is able to mimic, to a certain extent, the transactivating activity of XOTX2 and XOTX5b C-terminals, OTD, in the absence of the RS box, fails to properly target the gene sets that address retinal progenitors to their fates.

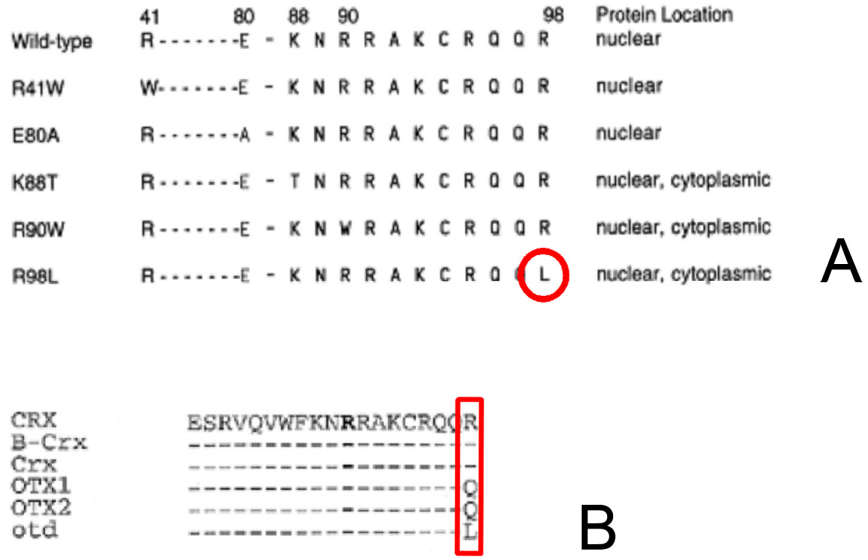


Figure 4.1. CRX mutations and cellular localization.

(A) Mutant CRX constructs and their partial amino acid sequences showing the location of the missense mutations. Interestingly, the R98L mutation (red circled L) produces a nuclear/cytoplasmic mislocalization of CRX protein (from Fei and Hughes, 2000).

(B) Amino acid sequence alignment of OTX/OTD proteins. Noteworthy, a L residue is present in OTD, corresponding to R98 in CRX (red boxed amino acid residues).

4.4 XOTX2 and XOTX5b differentially synergize with XNRL to transactivate the *Xenopus rhodopsin* promoter and differentially interact *in vitro* with XNRL

How can the RS box modulate the activity of XOTX2 and XOTX5b proteins? Two possibilities, not mutually exclusive, are that the box refines the DNA binding abilities of XOTX proteins towards different sets of promoters, or that the box modulates interactions with other molecular partners. Basically, the overall effects on cell fate by wild type and mutant XOTX2 and XOTX5b suggest that different sets of genes are activated depending on the type of RS box, driving the intrinsic program that will give rise to a neuron rather than another.

In our transactivation assay we showed that XOTX2 and XOTX5b differentially transactivate the *rhodopsin* promoter, synergizing with XNRL. This result is not obvious, since *rhodopsin* promoter contains OTX consensus and both XOTX2 and XOTX5b have the intrinsic ability to activate this gene (Viczian et al., 2003). Moreover, *rhodopsin* is a key photoreceptor gene, that is not expressed in bipolar cells, so our finding that XOTX5b (+XNRL) activate *rhodopsin* promoter with a significantly higher efficiency than XOTX2 (+XNRL) is consistent with *in vivo* context. In order to confirm the importance of the RS box, we demonstrated that XOTX2Mut3 mimics XOTX5b activity and, conversely, XOTX5bMut3 mimics the XOTX2 one.

We tested also OTD transactivation ability demonstrating that it is able to activate *Xenopus rhodopsin* promoter, even though with a lower efficiency respect to XOTX5b. This result is not surprisingly, since OTD is able to regulate *rhodopsin* expression in *Drosophila* (Tahayato et al., 2003) and recognizes the same DNA consensus of OTXs. Moreover, we found that replacing the C-terminal of OTD with that one of XOTX5b produces effects consistent with the *in vivo* results, OTD/XOTX5b significantly transactivating better than OTD.

The synergism in promoter transactivation is mediated by a direct protein-protein interaction, that we demonstrated among OTD/XOTX and XNRL. Significantly, we also found a clear difference in the interactive abilities of XOTX5b and XOTX2 towards XNRL, that is consistent with their respective roles in frog retinogenesis (Viczian et al., 2003) and with the results on the *rhodopsin* promoter activation (Peng and Chen, 2005 and present data).

4.5 XOTX2 and XOTX5b can form homo/heterodimers

After demonstrating by means of *in vivo* and *in vitro* experiments the instrumental role of RS box in modulating the biological functions of the XOTX2 and XOTX5b, we focus our attention on another molecular aspect of their action.

Viczian *et al.* (2003) made an interesting observation: when *Xotx2* and *Xotx5b* are co-lipofected in *Xenopus* retina there is an increase in the frequency of bipolar cells (Viczian et al. 2003). Moreover, they tested the ability of *Xotx5b* to transactivate the *Xop*-GFP by injecting the *Xenopus* embryos with the two constructs and found that XOTX5b is able to transactivate the *rhodopsin* promoter. XOTX2 is able to activate the expression of *Xop*-GFP, too. Surprisingly, when *Xotx2* and *Xotx5b* are co-injected in equal amounts, there is no expression of GFP, thus supporting the idea that *Xotx2* has a dominant negative effect on *Xotx5b* activity (Viczian et al., 2003).

On the basis of these observations we speculated that a kind of interaction between XOTX2 and XOTX5b exists. Previous work by Briata *et al.* (1999) showed that the homeodomain of the human OTX2 initially binds to target promoters as a monomer, and then a conformational change in the protein allows its dimerization. So, we investigated the possibility that XOTX2 and XOTX5b dimerize and we demonstrated that they really physically interact each other. This interaction impairs also the transactivation ability of XOTX5b, in particular affecting its ability to synergize with XNRL in transactivating the *rhodopsin* promoter. Actually, we think that the formation of homo/heterodimers between XOTX2 and XOTX5b could be an additional way for regulating their mechanisms of action. In particular, the dominant negative effect of XOTX2 may prevent possible action of the XOTX5b protein that, differently from earlier stages, is present in bipolar cells at post-larval stage. In this way, although XOTX5b is present in a subpopulation of bipolar cells (together with XOTX2) at post-larval stages, these cells are prevented from expressing key photoreceptor genes, such as *rhodopsin*.

4.6 Evolution of eye and retinal cell types

The evolution of eye is a fascinating topic for the same reason that had already enthused Darwin, who found it hard to explain that "the natural selection could produce… an organ so wonderful as the eye" (Darwin, 1859).

What was the most ancient precursor of an eye? Gehring and Ikeo have suggested a two-celled proto-eye made up of one photoreceptor cell and one pigmented cell (Gehring and Ikeo, 1999), resembling the two-celled eye that exists in polychaete trocophore (Arendt et al., 2002). This very simple eye could have accomplished some primitive form of vision by detecting the direction of light for phototaxis (Arendt, 2003). Additional cell types were added during subsequent eye evolution, such as lens cells, various kinds of support cells, muscle cells, etc.

In order to shed light on eye evolution, a comparative molecular cell biology approach has been attempted by focusing on the retinal cell type as the main unit of reference in eye homology research (reviewed in Arendt, 2003). According to this approach, a cell type can be defined as a "homogenous population of cells expressing the same set of orthologous genes for specification and differentiation, to implement a defined cellular phenotype". It is now well established that in the entire nervous system cell-type specification depends on the expression of specific combination of transcription factors, largely bHLH and homeodomain superfamily, as we have seen.

Comparative molecular cell biology explores the combinatorial code of gene expression to compare the cell types of a given species among themselves and to those of other closely, or even distantly related species. Comparing within a given species, some cell types will differ in their molecular characteristics only slightly, and will exhibit similar – but not identical – cellular phenotypes. Such similarities are indicative of a common evolutionary history, meaning that some ancestors of that species had a single precursor cell type that subsequently diversified into these cell types.

Detlev Arendt refers to the descendant cell types as “sister cell types”, defined as cells evolved from one common precursor by cell type diversification. Rods and cones of a given vertebrate species are a good example for sister cell types. On the other hand, it is possible to define “homologous cell types” as cells evolved from the same precursor in the last common ancestor of the compared groups.

For instance, photoreceptors cells can have two distinct morphologies. All photoreceptors enlarge the membraneous surface for the storage of photopigment, but the rhabdomeric photoreceptors do so by folding the apical cell surface, while the ciliary photoreceptors fold the ciliary membrane (Eakin, 1982). Rhabdomeric and ciliary photoreceptors co-exist in many bilaterian groups.

The most accredited hypothesis is that a common ancestor of all Bilateria, the Urbilateria, already possessed two distinct photoreceptor sister cell types. Some evidences support this hypothesis: first, two distinct opsin-employing photoreceptors have already diversified in Urbilateria (Arendt and Wittbrodt, 2001). Moreover, the construction of phylogenetic trees for the conserved molecules involved in phototransduction (opsin, α-G protein, arrestin, rhodopsin kinase) has given the surprising result that at least two distinct opsin paralogs exist in Bilateria and that invertebrate rhabdomeric and vertebrate ciliary photoreceptors deploy distinct paralogs. This is explained if one assumes that initially single pre-bilaterian photoreceptor precursor had diversified into two distinct types, paralleled by gene duplication of many cell-type specific genes (Arendt, 2003).

The comparison of molecules involved in the specification and differentiation of rhabdomeric photoreceptors with those of the different cell types of the vertebrate retina has revealed many resemblances not with rods and cones, but with retinal ganglion cells (Arendt et al., 2002; Frankfort and Mardon, 2002; Hsiung and Moses, 2002). This hypothesis is further supported by the finding that retinal ganglion cells express melanopsin (Hattar et al., 2002; Provencio et al., 2002), the vertebrate ortholog of invertebrate rhabdomeric opsins, and they have

recently been identified as additional photosensitive cells in the vertebrate retina, involved in entrainment of circadian rhythms and pupil constriction (Berson et al., 2002; Lucas et al., 2003).

Having identified retinal ganglion cells and invertebrate rhabdomeric photoreceptors as possible homologous cell types, considering both the specifying transcription factors and the cell-type specific effectors genes, it appears that ganglion cells, amacrine cells and horizontal cells are sister cell types (Arendt, 2003) (Fig. 4.2).

The evolutionary origin of bipolar cells is less clear (Arendt, 2003). However, the expression of some common transcription factors, such us *Otx* family, let us to speculate on their origin from an ancestral ciliary photoreceptor (Fig. 4.2).

In this regard, it has been demonstrated that transgenic mice generated using a region of *S-opsin* promoter fused to a reporter express the signal in S-cones but also in bipolar cells, reflecting regulatory mechanisms that are common to bipolar cells and cone photoreceptors (Chen et al., 1994). Moreover, photoreceptors and bipolar cells elaborate an unusual structure in their terminal, the ribbon synapse, which, within the retina, is unique to only these two cell types (reviewed in Sterling and Matthews, 2005).

Furthermore, a recent microarray analysis has been performed to investigate the gene expression profile of photoreceptors; interestingly, the authors found that many cone-enriched genes show expression in additional retinal cell types, but mainly in bipolar cells (Corbo et al., 2007).

This common origin between photoreceptors and bipolar cells may explain the cell fate plasticity demonstrated between these cell types (see Introduction) and our results, in which we found that *Xotx2* and *Xotx5b,* and in particular RS box, are able to switch bipolar versus photoreceptor cell-fate in a quite precise manner.

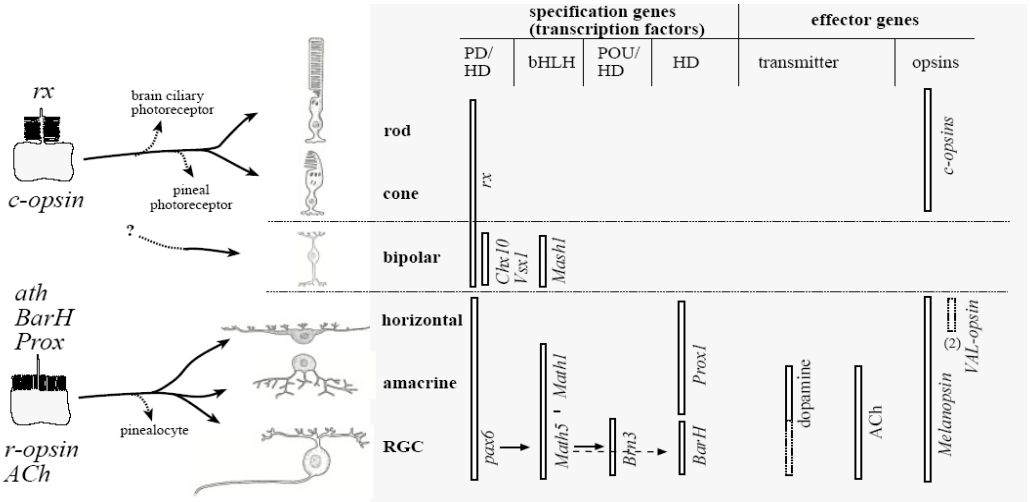


Figure 4.2. Diversification of cell types in the Vertebrate retina.

Molecular comparative cell biology indicates that rods and cones have evolved from a common ciliary photoreceptor precursor, while retinal ganglion, amacrine and horizontal cells have evolved from a rhabdomeric photoreceptor precursor. Black arrows represent cell type evolution. The origin of bipolar cells is less clear. The expression of transcription factors in the different cell types is shown (from Arendt, 2003).

Concluding remarks

We have investigated the molecular property of XOTX2 and XOTX5b during retinal neurogenesis. In particular, we have provided *in vivo* and *in vitro* evidences for different biochemical activities of XOTX proteins in the *Xenopus* retina, due to presence/absence of the Retinal Specificity (RS) box.

We suggest that the RS box allows XOTX2 and XOTX5b proteins to appropriately target gene sets involved in either bipolar or photoreceptor cell specification, respectively. This is particularly significant since OTX/CRX/OTD proteins are able to bind *in vitro* to the same consensus sequence TAATCC/T and yet they have significantly different effects in Vertebrate retina differentiation.

While still relatively little is known on what gives *in vivo* targeting specificity to homeodomain containing factors, our data show that the RS box of XOTX2 and XOTX5b is an essential and major domain of their functioning *in vivo* and is involved in providing such specificity in the developing *Xenopus* retina (Fig. 4.3).

We propose that RS box contributes to refine the correct combinatorial network of transcription factors that supervise the identity of a neuron.

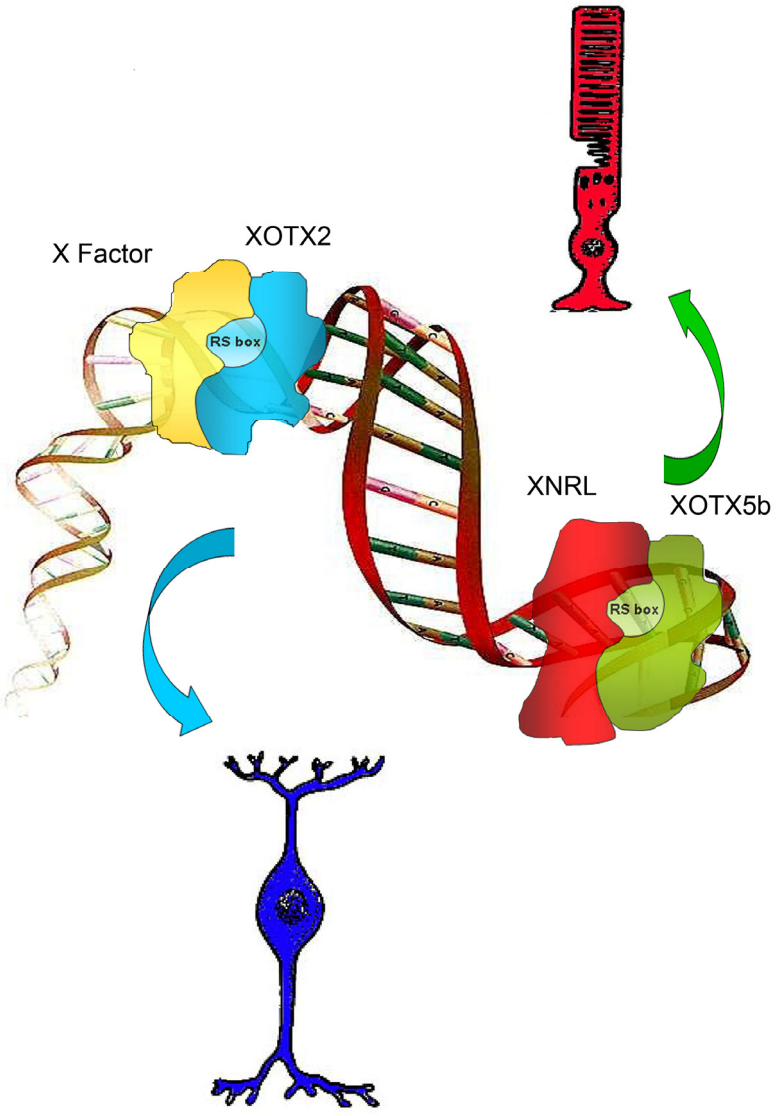


Figure 4.3. Schematic model of transcription regulation and retinal cell differentiation.

This drawing shows a model of the XOTX5b and XOTX2 transcriptional activity, modulated by RS box, in retinal cell fate determination.

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