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Development and molecular characterization of adult and larval eyes in *Platynereis dumerilii* (Polychaeta, Annelida, Lophotrochozoa)

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Abstract

The annelid worm *Platynereis dumerilii* (Lophotrochozoa) exhibits ancestral developmental, body plan and genomic characteristics and possesses two types of eyes: adult pigment cup eyes and larval two-celled eyes. *Platynereis* therefore represents a useful model organism for the study of eye evolution in annelids. My research goal has been to characterize the differentiating *Platynereis* adult and larval eyes on the molecular level in order to explore the evolutionary history of these two types of eyes and their cell types: rhabdomeric photoreceptor cells (rPRCs) and pigment cells (PCs).

This aim has been addressed by using the 'molecular fingerprint' (MFP) approach for the comparative study of cell types. I first identified specific molecular markers for each of the cell types in both types of eyes. These were then used to establish a comprehensive MFP of these cell types that included both effectors genes (differentiation genes expressed in eye and neuronal cell types) and transcription factors which play a role in eye and neuronal specification. This was achieved by means of gene expression studies, using wholemount double *in-situ* hybridization and 3D *in-silico* alignments.

The data obtained reveal that *Platynereis* adult and larval eyes are composed of six cell types, based on MFP comparisons: adult eyes ventral and dorsal rPRCs, adult eyes ventral and dorsal PCs, larval eye rPRCs and larval eye pigment cells. The distribution of the adult rPRCs and PCs into two (ventral and dorsal) cell types relates to the fact that *Platynereis* develops two pairs of adult eyes that appear to differ in terms of their molecular regulation.
It also revealed that many transcription factors regulating eye development in *Drosophila* and/or vertebrates are also expressed in the differentiating *Platynereis* eyes. Surprisingly, some of these are adult eye-specific and some are larval eye-specific, meaning that the adult and larval eyes of *Platynereis* show a distinct MFP, corroborating that they represent different types of eyes. On the other hand, some shared effector genes were identified between the rPRCs and PCs of the adult eyes, as well as of the larval eyes. This finding implicates that the rPRCs and PCs of *Platynereis* are sister cell types that can be traced back to a single ancestral multifunctional cell type precursor.
Hierarchical clustering analysis based on the MFP results mirrors the 'phylogeny' of the different eyes cell types, in which the larval eyes cell types cluster together as do the two types of adult eyes rPRCs and PCs.

In order to gain more insight into the developmental regulation of both eyes in *Platynereis*, I chose to assess the role of the conserved hedgehog (Hh) signaling pathway in *Platynereis* eye development. By using the antagonist cyclopamine to inhibit the hedgehog pathway in *Platynereis* embryos, I found out that *Platynereis* Hh pathway plays a role in adult but not in larval eye development. This adds another key distinction between the adult and larval eyes of *Platynereis*.

These results support the view that annelid eyes originated from one multifunctional single cell prototype eye that bore characteristics of both PRCs and PCs. It was first duplicated to give rise to adult and larval eye precursors to then diversify into the PRCs and PCs present in today's annelid eyes.

Zusammenfassung

Der Annelid *Platynereis dumerilii* (Nereididae, Annelida, Lophotrochozoa) ist durch zahlreiche evolutiv alte Merkmale, wie seine Entwicklung, seinen Bauplan und ein ursprüngliches Geninventar gekennzeichnet. Er besitzt zwei verschiedene Augentypen: die adulten Pigmentbecheraugen und zweizellige Larvalaugen. Daher ist *Platynereis* ein geeigneter Modellorganismus, um die Evolution von Augen in Anneliden zu untersuchen. Das Ziel meiner Arbeit ist die molekulare Charakterisierung der Differenzierung der Laval- und Adultaugen von *Platynereis*, um die Evolution dieser beiden Augentypen und ihrer Zelltypen – rhabdomere Photorezeptorzellen und Pigmentzellen – zu verstehen.

Zur vergleichenden Analyse der Zelltypen habe ich die Methode des Vergleichs von molekularen Fingerabdrücken genutzt. Dazu habe ich zunächst spezifische molekulare Marker für jeden der Zelltypen in den Laval- und Adultaugen identifiziert und damit anschließend eine umfassende Analyse des molekularen Fingerabdrucks dieser Zelltypen vorgenommen. In diese Analyse sind sowohl Differenzierungsgene als auch Transkriptionsfaktoren, die in den Augen und neuronalen Zellen expremiert werden, mit einbezogen worden. Die Expression dieser Gene habe ich mit Hilfe der Whole-mount-doppel-in-Situ-Hybridisierung und der 3D-in-silico-Alinierungstechnik untersucht.

Die Auswertung des molekularen Fingerabdrucks hat ergeben, dass die Laval- und die frühen Adultaugen von *Platynereis* aus sechs Zelltypen zusammengesetzt sind: den ventralen und dorsalen rhabdomeren Photorezeptorzellen der Adultaugen, den ventralen und dorsalen Pigmentzellen der Adultaugen, den larvalen rhabdomeren Photorezeptorzellen und den larvalen Augenpigmentzellen.
Die Aufteilung der rhabdomeren Photorezeptorzellen und der Pigmentzellen der Adultaugen in ventrale und dorsale Zelltypen ist auf die Bildung von zwei Paar Adultaugen zurückzuführen, die sich in ihrer molekularen Regulierung unterscheiden.

In meiner Arbeit konnte ich zeigen, dass zahlreiche Transkriptionfaktoren, die die Augenentwicklung in *Drosophila melanogaster* und Vertebraten regulieren, auch in den sich differenzierenden Augen von *Platynereis* expremiert werden.
Überraschenderweise handelt es sich dabei sowohl um adultaugenspezifische als auch um lavalaugenspezifische Gene, was zu der Schlussfolgerung führt, dass die Adult- und Larvalaugen von *Platynereis* unterschiedliche molekulare Fingerabdrücke haben. Damit wird die Annahme unterstützt, dass es sich um unterschiedliche Augentypen handelt.
Andererseits konnte ich auch Differenzierungsgene identifizieren, die sowohl in den rhabdomeren Phororezeptorzellen als auch in den Pigmentzellen der Adultaugen expremiert werden bzw. von beiden Zelltypen im Larvalauge expremiert werden. Diese Ergebnisse lassen den Schluss zu, dass es sich bei den rhabdomeren Photorezeptorzellen und Pigmentzellen von *Platynereis* um Schwesterzelltypen handelt, deren gemeinsamer Ursprung eine multifunktionelle Augenvorläuferzelle war.
Eine hierarchische Clusteranalyse, die auf den Ergebnissen des molekularen Fingerabdrucks beruht, spiegelt die “Phylogenie“ der verschiedenen Augenzelltypen wieder. Die Zelltypen der Larvalaugen bilden die eine Gruppe des Clusters. Innerhalb der anderen Gruppe, die alle Zellen der Adultaugen umfasst, bilden die ventralen und

dorsalen rhabdomeren Photorezeptorzellen eine Gruppe, eine weitere setzt sich aus den ventralen und dorsalen Pigmentzellen zusammen.

Um mehr über die (differenzielle) Entwicklung der Augen von *Platynereis* zu erfahren, habe ich den Einfluss der konservierten Hedgehog (Hh)-Signal-Kaskade auf die Augenentwicklung von *Platynereis* untersucht. Experimente mit Cyclopamin, einem Antagonisten der Hh-Signal-Kaskade, zeigten, dass Hh zwar die Entwicklung der Adultaugen, nicht aber die der Larvalaugen beeinflusst. Damit konnte ich einen weiteren grundlegenden Unterschied zwischen den beiden Augentypen von *Platynereis* aufzeigen.
Die Ergebnisse unterstützen die Auffassung, dass die Augen von Anneliden von einem einzelnen multifunktionellen Augenvorläuferzelltyp abstammen, der sowohl Merkmale der Photorezeptorzellen als auch der Pigmentzellen aufwies. Dieser Vorläufer wurde im Lauf der Evolution zunächst dupliziert und differenzierte später in Adult- und Larvalaugenvorläufer, die dann zu den Photorezeptorzellen und Pigmentzellen wurden, wie sie aus rezenten Anneliden bekannt sind.

Introduction

1.1 Overview

"To suppose that the eye, …., could have been formed by natural selection, seems, I freely confess, absurd in the highest possible degree. How a nerve comes to be sensitive to light, hardly concerns us more than how life itself first originated; but I may remark that several facts make me suspect that any sensitive nerve may be rendered sensitive to light, …" (*Darwin, The origin of species, chapter 6, 1882*)

The origin of the eye was and still is a fascinating mystery causes a huge debate. Our view on eye evolution have gone considerable number of changes in the last century. I would first like to mention some milestones in this path.

In 1979, Hansjochen Autrum (Autrum 1979) first argued that all eye share a common evolutionary origin (the monophyletic origin hypothesis) through the consistent use of membrane bound rhodopsin as a photopigment. He also noted that in all animals two main kinds of photoreceptors are present, rhabdomeric and cilliary, which coexist in the major branches of the phylogenetic tree.

However there were three main serious challenges to this view, first was a survey of photoreceptor ultrastructure that claimed independent evolution in 40 to 65 cases in separate phyletic lines (the polyphyletic origin hypothesis) (Salvini-Plawen and Mayr 1977) .The second is the different embryonic origin of the different structure in the vertebrates and cephalopods eyes (Nilsson 1996) . the third challenge came from molecular comparisons indicate an ancient dichotomy between the ciliary and the rhabdomeric photoreceptors types (Arendt and Wittbrodt 2001): The two major classes of opsins are neatly distributed in each of the two receptor-types, the transduction machinery and response termination also differ between ciliary and rhabdomeric receptors.

A decade later the discovery of homologous genes controlling eye development in vertebrates, insects and other animals supported the idea that all eyes share a common prototype eye ancestor. However the monophyletic eye hypothesis continued to suffer criticism relying mainly on the above third argument.

Two recent discoveries (Arendt, Tessmar-Raible et al. 2004; Panda, Nayak et al. 2005) illustrated that the two classes of animal photoreceptors, are likely to share an ancient common ancestor and have been evolving in parallel since their duplication over 600 million years ago. Arendt et al. identified cilliary photoreceptor (molecularly, regulatory and morphologically) in an invertebrate. Panda et al. followed the work of (Berson, Dunn et al. 2002; Hattar, Liao et al. 2002) and showed that the melanopsin (a putative opsin-family photopigment) expressed in the vertebrates retinal ganglion cells, encodes a fully functional opsin that signals more like an invertebrate opsin than like a classical vertebrate rod-and-cone opsin. Both studies support the indication that the last common ancestor of *Bilateria* must already have possessed two types of photoreceptor cells, ciliary and rhabdomeric.

In my PhD I was interested to investigate this controversy issue further and to explore eye evolution on the molecular level. Many indication have accumulated for shared genetic control of eye development, however, these came mainly from the two

branches of bilateria: the Deuterostomes and Protostomes. I chose Platyneries as a representative of the third branch: the Lophotrochozoa for a comparative study.

By establishing a molecular fingerprint (MFP) of *Platynereis* adult and larval eye cell types, I would like to explore the level of conservation of different genes in the development of different eye cell types, and two different eyes: Adult versus larval eyes of Platynereis.

1.2 Variety of eyes

Platynereis possess two types of eyes: adult pigment cup eyes and larval (simple prototype) eye. In the light of the comparative study I would also like to understand the molecular difference between these eyes and what it reflects. Can we learn from it something about the significance of today's astonishing bilaterians eyes repertoire? I would therefore like to briefly explore the topic of eye variety.

The simplest eye like function - phototactic function (the capability of orientating with respect to the direction of light) can be traced back to as simple organisms as unicellular flagellate algae (Witman 1993) . Another example of a very simple "eye" is found in Planula larvae of *Tripedalia* in the form of single cell, pigment cup ocelli, lacking neuronal connection. These light sensors have photosensitive microvilli and a motor-cilium. They respond directly to light and may act as a rudder to steer the larva (Nordstrom, Wallen et al. 2003).

Shading pigment is essential for a photoreceptor cells in order to detect the **direction** of the light. Therefore photoreceptor cells that are associated with pigment are considered 'eyes' whereas the ones that don't, are not considered as 'eyes'. The simplest eye we can imagine will consist of both cell types, however even simplified version, that contain both shading pigment and folded membrane with photo-pigment, in one cell, exist.

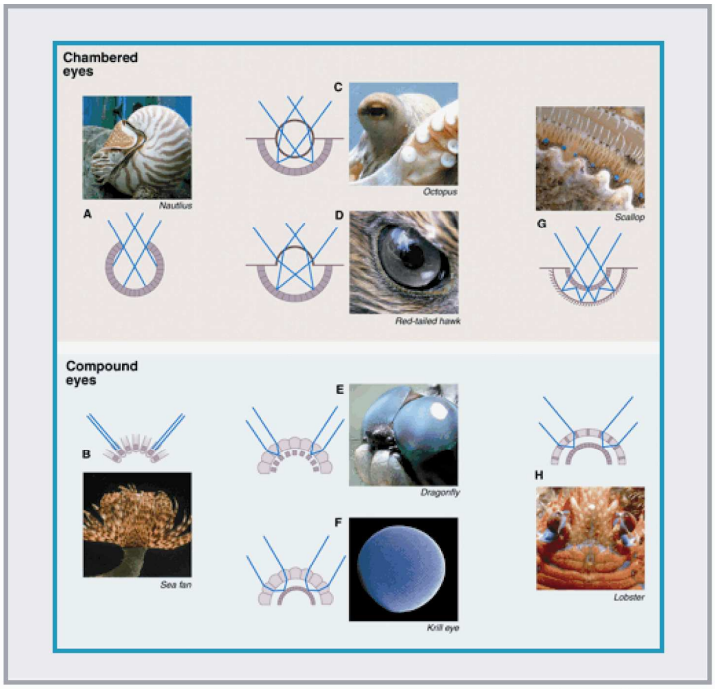
If we explore eye variety in detail we will find out that of ca.33 phyla, about a third have no specialized organ for detecting light. Another third have light sensitive organs and the remainder are animals with what we would consider eyes. Image forming eyes evolved in six of the 33 extant metazoan phyla : *Cnidaria, Mollusca, Annelida, Onychophora, Arthropoda and Chorodata.* These six phyla contribute about 96% of the known species alive today, pointing on the importance of eyes in our world. There are several features common to all eyes and arising from constrains regarding their physical properties - the ability to collect and focus light (land MF 2002).

These constrains restrict the eye variety to eight types of eye optics:

Both **chambered eyes** and **compound eyes** form images using shadows, refraction , or reflection . The simple **pit eye** led to the **lensed eyes** in fish ,cephalopods and terrestrial animals.

The **apposition compound eye** found in bees, crabs, and fruit flies; the **refracting superposition compound eye** found in moths and krill; and the **reflecting**

superposition eye found in decapod shrimps and lobsters. (Fernald 2006). Figure1shows an example of the chambered and compound eyes.



(Fernald, 2006)

Figure 1: Eight major types of optics in animal eyes.
Both chambered eyes (top) and compound eyes (bottom) form images using shadows (A and B), refraction (C to F), or reflection (G and H).
Light rays shown in blue, photoreceptive structures are shaded. The simple pit eye (A) (chambered nautilus) led to the lensed eyes in fish and cephalopods (C) and terrestrial animals (D). Scallop eyes (G) (bay scallop) are chambered but use concave mirror optics to produce an image. The simplest compound eye (B) found in bivalve molluscs , the apposition compound eye (E) found in bees, crabs, and fruit flies; the refracting superposition compound eye (F) (Antarctic krill) of moths and krill; and the reflecting superposition eye (H) found in decapod shrimps and lobsters. (Fernald, 2006)

Beside eyes optics, classification of eyes can also rely on their complexity. ‘Ocelli’ are simple, multicellular eyes composed of a photoreceptor cell, pigment cell and optionally support cells. Structurally they often resemble ‘pigment cup eye’. ‘Compound eyes’ are composed of number of distinct basic units called ommatidia, found in *Polychaeta, Bivalvia* and *Arthropoda*. An individual ommatidium resembles a pigment cup ocellus, on the structural level. ‘Complex eyes’ composed of cornea, iris, lens and retina. They are found in *Cephalopoda and Vertebrata.*(Arendt and Wittbrodt 2001)

Invertebrates have the greatest variety of eyes types, having both camera eyes (in e.g. *Cephalopods*) and compound eyes. Moreover, the number and location of eyes is highly varied in invertebrates - one can find multiple non-paired eyes and eyes in remarkable locations while vertebrates usually have paired chambered eyes with lenses (Fernald 2004). This remarkable structural variety reflects off course physical

properties variety in terms of the eye's function. While very simple eyes provide limited information regarding light intensity and direction, more advanced eyes can provide image reception. The capabilities of eyes as a function of their structure and therefore the different specificity of their developmental programs vary a lot. As an example the resolution of an image as measured in subtended degrees differs by about 13 fold among vertebrates and even more between vertebrates and invertebrates. Eagles posses the greatest acuity, which is around 10,000 fold greater than that of certain *planaria*. (land MF 2002)

Another interesting distinction between vertebrates and invertebrates eyes repertoire is their embryonic origin. The cephalopod eye forms from epidermal placode through successive infolding while the vertebrae eye develop from the neural plate, induces the overlying epidermis to form the lens, it is therefore considered as an invagination of the brain. Another interesting distinction is the lack of cornea in cephalopod eyes, which is present in all aquatic and non aquatic vertebrates.

1.2.1 The basic units of the eye

After exploring the great variety of eyes in *Bilateria*, it is astonishing to realize that they are all built from same 'cornerstone' – the photoreceptor and pigment cells. In the following section I will introduce them in detail.

1.2.1.1 Photoreceptor cells

In order to fulfill their function, photoreceptor cells have a clear bias to enlarge their membrane surface in order to collect light. They achieve this by enlarging either their apical cell membrane or their ciliary membrane. By this, two distinct groups of photoreceptor cell types are defined: the Rhabdomeric versus Ciliary photoreceptors (PRCs). In annelids a third type is present, the phaosomous photoreceptor cells.

In rhabdomeric PRCs the photoreceptive membrane are microvilli, finger like extension of the apical plasma membrane (Eakin and Hermans 1988). They are usually highly ordered, densely arranged and parallel to one another.

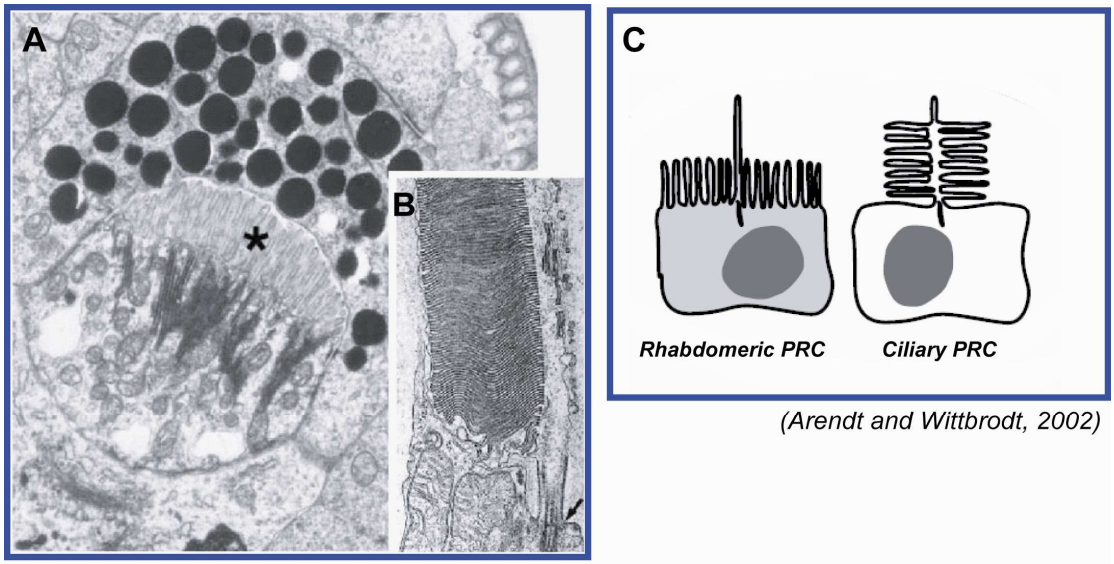
Ciliary PRCs bear an expansion of the cell membrane in the form of cilia projecting into an extracellular cavity (Eakin and Hermans 1988) .They are usually multi ciliated with either branched or un-branched cilia.

The phaosomous PRCs are seemingly intracellular single cell vacuoles into which the photoreceptive processes project (Eakin and Hermans 1988). It arises during development by invagination of the apical cell membrane and therefore provides a large surface from which the receptoral processes may extend. This vacuole may either remain open or closed upon completion of its development. Its photoreceptive membranes are usually microvilli although cilia may be present as well. Although extremely rare in *polychaeta*, they are most likely the only type of PRCs present in *clitellata* (except for ciliary ones found in their brain).

Both rhabomeric and ciliary PRCs coexist in the three branches of the Bilaterian tree: *Lophotrochozoa, Ecdyzoa and Deuterostomia* . Their tissue distribution, however, is not random, pointing at a bias for Rhabdomeric PRCs in cerebral eyes versus Ciliary

PRCs in non cerebral eyes or as ciliary brain PRCs that are not associated with pigment cells and therefore do not represent eyes. Some exceptions, however, exist in every branch.

Ciliary PRCs in non cerebral eyes are found at highly divergent positions, for example in polychaetes they are have been detected in the branchial crown eyes (Eakin and Hermans 1988) and in some mollusks they form part of the mantel edge eyes and optic tentacles (Barber, Evans et al. 1967; Barber and Wright 1969; Hughes 1970). In Deuterostomes, PRCs in the apical eyespots of the *tornaria* larva are rhabdomeric (Brandenburger, Woollacott et al. 1973), pointing that larval cerebral eyes with rhabdomeric PRCs (like the larval eyes of *Platynereis*) might have existed at the very root of the *Deuterostomia*. Remarkably, as apposed to the vast majority of *Bilateria*, in chordates, cerebral eyes have **ciliary** PRCs. The vertebrates are in fact the only deuterostomes that don't posses any rhabdomeric PRCs (Vanfleteren and Coomans 1982). (See figure 2 for examples of rPRCs and cPRCs)



(Arendt and Wittbrodt, 2002)

(Lacalli, 2004)

Figure 2: Rhabdomeric and ciliary photoreceptors (PRCs).

(A) An example of rhabdomeric photoreceptor (from *Platynereis* larval eye) showing the parallel array of microvilli (the Rhabdome is marked by asterisk) which is employed to increase the surface available for light reception. (B) The vertebrate eye contains ciliary photoreceptors, in which membranous lamellae are stacked within the body of a modified cilium, the base of which is arrowed (Lacalli, 2004) . (C) schematic drawing of the two types of PRCs (Arendt and Wittbrodt, 2002).

1.2.1.2 Pigment cells and pigment synthesis

Animal pigments are formed through the melanin, pteridine, ommochrome and papiliochrome synthesis pathways. Among these, the first three are the main contributors to body coloration. It is known that melanin is widely distributed throughout the animal kingdom. However, insects significantly differ from vertebrates in the mechanism of melanin formation, they use dopamine rather than dopa as the major precursor of melanin formation (Sugumaran 2002).

The variety of eye color patterns observed in the fruit fly, *Drosophila melanogaster*, for example (Grell 1968), is a result of accumulation of xanthommatin (ommochrome) and pteridines .
Ommochromes are distributed among Protostomes, but are not found among Deuterostomes. The ommochrome pathway is the most important route for elimination of tryptophan metabolites, which are toxic in the presence of excessive quantities. In this respect, it is important to emphasize that the general metabolic pathway of Tryptophan is typically very different between vertebrates and insects. In the former, the pathway leads to Serotonin and other products with a defined role in the synthesis of for example: nucleotides. In insects, on the other hand, the process leads to the production of Kynurenine and 3-hydroxykynurenine which condenses to give rise to a large amount of dermal and eye pigments. Two important groups of pigments derived from Tryptophan: Ommatins and Ommins. Ommatins are represented by (a) xanthommatin, the simplest basic pigment formed by condensation of two molecules of 3-hydroxylkynurenine forming a phenoxazone structure; (b) rhodommatin, ommatin C and D. (See figure 3 for the Ommochrome pathway) Ommins may be mixture of different sulfur-containing pigments and their molecules are more complicated . Ommatinas are present in almost all insects investigated, Principally in the eyes and the epidermis. Ommins are generally less frequently found. (Fuzeau-Braesch, 1972)

Pterins.- This group of substances is a very large one which has as its basic structure a double pyramidic-pyrazinic ring system, including 4 atoms of nitrogen. Pterins are very common in insects. Some principal members of the family are: pterin (white), pteridine (clear yellow), xanthopterin (yellow), erythropterin (red), ehrysopterin (yellow), isoxanthopterin (colorless), leucopterin (white), biopterin, drosopterin (red), and sepiapterin. To this type, we must add the "conjugated" pterins (C-6 substitution), like folic acid or pteroic acid, and the dehydrogenated pterins . New compoundsa are continually described in an increasing number of insects and it seems that pteridines are often present in all the body tissues throughout the life of the animal. (Fuzeau-Braesch, 1972)

The eye pigment of *Platynereis dumerlii* was isolated from adult worms and was identified as a mixture of three different pterin molecules (Viscontini, Hummel et al. 1970):
1. A dimer of Platynerepterin (a novel pterin, red colure pigment)
2. Nerepterin (a novel pterin, yellow colure pigment)
3. Neopterin
However in my Phd, I have accumulated data that support the synthesis and presence of ommochorme as well, in both eyes of *Platynereis dumerlii.*

Pteridines are synthesized from GTP with participation of a number of enzymes such as GTP-cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase , sepiapterin deaminase and **sepiapterin reductase**. I have identified and characterized the last enzyme on the list, as a marker for *Platynereis* adult eyes pigment cells. (See figure 3 for the pteridine pathway)

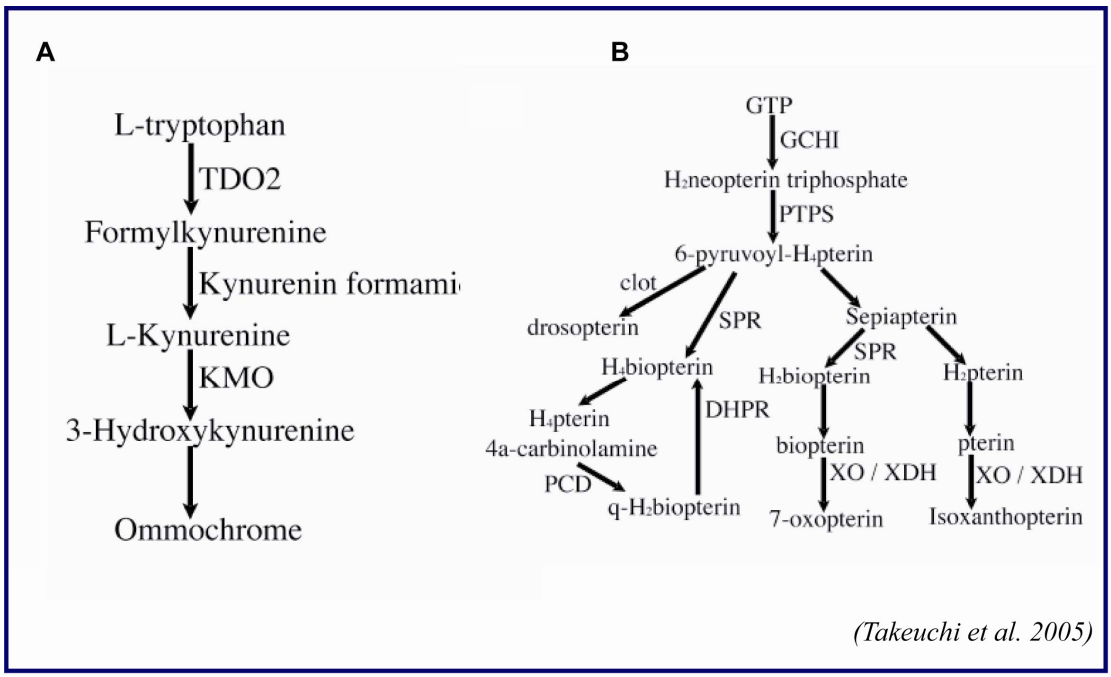


Figure 3: The ommochrome and pteridine synthesis pathways.
(A) The ommochrome synthesis pathway. TDO2, Tryptophan 2,3 dioxygenase; I identified TDO2 in *Platynereis* and confirmed it as a pigment cell marker of both adult and larval eyes. (B) The pteridine synthesis pathway. SPR, Sepiapterin reducatse (also known as Sepiapterin synthase). I identified SPR in *Platynereis* and confirmed it as a pigment cell marker of the adult eyes.

Insects usually use a mixture of both pterin and ommochore as their eye pigment. The pigment in pigment cells is stored in pigment granules. In certain polycaetes, their photoreceptor cells also contain pigment granules in their long processes (Fischer and Brökelmann 1966; Hermans and Eakin 1974), but the pigment granules in both cell types probably have different chemical properties and origins (Rhode 1992). The function of this pigment is very likely to prevent admittance of light through parts of the pigment cup. Another key but often overlooked function of pigment cells is that of cell and tissue support by means of insuring the PRCs' optical alignment and position.

1.2.2 The phototransduction cascade in Ciliary versus Rhabdomeric photoreceptors

On top of the ultrastructure differences between ciliary and rhabdomeric photoreceptor, they also differ in their phototransduction pathway:the molecules and mechanisms they use in order to transmit and process light information. I would like to describe the two mechanisms:

Phototransduction begins with the absorption of light by rhodopsin, triggering the *11-cis* to *all-trans* photoisomerization of the chromophore (retinal or 2-dehydro-retinal in vertebrates, 3-hydroxy-retinal in flies) and formation of the activated metarhodopsin state. In vertebrates, *all-trans* retinal subsequently dissociates and must be re-isomerized through a lengthy and time-consuming enzymatic pathway. Invertebrates' metarhodopsin is usually thermostable, and can be directly re-isomerized back to

rhodopsin by absorption of longer wavelength light. In vertebrate rods, the heterotrimeric G protein transducin activates a phosphodiesterase (PDE) resulting in hydrolysis of guanosine 3,5-cyclic monophosphate (cGMP) and closure of the transduction channels . In *Drosophila*, as in most invertebrates, rhodopsin activates a distinct G-protein isoform, Gq, which activates, instead of PDE, a phospholipase C isoform (PLCβ4, encoded by the *norpA* gene). This leads to opening of two classes of Ca2+-permeable light-sensitive channels: transient receptor potential (TRP) and TRP-like (TRPL) channels.

Surprisingly, vertebrate photoreceptors also express a PLCβ4 isoform, which is more closely related to *Drosophila norpA* than it is to other vertebrate PLC isoforms, perhaps indicative of a common ancestral photoreceptor in the distant evolutionary past.

The substrate re-synthesis also differ: In vertebrates, cGMP is re-synthesized by guanylate cyclase (GC) and GC-activating protein (GCAP), which is inhibited by Ca2+. In *Drosophila*, DAG is converted to phosphatidic acid (PA) by DAG kinase (DGK). PA is converted to PtdIns(4,5)P_2 by a multienzymatic pathway.
Metarhodopsin inactivation: Metarhodopsin is phosphorylated by rhodopsin kinase (RK) and capped by arrestin. RK is inhibited by recoverin in presence of Ca2+ (vertebrates only).
Inactivation of G protein and effector: Effector enzyme and Gα are inactivated by the GTPase activity of the G protein, leading to reassociation with $G_{\beta\gamma}$. Accelerated by the GAP activity of RGS9/Gβ5 and PDE (rods) and PLC (*Drosophila*) (Hardie and Raghu 2001).

1.2.3 The lens

Complex eyes with cellular lenses are found from jellyfish to man. Generally, the role of the transparent lens is to focus an image on the photoreceptor cells or the eye, or simply to gather light (especially in some invertebrates). Lenses of different species share many properties such as loss of organelles and accumulation of water-soluble proteins called crystallin. Crystallin account for about 90% of the water-soluble proteins of eye lenses, they contribute to transparency and affect refraction by forming a uniform concentration gradient, with the highest protein concentration at the center of the lens.

Lens proteins

Since the structure and function of lenses are highly similar among animals it was expected that their crystallin would be correspondingly similar. This turned out to be different then expected. Comparative studies revealed high heterogeneity and diversity among lens crystalline (Wistow and Piatigorsky 1988).

In vertebrates, α-crystallins found in all vertebrates lenses are homologous to the small heat shock proteins of *Drosophila* (Ingolia and Craig 1982) and are able to act as chaperones to protect against physiological stress (Horwitz 1992). It was also found that taxon-specific crystallins are similar or identical to metabolic enzymes. In many cases the taxon-specific enzyme-crystalline have enzymatic activity. Consistence with the later, they are also expressed in small amounts outside of the lens.

Invertebrates' lenses also contain crystallins that are homologous to metabolic enzymes. Two examples are: S-crystallins of cephalopods are related to glutathione S-transferase (Wistow and Piatigorsky 1987) and Ω-crystallin of cephalopods (Tomarev, Zinovieva et al. 1991) and scallops (Piatigorsky, Kozmik et al. 2000) is homologous to aldehyde dehydrogenase class 1 proteins. Unlike the situation in the vertebrates, these crystallins have lost enzymatic activity by exon shuffling and amino acid substitutions. Possibly the inactivity of these invertebrates enzyme-crystallins is a consequence of more ancient recruitment for crystalline role in the lens. Therefore the principle of accumulating proteins with non-lens functions as lens crystallins began early in eye evolution in invertebrates (Piatigorsky 2003).

Crystalline recruitment from ubiquitously expressed genes has occurred both with and without gene duplication; there is therefore no uniform rule that explains the evolutionary strategy of crystallins gene requirement. Since gene duplication is not necessary for the innovation of a crystalline role by a ubiquitously expressed protein such as an enzyme or small heat shock protein (Piatigorsky and Wistow 1991), It seems more likely that crystalline recruitment has taken place by changes in gene regulation of single copy gene and that in some cases, it was followed by gene duplication, resulting in both genes being highly expressed in the lens. However the opposite scenario might also exist.
Experiment using transgenic mouse have shown that lens expression of crystallin genes is controlled at the transcriptional level (Piatigorsky 1992). It was also shown that lens specificity is often species-independent (Takahashi, Hanaoka et al. 1994). Numerous studies have found that vertebrates crystalline genes are regulated by a limited and similar set of developmentally important transcription factors such as Pax6 and AP-1 (Cvekl and Piatigorsky 1996). However the *cis*-regulatory elements are often distributed differently in the promoters and enhancers of different crystalline genes (Ilagan, Cvekl et al. 1999).
Data is currently accumulating, supporting the idea that the convergent recruitment of crystalline genes have operated by similar mechanisms in invertebrates and vertebrates.

Lens structure

The literature suggests that definition of the lens involves some difficulties. This arises because of the mostly unknown physical properties of this structure in many marine invertebrates, for example can it form image or does it only conduct light? Hesse (Hesse 1899) described variabley shaped lens-like structure of unknown function in polychaete eyes. He tried to replace the term '*lens'* with the terms *Glaskörper* (=vitreous body) or *Füllmasse* (=filling material). In *Platynereis* this structure was defined as *Füllmasse* by Fischer and Brökelmann (Fischer and Brökelmann 1966). At 1988, Eakin and Hermans (Eakin and Hermans 1988) joined the discussion and proposed the term '*vitreous body'* as an alternative that was accepted. They also provided a great survey of various lens structures and could make the general distinction between 3 types: 1. lens that is formed by supporting/pigment cells processes. 2. Lens that is formed as a secreted body from supporting/pigment cells. 3. Lens that is formed by granules secreted from specialized lenticular cells. In all nereids studies so far (5 species), as well as in Platynereis, the vitreous bodies are composed of extensions of supporting/pigment cells processes knitted together by septate desmosomes. However no data exists about the physical property of the tubules found in these extensions, nor we know if they contain protein. Therefore we can only speculate about their function. In my PhD I was interested to isolate and identify the proteins used as lens proteins in *Platynereis* eyes.

1.3 Cell type comparison and molecular fingerprint concept

The main interest of my Phd is to gain more insight about how did the eye or eyes of *Urbilateria* , the last common ancestor of *Protostomia* and *Deutrostomia*, look like, and what were their molecular and cellular characteristic and complexity?

In general, how can we elucidate the historical course of evolution? By two main strategies, fossil records and the identification of homologous structures between today's living forms. Traditionally, comparative anatomists have looked at homologies at the organ and tissue level, however they mostly restricted themselves to comparisons within the vertebrates or arthropods and couldn't establish proper neuroantomical homologies between *Protostomia* and *Deutrostomia*, or at the best at the cell type level. For the eye, as mentioned in section *1.1*, this type of comparison (both on the organ and cell type level) ended up in three contradicting hypothesis regarding the origin of the eye, that were not resolved. Clearly, the homology approach reached a point where new qualities of data are required. The new data, or new level of comparison, apply to gene expression information (Arendt 2005).

If one combines the classical morphological comparisons with the study of ortholgy and paralogy of neuronal transcription factors and effector genes (downstream targets) and the comparative analysis of their function and expression, it is possible to establish a new level of molecular comparison of neuronal cell types. This is done by mean of defining a *'molecular fingerprint'* to a given cell type: *"the unique combination of genes active in a given cell type"* (Arendt 2005).

One example for this approach is the work of (Arendt, Tessmar-Raible et al. 2004), where they were able to indeed combine all the above mentioned criteria, in order to define a new cell type, formally undescribed, for *Platynereis dumerilii.* These are the brain ciliary photoreceptor described at the morphological level (electron microscopy), the transcription factor *Rx* expression and the expression of the effector gene *c-opsin.*

In my Phd I have adopted these approaches for the study of eye evolution in the model organism *Platynereis dumerilii.*

1.4 *Platynereis dumerilii* as a model organism to study evolution and development

The polychaete worm *Platynereis dumerilii* has been chosen for the study of evolution and development for several reasons. As a polychaete, it belongs to the phylum Lophotrochozoa, the third branch of the bilaterian phylogenetic tree that comprises many marine animals that are so far underrepresented in molecular studies in comparison to Ecdysozoa (insect and nematode as molecular model organisms) and Deuterostomia (vertebrates, ascidians, lancelets, and sea urchins as molecular model organisms).

1.4.1 Why choosing a Lophotrochozoa as a model organism?

Recent molecular phylogenies based on 18S rRNA and hox cluster gene relationships have revolutionized classification of bilaterian animals (Adoutte, Balavoine et al. 2000) now groping the bilaterians into three main branches: the ***Deuterostomia*** and two large groups of related protostomes, the **Ecdysozoa** and the **Lophotrochozoa** (mostly animals displaying a spiral cleavage of the egg and a trochophore-like larva, including annelids and mollusks).
Conventional model organism for the study of developmental processes and control all (except leeches) belong to either the deuterstomes (vertebrates, sea urchine, *Ciona*) or to the ecdysozoans (*Drosophila* and *Anopheles* as insects, *C.elegans* as nematode). Therefore, the *Lophotrochozoa* as a whole group and in particular its marine branch, were neglected. This group, however, exhibit an astonishing variety of developmental patterns, mechanisms and biological phenomena, for example: asexual reproduction, regeneration (in planarians and annelids) (Bely and Wray 2001; Salo and Baguna 2002) and diurnal, tidal, lunar and annual rhythmicity in marine polychaetes (Bentley and Olive 2001).

But in addition to this, the major reason to study *Lophotrochozoa* in many cases is their ancestral developmental, body plan and genomic characteristic. According to fossil records, the earliest bilaterians found were marine worms of considerable size, with morphology somewhat in between the body plans of today's polychaete annelids, mollusks and brachiopods (Conway-Morris 2003). A long-standing discussion on the basis of comparative morphological arguments points as well that urbilaterians may have resembled annelids (Dohrn 1875; Arendt and Nübler-Jung 1994; Nübler-Jung and Arendt 1994).
All these features are in contrary to conventional protostomes models that have been chosen for their short generation time and that occupy highly specialized ecological niches and are thus evolutionary derived.

1.4.2 *Platynereis dumerilii* (Polychaeta, Annelida) as model organism – in general

A group of animals is considered 'evolutionary ancestral' the more it lacks group specific (derived) characters that distinguish it from its relatives. Among Lophotrochozoans, this is particularly true for polychaete annelids, and best understood by the difficulty to define a polychaete. It appears impossible to delimit features that other Lophotrochozoans, or sometimes even other bilaterian branches, would not share. For example, their spiral cleavage is shared by molluscs and many other Lophotrochozoans (Nielsen 1995; Shankland and Seaver 2000). The polychaete as well as the mollusc trochophora-type primary ciliated larvae bear a striking resemblance to their deuterostome counterparts.

Recently the genomic complexity of *Platynereis dumerilii* was analyzed and compared to both vertebrates and invertebrates species. Remarkably, *Platynereis*, on the gene structure level (intron numbers) is more similar to vertebrates than to any ecdysozoan and further, on the intron and exon level, *Platynereis* and humans can be regarded as similarly slow-evolving representatives of protostomes and deuterostomes, respectively (Raible, Tessmar-Raible et al. 2005). This analysis shows that *Platynereis* has retained ancestral bilaterian genomic features, while other common model organisms have experienced extensive gene loss.

In addition to the above mention advantages, *Platynereis dumerilii* is easily cultured (Hauenschild and Fischer 1969) with a moderately rapid life cycle (well compared with vertebrate standard models like fish and mouse), embryos and adults are transparent, it has a simple cell lineage and it spawns in a way that produces thousands of synchronously developing embryos. All stages of development are accessible to wide array of imaging techniques and functional analysis including expression studies, immunohistochemistry, live cell imaging and laser cell ablation.

1.4.3 For the study of eye development and eye evolution

Platynereis has three important advantages for the study of eye development and eye evolution:
1. The concurrently presence of two different types of eyes in the embryonic stages: adult and larval eyes. One of the questions I wanted to answer in my PhD is what is the significant of the distinct occurrence of both eye types? Are the adult eyes representing multiplications of the larval eyes or do they represent two distinct types of eyes, controlled by distinct molecular mechanisms? And therefore are composed of distinct cell types?

2. The larval eyes correspond to the definition of a prototype eye, composed of a single rhabdomeric photoreceptor and single pigment cell. They are actually the simplest eye that is conserved across bilateria. They are also found in deuterstomes, in *tornaria larva,* where they have the same structure and location (left and right of the apical organ).

3. The adult eyes, in the embryonic stages, are composed of very few rhabdomeric photoreceptors, pigment cells and support cells, providing an excellent simplified model for the study of the MFP of the different cells types.

1.4.4 Eye development of *Platynereis dumerilii*

The information described here is based on the work of Dr. Birgit Rhode from 1992, who used TEM (transmission electron microscopy) to study the eye development of *Platynereis dumerilii* at several stages of development. Another work describing *Platynereis'* eye development is the work of Fischer and Brökelmann from 1966 that used light microscopy. The following paper of Arendt et al. (2002) about Platynereis eyes was the first one to include molecular data (gene expression) as well.

Platynereis dumerilii possesses two types of eyes: adult and larval eye. The adult-post trochophoral eyes are two pairs of eyes with one vitreous body ('lens') each. The existence of different types of eyes during development is a commom feture of marine invertebrates. Changing life habits during ontogenesis require alterations of existing sense organs or replacement by others. Larval eyes have been described for some polychaete species and apart from the one of the spionid larvae, all other trochopores eyes are two celled eyes composed of rhabdomeric photoreceptors (Rhode 1992).

The larval eyes: from 24hpf onwards, the free swimming trochophore exhibits a pair of larval eyes in the upper episphere. These eye spots lie within the epidermis close beneath the cuticle. Each of these eye spots was described by Rohde as composed of one sensory cell and one pigment cell. The distal end of the sensory cell bears the photo sensory apparatus, an array of parallel arranged microvilli which are oriented towards the concavity of the pigment cell and parallel to the epithelial surface. The pigment cell distal region has a crescent shape and is filled with electron-dense pigment granules. (See figure 4 for illustration)

***The adult eyes*:** at 43hpf, the late trochophore or metatrochopore, respectively, begins to develop the adult eyes. In **early 2 days** old larvae, epidermal cells directly beneath the larval cuticle indicate the developing eye anlage in the region of the dorsolateral prototroch. A few pigment granules are accumulated in the apex of one of these cells. Two peripherally located cells, pigment cells, enclose two other cells, sensory cells. Both cell types bear pigment granule but with different appearance. The origin of these two types of pigment granule was not clear. During my PhD I have identified molecular markers that can account for this (see "Results"). At this developmental stage, each eye anlage, which will give rise to two adult eyes (anterior and posterior), comprises four cells. In **late 2 day** old larvae, the eye anlage sink below the epidermis and the number of cells in each eye anlage doubles (four pigment cells and four sensory cells). In the following 24 hours the whole complex rearranges so that in each case, two pairs of pigment cells encloses two pairs of sensory cells. In early 3 day old larvae the anlage of each eye is composed of two pigment cells and two sensory cells. (See figure 4 for illustration). In **late 3 days** old larvae the eye anlagen of the anterior and posterior eyes are separated and each develops further independently. The posterior eye tilts its long axis slightly oblique to the surface of the epithelium. Pigment cells accumulate pigment granules about 0.3 µm in diameter at the distal region to create the pigment cup. In adition each of the pigment cells send long

process that dilates and forms a sac that accumulates electron dense vesicles or tubules. The tubule sacks of two adjoining pigment cells are connected via septate desmosomes thus forming the ocular cavity between them and the pigment cup. This apical assembly of supporting cells forms a sort of a lens/vitreous body. The sensory cells form small necks passing through the pigment cup and contributing to it with their pigment granules.

The grow of *Platynereis dumeriliis'* adult eye continues along it's life span, in the fashion described here. However, the striking enlargement of the eye volume during epitokous metamorphosis happens by extensions of the cells. The sensory cells processes elongate to more then double their original size and the vitreous body increased its volume many times (Fischer and Brökelmann 1966).

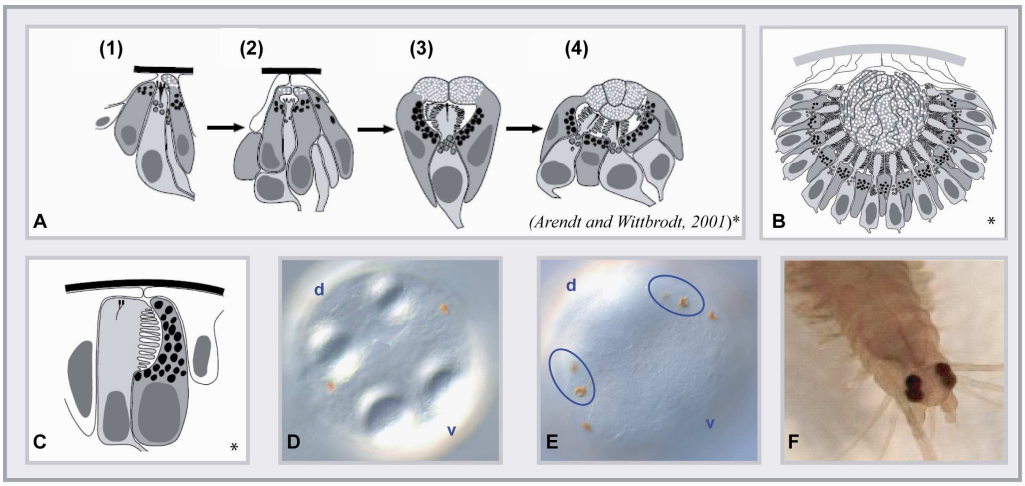


Figure 4: *Platynereis* developing Adult and Larval eyes.
(A) *Platynereis* developing adult eyes in: (1) early-two-day-old larvae; (2) late-two-day-old larvae; (3) late-three-day-old larvae and (4) three-week-old young worm. Light gray,Photorecptor cells (PRCs); dark gray, pigment cells; white circles, lens vesicles; black circles, PRCs pigment vesicles. (B) *Platynereis* adult eye of an adult worm. Note the lens like structure that is formed by long process of the PRCs. Note the pigment cup structure that formed of pigment vesicles from both PRCs and pigment cells. (C) *Platynereis* larval eye. Light gray,Photorecptor cell (PRC); Dark gray, pigment cell; Black circles, pigment cell pigment vesicles. (A-C: *Arendt and Wittbrodt, 2001*)
(D) Apical view of live *Platynereis* 24h larva. D.dorsal; V,ventral. Orange dots are the larval eyes. (E) Apical view of live *Platynereis* 72h larva. D.dorsal; V,ventral. The dots circled by a blue circle are the adult eyes. The ones without a circle are the larval eyes. (F) Adult *Platynereis* worm and its' eyes.

1.4.5 Introducing the investigated specimen – *Platynereis* embryos and life cycle

After fertilization of the oocyte, *Platynereis* shows typical spiral cleavage resulting in the appearance of smaller micromeres at the apical pole that will, by further cleavage, overgrow the larger macromeres in an epibolic fashion (Dorresteijn 1990). Then the embryo further undergoes gastrulation movements and starts differentiating into a spherical trochophore larva. It develops an apical tuft, a prototroch ciliary circle at the equator of the larva, and a posterior telotroch ciliated band (Dorresteijn, O'Grady et al. 1993). The un-segmented larva starts to develop metameric chaetal sacs that give rise to the chaetae (bristles) of the prospective parapodial appendages . At about 52hpf, the lateral bristles start protruding from the

metatrochophore that takes is being elongated . At 72hpf, the larva has transformed into an elongated juvenile young worm with protruding head appendages and three chaetae-bearing (chaetiferous) parapodial segments (Hauenschild and Fischer 1969). New homonomous segments are added constantly by budding from a growth zone located probably just anterior of the telotroch. After 3-4 weeks of development, *Platynereis* undergoes metamorphosis - cephalisation of the most anterior chaetiferous segment by transforming the parapodia into tentacular cirri (Hauenschild and Fischer 1969). New segments are constantly proliferating throughout the worm's life until sexual maturation occurs. At least 3-4 months after fertilization, the adult *Platynereis* worm undergoes sexual maturation that includes transformation of the body (e.g. loss of muscles, formation of additional blood vessels) from the atoke form into the epitoke form (Hauenschild and Fischer 1969; Fischer and Dorresteijn 2004). The sexually-mature epitoke worm releases eggs/sperm into the seawater after induction of males and females by pheromones. (See figure 5 for illustrations)

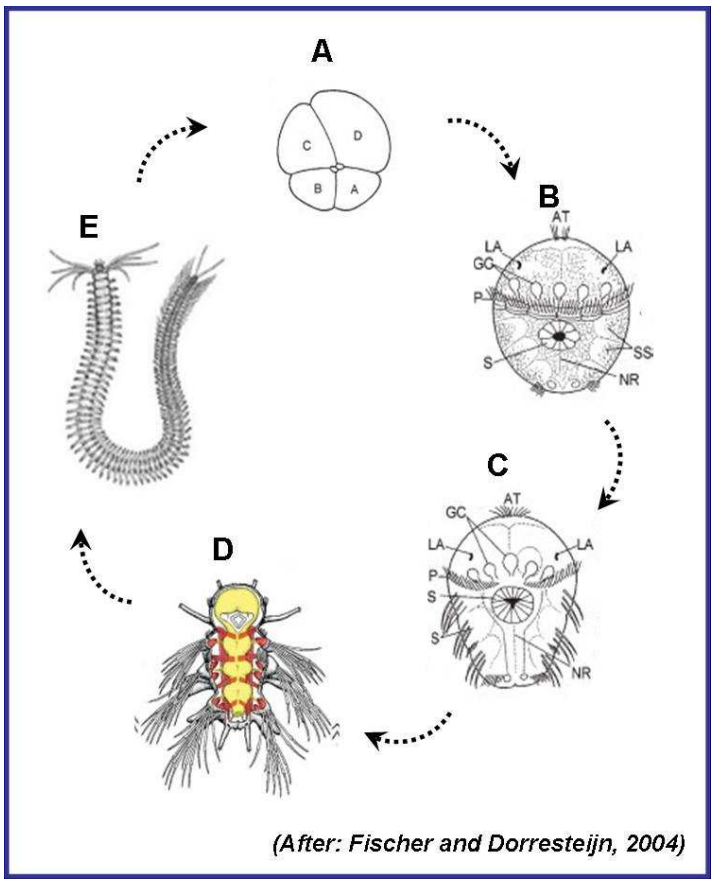


Figure 5. (A-E) Different stages of *Platynereis* development , schematized
(A) Four cell stage embryo showing the four quadrants. (B) Ventral view of 1-day-old trochophore larva. (A, antennae; AC, anal cirri; AT, apical tuft, GC, larval gland cells; LA, larval eyes; NR, neurogenic region; P, prototroch; Pa, palps; S, stomodeum; SS, setal sacs). (C) Ventral view of 2-day metatrochophore larva (same abbreviations as in B). (D) Dorsal view of three segmented 5-day-old juvenile, with the central nervous system marked in yellow and the musculature tissues marked in red. (E) An adult worm. (After: Fischer and Dorresteijn, 2004).

1.5 Molecular regulation of visual systems development

As described in the previous chapter, *Platynereis* eye development was studied in details on the morphological level, however the study of the molecular mechanism that control it was at it's genesis at the beginning of my PhD. Arendt et al. 2002 describe the cloning and expression of four *Platynereis* genes and postulate regarding their role in *Platynereis* eye development. The genes are: the effector gene: r-opsin and three transcription factors: Pax6, Six1/2 and Atonal.

My aim was to expand as much as possible the information regarding gene expression in *Platynereis* eyes, and moreover, to increase the "resolution" of it by means of distinguishing between the different cell types in the adult and larval eyes and their MFP. In the following chapter I will describe keystone conserved eye genes, gene network and one signaling pathway, which were candidate genes in the process of establishing the MFP of *Platynereis* eyes.

1.5.1 Key regulators of eye development

1.5.1.1 Otx

One of the earliest genes expressed in the anterior neural region is the homeobox gene Otx2. Otx homeobox genes play an important role in controlling specification, maintenance and regionalization of the vertebrate brain and together with the *Drosophila* **Otd** gene exhibit a reciprocal high degree of functional equivalence (Montalta-He, Leemans et al. 2002). Drosophila orthodenticle is required for photoreceptor cell development and is expressed at all stages of the developing visual system, including the photosensitive cells of Bolwig's organ, the ocelli, and the adult eye, it is a key player in the terminal differentiation of subtypes of photoreceptors by regulating rhodopsin expression, a function reminiscent of the role of one of its mammalian homologs, Crx, in eye development (Tahayato, Sonneville et al. 2003). Mouse Otx genes are also required for tissue specification in the developing eye (Martinez-Morales, Signore et al. 2001).

1.5.1.2 The Pax family of transcription factors

Pax transcription factors are defined by the presence of a highly conserved 128 amino acid DNA binding domain, the paired domain . The paired domain is a bipartite domain consisting of two independent subdomains: the amino-terminal PAI domain and the carboxy-terminal RED domain. Three amino acids (at positions 42, 44, and 47) within the PAI domain are responsible for the difference in the DNA-binding specificities between Pax2/5/8 and Pax6. Pax6 and its major contribution to eye development will be discussed in the context of the RDGN. However Apart from Pax6, additional Pax proteins are essential for normal eye development in Drosophila and mice. Two Drosophila Pax6-like genes, *eyg* and *toe*, might act in parallel to ey during eye formation. Recent results suggest distinct but coordinated roles for *ey*

and *eyg*. In the current state, *ey* provides eye specification whereas *eyg*, being genetically downstream of Notch signalling , regulates proliferation. Remarkably, Pax6(5a) protein, although playing a minor role in vertebrate eye development , can mimic Eyg in promoting tissue growth , which suggests at least biochemical equivalency of the two proteins.
Pax2 is another member of the Pax gene family that has unique functions during Drosophila and mouse eye development . Some similarities in nested expression patterns of Pax6 and Pax2 in developing eye discs of fly and vertebrate eyes have been noticed ,however, the genetic interaction between the two genes has only been observed in vertebrates. Mutual repression between Pax6 and Pax2 is responsible for the morphogenesis of the mouse optic primordium: Pax2 is crucial for the generation of the optic stalk while Pax6 is required for the development of the optic cup . Both genes seem to have partially redundant functions in the retinal pigment epithelium . In Drosophila, the sparkling (spa) function of Pax2 is expressed in the differentiating cone cells and primary pigment cells of late larval and pupal eye discs, whereas its shaven (sv) function is expressed in the developing eye bristles. In the context of specific roles for paired domain and homeodomain during eye evolution, I would like to preset 'the Paxcentric (PD-HD) model' for pax gene evolution. This model, introduced by Zbynek Kozmik, suggest that the modern Pax2 and Pax6 genes in bilateria evolved from a cnidarian PaxB-like ancestor by duplication and diversification. Since the PaxB gene in the cnidarian Tripedalia is expressed in the lens and retina and is able to activate both lens crystallin in addition to opsin reporter genes. The model suggest that Pax2 lost its homeodomain (HD), and Pax6 lost the octapeptide (yellow box) and changed the DNA-binding specificity of the paired domain (PD) by acquiring amino acids I42, Q44 and N47. The model predicts that the PD has been captured to function in the 'pigmentation' pathway as well as for driving morphogenesis ('eye design') through intercalary evolution, whereas the HD functioned in opsin expression. Meaning that two independent DNA binding domains within a single Pax transcription factor have
been co-opted for two essential features of the protoeye: production of a dark pigment (the 'pigmentation' program; paired domain-driven) and production of a photopigment (the 'opsin' program; HD-driven).
Few supporting evidence are brought here:
The Pax6 gene is expressed in the pigment cells of the prototypic planarian eye. Pax2 is required for the development of pigment cells in the Drosophila eye. In mouse, pax6 genes regulate retinal pigment epithelium (Pax6, Pax2), neural crest-derived melanocytes (Pax3) and in ascidians they regulate sensory pigment cells development (Pax6, Pax3/7) . Another supporting evidence is related to the Mitf transcription factor that has a conserved and fundamental function in the development of melanin producing cells. The loss of function of Mitf results in retinal pigment epithelium becoming an additional unpigmented neuroretina, whereas overexpression of Mitf induces a pigmented phenotype in neuroretina. Various Pax genes activate the Mitf gene promoter .In particular to our interest Pax6 directly interacts through its paired domain with Mitf protein and, is able to modify Mitf function through protein-protein interaction.

Drosophila Pax6 (ey) directly activates expression of rhabdomeric rhodopsin genes through homeodomain binding sites in their promoters, which might reflect an ancestral role of the homeodomain in opsin regulation. In vertebrates, Pax6 is not

expressed in ciliary photoreceptors and therefore no longer used for activation of opsins promoters. Remarkably, Pax6 expression remains in vertebrate retinal ganglion cells, which are considered to be a sister cells ancestral rhabdomeric photoreceptor cells. In the course of bilaterian evolution, additional paired-type homeodomain proteins, such as Crx in vertebrates or Otd in Drosophila, were co-opted for opsin regulation (Kozmik 2005). Our results regarding the molecular fingerprint of *Platynereis* eye cell types are in line with the proposed model in a way that Pax258 is expressed in pigment cells, of both adult and larval eyes, while Pax6 is expressed in all cells of the larval eyes. For further discussion see results and discussion sections.

1.5.1.3 Prox1/Prospero

The homeobox gene prospero was identified in Drosophila as a gene essential for CNS (Doe, Chu-LaGraff et al. 1991; Vaessin, Grell et al. 1991; Matsuzaki, Koizumi et al. 1992) and eye development (Oliver, Sosapineda et al. 1993).
In *Drosophila* prospero is detected in the R7 photoreceptor and the lens-secreting cone cells of the eye (Oliver, Sosapineda et al. 1993). *prospero* expression is required for proper connectivity of R7 photoreceptor axons to their synaptic targets (Kauffmann, Li et al. 1996). Prospero is also necessary and sufficient to repress R8 Rhodopsins In vivo, it therefore affects R7 versus R8 cell fate decisions (Cook, Pichaud et al. 2003).

Prospero/Prox form a separate family of proteins since their HD is highly atypical and is divergent from a classical antennapedia or any other HD. Prospero/Prox also contains a conserved C-terminal domain which was called the prospero domain (Burglin 1994). During vertebrate development, Prox1 is expressed in several tissues, including the retina. In the retina, Prox1 is first observed in a subset of proliferating retinal precursors, and is later present in differentiated horizontal, bipolar, and A_{II} amacrine cells (Belecky-Adams, Tomarev et al. 1997; Tomarev 1997; Dyer, Livesey et al. 2003). Dyer et al. have demonstrated that Prox1 is both necessary and sufficient for the formation of horizontal cells. Additional analysis revealed that cells expressing Prox1 are more likely to stop proliferating and undergo differentiation, suggesting that cells induced to express Prox1 exit the precursor pool to develop as horizontal cells, while those cells not induced continue proliferating until they encounter a rod or glia-inducing signal.
Thus, we hypothesis that Prox1 is a key regulator of both early and late stages of photoreceptor development, not only in insects but also *in* vertebrates.

1.5.1.4 Rx

the Rx/Rax (for Retinal homeobox) family is a paired-like homeobox gene family. each gene contains a highly conserved *paired*-like homeodomain and octapeptide typical of this homeobox gene subfamily . They also contain a conserved motif in the carboxy-terminal end of their proteins. This domain is present in several *paired*-like homeobox genes, and the consensus sequence of this region, which we call the *paired* tail. The structure of Rx genes is very conserved and since their discovery, they have been described in several vertebrate and invertebrate species including chicken, Xenopus, mouse, medaka, Drosophila, zebrafish, and human (Bailey, El-Hodiri et al.

2004). The number of Rx genes varies among different species, and generally ranges from one to three. The homeodomains of Rx proteins are well conserved.
The expression pattern of Rx genes in different species is partially similar. Here are few examples:

In ***Xenopus***, During neurulation, the retina is the primary site of Rx expression, but the pineal gland (epiphysis), and the ventral hypothalamus also express this gene . Initially the entire retinal neuroepithelium expresses Xrx to the same degree, but by the time the optic cup is formed, the Xrx RNA expression domain is restricted to the cells of the retinal ciliary margin. This region contains the multipotent retinal stem cells that continually generate the entire collection of retinal cell types throughout Xenopus life . Later in development, Xrx1 is reactivated in the photoreceptor cells (reviewed in (Bailey, El-Hodiri et al. 2004).

The **murine** Rx (Mrx) is first activated in the anterior neural plate of E7.5 embryos. At E10.5 its expression is confined to the developing retina and ventral forebrain. At E15.5 There is a uniform expression in the entire neuroretina. At later stages there is a progressive reduction of Mrx expression in the retina, and by P6.5 Mrx transcripts are present only in the photoreceptor and inner nuclear layer (Mathers, Grinberg et al. 1997).

Two Rx genes were found in **chicken**, cRax and cRaxL. cRax is expressed in the ectoderm anterior to Hensen's node at stage 4. During neurulation, cRax is expressed similarly to mice in the anterior neural folds in the prospective retina, and in the ventral forebrain (Ohuchi, Tomonari et al. 1999). cRaxL is expressed in the anterior neural ectoderm somewhat later than cRax. During the cellular differentiation of the retina, it is expressed in the initial stages of photoreceptor differentiation. cRax is not expressed in photoreceptor cells (Chen and Cepko 2002).

Drosophila Rx, DRx is expressed in the embryo in the procephalic region and in the clypeolabrum from stage 8 on and later in the brain and the central nervous system. The DRx expression pattern argues for a conserved function at least during brain development, but no expression could be detected in the embryonic eye primordial or in the larval eye imaginal discs (Eggert, Hauck et al. 1998).

In **planarians**, the Rx gene was isolated in *G. tigrina*, and named Gtrx. It does not show any expression in the planarian eye cells (Salo, Pineda et al. 2002).

Some indications regarding Rx function:
In mice, targeted elimination of the Mrx gene showed that Mrx-/- embryos have no visible eye structures, the ventral neuroectoderm is much thinner in mutants compare to w.t siblings, while the dorsal and lateral forebrain structures appear to be normal. The initial activation of Pax6, Otx2, and Six3 in Rx-/- embryos, in the anterior neural plate is not Rx dependent, but the specific up regulation of these genes in the retinal progenitor cells is Rx dependent (reviewed in (Bailey, El-Hodiri et al. 2004).
Of special significance is the lack of Pax6 expression in the retinal progenitor cells as it suggests that Rx is genetically upstream of Pax6. In contrast, there were no significant changes in Rx expression in the Pax6-/- background, demonstrating that both Rx expression and the initiation of eye development in mice is Pax6 independent (Zhang, Mathers et al. 2000).

The Mrx null embryos are unique in their failure to form an optic vesicle since even the *Small eye* mutant embryos develop optic vesicle to some extant (Hogan, Horsburgh et al. 1986) . This demonstrate even further that Mrx function is **essential** for eye development from its initial stages and that this gene has a unique role in eye development.
In Xenopus, embryonic Rx elimination leads to a reduction or a loss of eyes and anterior head .
In humans, RX has a critical role in eye formation as well as mutations in human RX gene cause anophthalmia and sclerocornea (Voronina, Kozhemyakina et al. 2004).

These examples serve as increasing evidence that Rx acts as a cell type specific proliferation factor in cells from which the retina and the ventral hypothalamus are derived. Regarding the later, a recent study that characterized and compared neurons that express the prohormone vasotocin (vasopressin/oxytocin)-neurophysin in the developing forebrain of *Platynereis* and zebrafish shows that *Platynereis* Rx gene is actually a part of the conserved molecular address (miR-7+, nk2.1+, **rx+**, otp+) that specify this conserved cell type (vasotocinergic extraocular photoreceptors). In addition it also shows the specific requirement of zebrafish rx3 for vasotocin-neurophysin expression (Tessmar-Raible, Raible et al. 2007).

However, the requirement for Rx function is not universal in all species, while the development of the vertebrate eye is dependent on Rx function, the development of eyes in lower animals, including the insect, is not.

1.5.2 The retinal determination gene network

The specification of the eye field in diverse organisms requires the expression of homologous members of the retinal determination gene network (RDGN), a group of transcription factors and cofactors. I would like to give an overview of the protein families that make up the RDGN. The proteins belonging to the PAX6, EYA (Eyes absent), SIX and DAC (Dachshund) families make up the key members of the RDGN. (See figure 6 for the genes structure and network illustration)

Eyeless/PAX6: *Drosophila eyeless* (*ey*) derives its name from the ‘eyeless’ phenotype that is caused by eye-specific, loss-of-function alleles of the *ey* gene (Bridges 1935). The isolation of null alleles of *ey* highlighted its broader functions in the development of the fly embryo and brain (Kammermeier and Reichert 2001). The cloning of *ey* revealed its homology to the vertebrate *Pax6* transcription factors, which encode a subgroup of the large family of PAX proteins that each contains two DNA-binding motifs: a PAIRED box and a HOMEOBOX (Quiring, Walldorf et al. 1994). The *Drosophila* genome also contains a second closely linked *Pax6* homolog, *twin-of-eyeless* (*toy*), which probably arose by gene duplication during insect evolution (Czerny, Halder et al. 1999). TOY and EY are independently required for eye development (Quiring, Walldorf et al. 1994; Kronhamn, Frei et al. 2002).
One of the most striking features of PAX6 family members is their ability to direct the formation of ectopic eyes upon overexpression (Halder, Callaerts et al. 1998). Consistent with this idea, TOY and EY act at the top of a transcriptional hierarchy, where they are required for the expression of downstream members of the RDGN

(Halder, Callaerts et al. 1998) . Like *toy* and *ey*, members of the RDGN encode transcription factors, and include the *Pax6*-like gene *eyegone* (*eyg*), which acts in parallel to *ey* (Jang, Chao et al. 2003) , and the downstream components of this network - *eya*, *sine oculis* (*so*) and *dac*. This transcriptional hierarchy is not absolute; ectopic expression of downstream members of the network, such as EYA or DAC, can also induce ectopic eye tissue and the expression of the upstream gene *eyeless* (Bonini, Bui et al. 1997; Shen and Mardon 1997).

EYA

Drosophila eya and its vertebrate homologs *Eya1-Eya4* have important roles in cell survival and differentiation, particularly during tissue specification (Bonini, Leiserson et al. 1993; Xu, Cheng et al. 1997; Xu, Woo et al. 1997; Bonini, Leiserson et al. 1998; Xu, Adams et al. 1999). The four mouse Eya genes have both unique and overlapping expression patterns, suggesting that their functions may not be wholly redundant (Xu, Cheng et al. 1997; Zimmerman, Bui et al. 1997).

EYA family proteins are characterized by a conserved C-terminal domain called the EYA domain (ED), while the N-terminus does not show conservation aside from the tyrosine rich EYA domain 2 (ED2), which is embedded within a proline/serine/threonine-rich region (Xu, Woo et al. 1997; Zimmerman, Bui et al. 1997). These N-terminal domains are crucial for the transcriptional co-activator function of EYA (Ohto, Kamada et al. 1999; Silver, Davies et al. 2003).

EYA has been best characterized as a transcriptional coactivator that is recruited to the DNA of target genes via its interaction with SIX family members (Ohto, Kamada et al. 1999; Silver, Davies et al. 2003). In addition, a second function has been described for EYA through the identification of the ED as a catalytic motif belonging to the haloacid dehalogenase enzyme family (Li, Oghi et al. 2003; Rayapureddi, Kattamuri et al. 2003; Tootle, Silver et al. 2003). recent studies have indicated that its second property is utilized in vivo during eye development in *Drosophila* (Rayapureddi, Kattamuri et al. 2003; Tootle, Silver et al. 2003).

SO/SIX

The SIX family contains three subgroups, SO/SIX1/SIX2, SIX4/SIX4/SIX5 and OPTIX/SIX3/SIX6, each with one member in *Drosophila* (underlined) and two members in vertebrates. All family members are characterized by two conserved domains, the SIX domain (mediates protein-protein interactions) and a homeobox DNA-binding domain (Seo, Curtiss et al. 1999; Kawakami, Sato et al. 2000). SIX family transcription factors are necessary for the development of many tissues and play an important role in regulating cell proliferation (Cheyette, Green et al. 1994; Dozier, Kagoshima et al. 2001; Carl, Loosli et al. 2002; Li, Perissi et al. 2002; Ozaki, Nakamura et al. 2004). The most divergent branch of the SIX family includes *Drosophila* OPTIX (Seimiya and Gehring 2000) and the vertebrate homologues SIX3 and SIX6, which, unlike the other two members of the subfamilies, do not interact with EYA proteins (Kawakami, Sato et al. 2000). Data from vertebrates suggests that SIX3/SIX6 act as transcriptional repressors that are crucial for proper eye and brain development, through their interactions with the GROUCHO (GRO) family of co-repressors (Kobayashi, Nishikawa et al. 2001; Zhu, Dyer et al. 2002; Lopez-Rios, Tessmar et al. 2003).

DAC

dachshund (*dac*) in *Drosophila*, and its vertebrate homologs, *Dach1* and *Dach2*, encode novel nuclear proteins containing two conserved domains, the DachBox-N

and the DachBox- C (Kozmik and Cvekl 1999; Davis, Shen et al. 2001). No specific DNA binding sites for DAC have been identified but it has been shown to bind naked DNA (Ikeda, Watanabe et al. 2002). The DachBox-C is thought to be a protein-protein interaction motif that interacts with the ED of EYA family members (Chen, Amoui et al. 1997). DAC synergizes with EYA to increase both the size and frequency of ectopic eyes when the two are expressed together (Chen, Amoui et al. 1997), supporting the model that these two proteins act in a complex to direct fly eye development.

While Drosophila dachshund mutants have abnormalities in eye, brain and limbs, **Mouse** Dach1 or Dach2 knockout mutants do not exhibit gross anatomical malformations in these tissues. In addition, Dach1/2 double homozygotes have intact eyes and limbs. Recent work (Davis, Harding et al. 2008) show that in Dach1/Dach2 double mutants, female reproductive tract (FRT) development is severely disrupted. **chicken** Dach1 is expressed in a variety of sites during embryonic development, including the eye and ear. In the developing eye of both chick and mouse, expression domains of Dach1 overlap with those of Pax6. Similarly, in the developing ear of both mouse and chick, Dach1 expression overlaps with the expression of another Pax gene, Pax2.

The second vertebrate homolog of the Drosophila dachshund gene, Dachshund2 (Dach2) is expressed in the developing somite prior to any myogenic genes with an expression profile similar to Pax3, a gene previously shown to induce muscle differentiation. Dach2 and Pax3 positively regulate each other's expression in vivo in the frame of the Pax3/Six1/Eya2/Dach 2 network and play a crucial role in regulating early myogenesis during somite development (Heanue, Reshef et al. 1999) (Kardon, Heanue et al. 2002). Of the RDGN members, DAC remains the least well mechanistically understood.

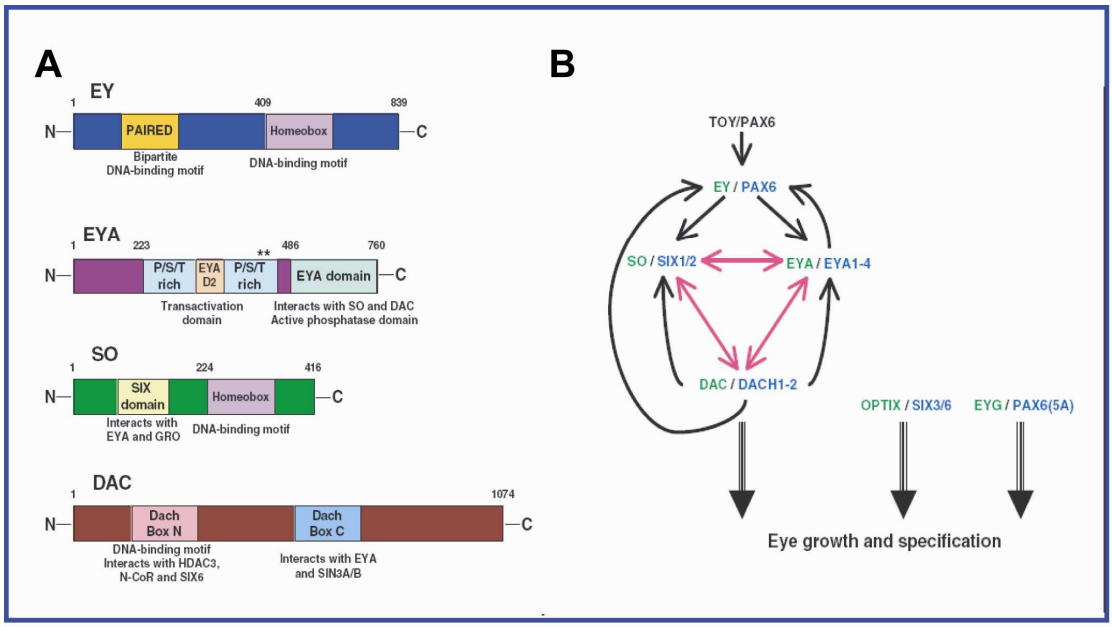


Figure 6 .(A) Domain structures of the retinal determination gene network (RDGN) members. Representative members of the PAX6 (EY), EYA, SIX (SO) and DAC families from Drosophila show the domain structure of RDGN members and their functions. Numbers represent amino acid number; **, conserved MAPK phosphorylation sites in EYA. C, C terminus; DAC, Dachshund; EY, Eyeless; EYA, Eyes absent; EYA D2, EYA domain 2; GRO, Groucho; HDAC3, Histone deacetylase 3; N, N terminus; N-CoR, Nuclear co-repressor; P/S/T rich, proline, serine and threonine rich region; SO, Sine oculis.

(B) Retinal determination gene network (RDGN).
The RDGN is expressed in a transcriptional hierarchy (black arrows), in which Twin of eyeless (TOY) leads to Eyeless (EY) expression, which leads to the expression of Sine oculis (SO), Eyes absent (EYA) and Dachshund (DAC). However, the hierarchy is not only linear, as the lower tier members EYA, SO and DAC contribute to positive feedback loops that ensure the continued expression of EY, and also physically interact with each other (pink arrows). Other RDGN members Eyegone (EYG) and OPTIX are required independently for proper eye development . Drosophila proteins are shown in green and their vertebrate homologs in blue. (PAX6(5A), 5A splice isoform of PAX6). (Silver and Rebay, 2005)

The potential conservation of the ey paradigm in vertebrate eye development has received tremendous attention. While homologues of all of the genes from the fly RDGN are expressed during development of the vertebrate eye, the function of each of these genes has not been strictly preserved. Two notable examples of non-conservation are:
1. The failure of mutations in Eya1 and Eya2 to produce an embryonic eye phenotype.
2. as mentioned above: Dach1 or Dach2 knockout mutants do not exhibit gross anatomical malformations in the eyes, brain or limbs.
It is intriguing to note, however, that the vertebrate genes are capable of many of the interactions present in the Drosophila eye, as evident from the vertebrate genes either rescuing Drosophila mutants or inducing ectopic eyes in the fly. This suggests that the orthologous vertebrate genes have maintained their molecular function but that the components have, to some extent, become uncoupled. In addition it is important to note that some aspects of the RDGN are well conserved. In particular, Pax6 is highly reminiscent of ey, while Six3 and Six6 have some characteristics of so. Thus, despite the lack of strict conservation of the RDGN, it is significant that several critical eye regulator genes have been preserved between the morphologically divergent fly and vertebrate eye (Donner and Maas 2004).

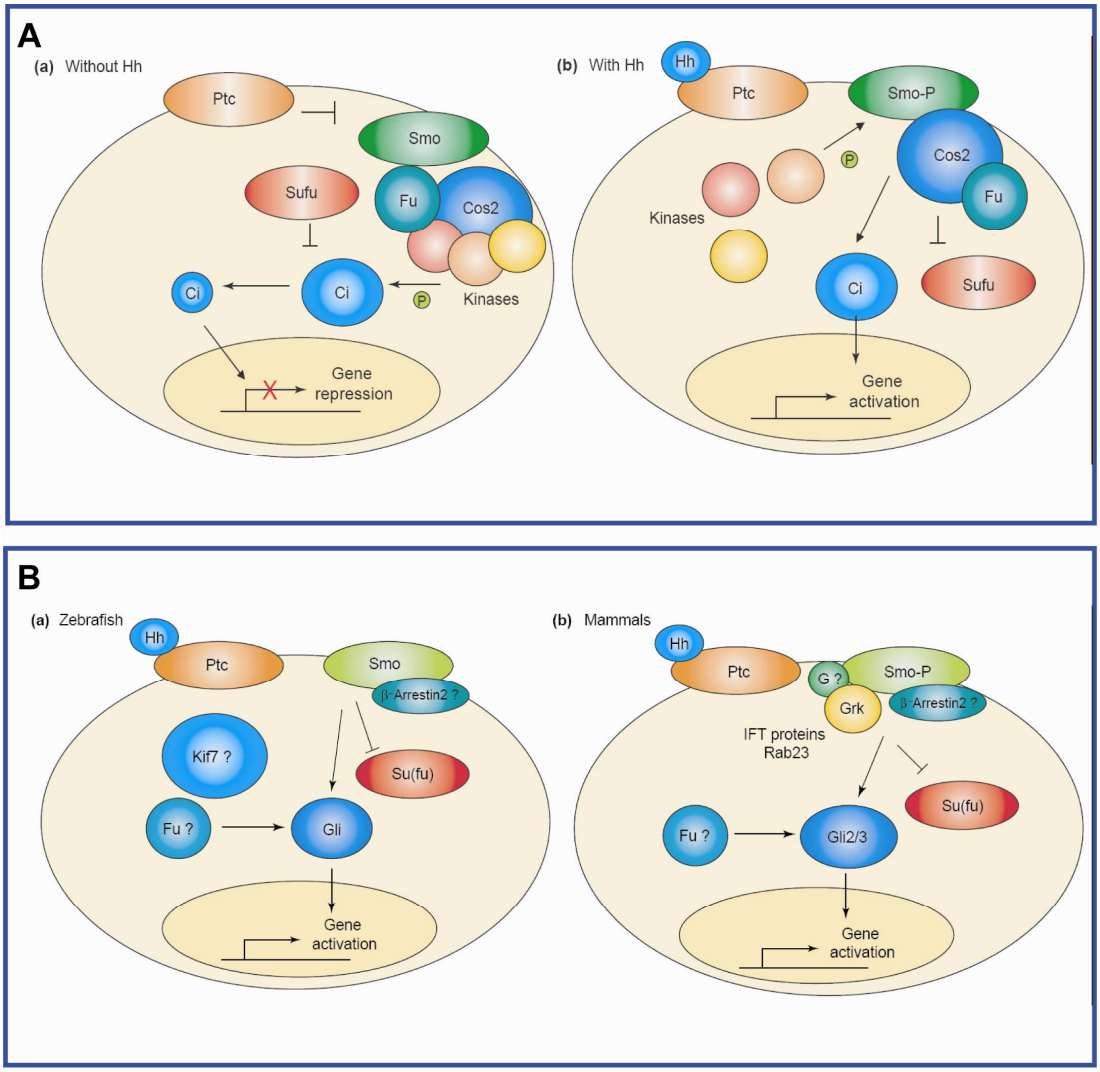
The RDGN nuclear factors do not act alone, but are employed coordinately by, and with, components of conserved signaling pathways to achieve the specificity of transcriptional response that is necessary for appropriate development. I would like to further focus on one of these signaling pathways, the Hedgehog (HH) signaling pathway, it's role and conservation in eye development and the link between it and the RDGN components.

1.5.3 The Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway is a well studied, highly conserved pathway that plays an important role in eye development of both vertebrates and invertebrates. I was interested to find out weather it also play a role in *Platynereis* adult and/or larval eye development.
In the following section I will briefly describe the pathway, its' components and thereafter its' role in eye development.

1.5.3.1 Pathway overview

Embryonic and post embryonic development processes are regulated by few evolutionary conserved signaling pathways (e.g. BMP, Wnt and Notch among others). These pathways are used repeatedly in different contexts to control many cell fate decisions in all animals. Although cellular context controls the final output of a signaling pathway, most experiments suggest that the core components of each pathway - ligand, receptor, cytoplasmic signal transduction machinery and transcription factor – are conserved in evolution. The Hh pathway, first elucidated in *Drosophila*, has been considered to be such a conserved cassette. [However, recent genetic studies have defined a surprisingly large number of proteins required for Hh signaling in vertebrates that have no apparent role in *Drosophila* Hh signaling]
The Hh family of secreted proteins regulates many developmental processes in both vertebrates and invertebrates. The *hh* gene was first identified in *Drosophila* because of its role in embryonic segment polarity and was later shown to act in other aspects of *Drosophila* development. Soon after , vertebrate homologs of Hh were identified in chick and mouse, and were implicated in patterning of the limb and the neural tube among other orans that were implicated later.
Drosophila has a single Hh ligand, which binds to its receptor, the multiple transmembrane protein Patched (Ptc). Ptc activity turns off the downstream signaling pathway in the absence of ligand, and binding of Hh relieves that repression. Smoothened (Smo), another transmembrane protein, acts downstream of Ptc and is an essential positive mediator of the Hh signal. Active Smo regulates the bifunctional transcription factor Cubitus interruptus (Ci). Full-length Ci protein can be modified in response to Hh and become a transcriptional activator. In the absence of Hh ligand, Ci is proteolytically processed into a shorter form (CiR) that acts as a transcriptional repressor of target genes. Both the proteolytic processing and the nuclear translocation of Ci are tightly regulated processes that involve a protein complex containing the atypical kinesin protein Costal 2 (Cos2; Cos – FlyBase), the serine threonine kinase Fused (Fu) and the novel protein Suppressor of fused [Sufu;Su(fu) – FlyBase]. (See figure 7 for illustration of the Hh pathway)



(Østerlund and Kogerman, 2006)

Figure 7. (A) Drosophila Hedgehog signalling pathway.
(a) Without Hedgehog (Hh), Ptc inhibits Smo and HSC is bound to microtubules or membranes and is associated with Smo. Ci is bound to this complex and can undergo phosphorylation (P) by the kinases (PKA, CK1 and GSK3) converting it to a transcriptional repressor. Sufu inhibits the full-length gene activator form of Ci.
(b) Stimulation by Hedgehog leads to the activation of Smo, release of the kinases from Cos2 and phosphorylation of the Smo C-terminal tail. Ci is no longer processed and can enter the nucleus as a full-length gene activator. Sufu is prevented from inhibiting Ci.

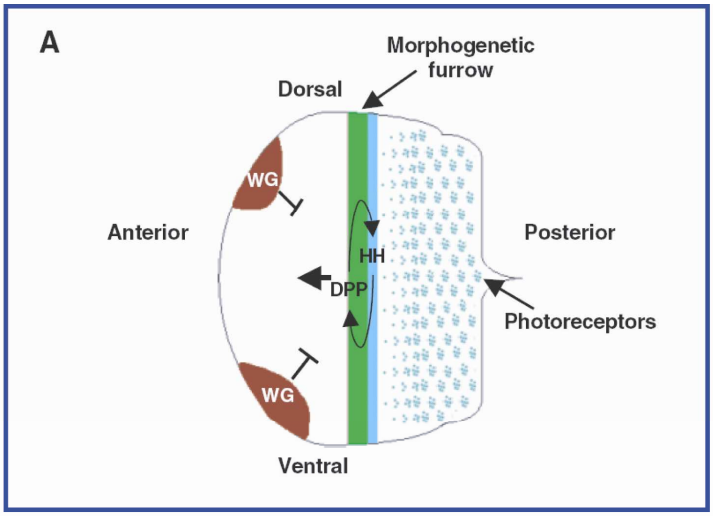
(B) Zebrafish (a) and mammalian (b) Hedgehog signalling.
Stimulation by Hedgehog (Hh) leads to activation of Smo and recruitment of b-Arrestin 2 in mammals, which is probably mediated by the phosphorylation of the Smo C terminal by Grk2 (Grk). Smo activation leads to the inhibition of Sufu and the activation of Gli2- and Gli3-mediated gene activation. (Østerlund1 and Kogerman, 2006)

Hh can act as an on/off switch that regulates the fate of immediately adjacent cells, it can also act as a short-range morphogen (over 10-15 cell diameters, ~20 µm) that controls three alternative fates as a function of its concentration, and as a third option, Hh can act as a long-range morphogen that controls several cell fates, as in the vertebrate neural tube, a field that spans over ~200 µm. (Huangfu and Anderson 2006).

1.5.3.2 Hh pathway and eye development

The Hh pathway has a conserved role in eye development. However, because of the different morphology of the eyes of Drosophila and vertebrates, I will divide the description in two parts dealing with them separately.

In *Drosophila*
The adult *Drosophila* eye contains ~ 800 ommatidia organized in a precise hexagonal array. each ommatidium comprises eight photoreceptors and 12 accessory cells, including four cone cells, six pigment cells and one mechanosensory bristle. The adult eye develops from an epithelial monolayer called the eye imaginal disc. Photoreceptor differentiation is initiated in early third instar larvae at the posterior margin of the eye disc and proceeds anteriorly following a synchronous wave of cellular changes termed the morphogenetic furrow (MF). Alterations in cell shape, cell cycle and patterns of gene expression occur within the MF, and these changes ultimately generate differentiated photoreceptors that are left in its wake. Therefore, a crucial event during *Drosophila* eye development is the initiation of the MF (reviewed in (Donner and Maas 2004).
hh signaling is required for the initiation of the morphogenetic furrow. Loss of *dpp* signaling in the eye imaginal disc also blocks initiation of photoreceptor differentiation. The major role of Hh signaling during *Drosophila* eye development is to alleviate the repression of *dpp* and *eya* by Ci^{rep}. *Eya* is the critical tissue-specific target of Hh signaling during the initiation of normal photoreceptor differentiation in *Drosophila* (Pappu, Chen et al. 2003).
Hh contribute to the propogation of the MF in the following manner: Hh protein is secreted by the newly differentiated ommatidia, stimulates the differentiation of the neighboring, but nonadjacent, immature ommatidia through the activation of *atonal* expression, a basic helix-loop-helix transcription factor . *atonal* promotes the differentiation of the first cell type, the R8 photoreceptor that recruits the other cell types, completing the ommatidial differentiation. These newly born ommatidia will in turn secrete Hh, thereby propagating the wave of differentiation (Tio, Ma et al. 1996). (See figure 8 for illustration)



(*Silver and Rebay, 2005*)

Figure 8. Expression of signaling molecules during Drosophila eye development.
A schematic of the eye disc that shows morphogenetic furrow progression. The eye disc undergoes waves of differentiation as the morphogenetic furrow, which is driven by the cooperative actions of the Hedgehog (HH, blue) and Decapentaplegic (DPP, green) signaling pathways, moves from the posterior to the anterior of the eye disc. The most posterior cells have differentiated into the photoreceptor cells, while anterior cells are still proliferating. Wingless (WG) expression in the dorsal- and ventral-most anterior regions of the disc prevents eye tissue formation in that region, leading to head cuticle formation. (Silver and Rebay, 2005)

In vertebrates

Extraretinal derived Hh signal

In vertebrates, the eye field derives from a single morphogenetic field that later splits into two lateral optic primordial under the influence of the precordal plate. This early event establishes the proximo-distal (P-D) axis of the optic vesicle: the distal-most region will invaginate, forming the optic cup and then the retina, while the proximal region will give rise to the optic stalk. Shh, secreted from the ventral midline, plays a primary role in this process. It is involved in both P-D and D-V axis establishment in the protruding optic vesicles. Shh specifies proximal territories (i . e. optic stalk) by promoting the expression of *Pax2*. *Pax2* transcriptionally represses *Pax6* expression. *Pax6* specifies distal territories (i. e. neural and pigmented retina). *Pax6* and *Pax2* forming a boundary between the retina and the optic stalk. In addition, Shh, together with other factors such as FGFs and retinoic acid (RA), contributes to the activation of *Vax2*, which specifies the ventral part of the retina. BMP4, expressed in the dorsal part of the retina, inhibits the ventralizing effect of Hh signaling through the activation of Tbx5 expression.

Endogenous sources of Hh signaling

Shh is secreted from postmitotic ganglion cells, while Indian or Desert Hedgehog orthologs proteins, are secreted from RPE cells. Downstream components of the cascade (i.e. *Patched*, *Smo* and *Gli* genes) are mainly expressed by undifferentiated precursors or proliferating retinoblasts.

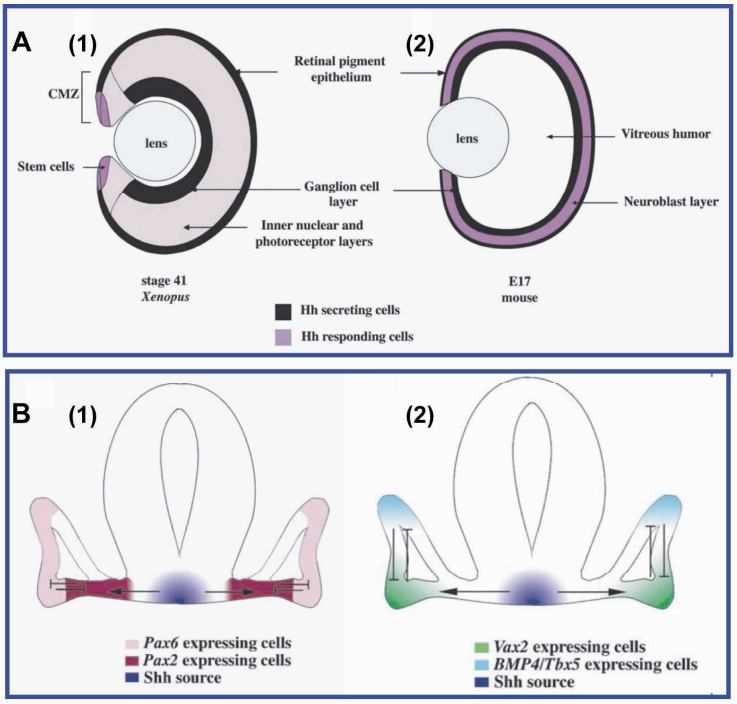
Retinal ganglion cells (RGCs) are the first neurons to be born in the retina, followed by interneurons and photoreceptors and Müller glial cells. RGC differentiation begins roughly in the central part of the retina and proceeds towards the periphery along a

central to peripheral pattern . This wave of differentiation is reminiscent of the neurogenic wave that occurs during *Drosophila* eye differentiation. It has been shown that Hh signaling controls this process in a similar way in both *Drosophila* and zebrafish. The first patch of postmitotic neurons is found ventronasally, close to the optic stalk. Two waves of gene expression spread from these newly formed RGCs: a wave of *ath5*and a wave of *Shh*. *ath5* is an *atonal* homologue, its protein is transiently sweeps across the differentiating retinoblasts and then is maintained in the periphery of the retina, where cells keep differentiating. *Shh* expression is initiated first in differentiated RGCs and then extends as their differentiation proceeds. Both *Shh* and *ath5* waves are necessary to the propagation of retinogenesis. After retinal differentiation has occurred, Hh has an opposite effect on RGCs: it inhibits the ultimate differentiation of retinal precursors, and by this controls the number of RGCs.

RPE cells

Hh expression has been reported in the RPE in almost all vertebrates studied: rat, mouse, chick, *Xenopus*, newt and zebrafish .In mouse, Ihh expression has also been reported in the mesenchymal cells surrounding the eye. This area of expression is situated on the opposite side of the retina from the ganglion cell layer. It is possible that cells situated in between these two sources of Hh, i. e. in the photoreceptor layer and in the inner nuclear layer of the retina, can sense and be influenced by these two Hh sources. In zebrafish it has been shown that Hh signaling also plays a role in photoreceptor genesis. Experiments in rat also suggesting a possible role for Hh in mammalian photoreceptor differentiation.

Hh signaling in the RPE is also essential for differentiation of the RPE itself. (Amato, Boy et al. 2004) (See figure 9 for illustration)



(Amato et al. 2004)

Figure 9. (A) Examples of Hedgehog gene expression in vertebrate retina.
Hedgehog proteins are expressed in vertebrate retinas by postmitotic RGCs and RPE cells. Downstream components of the cascade (i.e. Patched, Smo and Gli genes) are mainly expressed by undifferentiated precursors or proliferating retinoblasts. (1) Example of a stage 41 Xenopus retina. Shh is expressed in the RGC layer, while the two other members of the family, Bhh and Chh, are expressed in the central part of the RPE. Stem cells, situated at the edge of the CMZ, strongly express Smo, Gli2 and Gli3 genes, being potential responding cells to RGC and RPE derived Hh signals.
(2) Example of E17 mouse retina. The source of Shh is represented by the RGCs. RPE and surrounding mesenchymal cells (not shown) express Ihh. At this stage the signal is received by cells expressing Patched and Gli1 in the still proliferating neuroblast layer. CMZ, ciliary marginal zone; RPE, retinal pigment epithelium; RGCs, retinal ganglion cells.
(B) Functions of Hedgehog signaling in eye vesicle patterning.
Ventral derived Shh is involved in both P-D and D-V axis establishment in the protruding optic vesicles. (1) Shh specifies proximal territories (i . e. optic stalk) by promoting the expression of Pax2 which transcriptionally represses Pax6 expression. (2) Shh, together with other factors such as FGFs and retinoic acid (RA), contributes to the activation of Vax2, which specifies the ventral part of the retina. BMP4, expressed in the dorsal part of the retina, inhibits the ventralizing effect of Hh signaling through the activation of Tbx5 expression.
(Amato et al. 2004)

1.5.3.3 The use of Cyclopamine for inhibiting the Hh pathway

Cyclopamine is a plant steroidal alkaloid which induces cyclopia in vertebrate embryos *(Keeler, R.F. & Binns, 1968).* It has been shown to act by inhibiting the cellular response to the Shh signaling pathway (Cooper, Porter et al. 1998) (Incardona, Gaffield et al. 1998). This inhibitory effect is mediated by direct binding of cyclopamine to the heptahelical bundle of Smoothened (Smo) (Chen, Taipale et al. 2002).
Cyclopamine was also use to block the Hh in invertebrates (Kang, Huang et al. 2003).

In order to functionally interfere with *Platynereis* Hh pathway, we used the commercially available cyclopamine drug and incubated Platynereis embryos with the drug in two different time intervals:
1. Targeting the adult eye development
2. Targeting the larval eye development
The treatment was followed by fixation and wholemount *in-situ* hybridization for certain adult/larval eye cell type markers and potential adult/larval eye regulators. We found out the adult but not the larval eyes were severely affected by the treatment (for further details see 'Results'). We therefore propose a role for the Hh pathway in *Platynereies* adult eye development. On top of this, this result adds another molecular distinction between the two eyes of *Platynereis* (for further details see 'Discussion').

Results

2.1 Identification of *Platynereis* eye specific genes

2.1.1 EST collection screen for molecular markers of *Platynereis* eyes.

Platynereis larval and adult eyes have been described by light (Fischer and Brökelmann 1966) and EM microscopy (Fischer and Brökelmann 1966; Rhode 1992). However not much was known about their molecular properties. The only markers that were previously known for *Platynereis* eyes are: r-opsin (Arendt, Tessmar et al. 2002) and $G_q\alpha$ (Gaspar Jekely and Detlev arendt, unpublished) , both expressed in the adult but not the larval eyes (r-opsin is detectable in the larval eyes only at 48hpf, a day after they have formed). Therefore it was clear that as a starting point for my research, I will look for new specific markers for both eyes and for markers that will make it possible to distinguish between the different cell types of the eyes.

The *Platynereis* EST collection contains 21,762 EST clones. I have searched the literature and made a list of candidate "eye genes" both from Drosophila and vertebrates. The list was composed of: signal transduction molecules, pigment synthesis enzymes and neuronal components of neurotransmission.
Using FlyBase and NCBI databases the translated sequences of the genes were extracted. These were then BLASTed against the *Platynereis* EST collection (with the kind help of Dr.Gaspar Jekely, Dr. Florian Raible and Raju Tomer) to identify potential orthologous. Each promising candidate was BLASTed back against the non redundant nucleotide collection at NCBI to see if it was indeed the orthologous gene.

The candidate genes that proceeded to an in-situ screen were the following:

Source organism	**Name of the orthologous gene**	**PEPD ID**	**Clone ID**
Dm	guanine nucleotide-binding protein gamma subunit	48-26-22-F	CL360Contig1
Dm	**Eye-specific diacylglycerol kinase**	48-27-10-N	IB0AAA27DG05FM1
Dm	Centaurin gamma 1A protein		IB0AAA42BA08EM2.SCF
Dm	CG17645-PA		IB0AAA38CC09EM1
Human	tyrosinase-related protein 1	48-15-15-M	IB0AAA15BG08EM1
Human	activin receptor	48-19-23-F	IB0AAA19CC12EM1
Human	NADPH:quinone reductase	48-19-03-D	CL327Contig1
Human	11-cis retinol dehydrogenase		CL1125Contig1
Human	Cellular retinaldehyde-binding protein		IB0AAA20AE04EM1
Mouse	RAB27A protein	48-24-17-P	CL957Contig1
Human	glutathione S-transferase M5	48-30-04-B	IB0AAA30DA02EM1
Human	guanylate cyclase activator	48-16-16-I	IB0AAA16BE08EM1

	1A		
Human	vimentin	48-23-02-B	CL65Contig3
Dm	xanthine dehydrogenase		IB0AAA29AH04EM1
Dm	white - ABC family transporter		IB0AAA35CA10EM1
Rat	c-fos	48-29-24-C	IB0AAA29BB12EM1
Rat	jun-B		IB0AAA34AH12EM1
Gallus gallus	Calretinin	48-16-06-D	IB0AAA16DB03EM1
Danio rerio	kit receptor tyrosine kinase		IB0AAA24CG01EM1
Danio rerio	homeodomain protein lim1	48-21-05-H	IB0AAA21CD03EM1
Danio rerio	homeodomain protein lim1	48-30-15-P	IBOAAA30CH08EM1
Danio rerio	carbonic anhydrase	48-26-2-B	CL580Contig1
Danio rerio	carbonic anhydrase	48-28-1-N	IB0AAA28CG01EM1
Xenopus laevis	calbindin D28k	48-21-C	CL153Contig1
Xenopus laevis	calbindin D28k	48-31-04-D	IBOAAA28CG01EM1
Gallus gallus	photoreceptor-specific nuclear receptor [Gallus gallus]		IB0AAA17BA07EM1
	gl-PA		IB0AAD9YM23CM1
	CG12559-PC		IB0AAA28BA09EM1
	Phosphlipase C		IB0AAA27AE01EM1
	Synaptotagmin		IB0AAA36AD09FM1
Human	ETS-2 Protein		IB0AAA20BB09FM1
	Neuroregulin		IB0AAA15DB12EM1
	Kr-PA		IB0AAA42CB05EM2.SCF
Dm	Myo6		CL735Contig1
Dm	Ser/Thr Kinase	48-41-12-G	IB0AAA41BD06EM1
Dm	Lim-7		IB0AAA22CC11EM1
Ovis aries	activin receptor type II		IB0AAA19CC12EM1
	BDNF/NT-3 growth factors receptor precursor		CL1423Contig1
Danio rerio	zinc finger transcription factor Gli3		CL982Contig1
Mouse	GTP cyclohydrolase 1/Punch		IB0AAA17DA05EM1
Dm	**sepiapterin synthase A**		Pdu_48_8_H11
	Tryptophane 2,3 dioxygenase		Pdu_48_11_03_B *
	Intermediate filament gliarin		Pdu_48-11-04-D *
	FVRI		IBOAAA17B08 **
	Acetylcholine receptor 7/8 (9/10)		IBOAAA19CA10 ***

* Separate random screen done by Heidi Snyman. ** Separate screen done by Dr. Kristin Tassmar Raible. *** Separate screen done by Dr.Gaspar Jekely

The most specifically expressed genes are highlighted in light blue.
The genes that are further highlighted with bold letters were potential eye markers - described in the literature as signal transduction molecules, pigment synthesis enzymes or neuronal components of neurotransmission, genes described in “flybase” database as being expressed in the eyes or Bolwig's organ (the larval eye) of drosophila. see the following section for further details and figure 1-3 for some examples of their expression patterns.

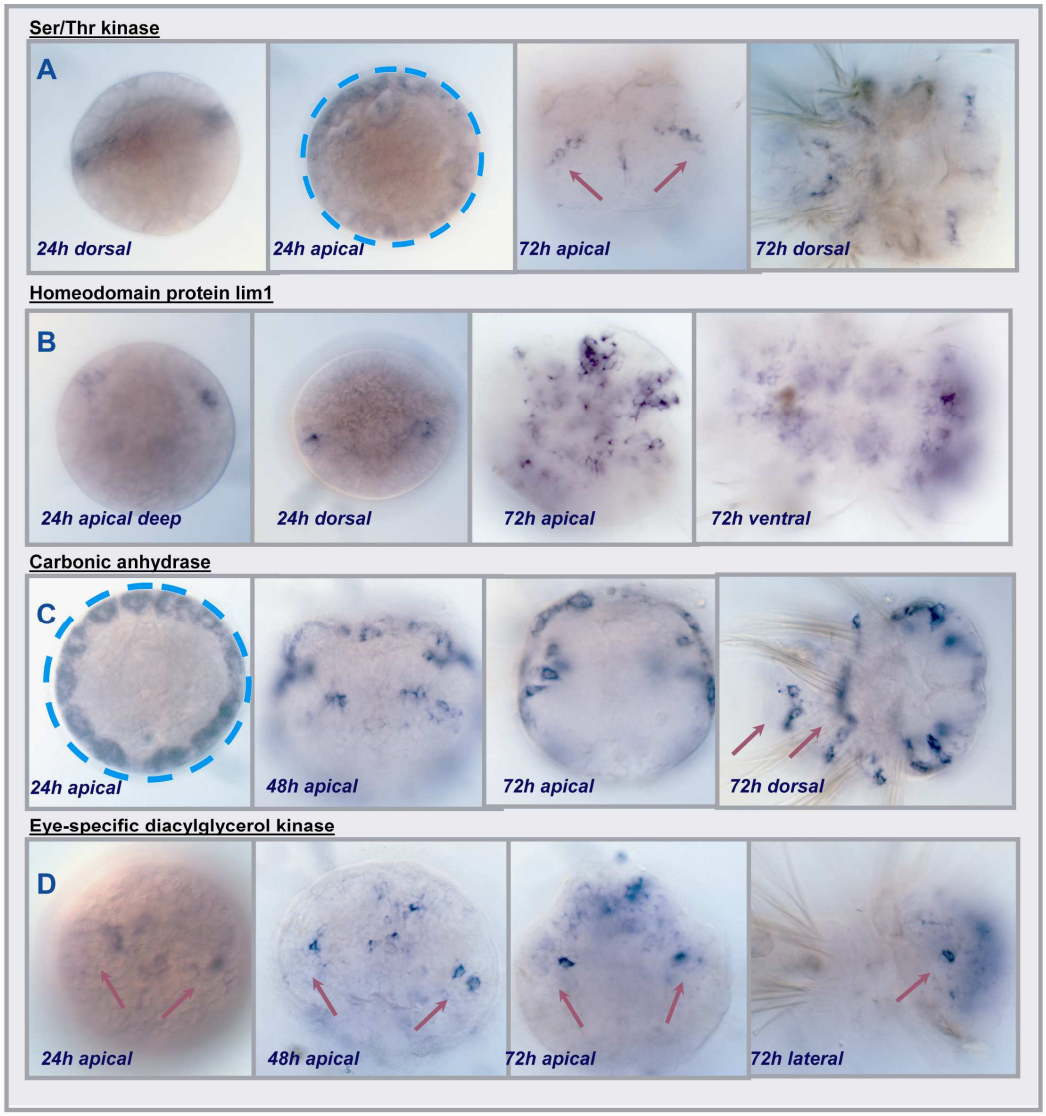


Figure 1. *In-situ* **hybridization screen for eye candidate genes from** *Platynereies* **ESTs collections.**
(A) Ser/Thr kinase, clone ID: IBOAAA41BD06 note expression at 24h in the ciliated cells of the prototroch ring (see shaded circle). At 72h in two symmetrical group of cells at the dorsal lateral brain (see arrows).
(B) Homeodomain protein lim1, clone ID: IBOAAA21CD03 note expression at 24h at two group of cells below the prototroch and at 72h in the dorsal, central and lateral brain as well as in the ventral plate.
(C) Carbonic anhydrase, clone ID: IBOAAA28CG01 note expression at 24h in the ciliated cells of the prototroch (see shaded circle) as well as in ciliated bands on the dorsal side of 72h embryo (see arrows).
(D) Eye-Specific diacylglycerol kinase, clone ID: IBOAAA27DG05 note expression at 24h, 48h and 72h in the larval eye region (see arrows). In addition, at 48h and 72h, in the dorsal medial brain. This marker was later confirmed as being expressed in the larval eyes.

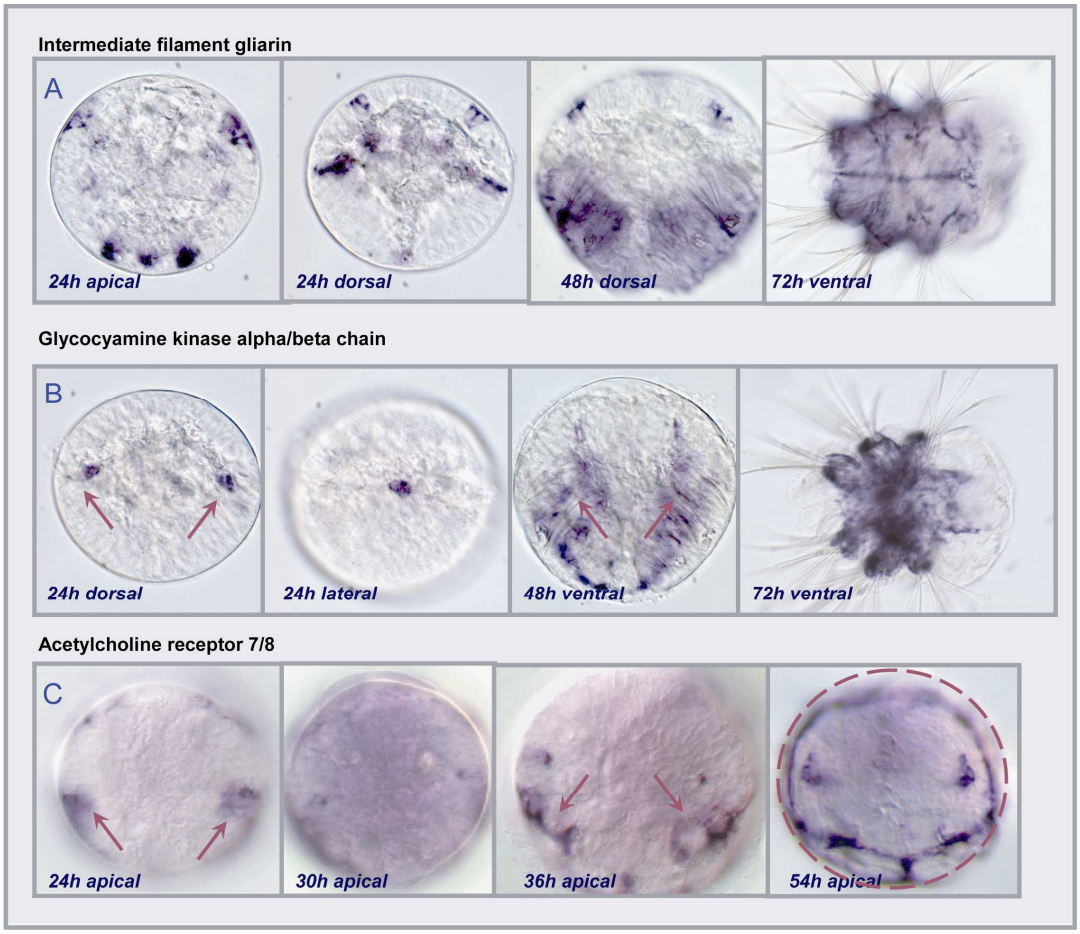


Figure 2. ***In-situ*** **hybridization screen for eye candidate genes from** ***Platynereies*** **ESTs collections.**
(A) Intermediate filament gliarin, clone ID: 48-11-04-D. Note expression at 24h in two symmetric groups of cells at the dorsal brain as well as in the ventral side of the brain. Note the expression at the periphery of the ventral nerve cord at 48h and 72h. (B) Glycocyamine kinase alpha/beta chain, clone ID:48-11-05-G. Note the highly specific expression in two symmetrical group of cells (2-3 cells) at the prototroch level (note arrows) . Later, at 48h and 72h, it is expressed in cells adjacent to the stomodeum (note arrows) and in the ventral plate. (C) Acetylcholine receptor 7/8, clone ID: IBOAAA19CA10. Note the expression at 24h in the ventral-medial brain, in the region of the developing larval eyes (note arrows) . Later at 36h it is expressed in additional group of cells in the ventral brain (note arrows) and at 54h a type of a ring is formed from peripherally located cells that express the gene (note shaded circle). This marker was later confirmed as being expressed in the larval eyes.

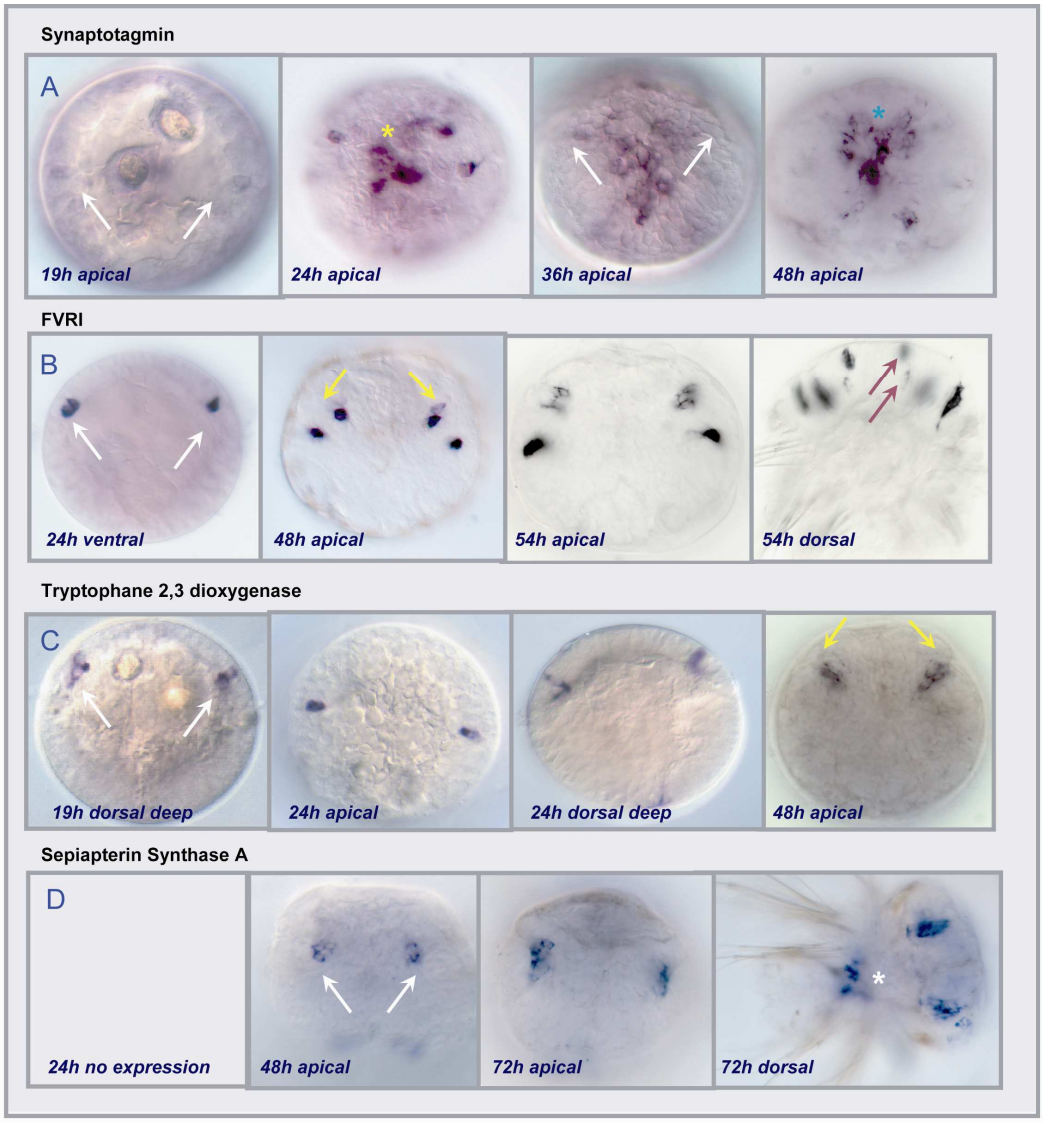


Figure 3. *In-situ* hybridization screen for eye candidate genes from *Platynereies* ESTs collections.
(A) Synaptotagmin, clone ID: IBOAAA36AD09. Note the early expression in the larval eye cells at 19h, indicated by white arrows. Note the expression at the apical organ region marked by a yellow asterisk. This expression region is later expanded to the developing adult eyes (marked by white arrows at 36h) and the developing nervous system (marked by a blue asterisk at 48h). (B) FVRI, clone ID: IBOAAA17B08. Note the specific expression in the larval eye cells at 24h (indicated by white arrows). At 48h expression also appears in the adult eyes cells (indicated by yellow arrows). At 54h more cells are expressing FVRI in both adult and larval eyes. In addition more cells, located apical and have a flask shape express the gene, see the 54h dorsal view, cells marked with purple arrows.
(C) Tryptophane 2,3 dioxygenase, clone ID: 48_11_03B. Note the highly specific expression in two cells of the larval eye indicated by white arrows at 19h and 24h. At 48h expression is seen in the adult eyes (indicated by yellow arrows) but not in the larval eyes. (D) Sepiapterin Synthase A, clone ID 48_8_H11. Note the specific expression in the adult eyes cells at 48h and 72h. At 72h it is also expressed in cells adjacent to and bellow the stomodeum, indicated by white asterisk.

As part of our laboratory consortium to integrate as many EST clones as possible to the PEPD (**http://ani.embl.de:8080/pepd/**) , I also took part in it by analyzing and integrating 18 genes.
This database contains gene expression data of *Platynereis dumerilii* examined through whole mount in situ hybridization. Sequences, images and expression pattern descriptions are freely accessible there.
The database is searchable by either of the following options:

a. Clone identity
    a. Clone accession number
    b. Keyword in blast results
b. Expression pattern
    a. Stained structure
    b. Intensity
    c. Category
c. Sequence
    a. Blast a nucleotide or protein sequence against PEPD

These 18 genes (the ones that have a PEPD ID in the table), were submitted to the PEPD database and I have submitted 123 pictures in total. These pictures contain the expression patterns at 3 stages: 24hpf, 48hpf and 72hpf in three different orientations: apical, ventral and dorsal.

2.1.1.1 The identification and verification of several eye markers: DAG kinase, Synaptotagmin, Sepiapterin Synthase A Tryptophane 2,3 dioxygenase, , FVRI

DAG Kinase
In *drosophila* DGK2 is exclusively expressed in adult fly retina. It has a role in the response termination of the photo transduction pathway, and its activity is essential for the maintenance of the photoreceptor. It accomplish this role by controlling **DAG** levels and converting it to phosphatidic acid and is also involved in the re-synthesis of PIP2 (Posphatidyl inositol 4,5 bi-phosphate) (Raghu et al. 2000). Pdu DAG Kinase is expressed at 24hpf in few cells in the region of the larval eyes. At 48hpf additional cells in the dorsal medial brain and in the adult eye region are stained. (See Figure 1D)

Synaptotagmin
Synaptotagmin is a synaptic vesicle-specific protein, known to bind Ca2+ in the presence of phospholipids, has been proposed to mediate Ca(2+)-dependent neurotransmitter release (Littleton and Bellen ,1995; Koh and Bellen, 2003) and therefore serve as a neuronal marker. By gene expression analysis - Wholemount *in-situ* hybridization- I found Pdu Synaptotagmin to be a neuronal marker, since it is indeed expressed in differentiated neurons, labeling among other differentiated neurons, the developing larval eyes (as will be illustrated in section 2.2).
Its expression begins at 15hpf in few cells at the apical organ. Staining in the larval eyes region appears at 17hpf, its expression broadens to all the differentiated neurons

of the developing nervous system. (See figure 3A for expression at stages 19,24,36 and 48h).

Most insects use a mixture of Pterin and Ommochrome derivatives for their pigment (Fuzeau-Braesch 1972). I identified an enzyme from each of the pathways, to be expressed in Pdu eyes:

Sepiapterin Synthase A.
The eye pigment of *Platynereis dumerlii* was isolated from adult worms and was identified as a mixture of three different pterin molecules (Viscontini, Hummel et al. 1970):
1. A dimer of Platynerepterin (a novel pterin, red colure pigment)
2. Nerepterin (a novel pterin, yellow colure pigment)
3. Neopterin
Sepiapterin Synthase A is one of the enzymes involved in the complex biosynthesis pathway of different pterins. It catalyses the conversion of GTP into H-4-biopterin (Ichinose, Katoh et al. 1991) (see figure 3). It is a highly conserved enzyme, found in both vertebrates and invertebrates. Pdu Sepiapterin Synthase A is expressed specifically in the adult eyes (and not in the larval eyes) from 48hpf (as will be illustrated in section 2.2) . (See figure 3,D for expression at stages 48 and 72h)

Tryptophane 2,3 dioxygenase.
Tryptophane 2,3 dioxygenase is involved in the biosynthesis of Ommochrome by catalyzing the degradation of L-Tryptophan into L-Formylkynurenine (Linzen 1967) (see figure 3). Pdu Tryptophane 2,3 dioxygenase is expressed in both larval and adult eyes,However in a dynamic mode (as will be illustrated in section 2.2) . It is expressed in two cells of the larval eyes as early as 15hpf, the expression in the larval eyes abolishes at 30hpf and starts to be expressed in the adult eyes between 36- 48hpf and continued to be expressed there afterwards (latest stage examined 72h) . (See figure 3,C for expression at stages 19,24 and 48h)

FVRI.
Peptides are a diverse and important class of messengers and hormones that transmit and regulate numerous behavioral, developmental, and physiological processes. FVRI is a member of a large family of peptides (7aa length) with RFamide C terminus and with unique N-terminal extensions. First identified in a mollusk (clam *Macrocallista nimbosa*) as a cardioexcitatory peptide, FMRFamide-related peptides (FaRPs) are now known to affect a wide range of processes from behavior to physiology in invertebrates and vertebrates (heart rate, gut motility and synaptic activity). The number of times FMRFamide containing peptides are encoded in a gene differs between animal species (Nichols 2003).

The Pdu FVRI gene contains 12 peptide 5 with identical N-terminal and 5 different ones. They are being cleaved by dibasic cleavage sites. Accordingly, an additional peptide can be expected to be processed from the precursor : pENRQSPamide. (Guenter Plickert, personal communication).

The expression of this peptide in Pdu is highly specific, in both the larval and adult eyes (as will be illustrated in section 2.2). First expression appears at 20hpf in the larval eyes, where it persists, and at 48hpf expression in the adult eyes appear. Later - at around 54hpf additional cells at the apical organ express FRVI as well. All FVRI expressing cells, as seen by wholemount *in-situ* hybridization, have a flask shape. (See Figure 3-B for expression at stages 24,48 and 54h).

2.1.2 Constructing brain-eye specific library

(The whole process was done together with Dr.Gaspar Jekely.)
We were interested in creating a cDNA library that is enriched with genes expressed in the nervous system and the eyes of *Platynereis*. For this we have dissected ~100 adult worms that were first devoted of food and treated with anti-algae agent (in order to prevent contaminations). Detailed description of the library construction steps is found in Material and Methods, section 4.5.
The quantification of the material was as following:
The **total RNA** read was 1µg/µl (in a volume of 100µl).
The **mRNA** read was 0.1µg/µl (in a volume of 10µl).
The second strand yield counting was: 0.125µg.

After the size fractionations steps, we have decided to keep fractions number: 6,7,8,9,10
Two ligations were made and transformed:

1. combining fractions 6,7,8
2. combining fractions 9, 10

We have estimated the **Complexity** of the library (made from 2 transformations together) to be 287,000 clones. With the **average insert size** of 870bp from the first ligation and 1190bp from the second ligation.
An analysis (minipreping and sequencing) of a sub-set of 48 colonies revealed the following:

- 3 colonies were empty.
- 3 colonies were very <u>short</u> in length.
- 24 colonies got no hit in GeneBank, when blasted against our existing ESTs collection: only 5 did **not** get any hit, the ones that got hit were in average 430 bp <u>shorter</u> then the existing EST clone.
- 4 colonies got gene hits in GeneBank.
- ~10% got hits of ribosomal proteins.

From these features we have concluded that the library's quality is not sufficient in order to incorporated it into the sequencing pipeline of the EST's.

2.1.3 Eyes absent 3’ RACE

An initial fragment of the Eyes absent gene was cloned in the lab by Patrick Steinmetz . I have done a 3’ and 5’ RACE PCR in order to obtain a larger fragment. Detailed description of the process is given in “Materials and methods”, section 4.2.3. A promising band was obtained and TOPO cloned. 30 colonies were analyzed by “Insert PCR”. Six colonies had the expected size (~850bp) and one of the three was indeed the Eyes absent 3’ RACE sequence. this fragment was TOPO cloned and put into the lab’s plasmid database.

2.1.3.1 Eyes absent expression pattern

Wholemount *in-situ* hybridization was carried out with a new riboprobe made of the extended cloned fragment. The following stages of embryos:24, 36, 48 and 72hpf were analysed. At 24h it is expressed extremely week in the dorsal brain, at 36h the expression becomes more apparent at that location. At 48h it is expressed in the dorsal brain with a narrow gap between the two sides (left and right of the brain), and in the stomodeum. The expression at 72h resembles that of the 48h. (See figure 4)

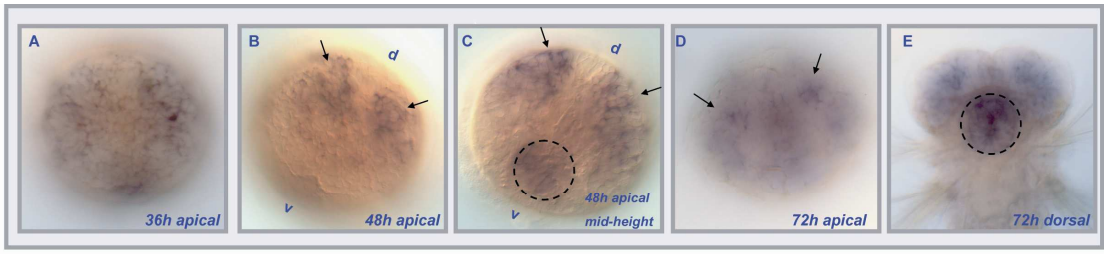


Figure 4. Eyes absent expression.
(A-E) Whole mount *in-situ* hybridization with an Eyes absent riboprobe. The expression at 24h is very weak and diffused (not shown), at 36h the expression in the dorsal lateral brain is visible, however still in a diffused manner (A). At 48h expression becomes more restricted to the dorsal brain with a gap between right and left side (B, high apical view, expression domains are pointed by a black arrows. Note the orientation: d, dorsal; v,ventral) and the stomodeum (C, mid-height apical view, the expression in the stomodeum is circled by a dashed circle. Dorsal brain domains are pointed by black arrows). At 72h the expression in the brain is expanded, however it remains stronger on the dorsal side, specially in the region of the adult eyes (D, pointed by black arrows). The expression in the stomodeum remains as well (E, dashed black circle).

2.1.4 Prox1 cloning and expression - collaboration project

Professor Zbynek Kozmik has initiated a collaboration between his laboratory and Detlev Arendt’s laboratory regarding the Prox1/*Prospero* gene. (See “Introduction” for the background and the reason for this study). I have synthesized *Platynereis* first strand cDNAs of the following stages: 24hpf, 48hpf and 5 days (RNA was donated by Kristin Tessmar-Raible) by using the SMART RACE cDNA amplification Kit (Clonethech). The integrity of the cDNAs was confirmed by PCR using commonly used primers of the Pax6 gene. The cDNA libraries were sent to the laboratory of Zbynek Kozmik for the cloning of Platynereis Prox1 gene.

An initial fragment was cloned and followed by a successful 3' RACE (Pavel Vopalensky). The RACE fragment of the C-terminal size is 1.17kb. An Alignment for the Prospero domain sequence was done by ClustalX algorithm and a Maximum-likelihood (ML) tree was constructed as well (Pavel Vopalensky), both are presented in figure 5.

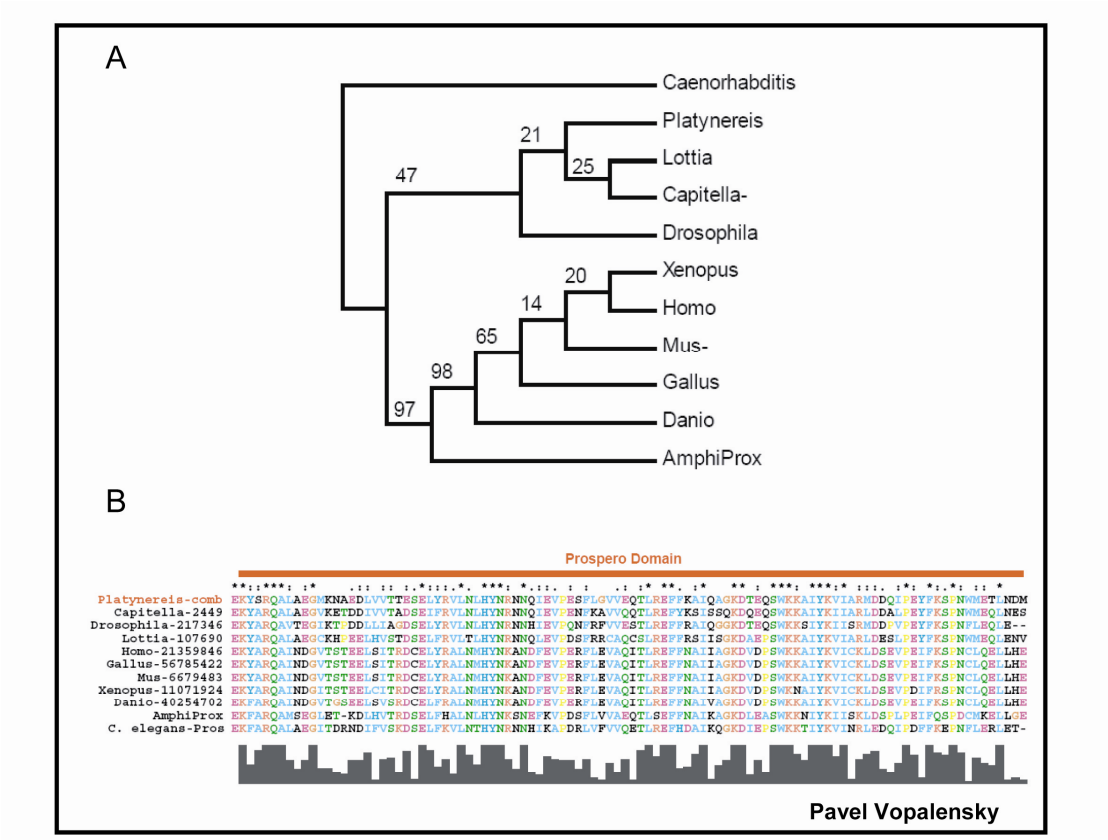


Figure 5. ***Platynereis*** **Prox1 gene cloning results (in collaboration with Pavel Vopalensky).**
(A) Maximum likelihood (ML) tree of *Platynereis* Prox1 (Pdu Prox1) C-terminus (~1.2KB) .C. elegans is used as the outgroup. Pdu Prox1 clusters with *Capitella* and *Lottia* sequences of Prox1 sequence. And the three cluster with the *Drosophilas'* Prox1 sequences. The different numbers shown on the branches represent percentages of 1000 bootstrap samples (for example 29 corresponds to 290) for ML method .(B) ClustalX Alignment of *Platynereis* Prox1 showing the high degree of conservation in the Prox1 domain.

I have synthesized an RNA probe and did wholemount *in-situ* hybridization to see the expression pattern of the gene.
Pdu Prox1 is expressed at 24hpf in the region of the larval eyes in 2-3 cells on each side , in few spots in the brain (relatively superficial) and in some cells at the dorsal and ventral brain at the level of the larval eye staining (See figure 6,A,B). At 48hpf it is expressed in the adult and larval eye regions, in the ventral plate and in a dorsal lateral region (See figure 6,D-F).
At 72hpf the brain expression is expanded in addition to very few cells in the ventral plate(See Figure 6,C).

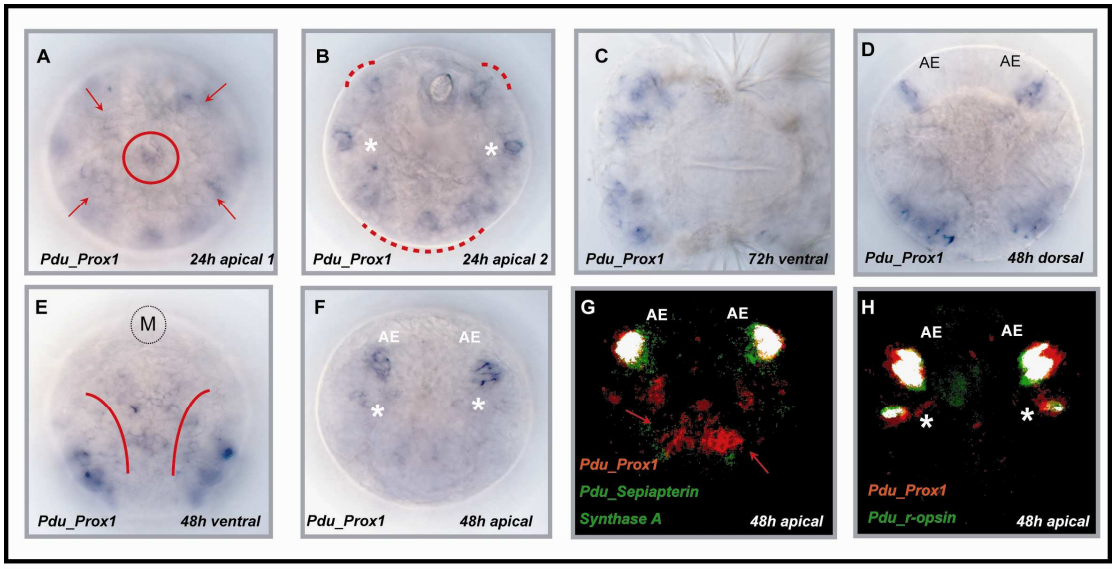


Figure 6. *Platynereis* Prox1 expression analysis.
(A-F) whole mount *in-situ* hybridization with a Prox1 riboprobe. (A) 24h embryo stained with Prox1 probe, high apical view. Note the expression in 4 peripheral regions pointed by red arrows and a central region (resembles the apical organ area) circled by a red circle. (B) A slight deeper view then in A, showing Prox1 expression in the region of the larval eyes (indicated by white asterisks) and in peripheral regions marked by red dashed lines. (C) broader expression of Prox1 in the brain of a 72h embryo. (D-F) Prox1 expression in a 48h embryo with the indicated orientations. Note the expression in the adult eye (AE, Adult eyes) in D and F, in the ventral plate in E ,as indicated by two parallel lines bellow the mouth opening (M,mouth).
(G,H) 3D *In-silico* alignment of Prox1 and the indicated probe. Z projections of maximum intensity. White indicates co-localization. (G) Z projections of stacks 24-37 (stacks representing the AE pigment cells) showing Prox1 expression in the AE pigment cells as indicated by the white signal. Red arrows pointing at superficial medial-ventral brain expression . (H) Z projections of stacks 38-63 (AE PRCs representing stacks) showing Prox1 expression in the AE PRCs as indicated by the white signal. These stacks stained with r-opsin probe also show the expression of Prox1 in the LE r-opsin cell.

Incorporation of the gene into the 3D in silico expression profiling protocol (Raju Tomer and Detlev Arendt, unplished data):

In order to cinfirm Prox1 experssion in Platynereis eyes and to distinguish wich cells exactly expressed it, I have decided to take the advantage of the *in silico* expression profiling protocol (Raju Tomer and Detlev Arendt, unplished data).
An *in-situ* hybridization with a Prox1 riboprobe together with anti-acetylated tubulin antibody and DAPI was carried out and imaged in the confocal microscop by me.
Five successful scans of 48hpf stage were obtained; these were then incorporated by Raju Tomer into the 3D *in silico* expression profiling protocol (an average scan was generated from the 5 scans, and this was then aligned to a reference embryo).
To create a double *in-silico* expression data for:
Prox1 and r-opsin and:
Prox1 and Speiapterin reductase A
I have used the “colocalization analysis - colocalization highlighter” plug-in in ImageJ software. By this one can visualize the co-expressing pixels of two different staining (‘channels’) with the possibility to control the threshold parameter. In addition, this plug-in also generates a new channel that contain the information from the co-expressing pixels exclusively (it is called: ‘co localized points 8-bit’).

By this we could see that Prox1 is expressed, at 48hpf, in the photoreceptor and pigment cells of the adult eyes and in the r-opsin positive cell of the larval eyes. (See Figure 6,G,H).

2.1.5 MITF cloning attempts

Mitf (microphthalmia-associated transcription factor) encodes a transcription factor of the basic/helix-loop helix/leucine-zipper family and is a key regulator during the development of two different types of melanin-producing cell lineages, namely neural crest-derived melanocytes/melanophores, and the retinal pigment epithelium (RPE) - differentiated from the outer layer of the eye cup (Levy, Khaled et al. 2006).

Melanin production is an ancient biological process found in all living kingdoms, and is shared by pigment cells associated with Pax6-dependent photoreceptive organs in several invertebrates (Glardon et al., 1997, 1998; Callaerts et al., 1999). This raises the interesting question of how well conserved the association of Mitf with melanin production and/or pigment cell development is?

Drosophila Mitf (*Dmel/Mitf)* is expressed during embryonic development and in the eye antennal imaginal disc (Hallsson et al.,2004**)**. *In vitro*, transcriptional regulation by Drosophila Mitf, like its mouse counterpart, is modified by the Eyeless (Drosophila Pax6) transcription factor. *In vivo*, targeted expression of wild-type or dominant-negative Drosophila Mitf results in developmental abnormalities reminiscent of *Mitf* function in mouse eye development.

Because of the interesting correlation of this gene between vertebrates and invertebrates we were interested to find out whether *Platyneries* Mitf also exist and if so, where does it express and what is its potential function.
We therefore tried to clone this gene in *Platynereis* based on sequence homology at the bHLH-zip domain.
First attempt:
I have used primers designed by both myself and Dr.Gaspar Jekely for degenerate PCR reactions and further nested reaction. Primers stock numbers are the following: 1096-1099.The template I used was a mixture of 72hpf single strand cDNA and 48hpf 3' SMART RACE cDNA.The reactions were run on an agarose gel, blotted and then hybridized with a radioactive probe. The probe was made from the Medaka Mitf cDNA plasmid (540bp in size, a kind donation from Dr. Jochen Wittbrodt) and therefore a low stringent radioactive hybridization was done in order to allow mismatches in sequence. The radioactive signals were mostly smears without an indicative promising band for cloning.
Second attempt:
This time, I have decided to use cDNA from adult worms. The reason was that Platynereis eyes continue to grow along its life (eye cells continue to differentiate) and therefore Mitf might be highly expressed at the adult worm as well (in addition: there are more cells in the eye and therefore higher copy number of mRNA).
I have used the same primer set and did nested reactions as well. The reactions were run on a gel and blotted and I have used the Medaka Mitf cDNA as a probe. From this hybridization 4 promising bands at the expected size were chosen, run on a

preparative gel and cut. I have chosen the 2 more promising bands and TOPO cloned them. Then, I did a colony PCR followed by restriction enzyme analysis to choose the right size clones for sequencing. The sequences were however negative for the gene. At this point, since I was involved in few projects simultaneously, I have decided to put this project aside. However, all the information and material (e.g. primers) needed for continuing with the Mitf cloning are present and accessible in the laboratory.

2.1.6 Lens protein identification project

We have initiated collaboration with the laboratory of Joram Piatigorsky at the National Eye Institute Bethesda, Maryland, for the purpose of isolating the lens protein/s of Platynereis.
I have dissected the eyes of ~100 mature animals (since sexually-mature epitoke worms expand their eyes tremendously). The material was frozen at -80^{o}c and once the collection completed it was sent to Dr. Josef Horwitz, Our contact person for the project. The processing of the sample there was as following:

The proteins from the sample were subjected to SDS-polyacrylamide gel electrophoresis, the chosen band was excised and digested by treatment with trypsin. Then, single microcapillary reverse-phase HPLC was run, directly coupled to the nano-electrospray ionization source of an ion trap mass spectrometer. This instrument configuration is capable of acquiring individual sequence (MS/MS) spectra on-line at high sensitivity (<<<1 femtomole) for multiple peptides in the chromatographic run. These MS/MS spectra, (also referred to as CID, sequence or fragmentation spectra), were then correlated with known sequences using the algorithm Sequest (Eng *et al*, 1994, Chittum *et al*, 1998). MS/MS peptide sequences reviewed by a scientist for consensus with known proteins and the results manually confirmed for fidelity.

In the first trial, I have used headless *Platynereis* bodies as a control sample. The samples were loaded and run on an SDS gel (1D). In the second trial I have used *Platyneries* brain (devoted from eyes) samples as control sample. And in this trail the samples were loaded and run on a 2D gel system.
In both experiments actin and tubulin dominated the protein profile. For the second trail, lower molecular bands seen were common to both eye and brain samples.

From the first trail 3 bands at the sizes of: 44, 30 and 25 Kd were sent for the sequencing approach mentioned above. A substantial amount of actin/tubulin and other intermediate filament proteins were present in each of bands. No other apparent candidate was present in the sequenced peptides.
From Western blot experiment performed in Joram Piatigorsky's laboratory, our collaborators are convinced that *Platynereies* lens proteins are totally different and might not even be related to any of the known vertebrate or invertebrate lens proteins. *Platynereies* lens proteine/s are probably hidden in one of the minor bands.
See Figure 7 for the results summary.
From our communication with Dr. Joseph Horwitz it seems that the main problem is to micro-dissect a **pure** Platynereis **lens** and not the whole eye. It seems to me that for *Platynereis*, at least with common methods, this will not be possible because of two reasons:

1. The small size of the eye, and therefore also the lens.

2. The fact that the eyes are fragile, meaning that they leak immediately upon dissection.

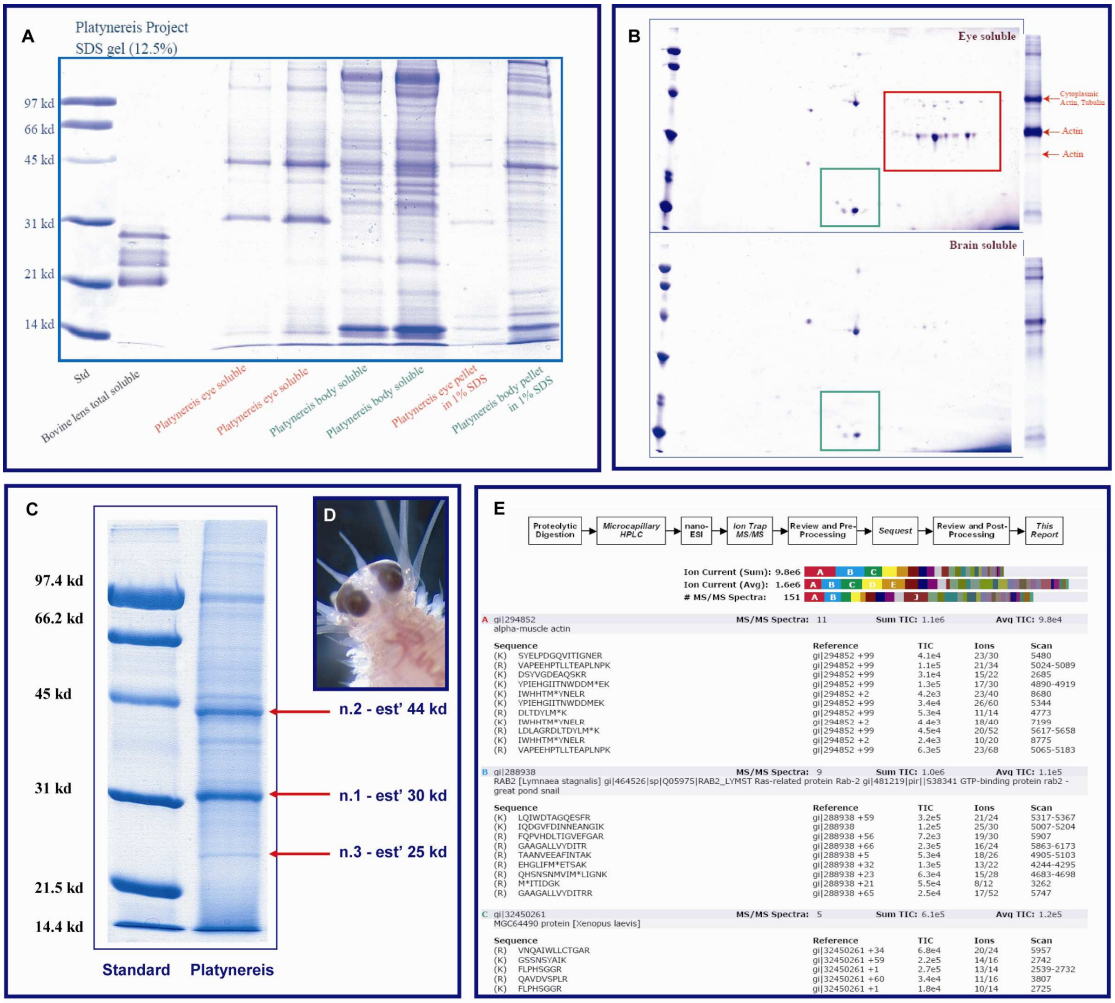


Figure 7: Lens protein identification project (Josef Horwitz).
(A) First trail of sampling run on 1D SDS gel. Headless *Platynereis* bodies serve as a control sample. (B) Second trail of sampling run on a 2D gel system. *Platyneries* brain (devoted from eyes) serves as control sample. In both experiments actin and tubulin dominated the protein profile (marked by red square in B). In the second trail, lower molecular bands (marked by green squares in B) were common to both eye and brain samples.
From the first trail 3 bands at the sizes of: 44, 30 and 25 Kd (C, pointed by red arrows) were isolated and sent for MS/MS peptide sequencing. (D) A picture of an adult *Platynereis* worm showing its' large adult eyes. Note their black colure which we hypothesis is due to accumulations of lens proteins.
A fraction of the sequencing results for band number 3 is shown in (E) as an example :on top is a flow chart of the procedure which the chosen bands go through. Below is the relative representation of the different peptides in the sampled band (the size of the colored cube is proportional to its' relative amount in the sample). The third part is the different hits that each peptide got using the BLAST "Sequest" algorithm. In this example the first three dominant peptides are: alpha muscle actin, RAB2 and MGC64490.

2.1.7 r-opsin II cloning.

Since the known Pdu r-opsin is detectable in the larval eyes only from 48hpf onwards. We have suspected that a different opsin is involved in light reception at earlier stages. This is a common feature of marine invertebrates and fish: a switch between 2 or more opsins during growth and changes in life habitats (e.g. Hope, Partridge et al. 1998; Cheng, Gan et al. 2007) Therefore I have decided to clone a second r-opsin

gene for *Platynereis*. I have used a 24h cDNA library, because of the following reasons:
1. Phototaxis behavior begins at this stage.
2. 24h stage is a day **before** the adult eyes forms, in which the known Pdu r-opsin is expressed.
I have used a set of degenerated primers (based on Patinopecten sequences for G_q proteins designed by Detlev Arendt). A single band at the expected size (~470bp) was obtained and cloned. 30 colonies were analyzed with the restriction enzymes: HinfI and EcoRI. 7 clones were sent for sequence. 6 of these clones were identified as a novel Pdu r-opsin. This gene was named Pdu r-opsinII. After several attempts of 5' and 3' RACE PCR with no success, I have decided to take a different approach and to combine the use of:

1. Sequence specific primers, from the sequence I have cloned (stock numbers: 1118, 1119, 1139) at the most 3' end of it.
2. New degenerated primers designed for an upper 5' location of the gene (stock numbers 1299).

By this I have successfully obtained a longer fragment of the gene, adding 303bp to the original fragment. In order to fuse the original and the extended fragment I have first used the "fusion PCR" strategy, however without success. Therefore I have used the "restriction site cloning" strategy (using the restriction enzymes: XbaI and MfeI, followed by ligating the two cut fragments) for fusing the two parts. By this I have obtained a 850bp fragment of the gene. This plasmid was put into the laboratory's plasmid database.

2.1.7.1 Pdu r-opsin II expression

Wholemount *in-situ* hybridization was carried out by me, using the initial 477bp fragment at several stages: 19, 24 and 48hpf (and in second round also on other intermediate stages). The only expression I could observe was a weak staining at 19hpf that was not consistent among all embryos. It was expressed in 1-2 cells in a location suggestive for the larval eyes but not on both sides. (See figure 8).
Additional Wholemount *in-situ* hybridization attempts with the elongated fragment and different modification of the protocol (e.g hybridization for 2 nights, using hydrolyzed probe) could not reproduce the results nor produce different results.

2.1.7.2 Pdu r-opsin II southern blot

Since the expression pattern was unresolved, I have developed doubts regarding the gene, is it indeed a *Platyneries* gene, could it be a contamination? In order to confirm that Pdu r-opsin II is indeed a *Platynereis* gene I have done southern blot hybridization using genomic DNA made from adult animals and the fused r-opsinII fragment for the probe. Negative (no probe) and positive (the plasmid of r-opsinII) controls were included.
In the first attempt I have used the DIG system (Roche) as an alternative for radioactivity (RA), this however was not sensitive enough to detect the gene. I have therefore used the classical methods using RA.
The result was positive meaning that this gene is indeed a true *Platynereis* gene. (See figure 8.)

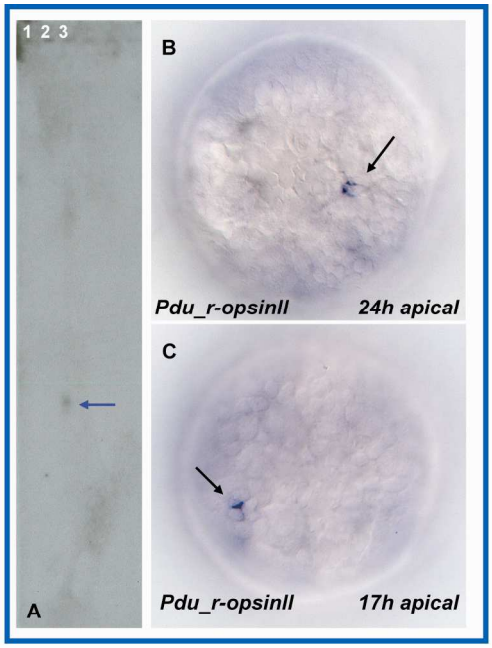


Figure 8. *Platynereis* r-opsinII Southern Blot and expression.
(A) Southern blot using *Platynereis* genomic DNA hybridized with a probe against r-opsinII. arrow indicats a positive signal. (B-C) Platynereis 24h (B) and 17h embryo (C) hybridized with a riboprobe for r-opsinII. Arrows indicating single cell expression in a position suggestive for the larval eyes.

2.1.8 BAC screening

For future applications such as functional interference, transgenesis etc, I have decided to obtain the upstream sequences of the following genes of interests:
Pdu r-opsin
Pdu r-opsin II
Pdu Pax258

2 BAC filters were screened for each gene. Screening was done using radioactive hybridization according to the protocol described in “Material and Methods”, section 4.2.1, under “High stringency Southern Blots /Radioactive hybridization”.

Two clones were identified and ordered for r-opsin and one for r-opsinII, the clones ID are the following:
For r-opsin: CH305-55E1, CH305-185P7
For r-opsinII: CH305-122F12

The BACs were plated and 3 colonies from each BAC were picked, mini-preped and digested with HindIII, EcoRI. The gels were blotted and hybridized to confirm the existence of the gene inside the BAC. By this I could see that both clones of r-opsin

were positive, but r-opsinII was negative. By the EcoRI and hindIII digestion/RA hybridization pattern we estimated that clone CH305-55E1 contain more of the r-opsin gene locus and therefore this clone was sent for the BAC's sequencing by the Genoscope institute, France.(See figure 9)

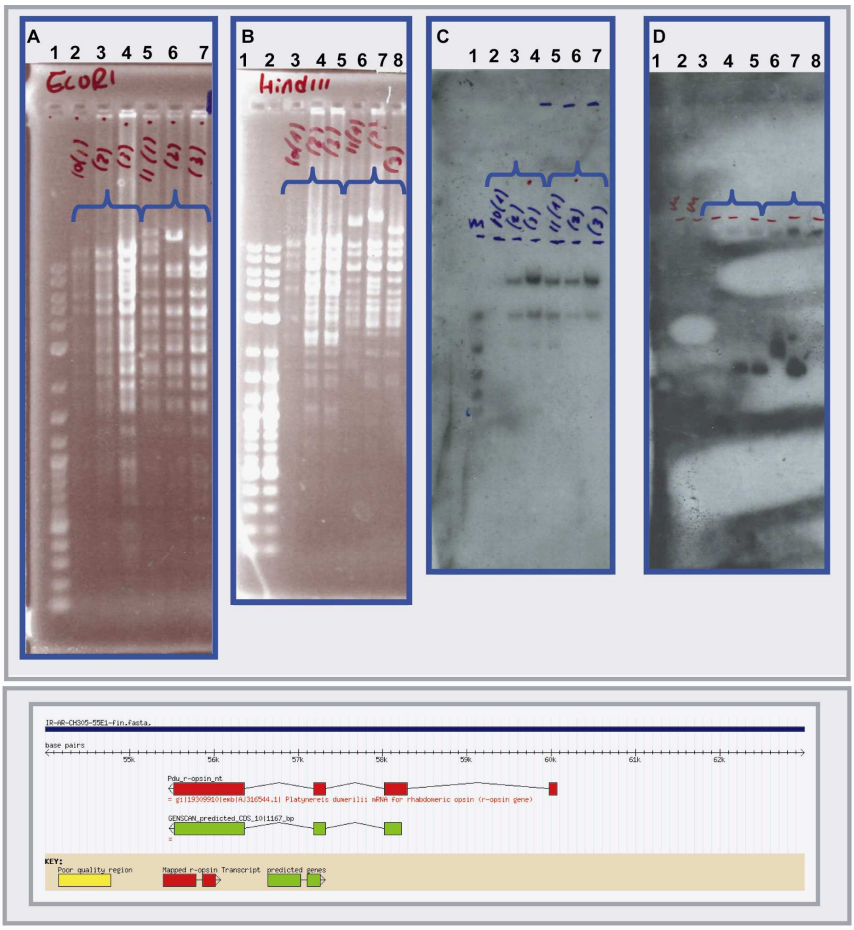


Figure 9 Top : r-opsin BAC confirmation - southern blot
(A,B) ECORI and HindIII digestions (respectively) of two *Platynereis* r-opsin BACs candidates. Loaded and run on an agarose gel. These candidates were identified by me from *Platynereis* BAC filters using southern blot protocol (hybridized with a Pdu_r-opsin probe). (A) lane 1 is a DNA size marker, lanes 2-4 are three colonies of BAC number: CH305-55E1, Lanes 5-7 are three colonies of BAC number: CH305-185P7. (B) lanes represent the same colonies loaded in the same order as in A with the only exception that both lanes 1 and 2 are loaded with a DNA size marker (in order to distinguish between the two gels). (C,D) Southern blots of the gels shown in A and B (respectively) using Pdu_r-opsin probe. All six colonies gave a positive signal. The signal from the first colony (lane 2 in A and C, lane 3 in B and D) was weaker then all others.

Figure 9 Bottom. r-opsin BAC confirmation (bioinformatics analysis - Dr. Florian Raible)
The published *Platynereis* r-opsin sequence (NCBI accession number AJ316544) was run against the isolated BAC sequence, using alignment software specialized in recognizing exon boundaries. The gene indeed mapped to this BAC sequence as seen by the red exon squares. In addition, a prediction algorithm, which predicts genes from the BAC sequence, was run and mapped the predicted r-opsin gene on the same BAC sequence (green exon squares). The sized of r-opsin BAC is 164313 nucleotides long (~164 KB). According to the mapping, the upstream sequence is around 55KB long and the down stream sequence is around 104 KB long.

The complete genomic r-opsin locus was annotated by Dr.Florian Raible, using a combination of gene prediction (Burge and Karlin 1997) and transcript mapping algorithms (Florea, Hartzell et al. 1998) (Wheelan, Church et al. 2001). The r-opsin

sequenced used for the analysis is the following: NCBI accession number AJ316544 (Arendt, Tessmar et al. 2002). (For visualized annotation see figure 9).

The 16,4313 nucleotides long *Platynereis* r-opsin BAC is now available for certain future applications (e.g. bioinformatics prediction of TFs binding sites, transgenesis etc.). it contains around 55KB upstream sequence and around 104KB downstream sequence of Pdu r-opsin gene.

2.2 Assignment of different eye markers to different cell types of the larval eyes

In collaboration with:
Dr. Gaspr Jekely :
1. Confocal microscopy of double *in-situ* hybridization for r-opsin and FVRI.
2. Triple immunostaining and confocal microscopy of: anti-Pdu_FVRI antibody,anti-acetylated tubulin and anti-Phalloidin antibody.
Dr. Harald Hausen :
1. Transmission Electron Microscopy.
2. 3D reconstructions.

At this point of my research I had 3 additional candidate markers for the **larval eyes**: Tryptophane 2,3 dioxygenase, FVRI and Synaptotagmin. In order to confirm their expression in the larval eyes I have performed the following experiments:

1. Double *in-situ* hybridization of Synaptotagmin and r-opsin (the already established marker for the larval eyes at 48h) at **48h**. From this double *in-situ* we could see that Synaptotagmin is indeed expressed in the larval eyes. The cell that expresses Synaptotagmin is laterally adjacent to the cell expressing r-opsin (See figure 10,A). This finding was un-excepted since we believed that the r-opsin positive cell is the rhabdomeric photoreceptor and therefore should be a differentiated neuron by itself.

I then wanted to confirm the other two new markers and performed the following experiments:
2. Double *in-situ* hybridization of FVRI and r-opsin. In light of the previous result and the fact that FVRI is a neuropeptide, I was not surprised to find that the cell expresses FVRI is adjacent to the r-opsin positive cell, and not the r-opsin positive cell itself. (See figure 10,B)

Since both FVRI and Synaptotagmin were expressed in a cell **adjacent** to the r-opsin positive cell, I wanted to find out if they are actually expressed in the same cell. For this I have done the following experiment:
3. Double *in-situ* hybridization of Synaptotagmin and FVRI at **24h**. From this experiment, I could see that both genes are co-expressed in the same cell. (See figure 10,C). The co-expression of two different neuronal markers in this cell strengthens the hypothesis that it is a functional neuron.

I then wanted to locate the pigment marker Tryptophane 2,3 dioxygenase in relation to the others markers confirmed. For this I have did the following experiment:

4. Double *in-situ* hybridization of Tryptophane 2,3 dioxygenase and FVRI at **24hpf**. From this experiment, I could see that Tryptophane 2,3 dioxygenase is expressed in two cells that one of them is co-expressing FVRI. The cell co-expressing FVRI is the lateral cell among the two (See figure 10,D). From this I concluded that the medial Tryptophane 2,3 dioxygenase positive cell is the pigment cell and the lateral Tryptophane 2,3 dioxygenase positive cell, that is also positive for FVRI and Synaptotagmin, is the rPRC. Similar result was obtained from the following experiment:

5. Double *in-situ* hybridization of Tryptophane 2,3 dioxygenase and Synaptotagmin at **24hpf**. In this staining I could re-confirm the location of the neuronal cell, lateral to the pigment cell (see figure 10,F, *in this staining Tryptophane 2,3 dioxygenase is stained with Fluorescent dye that is being shield by the NBT-BCIP of the Synaptotagmin and therefore seen only in one cell instead of two).

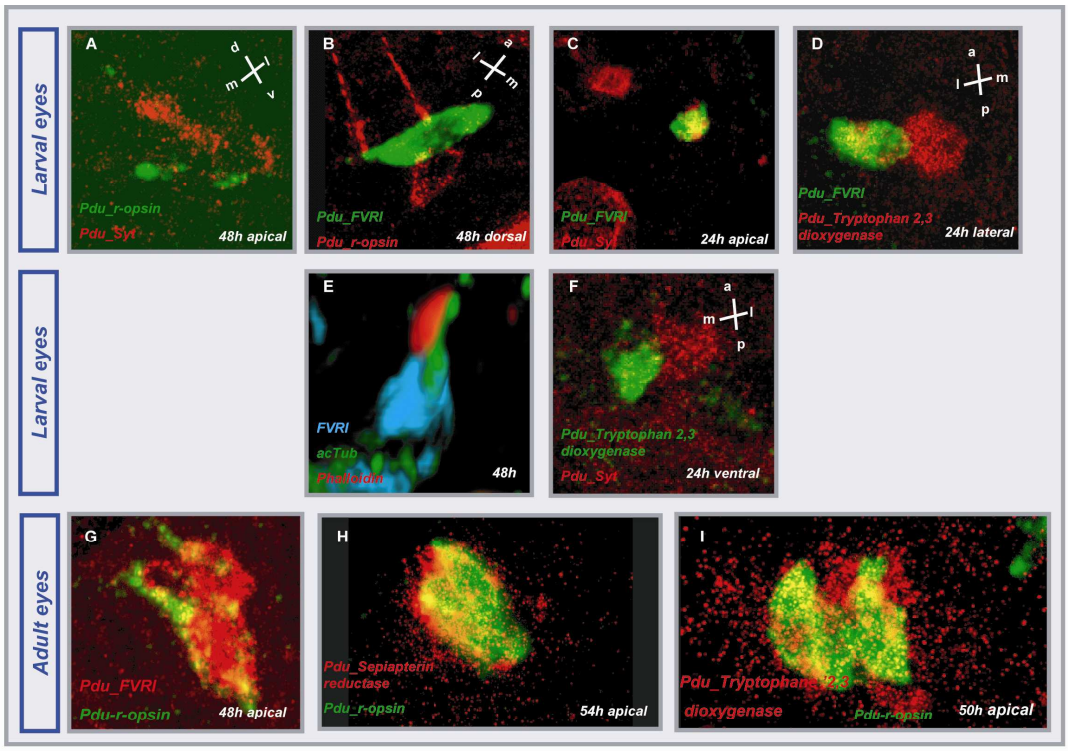


Figure 10. Verification of Adult and Larval eye markers

Whole mount *in situ* hybridizations imaged by reflection confocal microscopy (z projections of maximum intensity).

(A-F) Larval eye markers. (A) Apical view of *Platynereis* 48h embryo, focused on one larval eye, hybridized with indicated riboprobes. Syt, Synaptotagmin - is expressed in a cell adjacent to the r-opsin positive cell. (B) Dorsal view of 48h Platynereis embryo hybridized with the indicated riboprobes. Note that FVRI and r-opsin are not co-localized to the same cell. (C) Apical view of *Platynereis* 24h embryo hybridized with the indicated riboprobes, showing that FVRI and Synaptotagmine are expressed in the same cell, the rhabdomeric photoreceptor cell (rPRC). (D) Lateral view of *Platynereis* 24h embryo hybridized with the indicated riboprobes. FVRI is marking the rPRC and Tryptophan 2,3 dioxygenase is marking both the pigment cell and the rPRC. (E) Triple immuno-staining with indicated antibodies (experiment and microscopy performed by Dr.Gaspar Jekely) showing the FVRI cell body (azure) ,the Rhabdom (red) and a dendrite (green), all located to the same cell.

Note that the spatial orientation is indicated where needed: l, lateral; m, medial; a, anterior; p, postirior; d, dorsal, v, ventral.

(G-I) Adult eye markers. (G,H,I) apical views of *Platynereis* 48h,54h and 50h (respectively) embryo hybridized with indicated riboprobes. Showing the co-localization of the three markers with r-opsin in the adult eyes.

From the last 4 experiments, evidences supporting the view that r-opsin positive cell is actually not the rPRC, have accumulated. The candidate cell for the “real” rPRC was the adjacent FVRI positive cell.

To confirm this hypothesis, Dr. Gaspar Jekely performed and imaged the following experiment: triple immunostaining of anti-Pdu_FVRI antibody, anti-acetylated tubulin and anti-Phalloidin antibody. From this staining we could see that the FVRI positive cell is the cell containing the Rhabdom (by the Phalloidin staining) and a dendrite (by the acetylated tubulin antibody) - proving that it is indeed the rhabdomeric photoreceptor cell (see figure 10,E).

The rPRC mystery was solved, but we were still left with the mystery of which cell is the cell positive for r-opsin?

Based on double *in-situ* hybridization (FVRI + r-opsin) and TEM sections (Dr. Harald Hausen) of Platynereis larval eyes at 48h, two possible options emerged:

1. The r-opsin cell is one of the several flask shape cells found in close vicinity to the rPRC. (Observed **both** by TEM and by FVRI *in-situ* hybridization staining at >48h).
2. The r-opsin cell is the pigment cell.

By constructing two 3D models (Dr. Harald Hausen):
1. A 3D model based on a series of TEM sections of Platynereis larval eyes at 48h (see figure 11).
2. A 3D model based on confocal stacks of double *in-situ* hybridization (FVRI + r-opsin) (see figure 12).

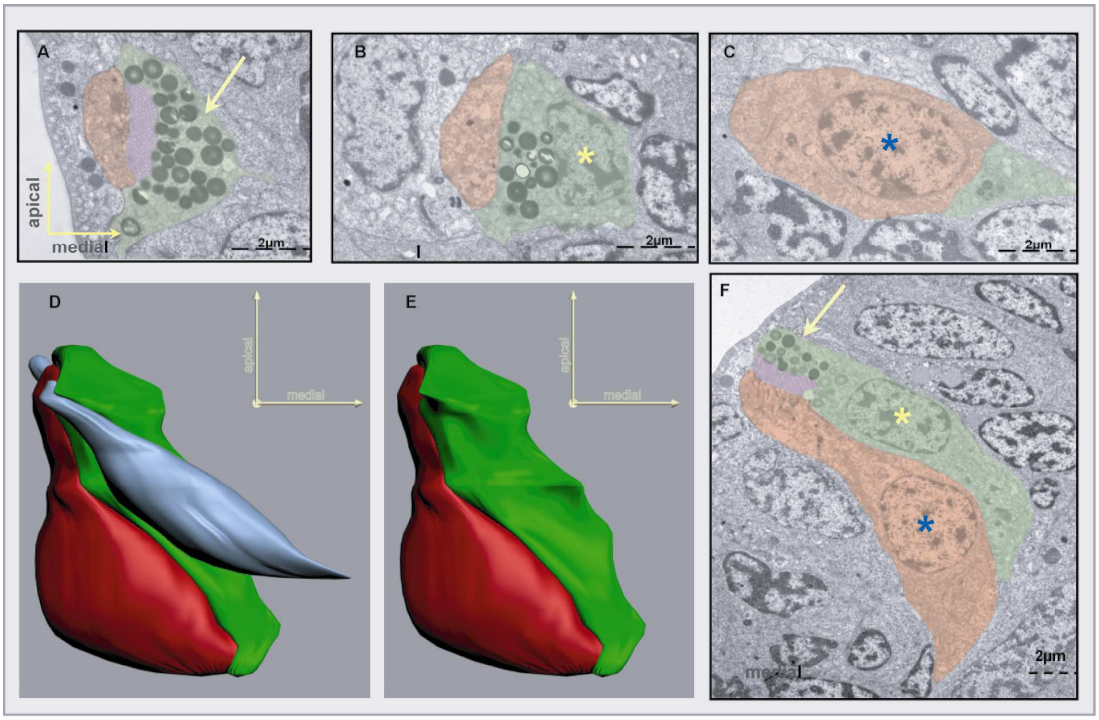


Figure 11. The identification of the larval eye r-opsin positive cell.
(A-C, F) Transmission electromicrographs (TEM) of *Platynereies* larval eye (Dr. Harald Hausen). The Orentation of the figures is indicated in figure A. The pigment cell is colored in green, the rPRC is colored in red and its rhabdome in purple.
(A-C) TEM consequent cross sections of three different layers of a *Platynereis* right larval eye .(A) Apical layer with rhabdom of rPRC and pigment shield of pigment cell. Yellow arrow is pointing at the membrane bound pigment granules of the pigment cell. (B) Mid layer with pigment cell nucleus (yellow asterisk). (C) Basal layer with nucleus of the rPRC (blue asterisk).
(F) Horizontal section of Platynereis right larval eye, showing the location and orientation of the rPRCc and the pigment cell , and their nuclei, being tightly adjacent.
(D,E) 3D reconstruction (Dr. Harald Hausen) of a complete series of TEM cross sections of *Platynereis* right larval eye from a ventral view. Note the orientation as implicated in the figure. The pigment cell is colored in green, the rPRC is colored in red. In D the flask shaped cells (here, two of them) that are adjacent to the PC and rPRC cells are also visible.

A clearer picture emerged. We found out that the pigment cell in the TEM model (recognized by its' pigment cup) is situated tightly adjacent to the rPRC (recognized by its' rhabdom). This arrangement is perfectly correlated to the spatial location of the FVRI cell and the r-opsin cell according to the 3D model of the *in-situ* hybridization

(compare figure 11,E and 12,C). We therefore concluded that the r-opsin positive cell is the pigment cell.

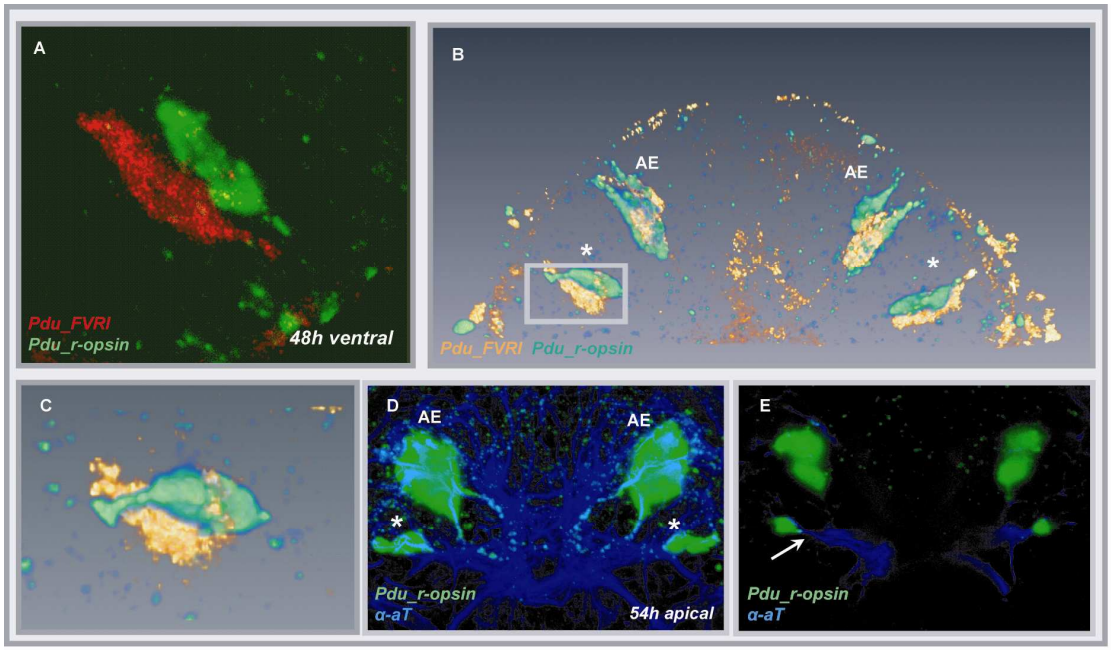


Figure 12. The identification of the larval eye r-opsin positive cell.
(A) Double whole mount *in-situ* hybridization with indicated probes showing *Platynereis* larval eye. (B,C) 3D reconstruction of the same staining as in (E), however this time imaged from apical view. (B) AE, adult eyes; White asterisks indicating the larval eyes.
(C) magnification of the marked eye in (B) showing the tight location of the rPRCc and r-opsin positive cell one relative to the other. (D, E) *In-situ* hybridization with r-opsin probe and anti-acetylated tubulin antibody (α-aT) (Dr. Gaspar Jekely).
(D) Z projection of the confocal scans, scanning depth: 110um. AE, adult eyes. White asterisks indicating the larval eyes. (E) Single confocal stack showing the direct connection of the larval eye r-opsin cell with an axon (pointed by white arrow).

2.2.1 The early expression of three LE markers: Synaptotagmin, FVRI, Tryptophan 2,3 dioxygenase.

In order to find out when are the larval eye cells markers start to be expressed I have done a set of early *in-situ* hybridization experiments using the above mentioned riboprobes. Synaptotagmin and FVRI as markers for the rPRC and Tryptophan 2,3 dioxygenase as a marker for the rPRC and the pigment cell. The earliest stage used was 15hpf. The results are presented in figure13.
The earliest expression of Synaptotagmine is detected in the larval eyes at 17hpf and is consistently expressed there (among other differentiated neurons) in later stages (see figure 13,A-D).
The earliest expression of FVRI is detected in the larval eyes at 20.5hpf and is consistently expressed there (among other cell) in later stages (See figure 13,E-H).
The earliest expression of Tryptophan 2,3 dioxygenase is detected in the larval eyes at 15hpf (the earliest stage examined). Its' expression there persist until the adult eye start to develop and at stage 30hpf it is no longer detected in the larval eyes(See figure 13,I-L). At 48hpf it is detected in the adult eyes.

Tryptophan 2,3 dioxygenase is therefore the earliest marker for the development of the larval eyes, however it is also the most dynamic one, being down regulated in the LE at around 28hpf.

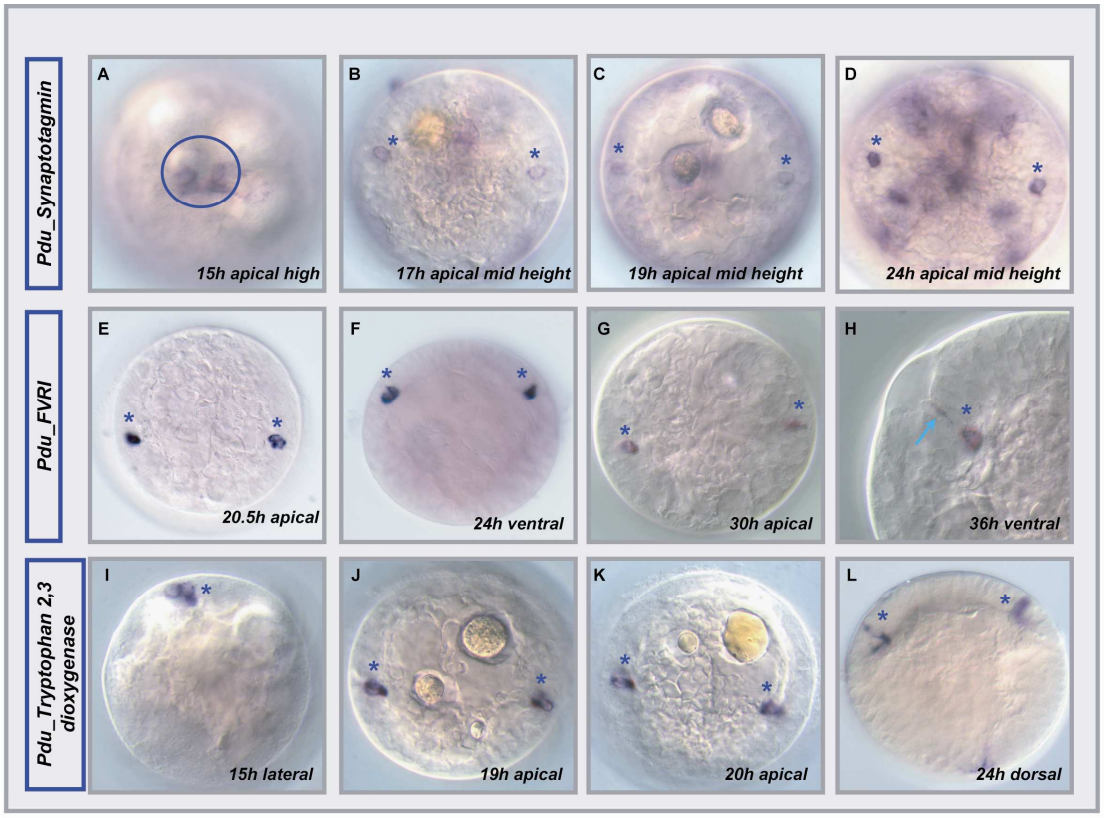


Figure 13. Larval eye markers at early stages of development.
(A-L) Whole mount *in-situ* hybridization with the larval eye markers indicated on the left side. Stage and orientation are indicated. Blue asterisks indicate the larval eye cells. 15h was the earliest stage included in the analysis.
According to the neuronal differentiation marker: **Synaptotagmine**, the larval eyes rPRC differentiate between stages 15h and 17h. At 15h there are very few cells stained in the apical organ region of the brain (A, marked by a blue circle). At 17h, the larval eyes are stained as well (B, indicated by blue asterisks). They continue to express Synaptotagmine as more other neurons differentiate in the brain (D).
The neuropeptide: **FVRI** is detectable in the larval eyes from stage 20.5h onwards (E-H). In (H) the dendrite of the FVRI, reaching the apical surface, is visible and pointed by an azure arrow.
The Ommochrome pigment marker: **Tryptophan 2,3 dioxygenase** is detectable in the larval eyes from stage 15h onwards (I-L). the latest detected expression in the larval eyes is at 28h. From this stage onwards this marker is expressed in the adult eyes.

2.3 Assigning the different eye markers to the different cell types of the adult eyes

In collaboration with:
Dr. Gaspr Jekely :
1. Confocal microscopy of double *in-situ* hybridization for r-opsin and FVRI.
Raju Tomer:
1. Wholemount *In Silico* Expression Profiling protocol (Raju Tomer and Detlev Arendt, unpublished data)

At that point of my research I had 3 additional candidate markers for the **adult eyes**: FVRI, Tryptophane 2,3 dioxygenase and Sepiapterin Synthase A. In order to confirm their expression in the adult eyes I have performed the following experiment:

1. Double *in-situ* hybridization of FVRI and r-opsin (the already established marker for the adult eyes at 48h) at **48h**. From this double *in-situ* hybridization we could see that FVRI is indeed expressed in the adult eyes (see figure 10,G). It is co-expressed with r-opsin and occasionally additional FVRI positive cells (that don't express r-opsin) are observed in close vicinity to the r-opsin positive cells. From this we concluded that FVRI is expressed in, and adjacent to the rhabdomeric photoreceptors of the adult eyes.

I then wanted to find out where do the two pigment markers expressed and therefore performed the following experiments:
2. Double *in-situ* hybridization of Tryptophane 2,3 dioxygenase and r-opsin. From this double *in-situ* hybridization we could see that Tryptophane 2,3 dioxygenase is expressed in the adult eyes (see figure 10,I). It is co-expressed with r-opsin and in additional cells (that don't express r-opsin and are found in higher confocal stacks then the r-opsin positive ones).
3. Double *in-situ* hybridization of Sepiapterin Synthase A and r-opsin. From this double *in-situ* hybridization we could see that Sepiapterin Synthase A is expressed in the adult eyes (see figure 10,H). It is co-expressed with r-opsin and in additional cells (that don't express r-opsin and are found in higher confocal stacks then the r-opsin positive ones).
From experiments 1,2 and 3 I concluded that :
A. The adult eye rhabdomeric photoreceptor cells express the following markers: r-opsin, FVRI and the two pigment synthesis markers: Tryptophane 2,3 dioxygenase and Sepiapterin Synthase A.
B. The two pigment synthesis markers: Tryptophane 2,3 dioxygenase and Sepiapterin Synthase A are also expressed in additional cells (not nesecarly the same ones) that are found somewhat above the rhabdomeric photoreceptor cells. I suspected these cells to be the adult eyes pigment cells (based on their location and the description of these cells by Rhode, 1992)

In order to see if the two pigment synthesis markers are co-expressed in the same cells I have performed the following experiments:
1. Double *in-situ* hybridization of Tryptophane 2,3 dioxygenase and Sepiapterin Synthase A, in two ways:

a. Using a florescent probe for Tryptophane 2,3 dioxygenase and a dig probe for Sepiapterin Synthase A.
b. Using a florescent probe for Sepiapterin Synthase A and a dig probe for Tryptophane 2,3 dioxygenase.

(Detecting the dig probe with NBT-BCIP and the florescent probe with florescent detection).

However, these genes are expressed relatively weak, the staining develops very slowly and by florescent detection I could not detect them. Therefore it was not possible to combine them in a double *in-situ* hybridization experiment.

Experiments 1-3 gave partial information regarding the cells that express each of the markers. However, it was almost impossible to correlate and integrate the information from the different double combinations in order to understand, at the cellular resolution, the complete 3D molecular-cellular composition of the adult eye.

To further resolve the co-expression data of the eye-specific genes in *Platynereies* adult eyes, together with Raju Tomer, we have integrated the gene expression information into the new Wholemount *in-silico* expression profiling protocol (Raju Tomer and Detlev Arendt, unpublished data) which allows the *in-silico* alignment of several single wholemount in-situ hybridization scans by image registration.
For the purpose of the adult eye analysis, I have scanned the Tryptophan 2,3 dioxygenase and Sepiapterin synthase A genes. Raju scanned the r-opsin and FVRI genes. Every 5 scans of a particular gene were integrated into an average scan and aligned to a reference embroy. Then the staining were combined in order to have the complete picture of the markers expression in the adult eyes.
For this I have used the "colocalization analysis - colocalization highlighter" plug-in in ImageJ software. By this one can visualize the co-expressing pixels of two different staining ('channels') with the possibility to control the threshold parameter. In addition, this plug-in also generates a new channel that contain the information from the co-expressing pixels exclusively (it is called: 'co localized points 8-bit').
The results were the following:

from combining **r-opsin and FVRI**: Most cells co-express both markers, a new 'channel' of co-expressing cells was created (the above mentioned 'co localized points 8-bit') to define the **photoreceptor cells (PRCs)**, they are found between stacks 38-63 of the scanned embryo marked by **r-opsin** staining (since FVRI expression is broader). With these stacks we could also see the larval eye r-opsin cell. (See figures 14,C and 15,A).

From combining **Tryptophan 2,3 dioxygenase + Sepiapterin synthase A**:
In the Upper stacks 24-34 **both** markers are expressed, in the same cells. In the deeper stacks only Tryp' 2,3 is expressed and in these deeper stacks, the newly formed PRCs 'channel' (see the section above) is also "expressed" (See figures: 14,C and 15,B-F)

Combining the data from EM of Platynereis eyes regarding the location of the pigment cells at 48hpf and their pigment granule composition (Platynereis pigment cells contain 2 types of membrane bound granules while the photoreceptor cells

contain 1 type of them,(Rhode 1992), I conclude that stacks 24-34 of **Sepiapterin synthase A** expressing cells represent the adult eyes **pigment cells.**

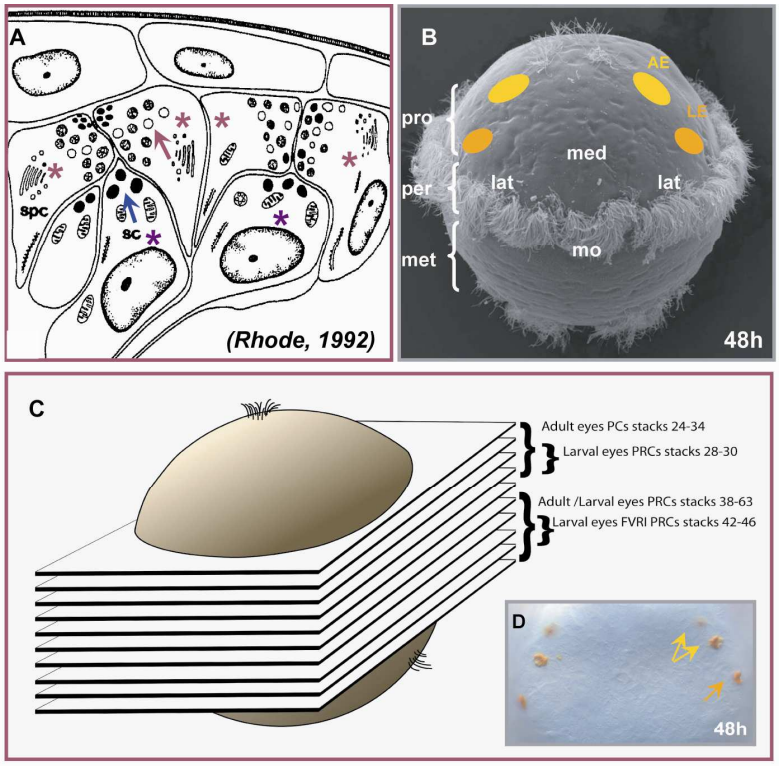


Figure 14. Assigning the spatial location of each of the eye cell types in the whole mount 3D *in-silico* model of 48h *Platynereis* embryo.
(A) Illustration based on TEM of eye from a 3-dyas-old *Platynereies* worm (Rhode, 1992). Blue asterisks indicating the rPRCc, purple asterisks indicating the pigment cells. Note that two pairs of pigment cells encloses two pairs of rPRC cells. In addition note that the pigment cells comprise two types (by appearance - more electron dense and less electron dense) of membrane bound pigment granule (marked by purple arrow) while the rPRCs comprise only one type. (B) Scanning electron microscopy (SEM, Dr. Harald Hausen) of *Platynereies* embryo. Pro, prostomium; Per, peristomium; met, metastomium; mo, mouth; lat, lateral; med, medial; AE, adult eyes; LE, larval eyes. (C) Illustration of the relative position of each of the eye cell types in the 3D whole mount *in-silico* model (PCs, pigment cells; PRCs, photoreceptor cells; stacks, confocal stacks). (D) An apical view of *Platynereis* embryo imaged by light microscopy with Nomarski optics showing the natural pigment of the adult eyes (two developing pairs indicated by orange arrows) and the larval eye (indicated by yellow arrow).

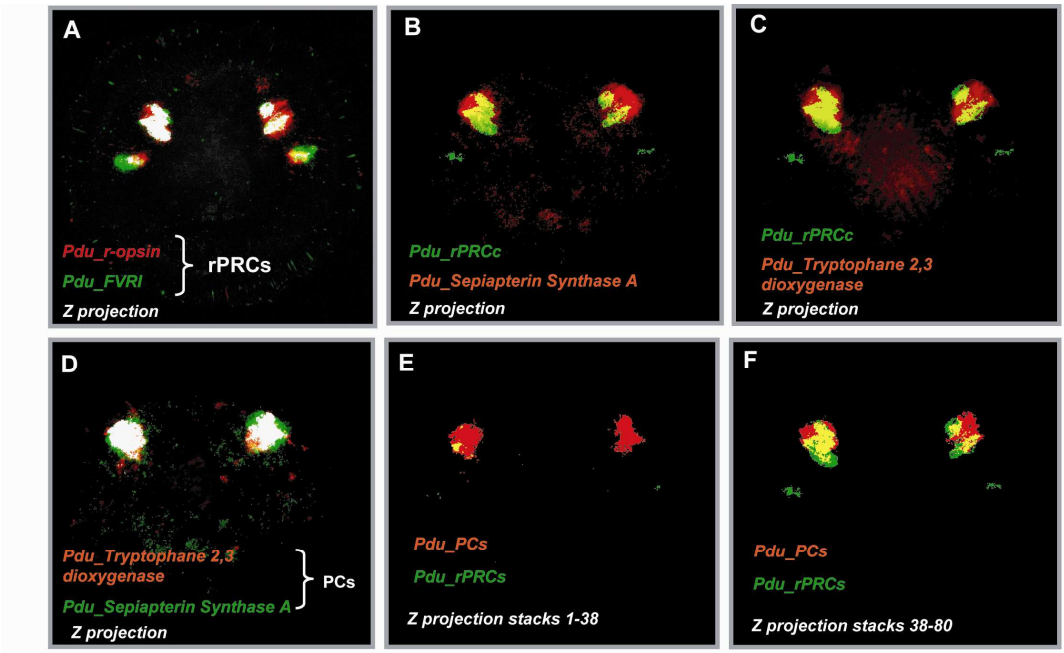


Figure 15. 3D whole mount *in-silico* expression profiling of the adult eye cell markers.
All images are z projection (maximum intensity) of a 48h embryo. White color indicate co-localization of the green and red channels in single stacks. Co-localization analysis was done by the "co-localization highlighter" plug-in for Image J software. By this plug-in we can created a new channel, representing the co-localized pixels exclusively. (A) Double *in-silico* expression analysis of the indicated probes showing that *Platynereis* rPRCs express both r-opsin and FVRI. We created a new channel of the co-localized pixels and named it "rPRCs". (B) Double *in-silico* expression analysis of the rPRCs from A and the pigment marker: Sepiapterin Synthase A. Yellow indicating co-expression, showing that *Platynereis* rPRCs express this marker as well. (C) Double *in-silico* expression analysis of the rPRCs from A and the second pigment marker: Tryptophane 2,3 dioxygenase. Yellow indicating co-expression, showing that *Platynereis* rPRCs express the second pigment marker as well. (D) Double *in-silico* expression analysis of both of the pigment markers. We created a new channel of the co-localized pixels and named it "PCs". (E,F) Double *in-silico* expression analysis of the both of the novel channels: the rPRCs and the PC. We noted that at stacks 1-38 only the PCs is expressed (E) while in the following, deeper stacks, **both** the PCs and the rPRCs are expressed (F). We therefore define stacks **1-38** as representing the **PCs** and stacks **38-80** as representing the **rPRCs**.

2.4 Establishing a molecular fingerprint of *Platynereis* adult and larval eyes

In collaboration with:
Dr. Gaspr Jekely :
1. Several joint confocal microscopy imaging of double *in-situ* hybridization for different transcription factors and eye markers were done by us, as a process of teaching me the confocal microscopy tools
Raju Tomer:
1. Wholemount *In Silico* Expression Profiling protocol (Raju Tomer and Detlev Arendt, unpublished data)
2. Hierarchical clustering analysis

Once the appropriate markers were found (effector genes) to each of the cell types of both adult and larval eyes I could start establishing the molecular fingerprint of them in terms of transcription factors. The transcription factors that were included in the analysis were cloned by current and previous members of the Arendt lab or found in the ESTs collection. We choose the set of TFs that will be analyzed according to the following criteria:

a. TFs known to be involved in eye development (e.g. Otx,Pax6)
b. TFs that have a role in brain regionalization (e.g. Lhx, Rx)
c. TFs that have a role in neuronal differentiation (Islet)
d. Signaling pathways molecules (e.g: Wnt, Hh)
e. Neuronal specification markers (AchRec7/8, GLT1)

Since Platynereis is an emerging model organism the methods I have used were also evolving during the time of my research. I have used, with this order, the following techniques:

1. Double fluorescent in-situ hybridization (Tessmar-Raible, Steinmetz et al. 2005).
2. Wholemount reflection confocal microscopy (Jekely and Arendt 2007).
3. 3D *in silico* expression profiling protocol (Raju Tomer and Detlev Arendt, unpublished).

The preliminary molecular fingerprint profile had emerged from the second technique and included a set of 14 transcription factors (TFs). The reflection confocal microscopy enables the detection of NBT-BCIP precipitate at the confocal microscopy in combination with a fluorescent staining.
In my experiments I have used the following combination:

1. A Dig probe for the transcription factor of interest and detected it with NBT-BCIP.
2. A Flu-probe for the eye marker of choice that was detected with the TSA fluorescent Systems (Perkin Elmer). (See figure16 and 17 for adult and larval eye examples, respectively)

By this I could also analyze weakly expressed TFs that would not have produce proper staining with a fluorescent probe.

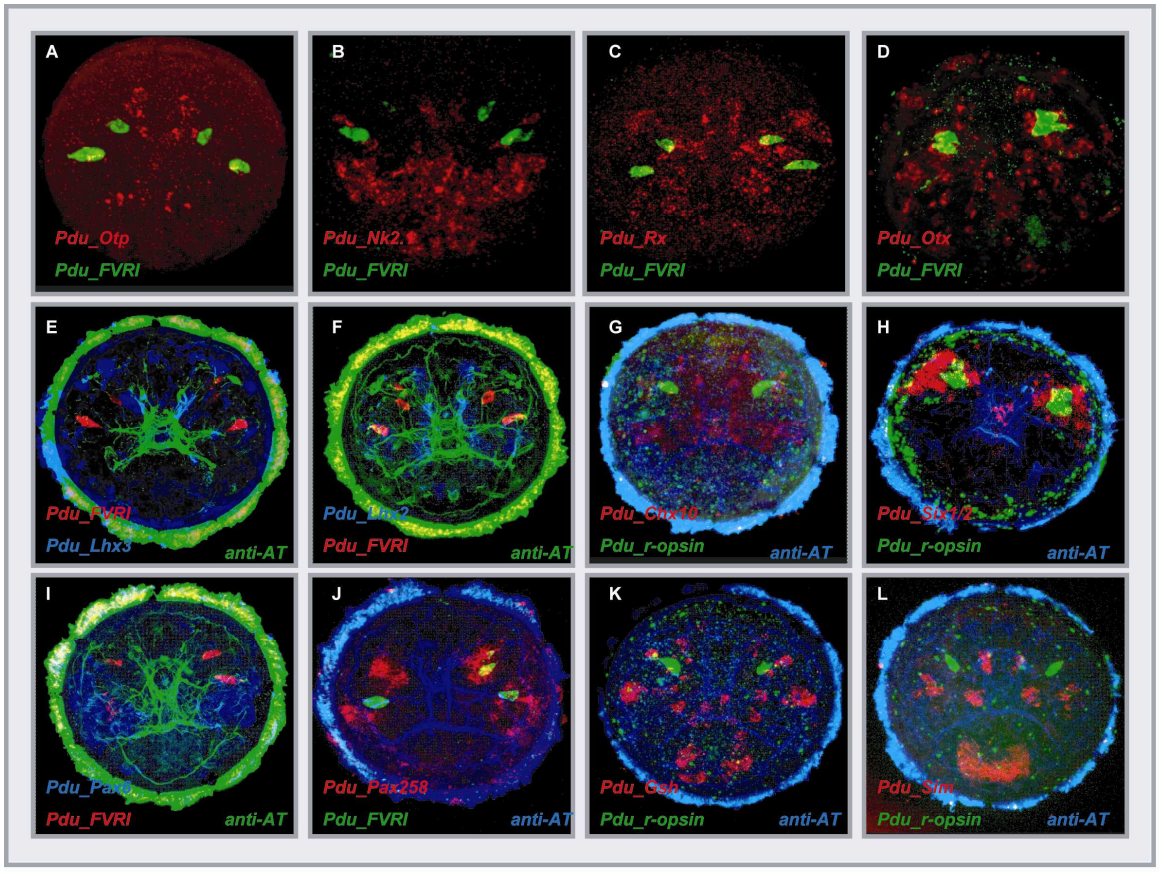


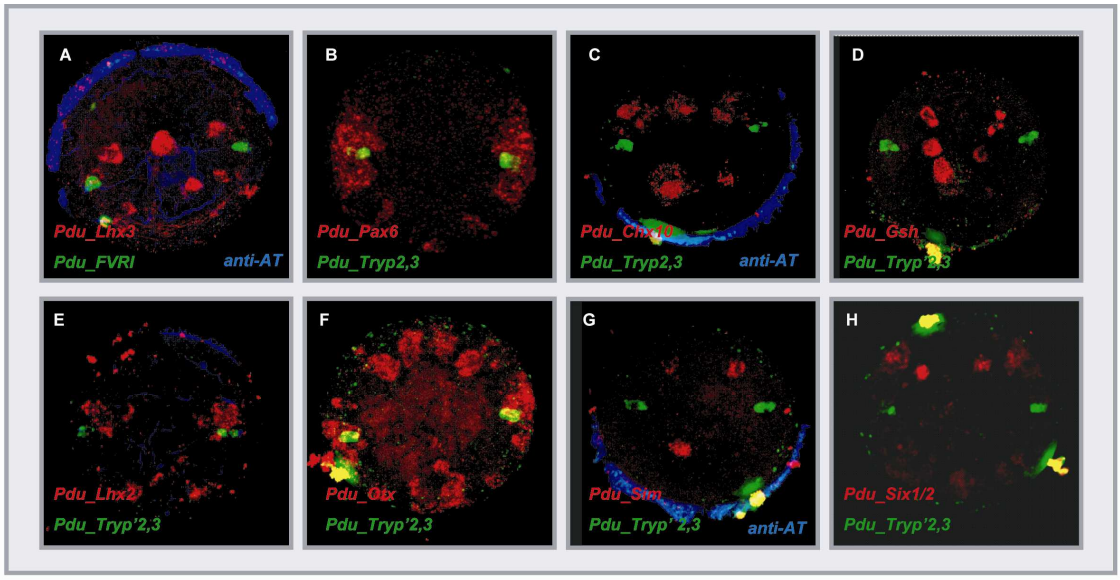
Figure 16: Wholemount double *in-situ* hybridizations of several transcription factors and adult eye rPRCs markers.

(A-L) Z-projections, apical orientations, made of confocal scans of the brain.

(A-D) Wholemount double **florescent *in-situ*** hybridizations with indicated probes on 48hpf embryos.

(E-L) Wholemount double *in-situ* hybridizations with indicated probes on 48hpf embryos ,using the reflection confocal microscopy method. For these *in-situ* hybridizations I have used the dig robe and NBT-BCIP for detecting the transcription factor, and a Fluorescein probe and the TSA fluorescent Systems for detecting the eye marker. Anti acetylated tubulin antibody was also included in these assays, as indicated in the figure.

Co-expression analysis was done on a single stack level. Note that the quality of the staining of the transcription factor varies due to different expression intensities. In addition, the Anti acetylated tubulin antibody staining was not yet optimized at this stage of my work, and therefore it also exhibits variable quality. This improved a lot by using the 3D in-silico alignment protocol for these staining.



I

Microsoft Excel - In Situ Data TFs.xls

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S17

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1		Nk2.1	Otp	Rx	Pax258	Chx10	Six1/2	Pax6	Gsx	Sim	Lhx3	Lhx2	Otx	Dach	Atonal
2	FVRI adult eyes	n	n	p*	p*	n		n		n &&&	p*	n	p	n&	
3	FVRI larval eyes	n^	p		n*	n	n	p		n/p *&&&	p*	p	p	n&	n
4	r-opsin adult eyes			n	n	n	n*		n	n			p		
5	r-opsin larval eyes			n	p*		n*	p	n	p *			p		
6	Tryp 2,3 Adult eyes		n	p			p						p	p &&	
7	Tryp 2,3 larval eyes	**	**		n	n	n	p	n	n	n	n	p		
8															
9															
10	Legend:	comments													
11	24h	The combination of rx/six12/otx for pigment cells also came out using *in-silico* alignment, independently.													
12	n negative														
13	p positive														
14	* not a clear case														
15	** roled out by position														
16		in-situ is in process													
17		will be analysed according to FVRI/r-opsin													
18	& at 54h however the apical dorsal FVRI do express Dach and all the surronding cells of the adult eyes FVRI														
19	&& at 54h and only one Tryp cell is positive														
20	*&&& at 54h, the tip of the cells are positive...														
21	^but 2 cells next to FVRI are positive, mediall and dorsal to it.														

Figure 17: Wholemount double *in-situ* hybridizations of several transcription factors and larval eyes cells markers.

(A-H) Z-projections, apical orientations, made of confocal scans of the brain. Wholemount double *in-situ* hybridizations with indicated probes on 24hpf embryos ,using the reflection confocal microscopy method. For these *in-situ* hybridizations I have used the dig robe and NBT-BCIP for detecting the transcription factor, and a Fluorescein probe and the TSA fluorescent Systems for detecting the eye marker. FVRI served as a marker for the larval eye rPRC and Tryptophan 2,3 dioxygenase (Tryp' 2,3) was used as a marker for the larval eye pigment cell.

Anti acetylated tubulin antibody was also included in A,C and G. However we found out that the nervous system at this stage is not yet well developed. In A, for example, one can note some axons, but this is rather rare. In the others one can note the ciliated prototroph ring stained.

(I) An example for data collection as was done by me for the double *in-situ* hybridizations, showing that in many cases, I got unresolved answer or had to repeat the experiments since Flurescein probes don't always perform well. Another cause for uncertainty is the shading effect of NBT-BCIP staining on a florescent staining that also ended up in unresolved cases.

There are however disadvantages for this method. Especially when one is aiming for as large as possible collection of TFs to be included. The main disadvantages are:

1. Weakly expressed genes do not perform well with fluorescent staining.
2. Shading effect - the NBT-BCIP precipitate can shade a fluorescent staining which is found in the same cell or in cells that are deeper. This can cause a lot of border line cases when looking for co-expression.
3. Laborious - limited number of genes can be analyzed.

In light of these disadvantages, it was clear that the Wholemount *In Silico* Expression Profiling protocol (Raju Tomer and Detlev Arendt, unpublished data) will be of a great benefit for the completion of the molecular fingerprint of *Platynereies* eyes cell types.
Therefore, together with Raju Tomer, we chose a set of 37 genes according to the above mentioned criteria. These genes were scanned by Raju Tomer (Prox1, Gli-1 and Sufu were scanned by me) and by combining them with the eye markers gene scans, we have obtained the molecular fingerprint of 6 different cell types of *Platynereies* eyes.
The generation of the double *in-silico* scans was automated using an algorithm written by Raju Tomer. For this we have defined the stacks range and the appropriate marker for each cell type (as mentioned before), these sub-series were then combined with the sub-series of 37 selected genes.
The algorithm generated two types of data:

1. avi. movies.
2. Z projection pictures of the double *in-silico* combinations:
   See the following figures for examples of:
   MFP of AE rPRCs - figure 18.
   MFP of AE PCs - figure 19.
   MFP of LE PC - figure 20.
   MFP of LE rPRC - figure 21.

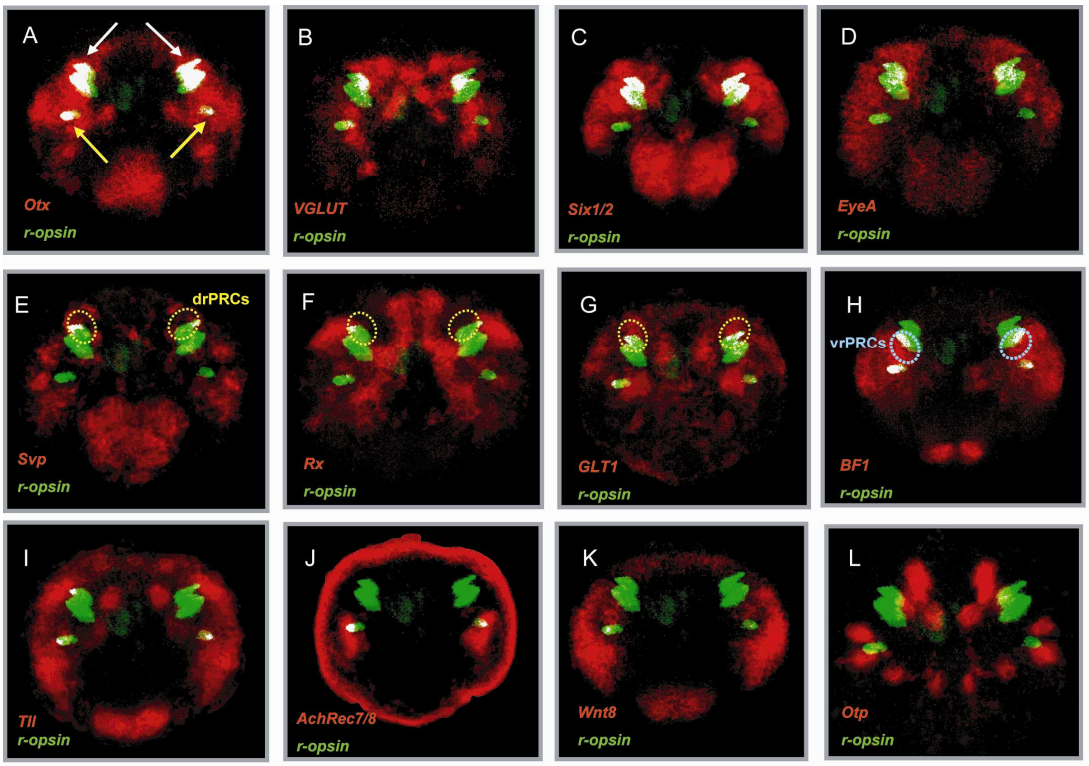


Figure 18. 3D whole mount *in-silico* expression profiling of the adult eye rPRCs - MFP
All figures are 3D *in-silico* alignments of the indicated probes on a 48h embryo. All figures are z projections, (maximum intensity), apical views, of the stacks representing the rPRCs (38-80). white colure indicates co-localization according to the co-localization highlighter plug-in of Image J. In all figures, as noted in figure A, adult eyes are indicated by the white arrows, larval eyes indicated by the yellow arrows.
(A-D) Examples for co-expression of the indicated TF and r-opsin in the mentioned stacks. VGLUT, Vesicular glutamate transporter; EyeA, Eyes Absent. (E-H) Examples for a **differential** co-expression of the indicated TF and r-opsin in either the dorsal rPRCs (E,F,G) - The positive cells are circled by a yellow dashed circle. Or the ventral rPRCs (H) - The positive cells are circled by a blue dashed circle. By the observation that the dorsal and ventral rPRCs have different MFP we have defined the adult eyes rPRCs as comprising two cell types: **dorsal rPRCs** (drPRCs) and **ventral rPRCs** (vrPRCs). Svp, Seven up; Rx, Retinal Homeobox; GLT1, Glutamate transporter 1; BF1, Brain factor 1. (I-L) examples of TFs not expressed in the adult eyes rPRCs. Tll, tailless; AchRec7/8, Acetyl choline receptor 7/8; Otp, Orthopedia.

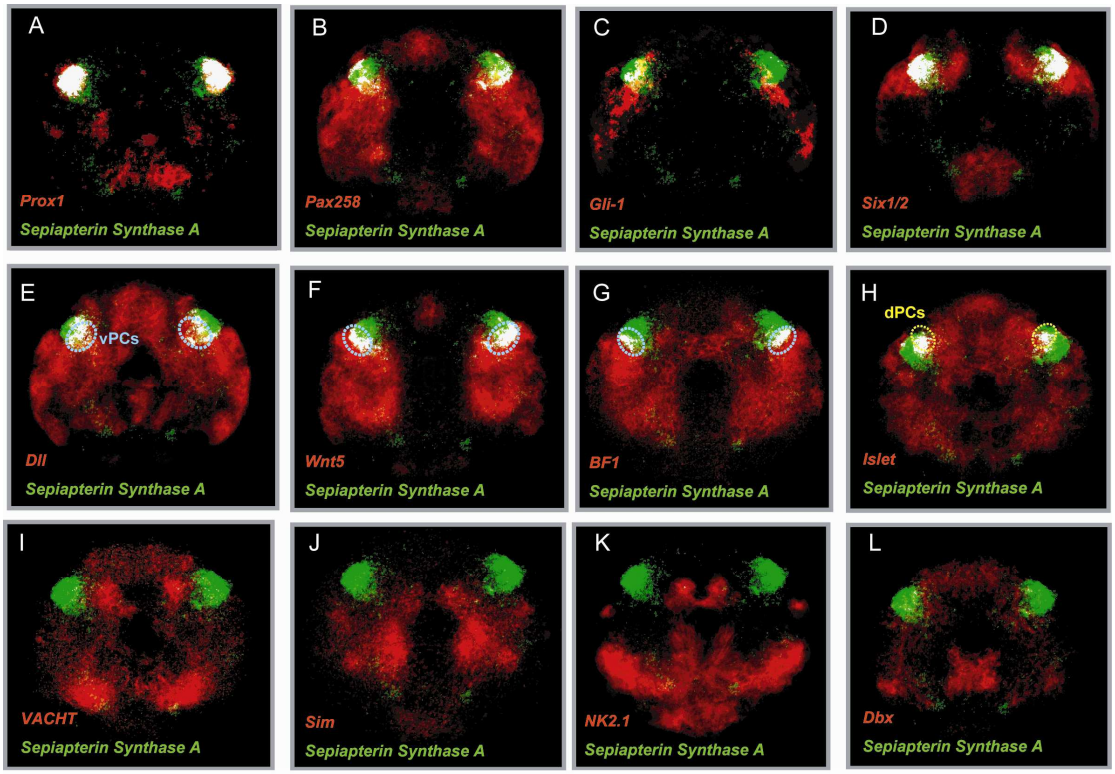


Figure 19. 3D whole mount *in-silico* expression profiling of the adult eye PCs - MFP
All figures are 3D ***in-silico*** alignments of the indicated probes on a 48h embryo. All figures are z projections (maximum intensity), apical views ,of the stacks representing the PCs (1-38). White colure indicates co-localization according to the co-localization highlighter plug-in of Image J.
(A-D) Examples for co-expression of the indicated TF and Sepiapterin Synthase A in the mentioned stacks. (E-H) Examples for a **differential** co-expression of the indicated TF and Sepiapterin Synthase A in either the ventral PCs (E,F,G)- the positive cells are circled by a blue dashed circle. Or the dorsal PCs (H) - the positive cells are circled by a yellow dashed circle. By the observation that the dorsal and ventral PCs have different MFP we have defined the adult eyes PCs as comprising two cell types: dorsal PCs (**dPCs**) and ventral PCs (**vPCs**). Dll, Distal-less; BF1, Brain factor-1. (I-L) examples of TFs not expressed in the adult eyes PCs. VACHT, vesicular acetylcholine transporter; Sim, single-minded; Dbx, developing brain homeobox.

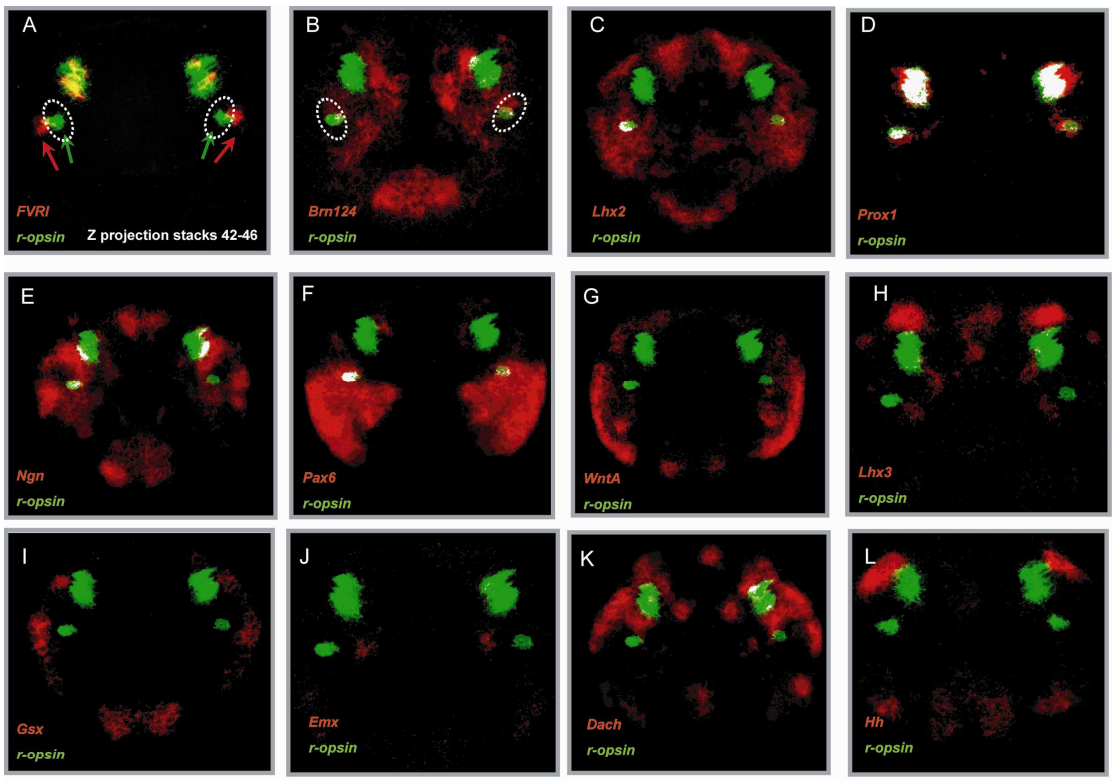


Figure 20. 3D whole mount *in-silico* expression profiling of the larval eye (LE) r-opsin positive cell (the pigment cell - PC) - MFP

(A) *in-silico* alignment of FVRI and r-opsin probes at 48h. Z projection of stacks 42-46. These stacks were chosen for the analysis of the MFP of the larval eyes since they comprise the cell bodies of both the LE FVRI cell (pointed by a red arrow) and the LE pigment cell (pointed by a green arrow). The cells analyzed in this section are circled by a dashed white circle (A, B).

(B-L) All figures are 3D in-silico alignments of the indicated probes on a 48h embryo. All figures are z projections (maximum intensity), apical views ,of the stacks representing the LE PC (42-46). White colure indicates co-localization according to the co-localization highlighter plug-in of Image J. (B-F) Examples for co-expression of the indicated TF and LE r-opsin in the mentioned stacks. Ngn, Neurogenin. (G-L) Examples of TFs not expressed in the LE r-opsin cell. Dach, Dachshund; Hh, Hedgehog.

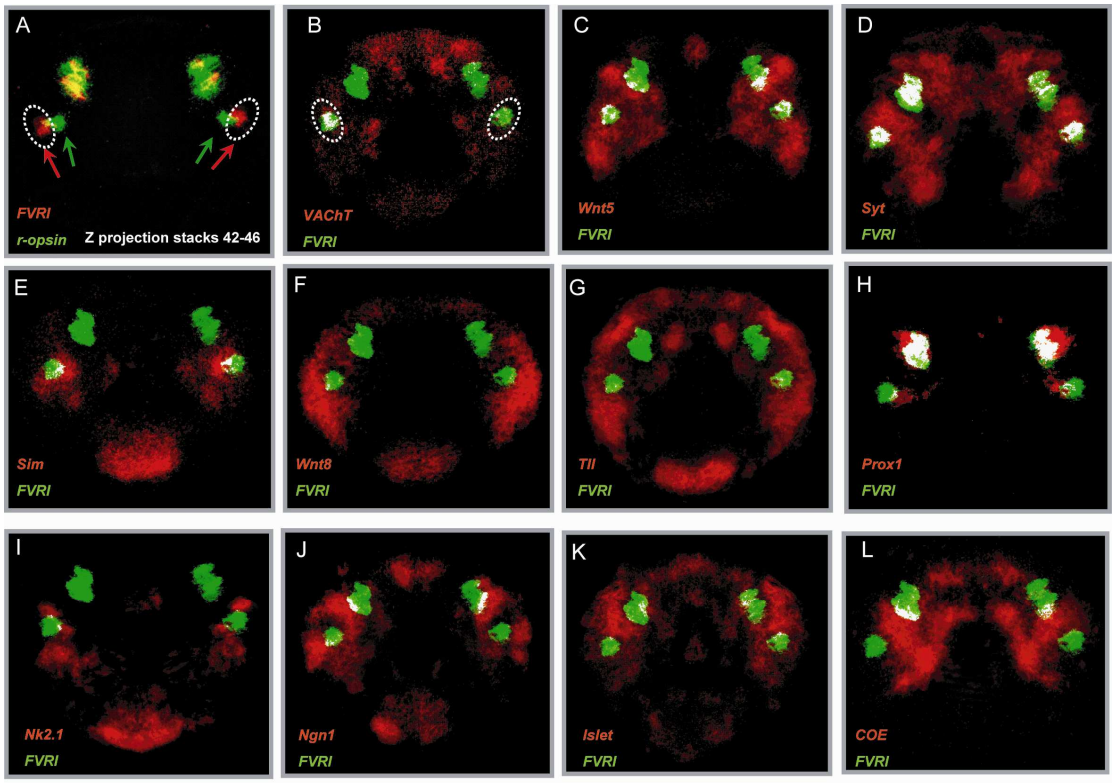
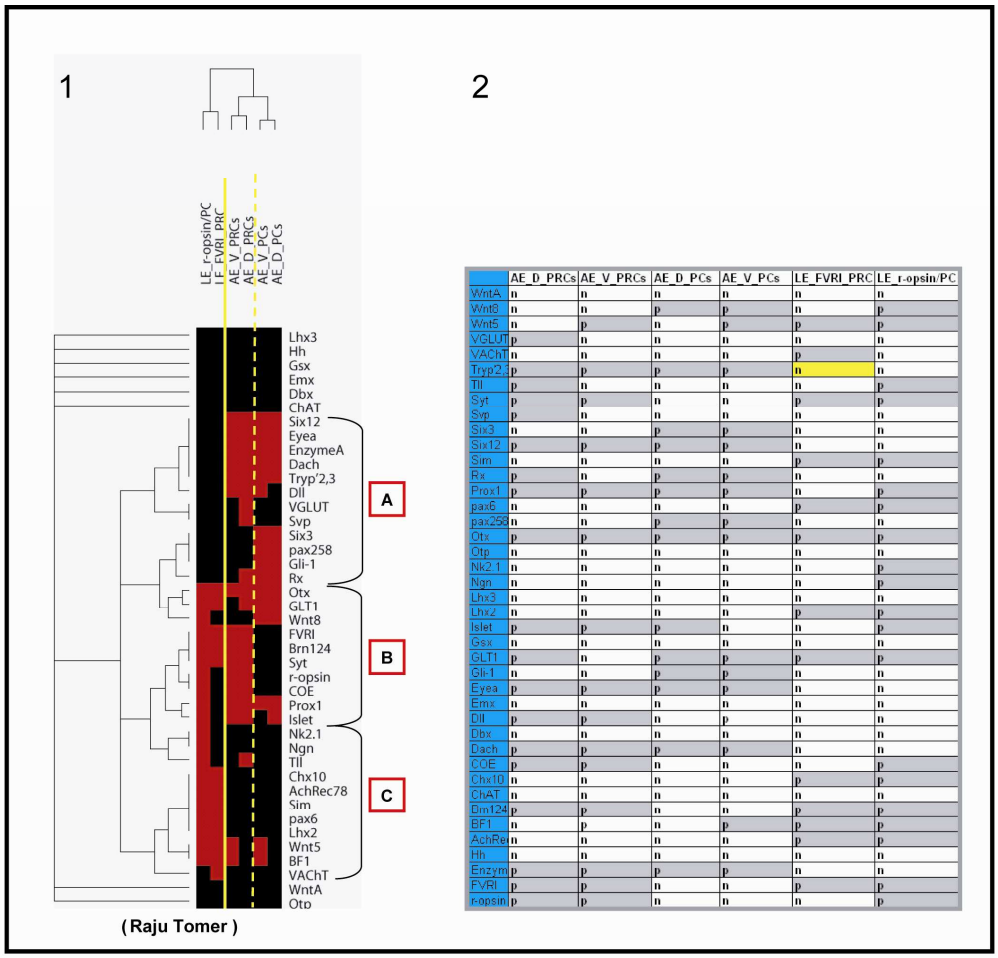


Figure 21. 3D whole mount *in-silico* expression profiling of the larval eye (LE) FVRI cell (the rhabdomeric photoreceptor cell, rPRC) - MFP

(A) *in-silico* alignment of FVRI and r-opsin probes at 48h. Z projection of stacks 42-46. these stacks were chosen for the analysis of the MFP of the larval eyes since they comprise the cell bodies of both the LE rPRC cell (pointed by a red arrow) and the LE pigment cell (pointed by a green arrow). The cells analyzed in this section are circled by a dashed white circle (A, B).

(B-L) All figures are 3D in-silico alignments of the indicated probes on a 48h embryo. All figures are z projections (maximum intensity), apical views ,of the stacks representing the LE rPRC (42-46). White colure indicates co-localization according to the co-localization highlighter plug-in of Image J. (B-E) Examples for co-expression of the indicated TF and LE FVRI in the mentioned stacks. VAChT, Vesicular acetylcholine transporter; Syt, Synaptotagmin; Sim, Single-minded. (F-L) Examples of TFs not expressed in the LE rPRC cell. Note that in these examples (F-L) the indicated TFs **are expressed in the LE PC** but **not in the LE rPRC**. Tll, Tailless; Ngn, Neurogenin; COE, Collier.

I visually analyzed the 148 combinations and constructed a raw data table of co-expression versus non co-expression results. (figure 22)
Using the data from this table, Raju Tomer performed a hierarchical clustering analysis of the molecular finger print. The clustering and its' analysis are presented in figures 22 and 23.



	AE_D_PRCs	AE_V_PRCs	AE_D_PCs	AE_V_PCs	LE_FVRI_PRC	LE_r-opsin/PC
WntA	n	n	n	n	n	n
Wnt8	n	n	p	p	n	p
Wnt5	n	p	n	p	p	p
VGLUT	p	n	n	n	n	n
VAChT	n	n	n	n	p	n
Tryp'2,3	p	p	p	p	n	n
Tll	p	n	n	n	n	p
Syt	p	p	n	n	p	p
Svp	p	n	n	n	n	n
Six3	n	n	p	p	n	n
Six12	p	p	p	p	n	n
Sim	n	n	n	n	p	p
Rx	p	n	p	p	n	n
Prox1	p	p	p	p	n	p
pax6	n	n	n	n	p	p
pax258	n	n	p	p	n	n
Otx	p	p	p	p	p	p
Otp	n	n	n	n	n	n
Nk2.1	n	n	n	n	n	p
Ngn	n	n	n	n	n	p
Lhx3	n	n	n	n	n	n
Lhx2	n	n	n	n	p	p
Islet	p	p	p	n	n	p
Gsx	n	n	n	n	n	n
GLT1	p	n	p	p	p	p
Gli-1	n	n	p	p	n	n
Eyea	p	p	p	p	n	n
Emx	n	n	n	n	n	n
Dll	p	p	n	p	n	n
Dbx	n	n	n	n	n	n
Dach	p	p	p	p	n	n
COE	p	p	n	n	n	p
Chx10	n	n	n	n	p	p
ChAT	n	n	n	n	n	n
Brn124	p	p	n	n	p	p
BF1	n	p	n	p	p	p
AchRe	n	n	n	n	p	p
Hh	n	n	n	n	n	n
Enzym	p	p	p	p	n	n
FVRI	p	p	n	n	p	p
r-opsin	p	p	n	n	n	p

Figure 22. Hierarchical clustering analysis (1, Raju Tomer) done on the raw expression data shown in the table (2).
In 1 and 2: LE r-opsin/PC, Larval eye r-opsin/pigment cell; LE FVRI/PRC, Larval eye FVRI/photoreceptor cell; AE_V_PRCs, Adult eyes ventral photoreceptor cells; AE_D_PRCs, Adult eyes dorsal photoreceptor cells; AE_V_PCs, Adult eyes ventral pigment cells; AE_D_PCs, Adult eyes dorsal pigment cells.
In 1: Note that the continuous yellow line draws a border between the larval and the adult eyes while the dashed line draws a border between the adult eyes PRCs and adult eyes PCs (in order to facilitate the overview of the analysis). Red square indicates positive for expression, black square indicates negative for expression.
In 2: The square highlighted in yellow indicates a known dynamic expression pattern - the LE FVRI/PRC expresses Tryptophan 2,3 dioxygenase at 24h but not at 48h. We included it as negative in order to be consistent with the chosen 48h stage.
Note that in the tree made from the clustering, the adult eyes cell types cluster together, so do the larval eye cell types, indicating that these eyes are different eye types, controlled by **different molecular mechanisms**. In order to further explore this finding I have grouped the analyzed genes into 3 groups, as indicated by A,B and C (1). This will be further discussed in the following figure (**figure 23**).

A first observation was the different signature of **dorsal** rPRCs vs. **ventral** rPRCs. And the same case for the PCs. Therefore the adult eyes are composed of 4 cell types (in terms of molecular fingerprint) and the larval eyes contain 2 cell types (rPRC and pigment cell).
Other features of the MFP (see figure 23) : Group A of genes is expressed (among the cell types included in this analysis) only in the adult eyes and not in the larval eyes. Sub-group A.1 are genes that expressed in both the AE PRC and the AE PC as apposed to sub-groups A.2 and A.3 that are expressed only in the AE PRCs and the AE PC, respectively.
Group B represents genes that are shared in expression between the adult and larval eyes. Note that Otx is expressed in all cell types of both AE and LE. The genes in sub-group B.1 (r-opsin and COE,) are expressed in the PRCs of both AE and LE.
Group C of genes is expressed (among the cell types included in this analysis) only in the larval eyes and not in the adult eyes (with the exception of Tll, Wnt5 and BF1). In this group we can distinguish between 4 sub-groups: C.1: genes that are expressed only in the LE r-opsin/PC. C.2: genes that are expressed in both cells of the larval eye. C.3: genes that are expressed in both cell types of the larval eyes as well as in the ventral adult eyes PRCs and ventral adult eyes PCs. C.4: (VAChT) is expressed only in the LE FVRI cell. Interpretation and conclusions of these results is discussed in the "Discussion" chapter.

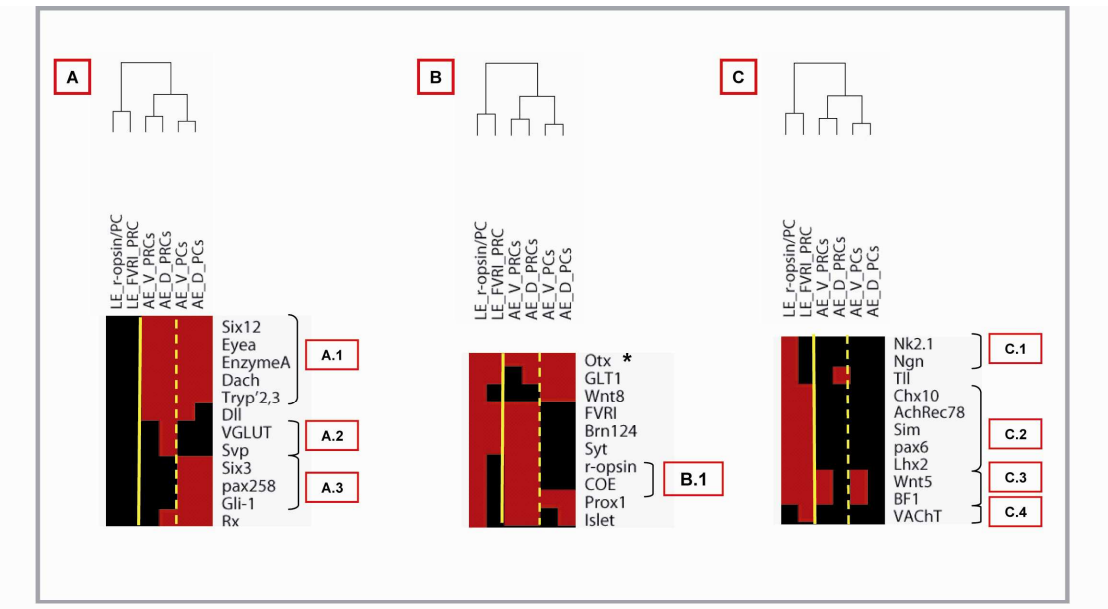


Figure 23. Hierarchical clustering analysis (Raju Tomer) - detailed view.
(A) This group of genes is expressed (among the cell types included in this analysis) only in the adult eyes and not in the larval eyes. (A.1) this sub-group of genes are expressed in both the AE PRC and the AE PC as apposed to sub-groups (A.2) and (A.3) that are expressed only in the AE PRCs and the AE PC, respectively.
(B) This group represents genes that are shared in expression between the adult and larval eyes. Note that Otx is expressed in all cell types of both AE and LE. The genes in sub-group B.1 (r-opsin and COE,) are expressed in the PRCs of both AE and LE.
(C) This group of genes is expressed (among the cell types included in this analysis) only in the larval eyes and not in the adult eyes (with the exception of Tll, Wnt5 and BF1). In this group we can distinguish between 4 sub-groups: C.1 genes that are expressed only in the LE r-opsin/PC. C.2 genes that are expressed in both cells of the larval eye. C.3 genes that are expressed in both cell types of the larval eyes as well as in the ventral adult eyes PRCs and ventral adult eyes PCs. C.4 (VAChT) is expressed only in the LE FVRI cell.

3 A role for the hedgehog signaling pathway in Pdu eyes development (In collaboration with Dr. Kristin Tessmar-Raible)

3.1 The expression of hedgehog, Gli-1, Smo and sufu

The following Platynereis hedgehog (Hh) pathway molecules were cloned in the lab by Dr.Kristin Tessmar-Raible and Fay Christodoulou: Sonic hedgehog (Hh) , Gli-1, Smoothened (Smo) and suppressor of fused (Sufu).
Dr.Kristin Tessmar-Raible and me looked at their expression pattern (by wholemount *in-situ* hybridization) around the start and end of the inhibition with Cyclopamine: 24,38 and 50hpf. (See Figure 24)

Smoothened expression:
At 24hpf it is expressed in 2 cells in the dorsal brain. At 38hpf it is expressed in the mentioned dorsal brain cells, 2-3 on each side. In addition expression in the ventral lateral brain is observed which is deeper then the dorsal brain staining. The ventral cord is also stained. At 50hpf the expression follows the one of 38hpf. The dorsal brain regions are now found closer to the ventral lateral ones (See figure 24,A-D).

Hh expression:
At 24hpf, Hh is expressed at symmetrically dorsal regions of the brain (in vicinity to the future adult eye anlage) and weakly in the developing stomodeum.
At 38hpf it is expressed in the mentioned dorsal brain regiones, in the stomodeum and in horizontal strips at the ventral plate.
At 50hpf it is expressed weakly at a very superficial medial brain area, in the mentioned dorsal brain regions, in the stomodeum and in symmetrically ventral brain regions (See figure 24,E-H).

Gli-1 expression:
We looked at Gli-1 expression as early as 19hpf, it is expressed at 2 posterior-dorsal cells. At 24hpf it starts to be expressed in the brain in a weak, diffused like fashion. At 38hpf its brain expression is gathered into two symmetrically dorsal-medial regions, between the adult and larval eye anlage, but quite superficial. It is also expressed weakly in the dorsal half of the stomodeum, and in the ventral plate – in horizontal stripes (ladder like). At 50hpf the expression is similar to the one of 38hpf and additional ventral lateral regions, deeper then the dorsal ones are also observed. (See figure 24, I-L).

Sufu expression:
We looked at Sufu expression as early as 19hpf, it is expressed in 2 apical cells. At 38hpf there are two small groups of expression in the brain: the one is in the dorsal medial brain, relatively deep. The second is in the region of the apical organ and very superficial. It is also expressed in few cells at the medial dorsal region. At 50hpf the expression remains as at 38hpf (See figure 24,M-P).

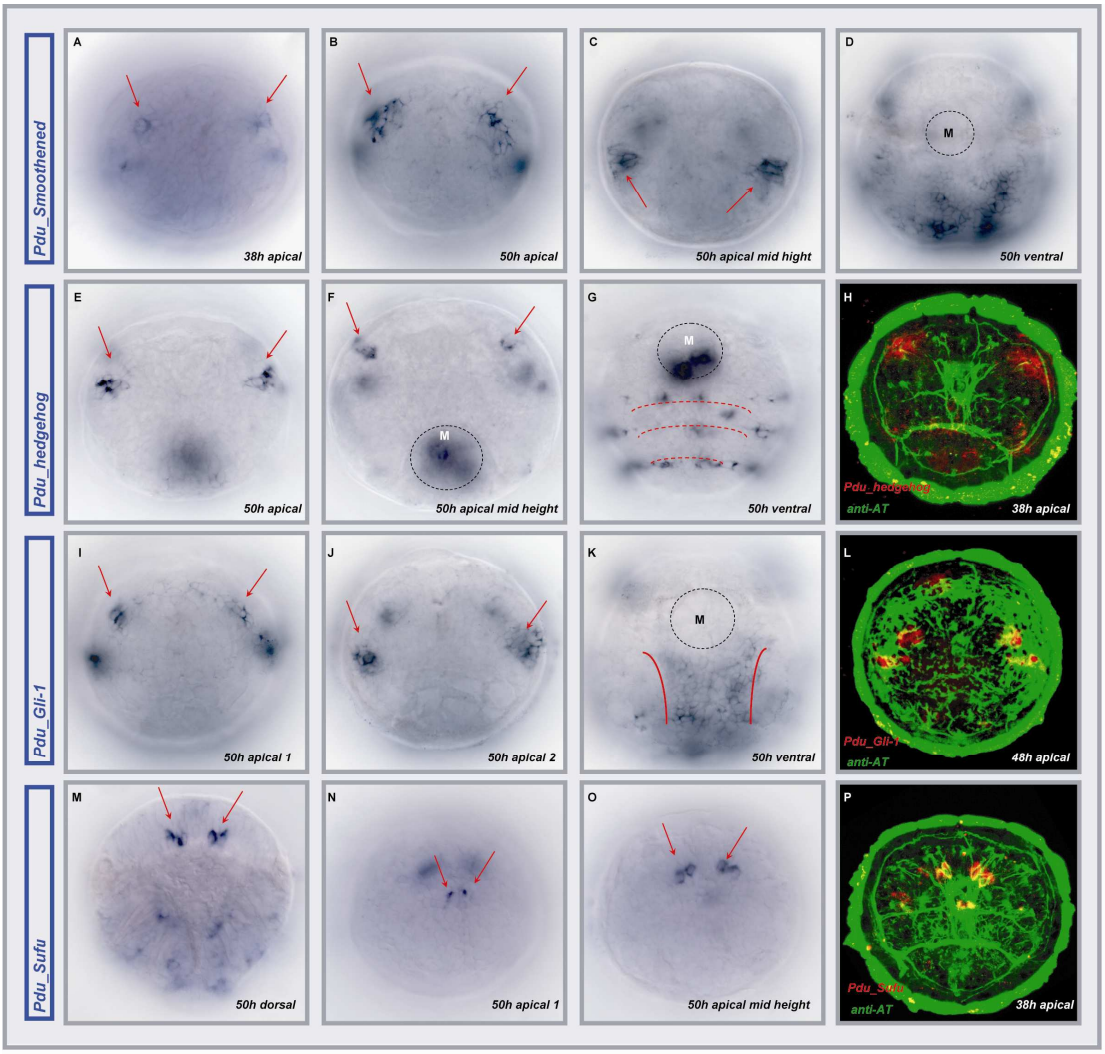


Figure 24. Expression patterns of *Platynereis* Hedgehog signaling pathway molecules.
(A-L) whole mount *in-situ* hybridization with the probes indicated on the left side. Stage and orientation are indicated. (H,L,P) Z projections of whole mount *in-situ* hybridization combined with anti-acetylated tubulin antibody scanned by confocal microscopy. (A-D) **Smoothened** expression: Note the two symmetrical expression domains in the brain, one in the dorsal brain (B, pointed by red arrows) the second in the medial brain, deeper then the first (C, pointed by red arrows). Both expressed also earlier (A). It is also expressed in the ventral nerve cord (D). **Hedgehog** expression: Note the two symmetrical expression domains in the dorsal brain, higher one (E, pointed by red arrows) and deeper one (F, pointed by red arrows). Note the expression in the stomodeum (F, marked by a dashed circle. M=mouth opening). Note the transverse stripes of expression in the ventral plate (G, marked by red dashed lines). **Gli-1** expression: note the two symmetrical expression domains in the brain, one in the dorsal brain (I, pointed by red arrows) the second in the medial-dorsal brain, deeper then the first (J, pointed by red arrows). Note the expression in the ventral plate (K, bounded by red lines. M=mouth opening). **Sufu** expression: note the restricted expression domains in the region of the apical organ. The most superficial expressing cells are shown in N (N, pointed by red arrows). The deeper ones are shown in M and O (pointed by red arrows). Additional expressing cells are found on the dorsal side (M).

In order to get more insight about the identity of the cells that express these genes and about co-expression of the pathway genes, I have started the process of inserting them into the pipeline of the Wholemount In Silico Expression Profiling protocol (Raju Tomer and Detlev Arendt, unpublished, currently available for 48hpf stage).
Gli-1 was scanned by me (x5) and Hh was scanned by Raju Tomer (x5). These genes were included in the molecular fingerprint analysis of the *Platynereis* eyes.

The following double *in-silico* alignment on 48h stage were done:

1. Gli-1 and Hh. From this alignment we could see that Gli-1 and Hh are co-expressed in a group of cells located at the dorsal lateral brain, in a region adjacent to the adult eye anlage. See figure 25,A.
2. Gli-1 and r-opsin. From this alignment we could see that Gli-1 and r-opsin are not co-expressed. See figure 25,B.
3. Sepiapterin synthase A and Hh. From this alignment we could see that Sepiapterin synthase A and Hh are not co-expressed. See figure 25,C.
4. Sepiapterin synthase A and Gli-1. From this alignment we could see that Sepiapterin synthase A and Gli-1 are co-expressed in the adult eyes pigment cells (found in stacks 24-37). See figure 25,D

Sufu and Smo are currently being processed and scanned and will also be included in the database.

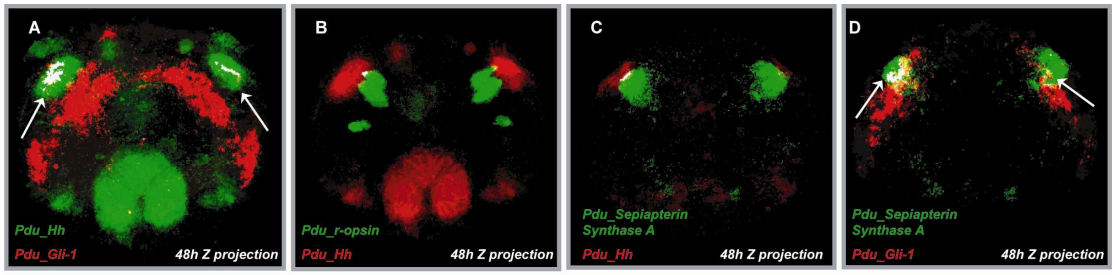


Figure 25. 3D *in-silico* alignments of Hh, Gli, r-opsin and Sepiapterin Synthase A.
All images are Z projection, Maximum intensity of different stacks (according to the cells analyzed, mentioned for each picture individually). White colure indicates co-localization according to co-localization highlighter plug-in of Image J. (A) *in-silico* alignment of indicated probes. Z projection comprising the whole brain (down to the level of the stomodeum). showing that cells in the dorsal lateral brain (pointed by white arrows), in close vicinity to the developing adult eyes, co-express hedgehog and the receptor Gli-1. the co-expressed cells are found in stacks 36-55 in which the PRCs are also present. (B) *in-silico* alignment of indicated probes, Z projection of stacks 38-63 (stacks representing the rPRCs with r-opsin). No co-expression is observed. (C) *in-silico* alignment of indicated probes, Z projection of stacks 24-37 (stacks representing the PCs with Sepiapterin Synthase A). No co-expression is observed. (D) *in-silico* alignment of indicated probes, Z projection of stacks 24-37 (stacks representing the PCs with Sepiapterin Synthase A). Note that the AE PCs co-expressing the receptor Gli-1 (indicated by white arrows).

3.2 Cyclopamine inhibition results

We have used Cyclopamine to block the Hh pathway in *Platynereis*. The method, drug concentration and the inhibition periods are described in “Materials and Methods” section 4.4.
For the analysis of larval eye development, larvae were fixed at the end of the inhibition and using wholemount *in-situ* hybridization I have looked at larval eye markers and potential regulators.

The markers I have used were:
FVRI
Tryptophan 2,3 dioxygenase (inhibition 12-24hpf)
Synaptotagmin (Syt)
Pax6
Eyes absent
Dachshund
The expression of the larval eye markers FVRI , Tryptophan 2,3 dioxygenase and Syt (also serves as a general neuronal marker) were not affected by the inhibition, the expression was similar to the control, indicating that the development of the larval eye is not affected by cyclopamine at this time and concentration of inhibition. Pax6 expression was reduced in the ventral medial brain (see figure 26,B,F). Dach expression was reduced in 3 different domains (see figure 26,C,G). EyeA was stained stronger in some of the embryos. See figure 26.

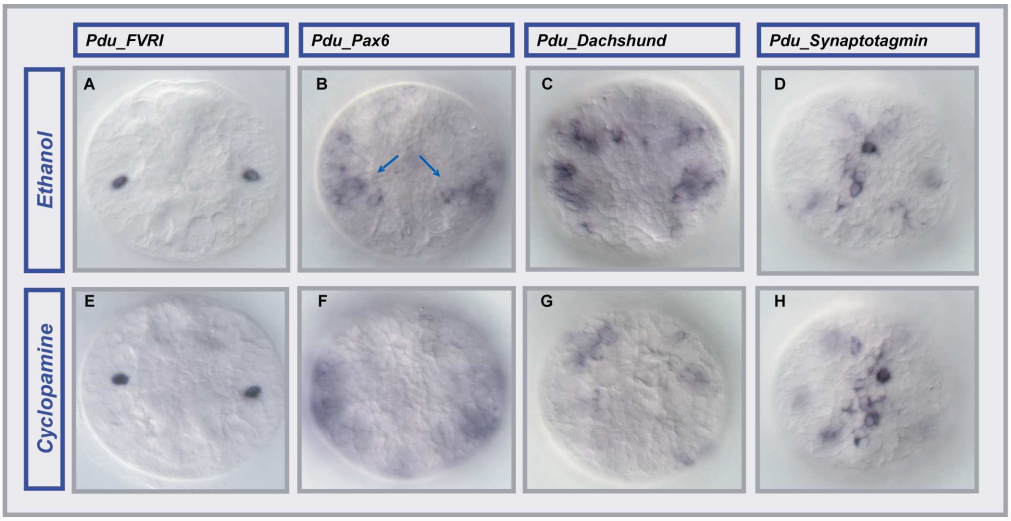


Figure 26. Cyclopamine inhibition stages 18h to 30h.
Whole mount *in-situ* hybridization with cyclopamine treated (E-H) and control embryos (A-D). The probes used are indicated on top. Note that according to the rPRC marker FRVI, the larval eye are present in the treated embryos, marked by black asterisk in both A (control) and E (treated). Note the reduced ventral medial expression of Pax6 in the treated embryos (F) as compared to the control (B) (affected region pointed by black arrows). Dachshund expression is affected as well, in two region: the lateral dorsal expression is reduced in the treated embryos (circled by blue circle in the control embryo, C), the ventral lateral expression is missing (pointed by black arrows in the affected embryo, G). Synaptotagmine expression is not significantly affected. In D and G one larval eye is visible, marked by black asterisk (because of focus plane), however in all treated embryos observed, larval eye staining was present.

For the analysis of adult eye development, larvae were fixed at the end of the inhibition and using *in-situ* hybridization I have looked at adult eye markers and potential regulators.

The markers I have used were:
FVRI
Tryptophan 2,3 dioxygenase
Sepiapterin synthase A
r-opsin
Synaptotagmin
Pax258
Eyes absent
Dachshund*

(*Initial analysis of Dach expression in cyclopamine treated embryos was done together with Raju Tomer)

The following adult eyes cell markers were severely reduced or not expressed in the adult eyes of the inhibited embryos:
r-opsin ,FVRI , Sepiapterin synthase A ,Tryptophan 2,3 dioxygenase (See figure 27,A-H).
Indicating that adult eye development is severely disrupted by cyclopamine inhibition.
Synaptotagmin expression was reduced in the lateral brain and ventral nerve cord (See figure 28,B,F).
Pax258 expression was differentially reduced in the brain but remained similar to the control embryos in the trunk (See figure 28,A,E)
Eyes absent expression was reduced both in the brain and stomodeum. (See figure 28,C,D,G,H).

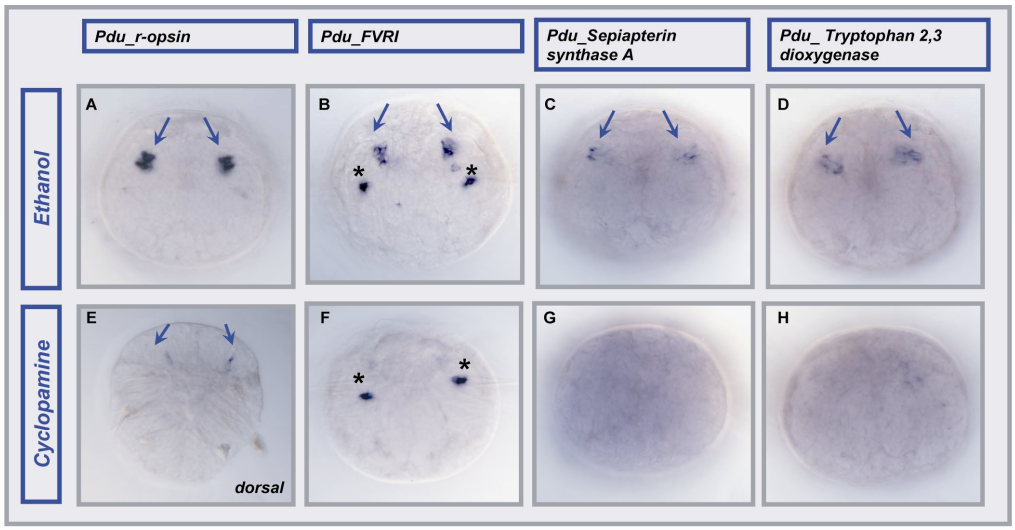


Figure 27. Cyclopamine inhibition stages 38 to 50h.
Whole mount *in-situ* hybridization with Cyclopamine treated (E-H) and control embryos (A-D). The probes used are indicated on top and represent the adult eye cell markers. All views (expect E-dorsal) are apical views. Note that according to the adult eye rPRCs marker r-opsin the adult eyes rPRCs are affected, note the reduced expression of the gene in the treated embryo (E) compared to the control embryo (A). The second rPRCs marker FRVI is differentially affected in the adult eyes of treated embryos (indicated by blue arrows, the larval eyes – indicated by asterisks - are not affected). According to the pigment markers: Sepiapterin Synthase A (C,G) and Tryptophan 2,3 dioxygenase (D,H), the adult eyes pigment cells are also strongly affected. Note that they are both not expressed in the treated embryos (G,H) compared to the control ones (C,D).

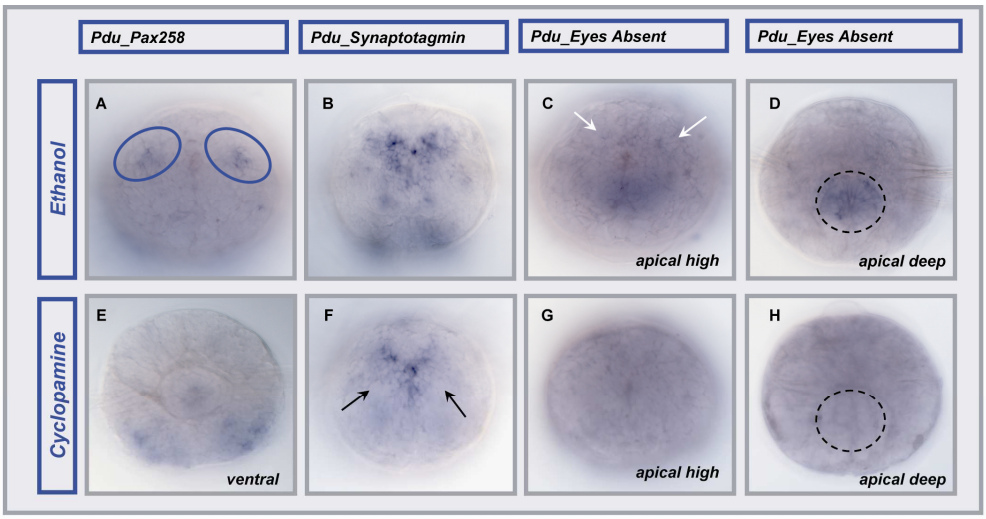


Figure 28. Cyclopamine inhibition stages 38 to 50h.
Whole mount *in-situ* hybridization with Cyclopamine treated (E-H) and control embryos (A-D). The probes used are indicated on top. All views (expect E-ventral) are apical views. Note that Pax258 expression is differentially affected in the brain (and not in the trunk) in treated (E) embryo compared to control (A, the affected regions are circled by blue circles, these expression regions are located between the adult and the larval eyes, on each side of the brain). The expression of Synaptotagmine is reduced in the lateral brain regions, as indicated by the black arrows (F). The expression of Eyes Absent is affected in the brain of treated embryos (G) compared to control (C) and in the stomodeum , circled by dashed circle (H compared to D).

Dach expression was reduced in several expression domains. Some were more affected then others. In order to better recognizing the affected regions, I first introduce the Dach 'wild type' expression pattern and name the different expression domains as following:
Ventral medial spots
Mushroom body anlage (Identified as such by Raju Tomer, unpublished data)
Adult eye anlage a
Adult eye anlage b
Ventral lateral spots
Dorsal medial spots
Stomodeum
(See figure 29, A-D and figure 30,A-C)
And then compare them to a one, representing, affected embryo (See figure 30,D-F).I found out that the ventral medial spots and ventral lateral spots were reduced in the treated embryo. Adult eye anlage a and b and mushroom body anlage were also reduced in the treated embryo. Dorsal medial spots were missing in the treated embryo. In figure 31, I present statistics of the different affected region in Dach expression.

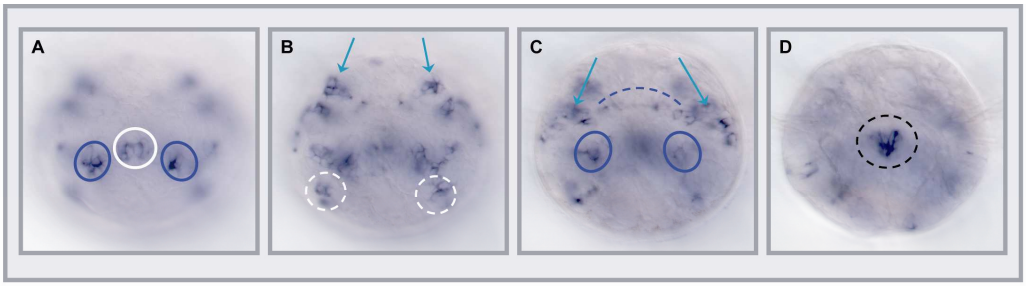


Figure 29. Dachshund expression domains at 50h.
(A-D) Consequent apical views of whole mount *in-situ* hybridizations with Dachshund riboprobe. A-upper most, D-deeper most. I have defined the expression domains as following: (A) **Ventral medial spots** (marked by a white circle), **Mushroom body analge** (Identified as such by Raju Tomer, marked by blue circles ,also in C). (B) **Adult eye anlage a** (pointed by azure arrows), **Ventral lateral spots** (marked by dashed white circle). (C) **Adult eye anlage b** (pointed by azure arrows), **Dorsal medial spots** (marked by dashed blue arch). (D) **Stomodeum** (marked by dashed black circle).

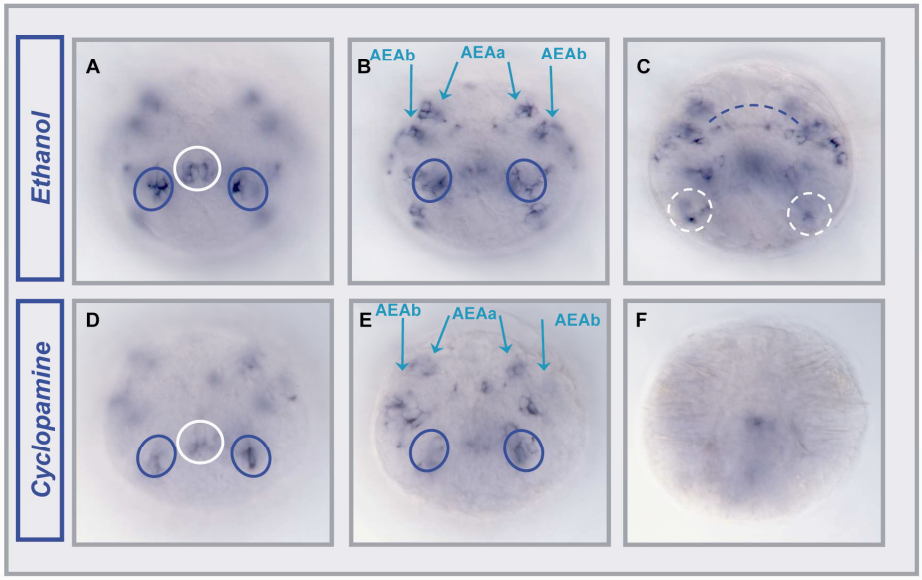


Figure 30. Dachshund expression domains affected by Cyclopamine inhibition between 38-50h.
(A-C) Consequent apical views of whole mount *in-situ* hybridizations with Dachshund riboprobe on control (A-C) and cyclopamine treated embryos (D-F). A,D-upper most, C,F -deeper most. The affected regions are marked as following: (A,D) **Ventral medial spots** (marked by a white circle) are reduced in the treated embryo. (B,E) **Adult eye anlage a and b** (AEAa, AEAb, pointed by azure arrows) are reduced in the treated embryo. **Mushroom body anlage** (marked by blue circles) are reduced in the treated embryo. (C,F) **Dorsal medial spots** (marked by dashed blue arch) are missing in the treated embryo. **Ventral lateral spots** (marked by dashed white circle) are reduced in the treated embryo.

Detailed statistic (scoring of the embryos) of the different adult eye markers expression is presented in figures 31,A-E. In all four adult eye markers: r-opsin, FVRI, Sepiapterin synthase A and Tryptophan 2,3 dioxygenase, the percentage of strongly affected embryos is higher then 30% and the percentage of non affected ones is lower then 13%. This indicates that the effect on adult eye development is profound and consistent among the different markers.
The differential effect on Dach different expression domains is quantified and presented in figure 29,E. It appears that the domains affected most severely are: the dorsal medial spots (DMS), the ventral medial and lateral spots (VMS,VLS) and the the adult eyes anlage b (AEAb).

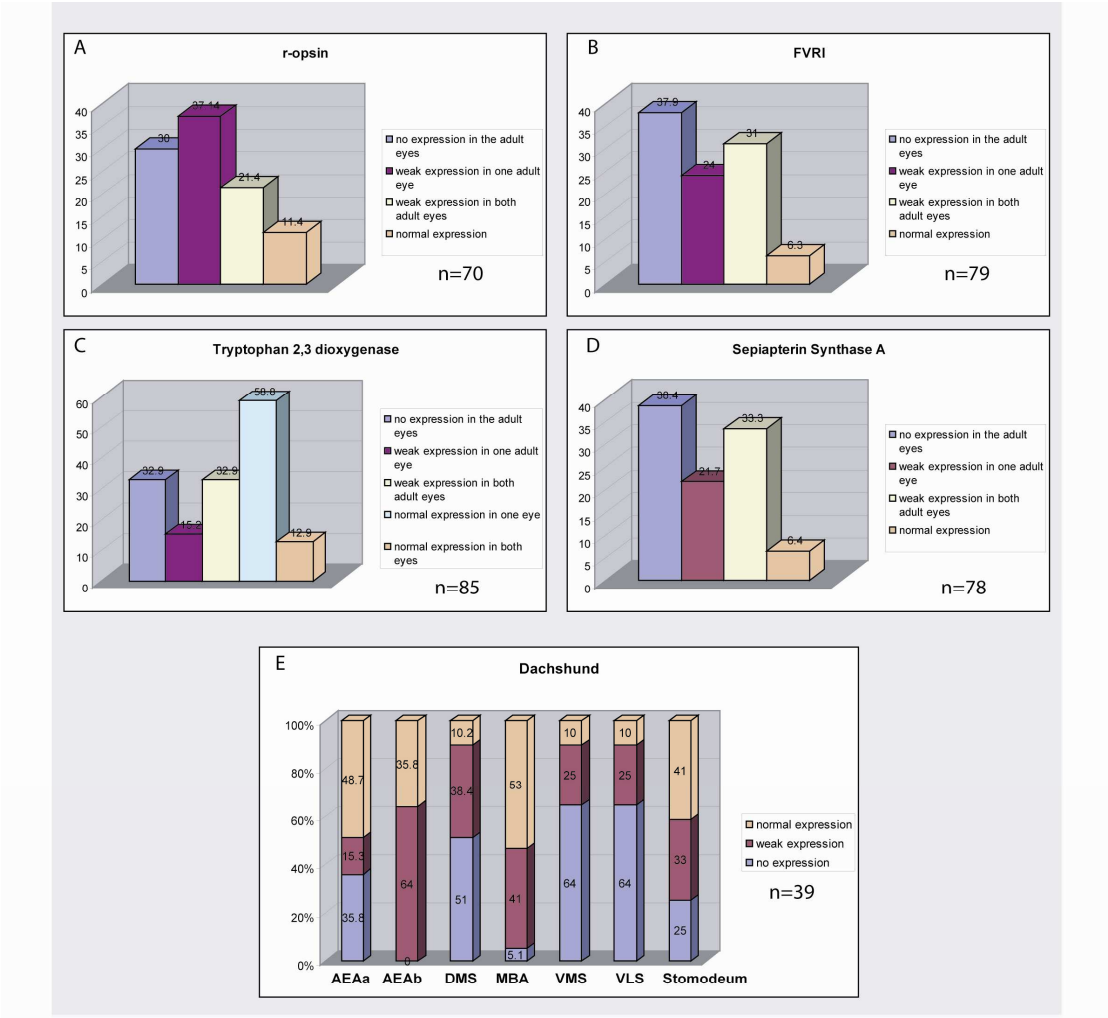


Figure 31. Scoring results of cyclopamine treated embryos phenotypes.
The number of scored embryos is indicated by n=( ).The numbers written on the bars are percentages of embryos showing the indicated phenotype.
A-D quantification of the phenotypes observed in Cyclopamine inhibited embryos at 38-50h, by whole mount *in-situ* hybridization with indicated probe. The probes used in A and B are adult eyes rPRCs markers. The probes used in C and D are adult eyes PCs markers. Note that in all four
markers the percentage of strongly affected embryos is higher then 30% and the percentage of non affected ones is lower then 13%. E - The effect of Cyclopamine treatment between 38-50h on Dachshund different expression domains: AEAa/b, adult eye anlage a/b; DMS, dorsal medial spots; MBA, mushroom body anlage; VMS, ventral medial spots; VMS, ventral lateral spots.

Detailed statistic (scoring of the embryos) of the effect on different adult eye potential regulators expression is presented in figures 32,A-E. It appears that the effect of cyclopamine treatment on them is alike, having above 50% of the embryos severely affected. Eyes absent is the most mildly affected (54% of embryos with weak expression) and Pax258 (94% of embryos having either no brain expression or weak brain expression) is the most severely affected.

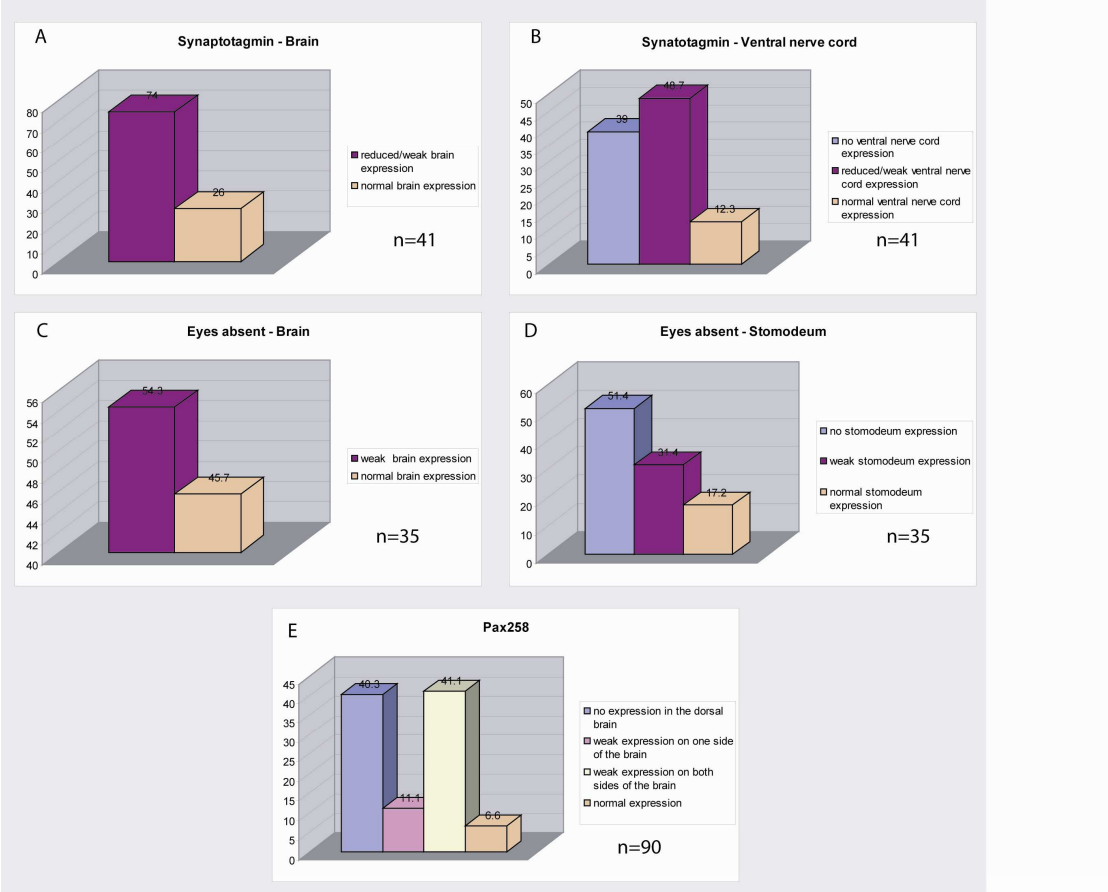


Figure 32. Scoring results of Cyclopamine treated embryos phenotypes.
The number of scored embryos is indicated by n=( ).The numbers written on the bars are percentages of embryos showing the indicated phenotype.
A-E quantification of the phenotypes observed in Cyclopamine inhibited embryos at 38-50h, by whole mount *in-situ* hybridization with the indicated probe. In (A,B) and (C,D) the differential effect on different expression domains of Synaptotagmin and Eyes absent (respectively) is observed. Pax258 is only affected in the brain, the ventral expression is not affected.

Discussion

3.1 *Platynereis* adult versus larval eyes

My main research interest has been to find out what is the evolutionary 'relationship' between *Platynereis* adult and larval eyes and what can we learn from it about the line of eye evolution in *Polychaetes*. At the beginning of my research we postulated that two scenarios are possible for explaining the evolution of adult and larval eyes and their cell types (photoreceptor and pigment cells) in *Polychaetes*:

1. Today's *Platynereis* eyes have evolved from an ancient eye that was initially composed of two cells: rPRC and a PC. This bicellular eye was duplicated to form the adult and larval eyes. In this scenario we would expect that the adult eye rPRCs and the larval eye rPRCs will be more related to each other than are the adult rPRCs and the adult PCs, for example. This would be because the split into adult and larval eyes would have occurred after the two celled eye was already present. (See figure 1 for illustration).

2. Today's *Platynereis* eyes have evolved from an ancient eye that was initially composed of a single cell. This cell had the characteristics of both rhabdomeric photoreceptor cell (rPRC) and a pigment cell (PC), therefore it had a rhabdom and pigment granules. This cell then duplicated into two identical cells, one that will later form the adult eye and the other, the larval eyes. The second step was the parallel diversification of each of these two identical cells into rhabdomeric photoreceptor cells and pigment cells which constitute both the extant larval and adult eyes. (See figure 1 for illustration)
In this scenario we would expect that the PCs of the adult and larval eyes will be less related to one another than are the PC and the rPRC of the larval eye, for example. This would be because the split into two different cell types would have occurred after the split of the ancestral cell into adult and larval eyes.

During my research I accumulated evidence that support the second hypothesis.
I would like to present and discuss them in the following sections:

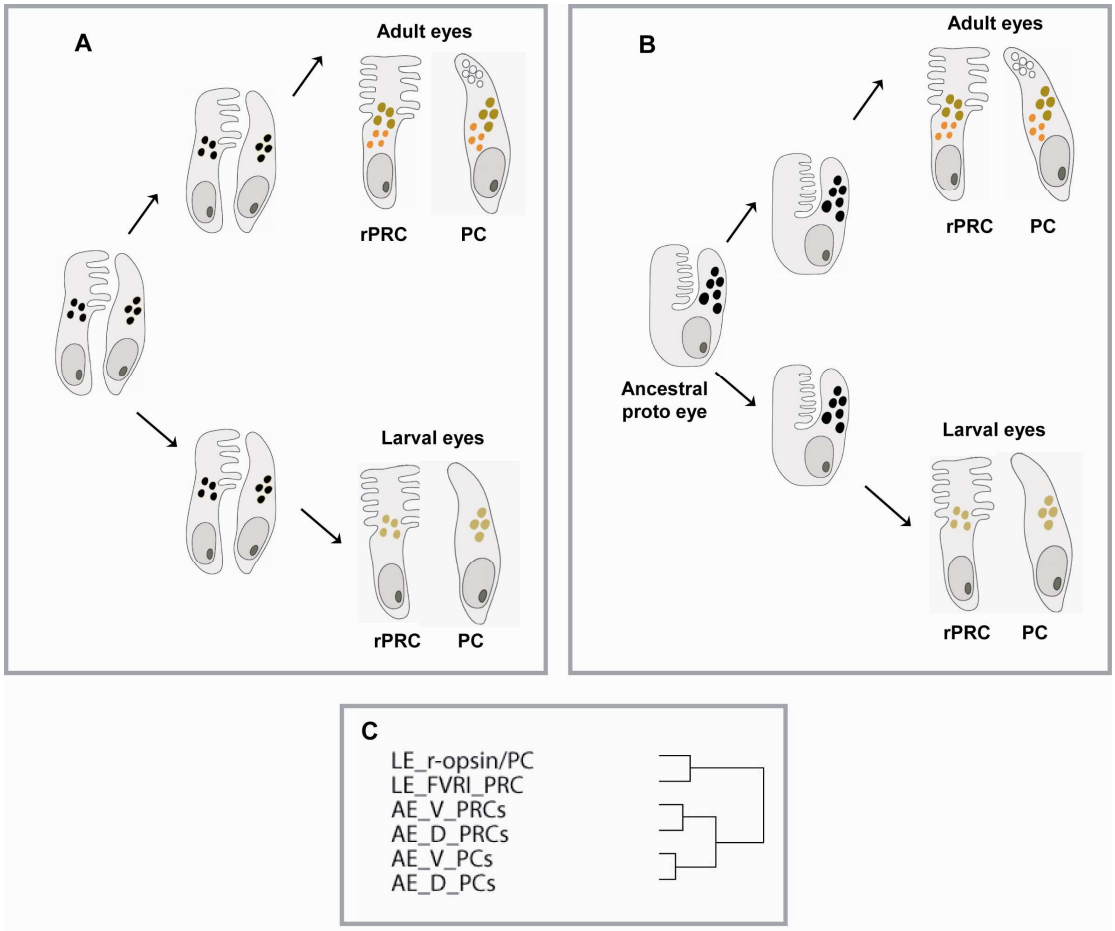


Figure 1: Two possible scenarios for the evolution of adult and larval eyes and their cell types in Polycheates.
(A) The first possible scenario where a bicellular proto eye was the ancestral eye. This proto eye was first duplicated to give rise to the adult and larval eyes and then diversified in each of the eyes and gave rise for the different cell types.
(B) The second possible scenario where a single cell proto eye was the ancestral eye. It was duplicated and diversified into the adult and larval eyes. This was then followed by the diversification of this cell into two different cell types.
In the ancestral proto eye: black circles represent pigment granules of unknown material.
In the adult eyes: Dark brown circles represent pigment granules originating from the Ommochrome synthesis pathway, Orange circles represent pigment granules originating from the Pterin synthesis pathway. White circles represent membrane bound granules filled with unknown material that accumulate at the apex of the PCs to create the lens.
In the larval eyes: light brown circles represent pigment granules originating from the Ommochrome synthesis pathway. I colored it in light brown since the Ommochrome synthesis marker is expressed in the larval eyes only until stage 30hpf. At 48hpf either this enzyme nor the Pterin synthesis enzyme are expressed in the larval eyes.
(C) A tree describing the evolutionary relationship between the different cell types of *Platynereis* adult and larval eyes, based on the hierarchical clustering analysis.
LE, Larval eyes; AE, Adult eyes; PC, Pigment cell; PRC, Photoreceptor cell; V, Ventral; D, Dorsal.

3.1.2 Molecular fingerprint of *Platynereis* adult and larval eyes

The hierarchical clustering analysis, based on the molecular fingerprint (MFP) of the different cell types of *Platynereis* eyes, revealed as a first finding that the adult rPRCs as well as the adult PCs are composed of distinct (by their MFP) dorsal and ventral cells. I would speculate that such distinction exists due to the fact that *Platynereis*

develops **two** pairs of adult eyes: anterior and posterior. Their anlagen are at first joint and will separate at late 3-day-old larvae (Rhode 1992). I would assume that even though in the examined stage (2-days-old larvae) their anlagen are still 'shared', a molecular regulatory distinction has already been imprinted. Among the genes that are expressed differentially in either the ventral or dorsal adult rPRCs and adult PCs, two genes actually share a common feature, being expressed only in the ventral rPRCs and the ventral PCs of the adult eyes as well as in both types of cells of the larval eyes. These are: Wnt5 and BF1. Therefore, they might represent 'ventral oriented' regulators of rPRCs and PCs (since the larval eyes are located in the ventral half of the brain).

The clustering analysis also shows that:

1. The adult eye ventral and dorsal rPRCs cluster together, as well as the adult eye ventral and dorsal PCs.
2. The two types of adult eyes rPRCs and two types of adult eyes PCs cluster together.
3. The larval eyes rPRCs and PCs cluster together.
(See figure 1,C for illustration)

This result supports the second hypothesis since indeed the cell types of the larval eyes are more closely related to each other then they are to any of the adult eye cell types. And this is the scenario we would expect if the course of evolution was one that originated from a single cell proto eye that first duplicated to give rise to the adult and larval eyes and only then diversified and gave rise to the different cell types existed today in both eyes.

3.1.3 Molecular differences between adult and larval eyes

If we look in more detail into the MFP we will find that key regulators of eye development are distributed in their expression so that we find transcription factors that are adult eye specific (e.g. Six1/2, Eyea, Dach, Rx) and others that are larval eye specific (e.g. Pax6, Sim, Chx10).
In addition, few effector genes are also adult (e.g. Sepiapterin Synthase A, Tryptophane 2,3 dioxygenase, VGLUT) or larval eye specific (e.g. Acetylcholine receptor7/8, VAChT). (See figures 22 and 23, in 'Results').
Therefore, the MFP clearly supports the view that the adult and larval eyes are different 'types' of eyes, which evolved independently and are controlled by different molecular regulators.

3.1.3.1 *Platynereis* larval eye r-opsinII

The expression of r-opsin adds another distinction in the MFP of *Platynereis* eyes, which is directly related to the function of the eyes. As mentioned before, *Platynereis* r-opsin is detectable in the adult eyes from their early differentiation stage : around 43hpf (Arendt, Tessmar et al. 2002), however in the larval eyes, it is detectable only one day after they have formed. I cloned a second *Platynereis* r-opsin, Pdu r-opsin II, from a 24hpf cDNA library.

Even though a clear and 'confident' expression pattern for this gene was not obtain, that will correlate this gene directly to the larval eyes rPRC, we do think that this gene is a true Platynereis larval r-opsin. Our arguments are:
1. According to sequence homology, Pdu r-opsin II clusters with a newly identified r-opsin from *Capitella capitata* (Tobias Kaller, unpublished data).

2. The newly identified ***Capitella capitata*** r-opsin is expressed in the larval eyes of ***Capitella*** (Tobias Kaller, unpublished data)**.**

3. I have confirmed Pdu r-opsin II as a true *Platynereis* gene by southern blot hybridization using genomic DNA.

Based on these arguments we postulate that Pdu r-opsin II is expressed at early stages (at least between 17h and 24h, since detection by wholemount *in-situ* hybridization was made at 17h,24h and the cloning of the gene was done from a 24hpf cDNA library) when phototaxis start to take place. We can't however determine if it is indeed the opsin responsible for the phototactic behavior and expressed in the rPRC (that is FVRI positive).

3.1.4 The ancestral single cell eye hypothesis

Regarding the distribution of the pigment cell markers (Sepiapterin Synthase A and Tryptophane 2,3 dioxygenase) expression, it is interesting to note that they are both expressed in both the rPRCs and the PC of the adult eyes. Tryptophane 2,3 dioxygenase is expressed both in the rPRC and the PC of the larval eyes, at 24hpf. This support the idea that *Platynereis* rPRC and PCs are actually sister cell types that trace back to a single ancestral cell which contained characteristics from both cell types: Photopigment as well as shading pigment.

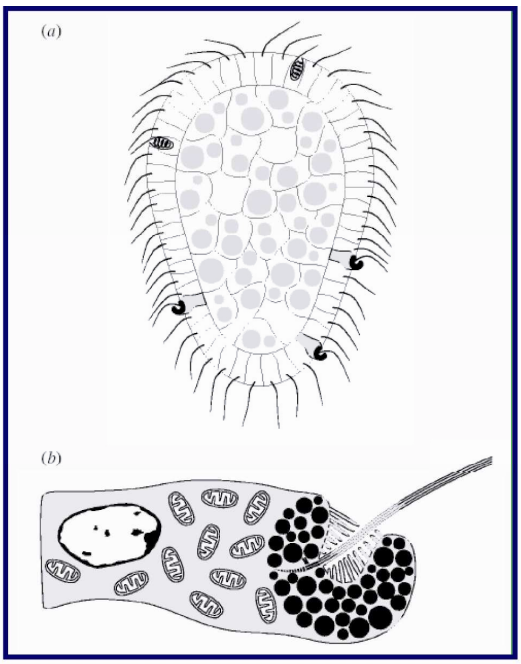
Such mechanism for cell type evolution is termed 'Segregation of functions in sister cell types' (Detlev Arendt, Nature Reviews Genetics, *in press*). It implies that in the beginning of Metazoan evolution, few cell types with multiple functions existed that then diversified by distributing their functions among the emerging sister cell types, resulting in greater number of specialized descendant cells. These events are reflected by a selective loss of expression of effector and regulatory genes resulting in selective loss of function.

This idea is in line with the finding of special ocelli in the planula larvae of a box jellyfish, *Tripedalia cystophora* .
cubozoan larvae reveals a simpler organization than in most other cnidarian larvae, having a radially symmetrical body plan. They have only two tissue layers, five cell types and no nervous system, cubozoan larvae are therefore among the most simply organized animal life-forms. Their only advanced feature is the presence of 10–15 pigment-cup ocelli, evenly spaced across the posterior half of the larval ectoderm.

These ocelli are single cell structures containing a cup of screening pigment filled with presumably photosensory microvilli. These rhabdomeric photoreceptors have no neural connections but each has a well-developed motor-cilium, appearing to be the only means by which light can control the behavior of the larva, see figure 2

(Nordstrom, Wallen et al. 2003). Their ocelli represent the one of simplest visual system described.

Combined with the larvaes' general simple organization, these ocelli support our hypothesis regarding the type of ancestral proto eye of polychaetes and even expands it to the base of Invertebrate linage: that the ancestral proto eye was a single multifunctional eye comprising three features: photopigment, shading pigment and a motor cilium (exactly as exhibited in the ocelli of the cubozoan larvae).
Regarding the third feature - the motor cilium, *Platynereis* larval eyes are also associated (spatially and functionally) with multi ciliated cells, as will be discussed in section 3.7. These findings support a single common origin for rPRCs ,PCs and ciliated cells.



(Nordström et al.2003)

Figure 2. Schematic drawings of (a) the planula larva of *Tripedalia cystophora* and (b) a close-up of an ocellus.
In (a) the extremely simple larval body has two tissue layers and a total of only five different cell types. The single cell ocellus (b) has a fully developed cilium, photoreceptive microvilli and a pigment cup. (Nordström et al.2003)

3.1.5 Comparing the MFP of *Platynereis* and *Drosophila* eyes

If we compare the MFP of *Platynereis* eyes to *Drosophila* eye regulation networks, we will find that for the retinal determination network genes (regulating the adult compound eye), for example, 3 out of 4 of them are actually shared with *Platynereis* adult eye MFP:
Six1/2, Eyea and Dach. The one that is not included is Pax6, it is part of the larval eye MFP.

An interesting observation is that also in *Droshophila*, there are interesting similarities and differences between PRCs subtype specification in the larval and adult eyes.
The larval eye consists of two distinct PRCs subtypes, PRCs containing blue-sensitive Rhodopsin-5 (Rh5) and PRCs containing green-sensitive Rh6. These two kinds of rhodopsin are found in the R8 PRCs of the adult eye (in a similar ratio), and are specified by the bistable loop of the *warts* and *melted* tumor suppressor genes.
Surprisingly, in the larval eyes, the distinction between Rh5 and Rh6 expression is regulated by a different mechanism:
Primary precursors, which give rise to the Rh5-subtype PRCs, signal to the surrounding tissue to develop as secondary precursors, which become the Rh6 subtype. EGFR signaling is required for the survival of these secondary precursors. The combinatorial action of the three transcription factors Sal, Svp, and Otd then direct the differentiation of the two PR subtypes (Sprecher, Pichaud et al. 2007).
Interestingly, even though larval PRs and the adult R8 have the same rhodopsin content, the mechanisms to establish their fates are remarkably different, a situation reminiscent of *Platynereis* adult and larval eyes, which express (at 48hpf) the same r-opsin, but have a different MFP.
The shared and distinct gene regulating adult and larval eyes in Drosophila are summarized in the following table (distinct genes are highlighted in gray):
As a conclusion from the comparison to *Drosophila*, we can say that, on the molecular level, the larval eyes of annelids and insects are different, as well as the adult annelids and adult insects eyes.

	Adult compound eyes	**Larval eye (Bolwig's organ)**
	Atonal	Atonal
	Eyeless	Eyeless
	Sine Oculis	Sine Oculis
	Eyes absent	Eyes absent
Signaling molecules	TGFα (Spitz)	TGFα (Spitz)
	Hh	Hh
		Tailless
	Dachshund	
Specifying rh5 vs. rh6 rhodopsin fates	warts melted	Spalt Seven-up Orthodenticle
Specifying rh7 vs. rh8 rhodopsin fates	Prospero	

Vertebrates eye development regulators are also part of *Platynereis* adult (e.g. Eyea, Rx, Six3) and larval (e.g. Pax6, Chx10) eyes MFP.

3.2 The fine structure of *Platynereis* adult and larval eyes basic units: rPRC and PCs

If we look closely at their described morphology (Rhode 1992) we will find some apparent differences between the same cell types of the two eyes:

1.The adult but not the larval PCs send long process containing granules filled with unknown material that accumulate at the apex of the PCs to creates the lens (also known as the 'vitreous body').

2. The adult PCs contain two types of pigment granules (described as such by electron microscopy study, Rhode 1992) while the larval eyes contain only one type of them. In this respect it is interesting to note that we can well correlate and explain to some extant the presence of these one/two types of pigment granules types: the adult eyes PCs express two pigment markers (Sepiapterin Synthase A and Tryptophane 2,3 dioxygenase) acting in two distinct pigment synthesis pathways: the pterin and the ommochrome (respectively) synthesis pathways. The larval eyes express only one of them, Tryptophane 2,3 dioxygenase.

3. The adult but not the larval eye rPRCs contain pigment granules (of one type). This can also be nicely correlated to the MFP since the adult rPRC express the two pigment synthesis markers mentioned above, while the larval eye rPRCs (at 48h) does not express either of them (it does, however express Tryptophane 2,3 dioxygenase between stages 15-24hpf).

Pigment granules in polychaete PRCs are common feature. Pigment granules in PCs as well as in PRCs have been reported for example in Vanadis tagensis (Hermans and Eakin 1974) and Archtomoe vitata (Singla 1975). The absence of pigment granules in larval eyes PRCs was also reported for O.ctenostoma (Verger-Bocquet 1983).

From these fine observations we can conclude that these eyes actually also differ based on the morphology of their basic units: the rPRC and the PCS.

In my PhD I have (together with Dr. Gaspar Jekely) identified a new cell type that is tightly associated with the rPRC of both adult and larval eyes. These cells are flask shape cells that express the amidated neuropeptide FVRI. This neuropeptide is also expressed in both adult and larval rPRC but more important, it is expressed in cells adjacent to the rPRCs of both eyes, representing a shared feature between the adult and larval eyes. We also observed that the number of FVRI cells found in close vicinity to the rPRCs increases during development, in both eyes. (See figures 3,10 and 13, in 'Results' for FVRI expression pattern).

In Cnidaria, Photoreceptive organs are well associated with neural cells showing immunoreactivity to RFamide peptides (the same family to which Platynereis FVRI peptide belongs to). I will mention one example, among few, to illustrate a possible role for these peptides associated with eyes:
A possible function of neuropeptides in **transmission of photic stimuli** was assayed by analyzing photic behavior in the cubomedusa *Tripedalia cystophora*, which has

highly developed eyes. In this assay light orientation was effectively prevented by RFamides administered to the animals in micromolar concentration. This result support the hypothesis that one possible function of RFamides in Cnidaria is to transmit photic stimuli to epitheliomuscular targets (Schneider 2003).
We therefore postulate that these cells in *Platynereis* also have a role in transmitting photic stimuli from the rPRCs to the operating organs/systems.

3.3 Larval and adult eyes respond differently to hedgehog antagonist

By looking at signaling/extracellular component of eye development - *Platynereis* Hh pathway - another distinction between *Platynereis* adult and larval eyes is added. Using Cyclopamine to inhibit the Hh pathway, I showed that *Platynereis* adult eyes development is severely disrupted, by the reduction in the expression of rPRC and PC markers. (For further details see 'Results' section 3.2).

In contrast, larval eyes development was not affected (according to the expression of rPRC and PC markers).In this respect I would like to mention that three candidate TFs (that were chosen for the experiment before the MFP analysis was completed) for the regulation of larval eyes, were affected. These are:
Pax6 was down regulated in the ventral medial brain, a region that doesn't include the larval eyes (they are laterally located). Dach and Eyea were down regulated and slightly up regulated (respectively), however we found out that they are not part of the larval eyes MFP, and therefore I assume that they don't play a role in their development.

We believe that the effect on adult eye development is due to the combined down regulation in the expression of the following transcription factors: *Pax258, Dach and EyesA* (see figures 28 and 30, in 'Results', for *in-situ* hybridizations, figures 31 and 32, in 'Results', for statistics). These TFs are three of the eight adult eyes specific TFs according to our MFP analysis. We also think that the effect of cyclopamine is specific and not the result of a general toxicity effect causing a developmental delay. Two supporting evidence for this are:

1. The differential down regulation of *Pax258* in the brain as apposed to the trunk, where it is not affected.

2. We could see that the treated embryos were not developmentally retarded by two observations:
   a. The fact that their chaetae were protruding out of the body, as characteristic for 50hpf stage.
   b. According to immunostaining with anti-acetylated tubulin antibody we could see that overall, their nervous system doesn't suffer from major abnormalities.

In addition the spatial and temporal expression of Pdu_Smo, Pdu_Gli-1 and Pdu_Hh is nicely correlated to the adult eye development: these three pathway components are expressed next to the proposed adult eye anlage (Arendt, Tessmar et al. 2002), at the dorsal lateral brain (see figure 24, in 'Results'). Their expression becomes prominent at around 30hpf, which is before the formation of the adult but after the formation of the larval eyes.

By several *in-silico* alignments of *Platynereis* Hh pathway players (see 'results' section 3.2) we also found out that:

1. Pdu_Hh and Pdu_Gli-1 are co-expressed in some cells at the dorsal lateral brain
2. Pigment cells express Pdu_Gli-1.
(See figure 25,A in 'Results').

Therefore they could be the cells receiving the Hh signal for further growth and differentiation of the adult eyes. We do however also see a severe effect of cyclopamine on the differentiation of the rPRCs (using r-opsin and FVRI as markers for there cells). I would therefore speculate that in *Platynereis* adult eyes, rPRCs and PCs differentiation are two dependent events, meaning that the future/committed rPRCs require some kind of a signal (maybe via cell-cell interaction) from the PCs, which receive the Hh signaling, in order to further differentiate into rPRCs.
This possible scenario might resembles the mechanism by which Hh signaling act in the vertebrate retina, where Hh proteins are expressed by postmitotic retina ganglion cells and retina pigmented epithelium cells, while downstream components of the cascade (Patched, Smo and Gli) are mainly expressed by undifferentiated precursors (See figure 9, in 'Introduction').
We could therefore speculate that for *Platynereis*, the signaling cells for adult eye development are the Hh positive ones found on the dorsal lateral brain, in close vicinity to the developing adult eyes, and as mentioned above, the cells that receive the signal are the PCs that send the signal further to the future rPRCs.

3.3.1 Differences in Hh signaling between *Drosophila* and Mammals

According to confocal scans of wholemount *in-situ* hybridization with a riboprobe for Pdu_Sufu combined with anti-acetylated tubulin antibody, it looks as if this gene is expressed, in few cells, in the region of the brain ciliary photoreceptor cells (dorsal medial brain, and not in the dorsal lateral region where all the other pathway members are expressed). Therefore Pdu_Sufu expression is somehow puzzling.
But, if we look closely at some comparative studies of the Hh pathway players we will find that Su(Fu), together with Smo and Cos2 are components of the pathway that have diverged remarkably between *Drosophila* and mammals. I will briefly describe the main differences:
Hh signaling is intact in *Drosophila* embryos lacking Su(Fu) function, and such embryos develop into viable and fertile adults (Preat 1992). Su(Fu) therefore exerts a weak negative influence on Ci activity. In contrast, *Drosophila* Cos2 is a critical component of the Hh pathway; it associates directly with the Hh receptor component Smoothened (Smo), and is essential for suppression of the transcriptional activity of Ci in the absence of ligand. Loss of Cos2 results in embryonic lethality due to constitutive activation of the Hh pathway (Grau and Simpson 1987; Sisson, Ho et al. 1997) .
However, despite the critical role of Cos2 in Drosophila, mammalian proteins acting equivalently to Cos2 have not been described.
A recent study explored the conservation of the above mentioned Hh pathway members between *Drosophila* and mammals. In brief, the mouse Smo (mSmo) C-terminal domain that in *Drosophila* is phosphorylated in response to Hh and binds to Cos2 is **not** required for mammalian Smo function. On the other hand RNAi-induced

loss of Su(Fu) expression in mammals results in a dramatic increase in Shh pathway activity. (Varjosalo, Li et al. 2006).
(See figure 7, in 'Introduction', for pathway illustration)
Based on theses finding and the expression pattern of Su(Fu) in *Platynereis*, I would postulate that *Platynereis* Su(Fu) probably does not play an essential role in *Platyenereis* Hh pathway.

The differential effect of cyclopamine inhibition on adult but not larval eyes adds another distinction between the adult and larval eyes in *Platynereis*.

3.4 Prox1 cloning and expression (collaboration)

The *Prox1/Prospero* collaboration project is aiming at performing a cross species comparison of *Prox1* expression and regulation. The hypothesis is that the association of *Prox1* with rhabdomeric photoreceptors is conserved across *Bilateria* and that Prox1 has a role in the regulation of r-opsin expression in these cells. The first indication for such regulation comes from *Drosophila* (Cook, Pichaud et al. 2003), where it was shown that Prospero is necessary and sufficient to repress R8 Rhodopsins *in vivo* (via direct interaction with Seq56 - an R7 repression element - in R8 rhodopsin promoters) and that it therefore affects R7 versus R8 cell fate decisions.
My contribution to the project was:

1. To show, by *In-situ* hybridization patterns, a correlation between Prox1 and r-opsin expression in *Platynereis*.
I have synthesized *Platynereis* cDNA that was used for Pdu-Prox1 cloning. Then, wholemount *in-situ* hybridization with a Pdu_Prox1 riboprobe on *Platynereis* 24, 48 and 72hpf embryos was done by me. The initial analysis of the expression pattern looked very promising for adult and larval eyes expression. This was then confirmed by including this gene in the 3D *in silico* expression profiling protocol (Raju Tomer and Detlev Arendt, unplished data). The results were that Prox1 is expressed, at 48hpf, in the photoreceptor and pigment cells of the adult eyes and in the r-opsin positive cell (the pigment cell) of the larval eyes, showing that the co-expression of Prox1/Prospero and r-opsin is conserved between Ecdysozoa and Lophotrochozoa.
(See figure 6, in 'Results', for Prox1 expression)

Amphioxus Prox1 is expressed in the Hesse cells and in the rhabdomeric photoreceptors located dorsally in the brain, the Joseph cells (Pavel Vopalensky, unpublished data) as revealed by double immunostaining with Amphioxus prox1 antibody and Amphioxus r-opsin antibody. This expands the conservation of this feature to the Deuterostomes as well.

2. For the purpose of studying the regulation of Pdu_r_opsin by Prox1 using reporter gene assays and DNA binding assays. I have isolated *Platynereis* r-opsin BAC (See figure 9, in'Results'). The sequence of the BAC will be studied in the laboratory of Professor Zbynek Kozmik.

3.5 Pax genes expression in *Platynereis* adult and larval eyes

The view that varied animals' eyes arise independently, multiple times during evolution, was challenged about a decade ago by the significant discoveries that Pax6, a highly conserved transcription factor, plays a key role in eye morphogenesis in both flies and mammals (Gehring and Ikeo 1999; Kozmik 2005). Recent studies have shown that other members of the Pax gene family also play a central role in eye morphogenesis (e.g. the *Eye gone* gene regulate Drosophilas' eye growth) (Jang, Chao et al. 2003).
It is therefore interesting to explore the distribution of *Pax* genes expression in Platynereis eyes.
Platynereis **Pax6** is expressed in the **larval eyes** pigment cell and photoreceptor cell (but not in the adult eyes). *Platynereis* **Pax258**, on the other hand, is expressed in the **adult eyes** pigment cells (and not in all other eye cell types). The remarkable finding that *Platynereis* adult eyes develop and exhibit life long growth and differentiation without the expression of Pax6 was discussed before (Arendt, Tessmar et al. 2002). Even though it was proposed that *pax6* genes have an ancestral direct role in photoreceptor cell differentiation (Gehring and Ikeo, 1999; Pichaud et al., 2001; Sheng et al., 1997), *Platynereis* adult eyes are a clear example (among others) that contradicts this view.
The observation that Pdu-Pax258 is expressed in adult pigment but not in photoreceptor cells is highly interesting in the light of the proposed 'Paxcentric (PD-HD) model' for Pax gene evolution (Kozmik 2005) . The model is introduced in the 'Introduction' section 1.5.1.2. In brief, it suggests that the modern Pax2 and Pax6 genes in bilateria evolved from a cnidarian PaxB-like ancestor by duplication and diversification in which Pax2 lost its homeodomain (HD), and Pax6 lost the octapeptide (yellow box) and changed the DNA-binding specificity of the paired domain (PD). The model predicts that the PD has been captured to function in the 'pigmentation' pathway as well as for driving morphogenesis ('eye design') through intercalary evolution, whereas the HD functioned in opsin expression. Meaning that two independent DNA binding domains within a single Pax transcription factor have been co-opted for two essential features of the protoeye: production of a dark pigment (the 'pigmentation' program; paired domain-driven) and production of a photopigment (the 'opsin' program; HD-driven).

Platynereis Pax258 is expressed in the adult eyes pigment cells which correlate nicely with the 'pigmentation' programme mentioned in the theory. Pax6 is expressed in the larval eye rhabdomeric photoreceptor **and** pigment cell. It is interesting to note that the larval eye pigment cell is unique in the sense that it expresses an opsin (Pdu-r-opsin). It is most likely that the larval eye rPRC also expresses an opsin (since it has a rhabdom and this cell was shown to mediate phototaxis, Jekely et al. 2008, *in rivision*), however it is not identified yet. We can speculate that since Pdu-pax6 inherited the 'opsin' program, **both** cells of the larval eyes (the rPRC and the PC) express an opsin. And, that the transcriptional control of adult eyes rPRCs specification is driven by a different factor, as speculated above. (an example for a similar situation in *Drosophila* is also mentioned above, section 3.1.5)

3.6 Candidate regulators for *Platynereis* adult eye development

If *Platynereis* Pax6 plays no role in transcriptional control of *Platynereis* adult eyes differentiation, (e.g. driving r-opsin or any other rPRC or PCs specific gene expression), which gene could fulfill this task?
We can speculate on the following candidates:
Otx
Drosophila orthodenticle is required for photoreceptor cell development and is expressed at all stages of the developing visual system, including the photosensitive cells of Bolwig's organ, the ocelli, and the adult eye (Vandendries, Johnson et al. 1996).
Crx , One of the four orthodenticle paralogs in mice, regulates cone and rod PRCs development as well as PR-specific gene expression (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). Mouse *Otx1* and *Otx2* genes are required in a dose-dependent manner for tissue specification in the developing eye (Martinez-Morales, Signore et al. 2001). Therefore, Otx plays a key role in eyes development in both *Drosophila* and mammals.
Platynereis Otx is expressed in **all cell types of both the adult and larval eyes** (as examined at 48hpf for both eyes and at 24h for the larval eyes). Among the 37 transcription factors that we have examined, it is the only one that shows such a feature. It is on the other hand not such a broadly expressed transcription factor, which strength our hypothesis that it plays a significant role in adult and larval eye development. It is expressed as early as 15h (the earliest stage examined) in both regions that would correspond to the adult and larval eye anlage. (See figure 3).

The second candidate is **Prox1**. As mentioned in the previous section, Prox1 has a conserved role in photoreceptor differentiation. At 48hpf, *Platynereis* Prox1 is expressed in the photoreceptor and pigment cells of the adult eyes and in the r-opsin positive cell (the pigment cell) of the larval eyes. At 24hpf it is expressed in the larval eye location, in two cells, that highly correlate to the larval eyes pigment and photoreceptor cell (See figure 8 in ‘results’). It is therefore another promising candidate to control adult and larval eye development in *Platynereis*.

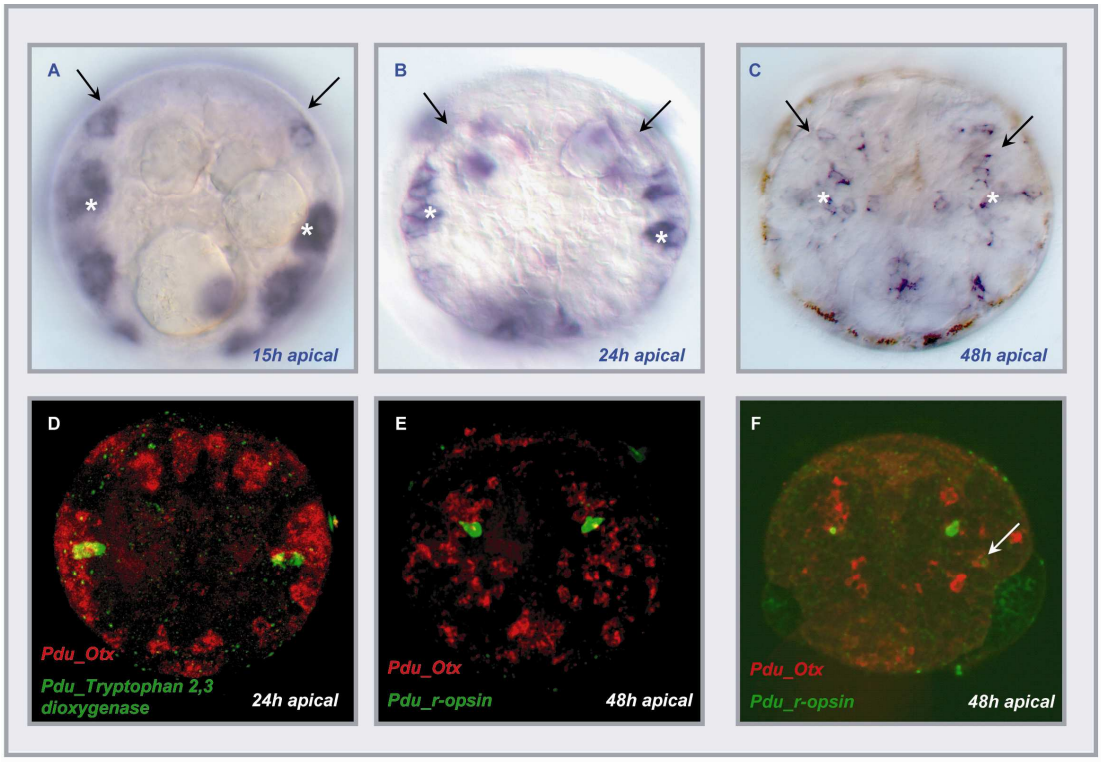


Figure 3: The expression pattern of *Platynereis* Otx, a candidate transcription factor for regulating adult and larval eyes development.
(A-C) Wholemount *in-situ* hybridization with an Otx riboprobe. Stages and orientations are indicated. Putative adult eyes anlage are pointed by black arrows. Putative larval eyes anlage are indicated by white asterisks.
(D-F) Wholemount double *in-situ* hybridization using the reflection confocal microscopy method. (D) Z projection of double *in-situ* hybridization with the indicated probes, showing Otx expression in the larval eyes FVRI cell/s.
(E) Z projection of double *in-situ* hybridization with the indicated probes, showing Otx expression in the adult eyes rPRCs. (F) single stack of double *in-situ* hybridization with indicated probes showing the co-localization of Otx and r-opsin in the adult and larval eyes. Larval eye is visible only on one side due to weak staining at this stage, it is indicated by white arrow.

3.7 The function of *Platynereis* adult and larval eyes

The adult eyes are though to be used for vision as an image forming eyes, based on their design as a pigment cup eye.

Regarding the larval eye function, I took part (by characterizing the expression pattern of the FVRI gene in *Platynereies* eyes) in a study describing the mechanism governing phototaxis by *Platynereis* larval eyes (Jékely et al. 2008, *in revision*). *Platynereis* larvae swim using the ciliary band consisting of two tiers of 12 large multiciliated cells. The ring nerve underneath the ciliary band shows axonal contact to the eyespots. The study demonstrates that *Platynereis* larval eyes are indeed responsible for the phototactic behavior and that the selective illumination of one larval eyespot changes the beating of adjacent cilia via direct **cholinergic** innervation resulting in locally reduced water flow. Computer simulations of larval swimming show that these local effects are sufficient to direct the helically swimming

trajectories towards the light. The computer model also reveals that axial rotation of the larval body is essential for phototaxis and that helical swimming increases the precision of navigation.
We therefore observed that also at the functional level, *Platynereis* larval eyes employ different function (phototaxis during vertical migration) compared to the adult eyes that serve for vision. In addition, as apposed to the **cholinergic** activity of the larval eyes we have evidence for the adult eyes being rather **glutamergic** (since the gene Vesicular glutamate transporter is expressed in the adult eyes rPRCs whereas the cholinergic markers: Acetylcholine receptor7/8 and VAChT are expressed only in the larval eyes).

Materials and methods

4.1 *Platynereis dumerilii* animals and embryos

4.1.1 *Platynereis dumerilii* culture

Platynereis dumerilii animals were obtained from an in-house culture at EMBL. The culture was established by Detlev Arendt and maintained by Heidi Snyman and Diana Bryant followed the procedure described by (Dorresteijn, O'Grady et al. 1993). Animals were held in glass aquarium at 18ºc, following a light cycle similar to the natural moon cycle: approximately one week of artificial moonlight followed by three weeks of darkness. Sexual maturation of both males and females occurred at 5-15 days after the moon was tuned off. The epitokes were fertilized in a small transparent cup filled with natural sea water (NSW), usually each fertilization resulted in several thousand synchronously developing embryos. The zygotes were washed once, 5 minutes after the spawning and were left at 18ºc to develop to the desired developmental stage. The embryos were washed after one day of development, using nylon net with a whole size of 100µm and NSW.

4.1.2 *Platynereis dumerilii* embryos handling

Platynereis embryos were collected at the desired stage by using the above mentioned nylone net. Non developed embryos were discarded. Fixation was done by incubation with 4% Paraformaldehyde (PFA) in PTW (phosphate saline buffer, or PBS, containing 0.1% Tween20) for two hours, followed by three washing steps of half an hour each in 100% Methanol. Embryos were stored at -20 ºc in 100% Methanol.

4.2 Gene cloning

4.2.1 General gene cloning techniques

Degenerated PCR

Novel gene fragments of *Platynereis dumerilii* were cloned by designing degenerated primers (Buck and Axel 1991) in conserved regions, based on the amino acid sequence
alignment of bilaterian of the gene of interest. The alignment was generated using the ClustalX software. The primers were designed with the help of the Oligo 6.44 software for Mac OS 9.

RACE- Rapid amplification of cDNA-ends

RACE can provide the sequence of an RNA transcript from a small known sequence within the transcript to the 5' end (5' RACE-PCR) or 3' end (3' RACE-PCR) of the RNA. The idea is to use a ‘fragment specific primer’ together with a ‘SMART RACE cDNA specific primer’ to obtain a bigger fragment to either the 5’ or 3’ direction.

The fragment specific primer should be designed in a way that will keep at least 100bp of the original fragment in order to later be able to detect positive bands using RA hybridization.

Fragment specific primers were designed using Oligo 6.44 program. RACE cDNA library was synthesized in the following way:
RNA was first extracted from specific developmental stages (e.g. 24hpf, 48hpf) using the RNAeasy kit (Qiagen) and this was used as a template for the synthesis of the SMART RACE cDNA.
5' and 3' RACE cDNA were synthesized using the SMART kit (Clonthech) according to manufacture's instructions.

RACE PCR program I used is the following:

95 ºc for 2 min' ('hot start')
Addition of: Taq DNA polymerase, 10x buffer
95 ºc for 1 min', **x** ºc for 2 min', 72ºc for 4 min'
Repeat 5 times
95 ºc addition of RACE primers
95 ºc for 1 min', **x** ºc for 2 min', 72ºc for 4 min'
Repeat 35 times
72 ºc for 10 min'

(**x** = according to the melting temp' of the primers)

I used a 24hpf 5' RACE cDNA and 24hpf 3' RACE cDNA for 5' and 3' RACE, respectively. I have also used a ZAP 24hpf and 48hpf libraries for both 3' and 5' RACE.

RACE PCRs were preformed using a specific primer for the gene of interest and a specific primer for the library used.

1. For first strand single-stranded cDNA synthesis: RaceAda
2. For SMART RACE libraries: UPM long&short mix, NUP (for nested reactions)
3. For Zap phage library: T7(70); T3(70)

In a first PCR reaction, the outer primers were used. From this reaction, 1ul was used in a "nested" PCR reaction to re-amplify extended fragments. This increases the chances of amplifying the desired fragment. All the products were run on an agarose gel , then transferred according to the Southern blot technique and hybridized with highly stringent conditions, using the existing fragment of the gene as a template to synthesize a P^{32}-labeled probe.

Fragments obtained by degenerated or RACE PCR were cloned by the following common procedure:

Gel extraction and purification

PCR reaction was run on a 1.5% agarose gel. Visualization of the band/bands was obtained by incubation in ethidiumbromide in TAE (1:10000 dilution) for 15 minutes. The desired band was cut with a razor blade under long-wave UV light (λ=366nm) and using the GFX™ Gel Band Purification Kit (Amersham Pharmacia Bioscience) according to the manufacturer's instructions.

TOPO cloning

The desired fragment was cloned into TOPO vector using TOPO TA Cloning® Kit according to the manufacturer's instructions.

Colonies amplification and analysis

Colonies were amplified in electrocompetent *E.coli* DH10B cells. Grown over night in 5ml LB-AMP medium. DNA was extracted using a QIAprep Spin Miniprep Kit® .

Colony analysis was done either by colony PCR or restriction enzymes.

Colony PCR

Colony PCR is a rapid way to find out which of the colonies contains an insert with the expected size. Primers used are designed according to the multiple cloning site (MCS) of the TOPO vector. A sample of each colony is taken directly from the LB plate and used for The PCR and in parallel gown slowly in LB-AMP medium.
Primers used are:
Stock number 820
Stock number 821
PCR program used is:
95 ºc for 2 min'
95 ºc for 30 sec',72 ºc for 30 sec'+ **x**
Repeat 35 times
72 ºc for 10 min'

(**x**=elongation time depends on the size of the expected insert, as a "role of thumb" 1min' for every 1KB)

Restriction enzyme analysis

"Multi cutter" enzymes, creating a "pattern" of small, different size fragments, and ECORI (as a cutter found both sides of the MCS – indicating the insert size) were used to Digest a sample from each of the DNA purified from the colonies. By this I could distinguish between similar and dissimilar colonies.

The next step would be sending few promising colonies for sequencing at the EMBL gene core facility.

However, if the desired fragment was not obtained (either the initial fragment or a RACE fragment), my next procedure was radioactive hybridization of the PCR products or the colonies that grow after cloning a suspected fragment.

High stringency Southern Blots /Radioactive hybridization

Synthesis of P^{32}-labeled probes

The fragment that used for probing was digested out of the plasmid and extracted from the agarose gel with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience). The fragment was then labeled using Megaprime DNA Labelling Kit (Amersham Bioscience, n. RPN 1604) and P^{32} dCTP radioactive nucleotide, according to manufacture's instructions.

Hybridization procedure for : **Colony-lifts** from bacterial plates , **DNA** loaded on an agarose gel and **BAC** filters.

After transfer of the DNA to nylon filters as described in a protocol of (Sambrook, Fritsch et al. 1989), the filters were shortly washed with 1xSSC and pre-hybridized for 15 min, 65 ºC in RapidHyb Buffer (Amersham Bioscience, #RPN 1636). Then, 25ul of the synthesized probe were denaturing for 5min at 95°C and added into the tube containing the filters. After hybridizing for 1,5h at 65°C, the filter was washed twice with 2xSSC containing 0.1%SDS, followed by a 30min wash in 0.1xSSC containing 0.1% SDS, 65 ºC. The blot was then exposed with an intensifier screen at -80 ºC until a clear signal was detected.

Southern blot of genomic DNA

Genomic DNA of adult *Platynereis* worms was extracted using NucleoSpin® Tissue kit (Clontech). Preior to extractions the worms were washed with NSW. ~50µg of genomic DNA was then digested with HindIII restriction enzyme (using 3 units of enzyme/ 1 µg DNA) over night and re-precipitated in the following procedure : Adding 0.1 volumes of 5M NACl + 3 volumes Ethanol, followed by incubating at -20ºc for 2.5h. the sample was then centrifuged for 30 min' at 14,000 rpm, the supernatant was discarded and the pellet was washed with 70% Ethanol for 5 min' followed by 10 min' centrifugation. The pellet was then eluted in 30µl of EB buffer.
The digested genomic DNA was loaded and run in different dilutions (1:10, 1:100, 1:1000) on a 1% agarose gel. From this step the procedure followed the above mentioned 'High stringency Southern Blots /Radioactive hybridization' protocol.

4.2.2 r-opsinII cloning

For the cloning of a novel *Platynereis* r-opsin gene, I have used G_q **degenerated** primers designed by Detlev Arendt. The primers sequences that I used are:

Stock number 73 - G_qRup1- CAYTGGACICARTTYCCICCIGT
Stock number 74 - G_qRlo1 - ATNGCYTCICKRWAYTTIGGRTG
Stock number 75 - G_qRup2 - CARACGCCAGCIAAYATGTTYATHATHAA
Stock number 76 - G_qRlo2 - CTCTGCGTADATDATIGGRTTRTGIAT

The **DNA templates** I have used were: 24hpf sscDNA library and 24hpf 5' RACE cDNA library (in separate reactions).

The **PCR program** was the following, according to the melting temperature of the Primers:

94 ºc for 2 min'
94 ºc for 1 min', 43.3 ºc for 2 min', 72ºc for 4 min'
Repeat 5 times
94 ºc for 1 min', 48.3 ºc for 2 min', 72ºc for 4 min'
Repeat 35 times
72 ºc for 10 min'

Stock number 1116 - up1 GACGTCTTGCGGCTTTGACTTCCT
Stock number 1117 - up2 CGGCTTTGACTTCCTGTCCCAAGA
Stock number 1118 - lo1 GCTGGATGCCGTACGCTTTGTTAG
Stock number 1119 - lo2 CGTATGCCAAATCCCGAGTGTTTT
Stock number 1137 - up1 TTGCGGCTTTGACTTCCTG
Stock number 1138 - up2 GCGGGATACTTGCCACAGT
Stock number 1139 - lo1 CTGAGCACAAGCGGATACAGACA
Stock number 1140 - lo2 CAACAAATACGCCTGGCAAAGAT
Stock number 1185 - up1 GTTTAATTACTGCATCTTCAGTTGTGGTT
Stock number 1186 - lo1 TACGCTTTGTTAGTAATGCTGAGCACAA

r-opsin II initial fragment ('28') and the obtained 5' RACE ('62_9') fragment were fused by restriction site cloning strategy. Both fragments were first cloned into TOPO vectors as described above. They were then digested with the following enzymes:
62_9 was double digested with *XbaI* (at the MCS) and *MfeI* (has a single site at the region of the overlap between the two fragments).
28 was digested with the same enzymes.
The digested fragments were run on a gel, the desired fragments for the fusion were cut and eluted and then ligated using a general ligation protocol (Sambrook, Fritsch et al. 1989).

4.2.3 EyeA RACE

I have obtained a 3' RACE fragment based an EyeA fragment cloned by Patrick Steinmentz. The sequence specific primers I used are:

Stock number 280 - EyeAU1 - gga gga ttg tta gga ccc cag aaa a
Stock number 281 - EyeAU2 - cag tgg tta caa ttg cga tca gaa a
Stock number 282 - EyeAL1 - ggc aag ttg atc cct gaa cga ttt t
Stock number 283 - EyeAL2 - tca gtg cca aag tca acc aat tgt c

The library specific primers I used are:

1. for 5' RACE: UPM (Universal Primer Mix) long primer stock number 439 + shorter primer stock number 440
   NUP primer stock number 207.

2. for 3' RACE: poly (A)-primer "RACE Ada"

The **PCR program** was the following, according to the melting temperature of the Primers:

95 ºc for 2 min'
95 ºc for 1 min', 60 ºc for 2 min', 72ºc for 4 min'
Repeat 5 times
95 ºc for 1 min', 60 ºc for 2 min', 72ºc for 4 min'
Repeat 35 times
72 ºc for 10 min'

As a template for 5' RACE, I have used a 24hpf SMART RACE cDNA library, for the 3' RACE I have used a 24hpf sscDNA library

Followed by the procedures described above.

4.3 Whole mount *in-situ* hybridization

4.3.1 General protocol – single probe detection with acetylated tubulin antibody and DAPI staining

RNA probe preparation

reagents:

NTP-Mix: ATP, CTP, GTP 15.4 mM each, UTP 10.0 mM (all Boehringer)
Digoxigenin-11-UTP 10 mM (Boehringer)
Fluorescein-12-UTP 10 mM (Boehringer)
RNasin 20-40 U/µl (Promega, Pharmacia)
T7-/SP6-/T3-RNA-Polymerase 20 U/µl (Boehringer)
5xTranscriptionbuffer (Stratagene)
DNaseI RNase-free 10U/µl (Boehringer)

1. linearize 10 µg of template with a suitable enzyme allowing as transcription (blunt or 5-prime overhang should be preferred to avoid snap back effects)
2. purify template from enzyme and digestion buffer (QiaQuick nucleotide removal kit, Qiagen)
3. control for a complete digest on an agarose gel
4. add in the following order to a total volume of 20 µl:

linearised template	1 µg
100 mM DTT	2 µl
NTP-Mix	1,3 µl
10 mM Digoxygenin-UTP/Fluorescein-UTP	0,7 µl
RNase inhibitor	0,5 µl
10xTranscriptionbuffer	2 µl
H_2O	ad up to 19 µl
RNA-Polymerase (T7 or SP6)	1 µl

1. Incubate at 37ºc for 3-4 hours.
2. add 1 µl DNaseI and incubate for another 15 min at 37°C
3. purify RNA using the Quiagen RNeasy kit
4. Take an aliquot of 4 µl and load in formamide loading buffer onto a 1.5%TAE agarose gel.
5. dilute the remaining probe in 150 µl Hyb-buffer and store at –20°C

Proteinase digestion and postfixation

reagents:

ProteinaseK: prepare a stock solution of 20 mg/ml and store frozen aliquots at -20°C.

4% PFA see above

all steps are performed at room temperature with shaking.

- rehydrate 5 min in 75% MeOH/PTW
- rehydrate 5 min in 50% MeOH/PTW
- rehydrate 5 min in 25% MeOH/PTW
- rinse 2 x 5 min each in PTW
- digest with ProteinaseK (final concetration100 µg/ml in PTW) without shaking for several minutes depending on the stage of the embryos
  <24hpf: 1min; 24hpf-48hpf: 1.5 min ; 48hpf-72hpf: 2min; 72hpf-96hpf:3min
- rinse 2 x shortly in freshly prepared 2 mg/ml glycine/PTW
- fix in 4% PFA/PTW for 20 min
- wash 5 x 5 min in PTW

Hybridization

reagents:

Heparin: make a stock of 50 mg/ml in H_2O, store at -20°C

Hybridization Mix: 50% formamide (Fluka, ultra pure), 5xSSC, 50 µg/ml heparin, 0.1%Tween20, 5 mg/ml torula RNA, store at -20°C,
for 50 ml of Hyb-Mix:

	stock	Hyb-mix
Formamide	100 %	25 ml
SSC	20 x	12.5 ml
Heparin	50 mg/m	l50 µl
Torula-RNA (Sigma)	solid	250 mg
Tween20	10 %	500 µl
H_2O		ad 50 ml

All steps are performed in a water bath or hybridization oven preheated to 65°C

- transfer embryos to 2 ml Eppendorf tubes
- pre-hybridize 1-2 hrs in 1 ml Hyb-Mix at 65°C
- denature probe (4-10 µl/200µl Hyb-Mix, as a rule of the thumb 4 µl of the probe will give a good staining) in 200 µl of Hyb-Mix for 10 min at 80°C
- Remove pre-hybridization solution leaving embryos slightly covered to avoid their desiccation, the embryos are very sensitive at 65°C
- Quickly add hybridization probe, mix gently and hybridize at 65°C overnight

Washes

Reagents:

4xSSCT: dilute 20xSSC to 4xSSC and add Tween20 to 0.1%

All steps are performed in a water bath, all wash solutions are pre-warmed to 65°C

- wash embryos 2 x 30 min in 1 ml 50% formamide/2xSSCT at 65°C
- wash embryos 2x 15 min in 1 ml 2xSSCT at 65°C
- wash embryos 2 x each 30 min in 1 ml 0.2xSSCT at 65°C

Detection

reagents:

BCIP (Boehringer): 50 mg/ml in 100% DMF

NBT (Boehringer): 75 mg/ml in 70% DMF/H_2O

SB: 100 mM TrisCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1%Tween20

for 50 ml of SB add:

	stock	1xSB
TrisCl, pH 9.5	2 M	2.5 ml
NaCl	5 M	1.0 ml
$MgCl_2$	1 M	2.5 ml
Tween20	20 %	250 µl
H_2O		ad 50 ml

Primary staining

1. block embryos 1(-2) hrs with 1ml of 5% sheep serum/PTW at room temperature
2. incubate embryos in 200 µl pre absorbed anti-Dig-AP F_{ab} fragments at 1 : 2000 dilution in PTW AND anti- acetylated tubulin AB at 1:250 dilution overnight at 4°C , shaking at 450 rpm.
3. transfer embryos to a 6-well dish and wash 6 x 10 min while shaking in PTW at room temperature
4. equilibrate 2 x 5 min in Staining buffer (SB)
5. dissolve 4.5 µl NBT (final 337.5 µg/ml) and 3.5 µlBCIP (final 175 µg/ml) in 1 ml of SB and add to the embryos
6. stain in the dark without shaking for up to 5 days, stop staining with SB ph 7.5
7. wash 3 x 5 min in PTW
8. if staining only with NBT-BCIP, fix for 20min in 4%PFA/2xPTW. If staining with anti-AT as well, do not fix and continue to the next steps (and keep in dark).
9. block embryos for 1h with 5% sheep serum/PTW at room temperature
10. incubate embryos with anti-mouse FITC/TRITC/CY5 (1:150,1:100,1:150) AND DAPI (final concentration of 1µg/ml) over night at 4°C , shaking at 450rpm.
11. wash with PTW 5X5.

Mounting

- transfer embryos to 87% glycerol OR DABCO glycerol (2mg/ml)
- leave in 87% glycerol at least overnight for complete equilibration
- mount in 87% glycerol OR DABCO glycerol
  in (*)viewing chamber and continue to microscopy.

* viewing chamber: Glue two or three stripes of tape on microscope slide, add ~80µl of glycerol with embryos and cover with cove slip. This enables to view the whole embryo and rotate it within the slide.

Microscopy

Bright field images were taken with the Zeiss Axiophot microscope with DIC optics. Depending on the stages, using 20 and 40x objectives. Pictures were recorded using a digital camera in a ".tiff" format. Embryos were usually images in few stacks and

orientations (apical, ventral, dorsal), depending on the staining. Images were processed using Image J software.

Confocal microscopy was done using a Leica TCS SP2 and Leica TCS SPE, with a 40x oil-immersion objective.

I have used:

1. The wholemount reflection CLSM (Jekely and Arendt 2007) that enables the detection of both NBT-BCIP precipitation and a fluorescent signal (either from antibody or RNA probe)
2. The common detection of two or more fluorescent signals.

Step size was 1µm. the volume scanned varied depending on the staining and the purpose of the scan.

4.3.2 Double fluorescent *in-situ* hybridization protocol

The protocol is similar except the following additions and modifications:

1. The synthesis of two probes, one Digoxigenin and one Fluorescein.
2. The detection of the probes is done separately for each probe, using the TSA fluorescent Systems (Perkin Elmer). Therefore the protocol for the detection is the following:

1st Detection

reagents:

Perkin Elmer Cyanine 3 TSA Plus System

Perkin Elmer Fluorescein TSA Plus System

Perkin Elmer Blocking Reagent (included in kits; Cat. No. FP1020 for separate order)

Perkin Elmer Amplification Diluent Plus (only inculded in "Plus" Systems; Cat.No. FP1135 for separate order)

TNT: 0,1M Tris-Hcl pH 7,5; 0,15M NaCl; 0,1% Tween 20

Anti-Digoxigenin-POD, Fab fragments (Roche, Cat. No. 1 207 733)

Anti-Fluorescein-POD, Fab fragments (Roche, Cat. No. 1 426 346)

All procedures are done in eppendorf tubes

1. block embryos 1(-2) hrs with 1ml of 1% Perkin Elmer Blocking Reagent/TNT at
2. room temperature
3. incubate embryos for 1(-2) hrs in 100 µl preabsorbed anti-Fluo-POD F_{ab} fragments at a 1 : 50 dilution in 1%Blocking reagent/TNT overnight at 4°C
4. wash 6x 5' in TNT

5. equilibrate 1x in 100ul TSA Plus Amplification
6. dilute Fluorescein Fluorophore Tyramide 1:25 in TSA Plus Amplification
7. add staining solution: 25ul/tube and stain in the dark without shaking for 2h-5h
8. check staining by transferring a few embryos in 3ml TNT in a 6-well plate; wash 3 times with TNT, mount and have a look under the microscope
9. wash 3x in TNT

POD enzyme inactivation

1. incubate 20' in the dark in 1%H_2O_2/TNT without shaking
2. wash 4x 5' in TNT

2nd Detection

All procedures are done in eppendorf tubes, in the dark.

1. block embryos 1(-2) hrs with 1ml of 1% Perkin Elmer Blocking Reagent/TNT at room temperature
2. incubate embryos for 1(-2) hrs in 100 µl preabsorbed anti-Dig-POD F_{ab} fragments at a 1 : 100 dilution in 1%Blocking reagent/TNT overnight at 4°C
3. wash 6x 5' in TNT
4. equilibrate 1x in 100ul TSA Plus Amplification Diluent
5. dilute Cy3 Fluorophore Tyramide 1:25 in TSA Plus Amplification Diluent
6. add staining solution: 25ul/tube and stain in the dark without shaking for 2h-5h
7. check staining by transferring a few embryos in 3ml TNT in a 6-well plate; wash once with TNT, mount and have a look under the microscope
8. wash 3x in TNT

4.3.3 Double detection: fluorescent probe and DIG probe

The technique described in detail by (Jekely and Arendt 2007). Briefly, the protocol follows the above **4.3.1** section. Then, continuing to additional fluorescent staining described above in **4.3.2** (only the first detection, adding then the required secondary antibody for acetylated tubulin and the DAPI).
At the confocal microscope I have used the 633nm laser to detect the NBT-BCIP signal, by tuning the diction window at the ***same*** wavelength as the laser used for shining and as small as possible. For detecting the fluorescent signal I have used the standard method of fluorescent microscopy, where I excite the flourophore with the appropriate laser line and detect the emitted light at a longer wavelength.

Scannings that were incorporated into the 3D *in-silico* alignment tool were taken with a zoom of 1.43 and 3 frames per averaging.

4.4 Cyclopamine inhibitions

The assay was established in the lab by Kristin Tessmar-Raible. In each experiment at least 3 batches were used, and split into inhibited and control. The embryos were all collected on one net and distributed equally into 24 well plates (around 100 embryos/well). The drug Cyclopamine was purchased from Toronto Research Canada (TRC), diluted with 95% Ethanol into a stock concentration of 2.4mM. From this stock I have used a range of volumes (see table) directly applied into the natural sea water, followed by a moderate shaking. The controls received 95% Ethanol in an equivalent volume. The embryos were incubated in cyclopamine/NSW for a period of time (see table). Incubation was done at 18ºc, in the dark (since the drug is light sensitive). The embryos were then fixed according to the above protocol for WMISH, for the analysis of changes in gene expression.

Table 1

	Targeting larval eyes development		**Targeting adult eyes development**	
Concentration (stock solution/ml NSW)	1-1.5ul/1ml	4ul/1.5ml	1-1.5ul/1ml	4ul/1.5ml
Inhibition period	22-48hpf	18-30hpf	22-48hpf	38-50hpf

4.5 Construction of brain-eye specific library

For the construction of brain-eye specific library, around 100 adult worms were abstained from food and were treated with algae killer to avoid contamination. The worms were dissected to separate the head and the samples were kept in Trizol at -80ºc prior to the following procedure.

4.5.1 Trizol extraction of total RNA

1. Homogenize extract first with pestle in 50µl of Trizol (GibcoBRL – Life Technologies, Cat # 15596-018) then with a 27G syringe in total volume of trizol of 1000µl.
2. Leave 5 min’ at room temperature (RT).
3. Centrifuge 10 min’, 4ºc, 14, 000rpm

4. Transfer supernatant to a new eppendorf, add 300µl Cholorophorm, vortex 15 sec, leave 5 min' at RT.
5. Centrifuge 15 min', 4ºc, 14, 000rpm
6. Save aqueous phase to a new eppendorf, add 300µl Chlorophorm, vortex 15 sec, leave 5 min' at RT.
7. Centrifuge 15 min', 4ºc, 14, 000rpm
8. Save aqueous phase to a new eppendorf, add 500µl Isopropanol, shake, leave 10 min' at RT
9. Centrifuge 10 min', 4ºc, 14, 000rpm
10. Remove liquid, wash pellet with 1000ul 70% EtOH, centrifuge 5 min', 4ºc, 14, 000rpm
11. Remove liquid and briefly air-dry the RNA pellet (be careful not to over dry it)
12. Add 100µl of H_2O to dissolve pellet

4.5.2 mRNA isolation using Dynabeads®

We have used the Dynabeads® mRNA DIRECT™ Kit, with the following modified protocol (for 100-200µl of total RNA):

1. All buffers except the 10Mm Tris-HCl should be brought to room temperature. The RNA sample should be thawed on ice.
2. Re-suspend Dynabeads by pipetting and take 250µl to a new eppendorf tube.
3. Place the eppendorf tube with the Dynabeads in the Magnet (Dynal MPC™ Magnetic Particle Concentrator MPC), wait 30 seconds and remove the supernatant.
4. Re-suspend Dynabeads in 250µl lysis buffer and place in MPC.
5. Add 1ml lysis buffer to RNA sample
6. Remove sup' from the Dynabeads.
7. Add RNA sample to Dynabeads, re-suspend Dynabeads and mix in Nutator for 5 minutes.
8. Place in the MPC, wait 30 sec' and save sup' to initial sample eppendorf and keep it on ice.
9. Add 1ml of buffer B to the Dynabeads, re-suspend them, place in MPC, wait 30 seconds and remove sup'.
10. Repeat the wash with buffer B.
11. Re-suspend in 20µl of 10mM Tris-HC, heat to 65ºc for 2 min'.
12. Save supernatant with mRNA and mix with the supernatant from step 8.
13. Wash Dynabeads with 250µl lysis buffer.
14. add lysis buffer and RNA to the Dynabeads, repeat steps 7-13, without saving the supernatant in step 8.
15. Save supernatante with RNA. Elute twice with 5µl, measure mRNA concentration.

4.5.3 cDNA library synthesis and cloning

We have used the SuperScript™ Plasmid System with Gatway® technology for cDNA Synthesis and cloning Kit from Invitrogene (Catalog n.18248-013). An overview of the main steps is given:

1. First strand synthesis
2. Second strand synthesis
3. Introducing Asymmetry into cDNA
4. Maximizing the ligation efficiency of cDNA to the vector by adapter addition.
5. Size fractionation of cDNA
6. Ligation of size fractionation cDNA to the plasmid vector and introduction into *E.coli*
7. Gatway cloning
8. Expansion of plasmid cDNA libraries

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