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Development and evolution: the case of eye development

Abstract – In «*The Origin of Species*» Charles Darwin (1859) discusses eye evolution in the paragraph «*Organs of extreme perfection and complication*» of Chapter VI, which has the significant title «*Difficulties of the Theory*». Darwin appears to be fully aware of difficulties on eye evolution: «*To suppose that the eye, with all its inimitable contrivances… could have been formed by natural selection, seems, I freely confess, absurd in the highest degree*». Indeed, the great variety of eyes in the animal word raises the question on their possible origin, by natural selection, from a simple prototypic ancestral eye. In fact eyes have also been considered an example of functional convergence or parallelism and the view on their possible polyphyletic origin has been authoritatively proposed. The recent emergence of an extensive conservation of the genetic network driving eye development in animals as distant as insects and mammals has brought new life to the debate on the monophyletic versus polyphyletic origin of the eye. A core question on this topic is whether the conserved genes build truly homologous eyes. To answer this question it is necessary to define what is an eye and its relation to a prototype eye. A recent proposal (Arendt and Wittbrodt, 2001), while supporting the homology of cerebral eyes in Protostomia, maintains the possible non-homology between the eyes of Chordates and non-Chordates due to the structural diversity of their photoreceptors and the biochemical diversity of their phototransduction cascade.

This paper presents the molecular genetics of eye development by comparing the gene networks controlling eye development in invertebrates and vertebrates: to this aim emphasis will be on two developmental model organisms, the fruit fly *Drosophila* and the frog *Xenopus*, respectively. In this context recent results from our laboratory, obtained in *Xenopus*, will also be presented. These data illustrate aspects of the molecular genetics of the early steps of eye development – namely, eye field specification – as well as of later events in retina development, such as proliferation and fate choice of the retina cells in vertebrates.

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Introduction

Vision and eye since long time attracted attention and thoughts of humans, particularly in relation to the process of formation of knowledge in the human mind. Plato compares the eye to the sun (Rep. 508B, 3-4); Augustine declares: «…oculi autem sunt ad cognoscendum in sensibus principes,…» (Confessiones X, 35.54). However, while the eye was clearly conceived to be the organ dedicated to vision in different organisms, many centuries had to pass by before we realized the amazing variety of the extant eye structures and asked the question if, and how, the different eye structures could relate to each other and what could be the evolutionary origin of the eye.

Evolution has generated at least three major different types of eyes: the camera-type eye, consisting of a single lens projecting onto a retina (found in vertebrates and cephalopods); the compound eye with multiple ommatidia, each formed by a set of photoreceptor cells and a lens of its own (insects and other arthropods); and the mirror eye which, in the case of the scallop (*Pecten*) uses both a lens focusing the light onto a distal retina and a reflecting parabolic mirror projecting the light onto a proximal retina. Eyes also differ for the optical solutions adopted to seeing; for the type of photoreceptors and their physiological responses; for their embryological origin (Fernald, 2000). Despite these striking differences, many striking similarities can also be found across various phyla, and this has caused controversial ideas on the evolutionary origin of the eye.

Darwin's view on eye evolution

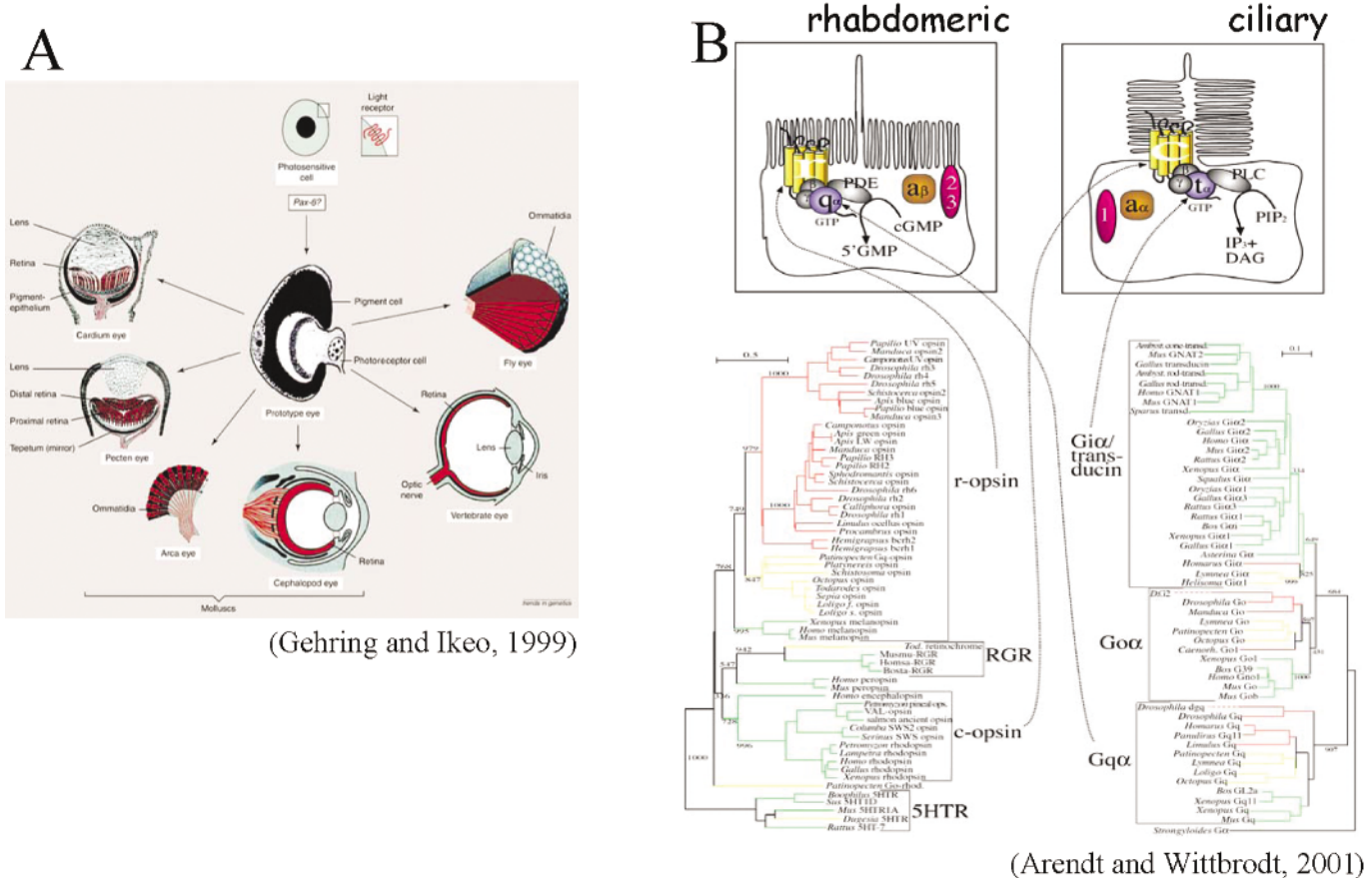
In «*The Origin of Species*» Charles Darwin (1859) discusses eye evolution in the paragraph «*Organs of extreme perfection and complication*» of Chapter VI, which has the significant title «*Difficulties of the Theory*». Darwin so formulates his major question concerning the eye: «*Can we believe that natural selection could produce… organs of such wonderful structure, as the eye, of which we hardly as yet fully understand the inimitable perfection?*» Darwin appears to be fully aware of difficulties on eye evolution, and in fact this is the «incipit» of his reasoning: «*To suppose that the eye, with all its inimitable contrivances… could have been formed by natural selection, seems, I freely confess, absurd in the highest degree*». Nevertheless, it appears to Darwin that it may be feasible to recognize a series of gradations in the eye structures of at least some animal groups (e.g., Crustacea), on which natural selection could have acted, starting from a prototype eye: «*With these facts,… which show that there is much graduated diversity in the eyes… I can see no very great difficulty… in believing that natural selection has converted the simple apparatus of an optic nerve merely coated with pigment and invested by transparent membrane into an optical instrument as perfect as…*» «*…and to admit that a structure even as perfect as the eye of an eagle might be formed by natural selection…*». We

cannot avoid noticing the cautious approach of Darwin to the view he comes to propose, as reflected in his affirming a «*very great difficulty*» and, to some extent, being driven «*to admit*» the intervention of natural selection. Here: «*…though I have felt the difficulty far too keenly to be surprised at any degree of hesitation…*» Darwin even sympathizes with skeptics. Finally: «*We should be extremely cautious in concluding that an organ could not have been formed by transitional gradations of some kind*», he concludes by suggesting to be cautious in «excluding» the formation of eyes by «*transitional gradations*», rather than by strongly supporting this view in a positive manner. In any instance, even though with great caution and awareness of the difficulties generated to his theory by the case of eye evolution, in the end Darwin appears to think that eyes originated by natural selection, starting from a simple prototype eye. Even though he appears to restrain from any clear-cut statement concerning the question of a monophyletic versus a polyphyletic origin of the eyes, in Darwin's view the origin of the eye appears to pose a similar problem as the origin of life itself: «*How a nerve comes to be sensitive to light, hardly concerns us more than how life itself first originated*;…»: thus, a rare event, that ought not to have occurred repeatedly in different phyla. A major stand in favor of the polyphyletic origin of the eyes is instead found much later in the landmark paper of Salvini-Plawen and Mayr (1977): based on a wide comparison of morphology, structure and embryology of eyes, the Authors reach the conclusion that eyes must have originated independently at least 40-65 times, thus providing a strong and circumstantiated support to the polyphyletic origin of eyes. As we shall see below, more recently this question has been re-examined on a molecular ground and is still lively debated at the present time.

What is an eye?

At its root, the question on the evolutionary origin of eyes means to ask whether all different eyes can derive from a simple, ancestral prototype eye and to identify such a simple eye (Fig. 1A). Here, again, we can refer to Darwin's definition: «*…the simple apparatus of an optic nerve merely coated with pigment and invested by transparent membrane…*» In fact, simple eyes are formed by a light-sensitive cell provided with a neural projection and associated to a pigmented cell that screens the deeper tissues from light. The eyespots of primary ciliary larvae such as the trocophora (Lophotrochozoa, Protostomia) and tornaria (basal Deuterostomia) larvae are similar to such prototype eye: these larvae are found at the root of development in different phyla of Bilateria, whose adult eyes display a variety of different morphologies and structures.

In order to discuss eye evolution and the degree of homology of eyes, it is necessary to define what are the eyes to be compared. The most promising candidates, in the context of relating eye development to eye evolution, are «cerebral eyes» (see Arendt and Wittbrodt, 2001 and references therein). These are eyes projecting



(Gehring and Ikeo, 1999)

(Arendt and Wittbrodt, 2001)

Fig. 1 - A) A hypothetical scheme of the evolution of various eye-types from a common ancestral prototype eye (from Gehring and Ikeo, 1999). B) Genetic cascades involved in phototransduction of rhabdomeric (invertebrate) and ciliary (vertebrate) photoreceptors (from Arendt and Wittbrodt, 2001).

to an anterior nervous center and located in the anterior body region, thus being comparable with respect to the same spatial references. The position criterion to define cerebral eyes is today complemented by the notion that they form in a region specified by the *otd/otx* patterning genes in a variety of organisms including planarians, polychaetes, insects, ascidians and vertebrates. In addition, these eyes should be capable of spatial vision, that is to compare/elaborate the different intensities of the light coming from different directions, and not only to perceive light. Most probably, eyes apt to spatial vision became necessary when animals achieved a bilateral symmetry; thus, asking whether eyes may have a monophyletic origin means to ask whether Urbilateria (the organisms considered to be at the root of Bilateria) were endowed with simple cerebral eyes, such as those of the primary ciliary larvae (the larval eyespots can be considered «cerebral» in that they form close to the developing brain). In this respect we may observe that planarians already possess simple eyes unable to focus images, but nevertheless capable of projecting to anterior nervous centers, thus being able to elaborate the different intensities and directions of light (Sakai *et al.*, 2000). Ancestrality of cerebral eyes is also supported by their occurrence both in Protostomia (Ecdysozoa and Lophotrochozoa) and lower Deuterostomia.

To explore the possible derivation of different eyes from a simple prototype eye we need to consider the homology of eyes in different animals, starting first from those structures enabling eyes to detect light, that is the photoreceptor cells.

Photoreceptors and phototransduction

Photoreceptors are cells detecting light and, in the search for homology, they can be analyzed from different viewpoints. For example, are photoreceptors in different eyes structurally and/or functionally related? What kind of photoreceptors did the Urbilateria possess, if any? What are the phototransduction molecular pathways in the different eyes? And, again, are these pathways traceable to a common origin? Recent work from Arendt and Wittbrodt (2001) deeply elaborated on these questions, and is mostly to this work I will refer here (see also references therein).

The starting point for photoreceptors is a ciliated epithelial cell that enormously expands its surrounding membrane. According to which aspect of the cell membrane is expanded, photoreceptors belong to one of two different kinds: in rhabdomeric photoreceptors the apical cell membrane folds into microvilli; ciliary photoreceptors expand instead their cilium membrane (Fig. 1B). There appears to be a net distinction between the two kinds of photoreceptors, since no intermediate structures are known. Both types may coexist in Lophotrochozoa, Ecdysozoa and Deuterostomia. Remarkably, however, their distribution is not random: cerebral eyes have rhabdomeric photoreceptors in Protostomia and in lower Deuterostomia, while those of chordates have ciliary photoreceptors; exceptions are very rare. In fact, vertebrates are the only deuterostomes not possessing any rhabdomeric photoreceptors.

Whereas on the only basis of ultrastructure and distribution it is not possible to argue on which is the genealogical relationship of the two photoreceptor types, further information can be obtained testing the homology of the molecules implicated in the light detection and phototransduction processes (Fig. 1B). The first step, photoactivation of rhodopsin, involves the isomerisation of covalently bound retinoids. Photoactivated rhodopsin activates a G-protein that in turn activates intracellular messengers to hyperpolarize or depolarize the photoreceptor cell. The subsequent quenching of phototransduction involves phosphorylation of photoactivated rhodopsin by rhodopsin kinase, followed by binding of arrestin, which competes with the G-protein for binding to photoactivated rhodopsin. Notwithstanding the conservation of such a general scheme, the two kinds of photoreceptors totally differ as for what molecular families they use for light transduction as well as for quenching of transduction. The two types of photoreceptors in fact employ different families either of opsins, G-proteins or second messengers for the light transduction pathway; in addition, different molecules work in the quenching process (Fig. 1B). Thus, two clearly distinct types of photoreceptors occur in Bilateria that employ non-orthologous systems for light detection and phototransduction. Whether both might already be present in Urbilateria, remains an open question. Based on a morphological and molecular comparative survey, it has been proposed that a two-celled eye precursor, endowed with a rhabdomeric photoreceptor and similar to the eyespots of the present day primary ciliary larvae, was present in the larvae of Urbilateria (Arendt and Wittbrodt, 2001; Arendt *et al.*, 2002).

Development and evolution: the case of the eye

The fields of development and evolution are convergent because both are rooted in the genomic regulatory programs for body plan formation. Evolutionary biology aims to understand how organisms evolve and how they change form; developmental biology seeks to understand how alterations in gene expression and function, during development, lead to changes in form. Although the two subjects are so tightly related, only in the past fifteen years they joined in a fruitful relationship which gave rise to a new discipline: evolutionary developmental biology, or «evo-devo». This new subject studies how developmental processes evolved; in particular, it studies how they can be modified by genetic changes and how such modifications produce the diversity of morphologies and body plans (Holland, 1999).

The main push to the growth of evolutionary developmental biology came from the discovery that animals as different as nematodes, flies and vertebrates use similar genes for similar functions. Amazing examples are the conservation of the homeodomain in transcription factors, the homology of the homeotic/*Hox* genes from *Drosophila* to mammals and, more recently, the identification of *pax6* as one of the master regulatory genes in eye development throughout the animal kingdom (McGinnis *et al.*, 1984; McGinnis, 1994; Quiring *et al.*, 1994). However, simply

documenting more cases of gene conservation does not help in shedding light into the matter of how evolution really took course. We need to establish when and in which organisms a gene appeared, how it was eventually mutated and which factors promoted mutation. In the end, this is the task of «evo-devo»: to clarify how developmental processes are modified during evolution and how this results in changes in animal morphology and body plan.

What the relationships between eye development and eye evolution? A major question is whether the gene activities controlling eye development are conserved across animal phyla, thus pointing to their common origin. In the following sections I will first report on seminal work in *Drosophila,* and then results will be compared to those obtained in vertebrate model systems; in this context work from our laboratory in the frog *Xenopus laevis*, a vertebrate developmental system, will also be presented. Only brief reference to other organisms will be made.

Eye development in Drosophila: *a conserved master control role for* pax6?

In recent years much has been learnt on the molecular-genetic control of eye development. Work uncovering the function played by the *pax6* gene in eye development in several species, spanning invertebrates and vertebrates, played a pivotal role in bursting interest on this subject. *pax6* – a gene belonging to the *pax* multigene family – codes for a transcription factor endowed with both a paired domain and a homeodomain. *pax6* is highly conserved in evolution and is expressed in the developing eye of organisms as diverse as planarians and man (Quiring *et al*., 1994; Callaerts *et al*., 1999). Curiously, *pax6* is also expressed in adult organisms with no eyes, such as the sea urchins, where its expression is however found in structures somehow related to light detection, such as the tube feet. Besides performing phototactic movements, the tube feet convey objects close to the skin struck by light at that moment, thus being involved in a covering reaction of the light-sensitive body surface (Minsuk and Raff, 2002).

A breakthrough on the function of *pax6* came from work in *Drosophila,* where *pax6/eyeless* is expressed in the primordium of the eye imaginal disc in the embryo, as well as in the eye anlage of the eye/antennal imaginal disc in the larva (Gehring and Ikeo, 1999 and references therein). In the *Drosophila eyeless* (*ey*) mutant, where no eyes are formed, no *pax6* expression is present during development, thus demonstrating *pax6* to be required for eye formation. Strikingly, when Walter Gehring and his collaborators expressed *pax6* ectopically in transgenic flies, eyes developed in ectopic sites such as antennae, wings and legs, thus indicating *pax6* also to be sufficient for eye development (Fig. 2A; Halder *et al*., 1995). Even more impressively, expressing *pax6* genes from various sources, such as squid, ascidia, mouse produced the same result in transgenic flies – that is, formation of ectopic eyes that were of the *Drosophila* kind, regardless of the origin of the *pax6* gene. These results led the Authors to suggest an extreme conservation of a «master»

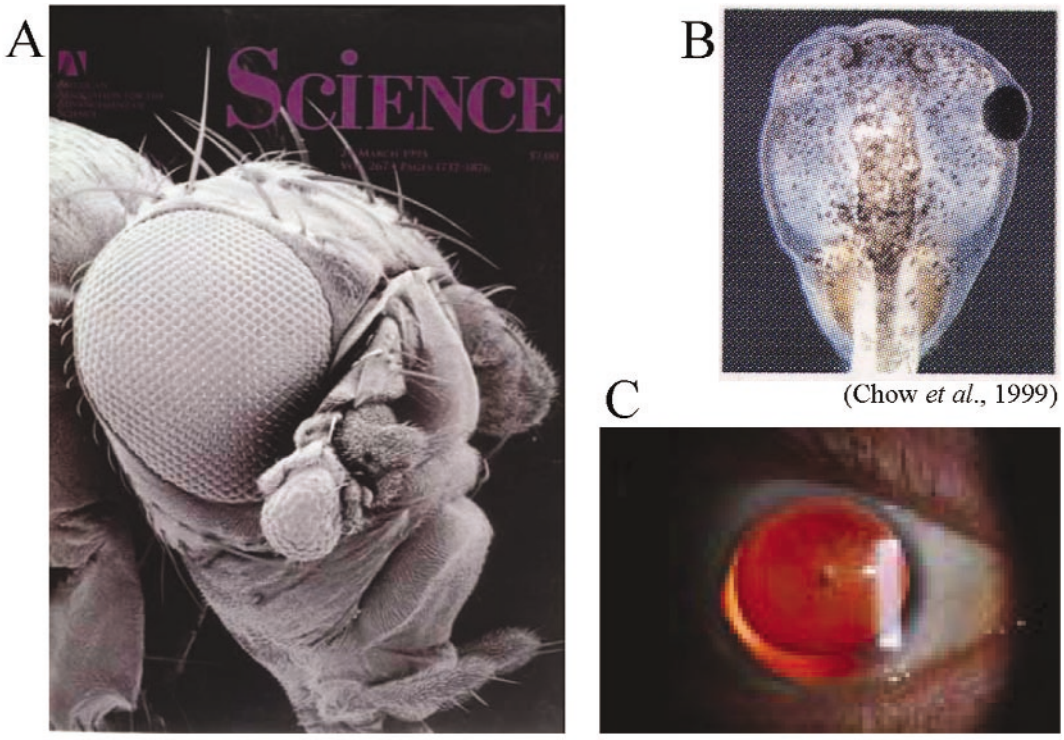


Fig. 2 - *eyeless/pax6* is both necessary and sufficient for eye formation in *Drosophila* and vertebrates. A) Transgenic flies expressing *pax6* in ectopic sites show development of eyes in antennae (from Halder *et al*., 1995). B) Overexpression of a dominant negative form of *pax6* in *Xenopus laevis* inhibits eye formation in the experimental side (from Chow *et al*., 1999). C) Aniridia in humans is caused by a mutation in the *PAX6* gene. Development of iris, lens, retina and cornea is disturbed (from Glaser *et al*., 1992).

role of *pax6* in eye development and evolution (Gehring and Ikeo, 1999). Although subsequent work restrained our view on the *pax6* role to some extent (see below), work in *Drosophila* paved the way to a flourishing of studies aimed to unravel the genetic network underlying eye development in both invertebrates and vertebrates.

How could *pax6* act in mastering eye development? According to a first model, by controlling a linear cascade of gene activities, where, like *pax6*, the genes in the cascade also code for transcription factor proteins. Both mutant and molecular analyses support this model. The subsequent discovery in *Drosophila* of a gene so similar to *eyeless* to deserve the name *twin of eyeless (toy*; Czerny *et al*., 1999), both necessary and sufficient for eye formation and acting upstream of *ey*, does not affect the substance of a model where a hierarchy of gene activities progressively drives eye development. More recently, this first linear model has been modified by

the integration of new data, showing that the gene products downstream of *ey* can in turn regulate *ey* itself, thus creating an auto-regulatory loop committing the cells to the eye fate (Fig. 3A). These genes – *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) – all code for transcription factors that are necessary for eye development (Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Mardon *et al.*, 1994**).** In misexpression experiments *eya, eya + so, dac, dac + eya* as well as *teashirt* (*tsh*) all can induce ectopic eye formation, but also upregulate *ey* expression as well as each other's expression (Chen *et al.*, 1997; Pignoni *et al.*, 1997; Shen and Mardon, 1997; Pan and Rubin, 1998). However, it should be emphasized that *ey* is a much more potent inducer of ectopic eyes than any single gene in the later group, suggesting that no single gene can recapitulate the entire spectrum of *ey* activities. In contrast both *eyegone (eyg*; a second *pax* gene) and *optix* (*opt*; a *so* gene family) are able to induce ectopic eye formation independently of *ey*, suggesting that during ectopic eye formation they can function in a partially different pathway (Seimiya and Gehring, 2000). Thus, an interactive network of transcription factor genes, more than a linear genetic cascade, appears to control eye development in *Drosophila* (Fig. 3A).

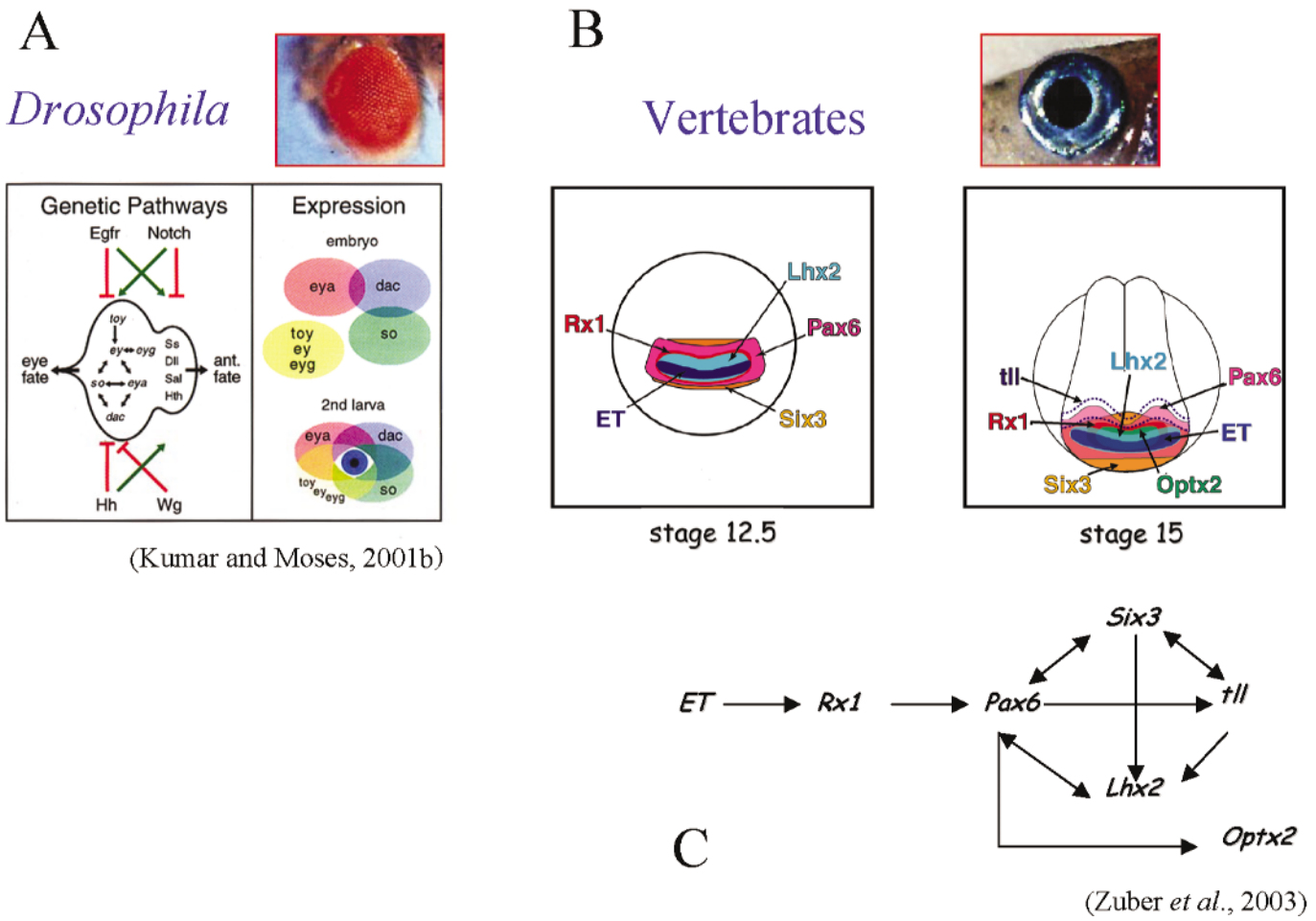
If a network of transcription factors does control eye specification in the fly, one prediction would be that these genes would show overlapping expression domains in the developing fly eye. Indeed, *toy*, *ey*, *so*, *eya*, *dac* and *eyg* are co-expressed in the eye field between larval stage 1 and 2 (Fig. 3A; Kumar and Moses, 2001a, b).

What does regulate the regulators? A satisfying answer involves the Notch and EGF receptor signaling. Thus, fly eye development at larval stage 1 and 2 has been shown to coincide with a critical period during which up-regulation of Notch signaling and down-regulation of EGF signaling in the eye portion of the eye-antennal disc is believed to specify eye fate (Kumar and Moses, 2001a, b). The observation that Hedgehog and Wingless signaling can also participate in eye-antennal fate decisions (Royet and Finkelstein, 1996, 1997) has led to the hypothesis that Notch, EGF, Wingless, and Hedgehog signals function upstream of at least some components of the network of transcription factors controlling eye specification; where the transcription factor gene network will be co-expressed, there an eye will form (Fig. 3A). Now, we are interested to see to what extent genes and networks have been shared in eye development throughout evolution.

Vertebrate eye development

The well established notion of *Drosophila* as a paradigm for the molecular genetics of embryo development established the approach of looking in other organisms for genes previously isolated in *Drosophila* and found to play a crucial function in development. Curiously, for *eyeless/pax6* the process went the other way around since *Pax6* was first isolated in the mouse and then found to be orthologous to the mutated gene in the *Drosophila eyeless* phenotype (Quiring *et al.*, 1994).

The study of the molecular-genetic control of eye development in vertebrates



(Kumar and Moses, 2001b)

(Zuber *et al.*, 2003)

Fig. 3 - Comparison between genetic pathways that lead to eye specification in *Drosophila* and vertebrates. A) Genetic hierarchy which leads to specification of the eye fate from the eye-antenna imaginal disc and expression domains of the eye field-specific transcription factors at different developmental stages in *Drosophila* (from Kumar and Moses, 2001b). B) Expression domains of the eye field transcription factors (EFTFs) at different stages of eye field specification in *Xenopus laevis*. C) Scheme showing epistatic interactions between the vertebrate EFTFs (from Zuber *et al.*, 2003).

takes advantage of model organisms such as the zebra and medaka fishes, the frog *Xenopus*, chick and mouse. Each organism contributes with knowledge on species-specific processes, but behind that a comprehensive picture is emerging underlying eye development in all vertebrates. In recent years our laboratory concentrated on the study of eye development in *Xenopus*, and what follows is a selection of outcomes in the frog model system. The results will be considered in the perspective of a comparison with knowledge in *Drosophila*.

Eye development in Xenopus: *eye field specification by a gene network*

An important difference should first be underlined between *Drosophila*, on one side, and *Xenopus* and vertebrates on the other, concerning the embryological origin of eyes: they are of ectodermic origin in *Drosophila*, while they are of neural origin in vertebrates (see Lupo *et al.,* 2000). This means that vertebrate eye formation is preceded by neural induction, where signals stemming from the mesendoderm address cells of the dorsal ectoderm towards a neural fate. Afterwards, a crucial step of specification occurs in the anterior neural plate due to the expression of the homeobox *otx2* gene in a wide domain comprising the presumptive forebrain and midbrain: vertebrate eyes will be formed within the expression domain of *otx2* (Fig. 4A). In fact, both classical data and more recent experiments have shown that a large part of the anterior neural plate can form eyes; this area is referred to as the «eye morphogenetic field» and is usually wider than the area that eventually will give rise to the eyes (Adelmann, 1929). When the earlier eye markers are activated, their expression entirely resides within the *otx2* positive area, in an internal aspect where the *otx2* expression is concomitantly turned off (Fig. 4B, C; see for example Casarosa *et al*., 1997; Andreazzoli *et al*., 1999; Zuber *et al*., 2003). Several transcription factors are activated in the area free of the *otx2* expression: it is the concerted action of these genes (the EFTFs: Eye Field Transcription Factors) responsible for the progressive specification of the eye field in the anterior neural plate (Fig. 3B, 4C).

Is there any relation between the vertebrate eye field genes and their counterpart in *Drosophila*? Besides *eyeless/pax6*, multi-gene families were found to correspond, in vertebrates, to the single *eya*, *so* and *dac* eye genes of *Drosophila* and both a resemblance in expression and a functional similarity were underscored. *pax6* was found to be necessary to make eyes in vertebrates as it is in *Drosophila* (Fig. 2B,C; Hill *et al*., 1991; Glaser *et al*., 1992; Jordan *et al*., 1992; Chow *et al*., 1999). Furthermore, in particular conditions *pax6* is sufficient to elicit formation of ectopic eyes in *Xenopus* (Chow *et al*., 1999). *six3*, a gene of the *six* family orthologous of *optix*, was also shown to induce eye formation in both medaka and *Xenopus*, and to be necessary for eye development in medaka, *Xenopus* and mouse (Loosly *et al.,* 1999; Carl *et al*., 2002; Lagutin *et al*., 2003; our unpublished results). To date, a number of transcription factor genes are known to be expressed early in the eye field of vertebrates, from fishes to man. Mutations in these genes produce absence,

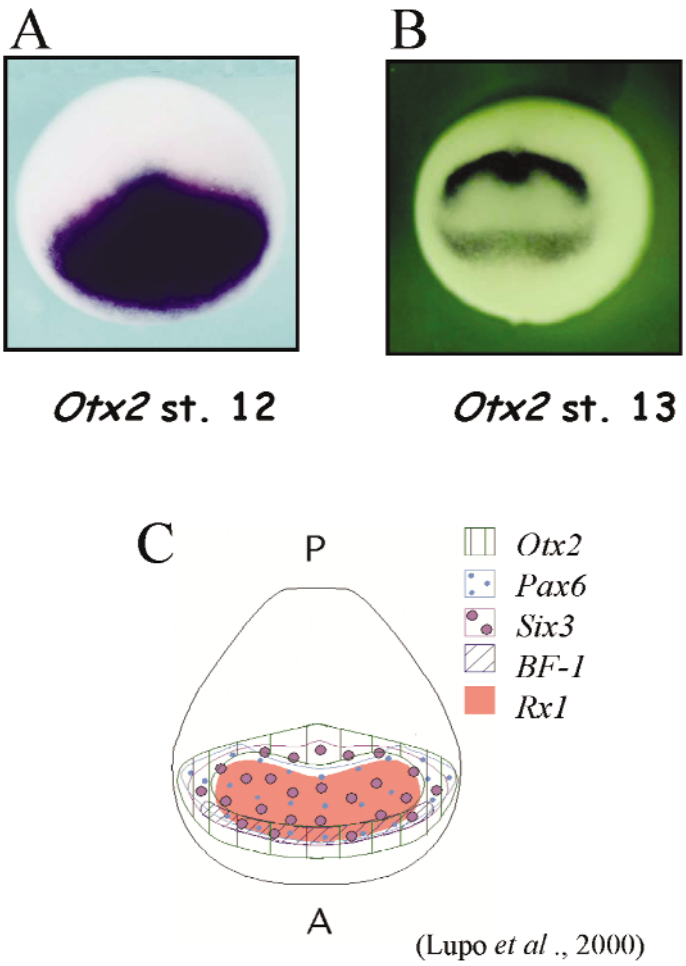


Fig. 4 - Eye field specification requires down-regulation of *otx2* expression in the presumptive eye field territory in concomitance with the onset of EFTFs expression. *Xotx2* expression before (A) and after (B) eye field specification in the anterior neural plate of the *Xenopus* embryo. C) Scheme illustrating EFTF expression in the anterior neural plate with respect to *Xotx2* expression (Lupo *et al*., 2000).

reduction, or malformation of eyes, showing all of them are necessary to eye formation; furthermore, their overexpression may induce expansion, or ectopic formation, of eye tissues.

Are the genes eliciting eye formation interconnected in a genetic network like the one found in *Drosophila*? Surprisingly, the basic answer is again positive, although similarity does not mean identity (the occurrence of multi-gene families in vertebrates versus single genes in *Drosophila* is in itself a difference affecting the

relationships among genes). In collaboration with the laboratory of William Harris in Cambridge, we contributed to answer this question by analyzing the spatio-temporal expression of eight transcription factor genes, all expressed in the eye field (*otx2*, *ET*, *pax6*, *six3*, *rx1*, *tll, lhx2* and *optx2*; Zuber *et al*., 2003). As for the timing, expression of the eye field genes follows a precise time sequence, with *ET* expressed first, *optx2* last and the others in between in a defined order. This result suggests the occurrence of a gene hierarchy that might reflect the temporal sequence of gene activation. Spatially, the gene domains of expression are partially overlapping and concentric: the domains of four genes (*ET*, *rx1*, *lhx2*, *optx2*) are essentially comprised within the eye field, while those of other genes (*otx2*, *pax6*, *six3*) cover other neural presumptive territories as well, surrounding the eye field (Fig. 3B, 4C). Among the early eye field genes *ET* – the first to be expressed – occupies the narrowest territory, which is included and encircled by the *lhx2* and *rx1* domains of expression. Alike the temporal pattern, also the spatial pattern of expression is dynamic and accommodates with time, as shown by comparing expression in early and late neurula (Fig. 3B).

To underscore the relationships between genes, expression of each eye field gene was analyzed in the presence of overexpression of each of the other genes in vivo. Together with the definition of the spatio-temporal gene expression, these results brought to the scheme in Fig. 3C: *ET*, followed by *rx1*, appears to be at the head of an auto-regulatory network of gene activities. In *Xenopus*, elimination of either *ET*, *rx1*, *pax6* or *six3* from a cocktail of EFTFs injected into the *Xenopus* embryo reduces the frequency of ectopic eye tissue formation; the most dramatic reductions in ectopic eye tissue were observed when *pax6* was removed. This meshes well with the general prominence given to *pax6* and its *Drosophila* homologues *ey* and *toy* as transcription factors centrally involved in early eye development (see Wawerisk and Maas, 2000).

In summary, similarly to what found in *Drosophila* expression of a network of interacting transcription factor genes may induce and/or stabilize eye development in the vertebrate eye field. The network of the vertebrate eye field appears similar to that in *Drosophila* not only conceptually, but also for the presence of orthologous genes and for similarities in gene interactions. As in *Drosophila*, the physical interaction of some of the encoded proteins – Pax6 with Rx1, Six3 and Lhx2; Optx2 (=Six6, Six9) with Dac – has been demonstrated biochemically (see references in Zuber *et al*., 2003). The occurrence of a gene network controlling eye development in both flies and vertebrates somewhat limits the primacy of *pax6*. It should also be considered that *pax6* is expressed in sites other than the eyes in all examined organisms. In addition, *pax6* is not expressed in the photoreceptors of either cephalopods or vertebrates and, while it activates the rhodopsin genes in *Drosophila*, it controls instead the activity of the crystallin genes in vertebrates (see Harris, 1997). Remarkably, the *paxB* gene from *Tripedalia cystophora*, a member of the ancient Cnidaria, also activates the lens J3-crystallin gene of the jellyfish ocelli

visual organs strikingly similar to the vertebrate eyes (Kozmick *et al.*, 2003). The discovery that *paxB* is a structural hybrid between the vertebrate *pax2/5/8* and *pax6* genes, together with its functional properties, brought the Authors to suggest a paxB-like protein to be the primordial pax protein of eye evolution. On this view, *pax6* genes may have evolved independently in Bilateria after their separation from Cnidaria.

A closer look on six3…

The *six3* gene was originally isolated because of its homology with the *Drosophila so* gene. More recent phylogenetic analyses brought to the inclusion of *Drosophila so* in the *six1/six2* subclass, while *optix* was considered to be the true orthologue of *six3/optx2*. Despite that, the expression pattern of the *Xenopus six3* during development is much closer to the *so* expression pattern than to the *optix* one (Oliver *et al.*, 1995a,b; Pignoni *et al.*, 1997). Functionally, mutations in the *Drosophila so* gene lead to defects of the entire visual system due to extensive cell death (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). Loss of function experiments in *Xenopus*, mouse and medaka fish revealed a *six3* similar requirement for the development of the whole visual system (our unpublished results; Carl *et al.*, 2002; Lagutin *et al.*, 2003). Moreover, while both *six3* and *optix* are able to induce ectopic eye formation in a competent region, this activity is not displayed by *so* (Loosli *et al.*, 1999; Seimiya and Gehring, 2000; Lagutin *et al.*, 2001). Interestingly, although *optix* does not require *eyeless* for the induction of ectopic eyes in *Drosophila*, *Xsix3* overexpression induces the expression of *Xpax6* at early stages of development: this suggests that in vertebrates *pax6* may represent an early target of *six3* (our unpublished results). Furthermore, *pax6* gain of function experiments, performed in *Xenopus*, and loss-of-function analysis in medaka fish and mouse suggest that *six3*, like *optix*, is not an early target of *pax6*. Later on, both genes cross-regulate each other. Combined, these data suggest that *pax6* works downstream of *six3* during the early steps of vertebrate eye specification. This is different from the counterpart in fly, where *so* is one of the direct targets of *ey* and *optix* is in a pathway distinct from the *ey* one (Niimi *et al.*, 1999; Punzo *et al.*, 2002).

…and rx1

rx genes are expressed in vertebrate forebrain and retina (Casarosa *et al.*, 1997; Mathers *et al.*, 1997) and are essential for eye formation (Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999). Differently, the *Drosophila* homolog *Drx* as well as the planaria *Gtrx* are expressed in the central nervous system but not in the eye (Eggert *et al.*, 1998; Salò *et al.*, 2002). This suggests that *rx* genes are involved in brain patterning processes in Protostomia, while they evolved a specific role in eye development in Deuterostomia.

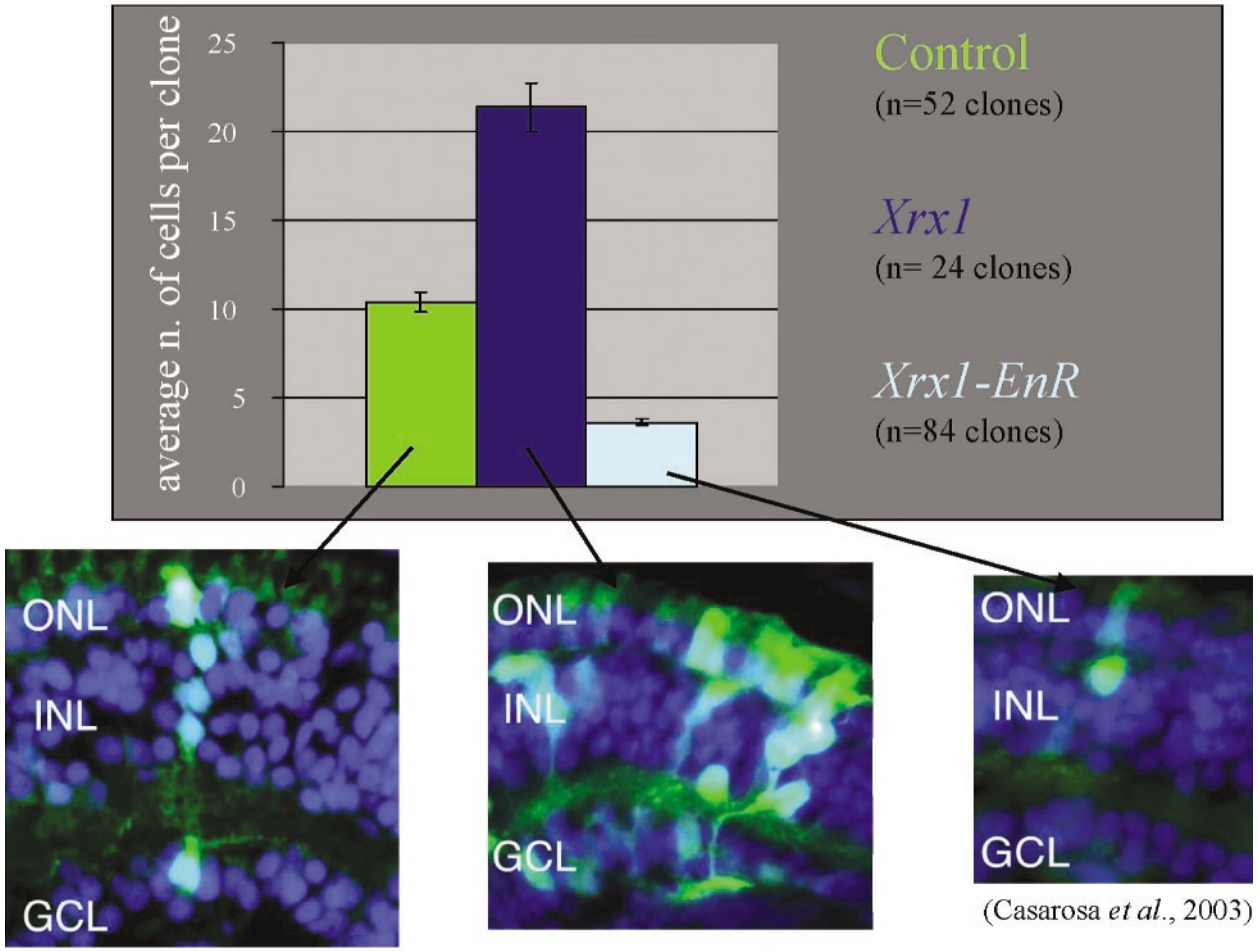


Fig. 5 - *rx1* supports proliferation of retinal precursors. Overexpression of *Xrx1* in single retinal precursors leads to the formation of larger clones, as compared to the control and to a dominant negative form (Casarosa *et al.*, 2003).

We recently contributed to show that *Xrx1* is one of the key players in maintaining an active proliferative state and preventing neurogenesis in its territories of expression, retina and forebrain (Andreazzoli *et al.*, 2003; Casarosa *et al.*, 2003). The role of *Xrx1* in promoting proliferation has also been demonstrated in single retinal progenitors: *Xrx1* overexpression remarkably increases the clonal proliferation of single retinal progenitors, while *Xrx1* functional inactivation has the opposite effect (Casarosa *et al.*, 2003) (Fig. 5). Interestingly, another key regulator of retinal development, the transcriptional repressor *Xoptx2*, also induces retinal overgrowth by supporting clonal proliferation of retinal progenitors, possibly acting downstream of *Xrx1* (Zuber *et al.,* 1999; Andreazzoli *et al.*, 2003). Emerging evidence suggests that a number of patterning genes can control cell proliferation in specific regions of the embryo, thus contributing to differential growth of embryonic tissues and organs (Cremisi *et al.*, 2003). Notably, *rx1, optx2* and *six3* are necessary for eye formation and sufficient for retinal growth, raising intriguing similarities with the gene complex regulating cell proliferation during *Drosophila* eye development. In fact, interaction and cross-regulation of the three *Drosophila* transcription factor genes *eyeless (ey), homothorax (hth)* and *teashirt (tsh),* account for cell proliferation of the *Drosophila* eye imaginal disc and prevent the expression of the transcription factors that at later stages will be responsible for photoreceptor differentiation (Bessa *et al.*, 2002). *ey, hth* and *tsh* then represent a functional gene complex coordinating cell proliferation and differentiation. Whether *rx1, optx2, six3* and possibly *pax6* constitute a similar complex remains to be investigated.

The role of *Xrx1* does not seem to be restricted to the control of cell cycle. Indeed, available data suggest that *Xrx1* can influence different molecular pathways than those controlled by *cdk2/cyclinA2* (Casarosa *et al.*, 2003). Furthermore, while *cdk2/cyclinA2* overexpression in retinal progenitors favors the generation of late-born cell types, *Xrx1* supports the maintenance of their multipotency, thus suggesting it may play a role in ensuring the subsequent differentiation of the various retinal cell types in the correct proportions. Now, let's turn to present a case of conserved regulatory genes playing both an early role in eye field specification, and a later role in retinal cell type specification.

Conserved roles for the otd/otx *genes in Drosophila and vertebrates*

The *Drosophila orthodenticle* (*otd*) gene is essential for the correct development of the fly head (Fig. 6A; Finkelstein and Perrimon, 1990). Significantly, the *otx* genes of vertebrates, homologous to *otd*, are expressed in the most rostral aspects of the brain and are necessary for the proper development of anterior neural structures (Simeone *et al.*, 1992; Matsuo *et al.*, 1995; Acampora *et al.*, 1995, 1996; Ang *et al.*, 1996).

Rescue experiments of *Otx2*$^{-/-}$ mutant mice demonstrated an extensive conservation of the *otd/otx* gene function in building the anterior body regions of organ-

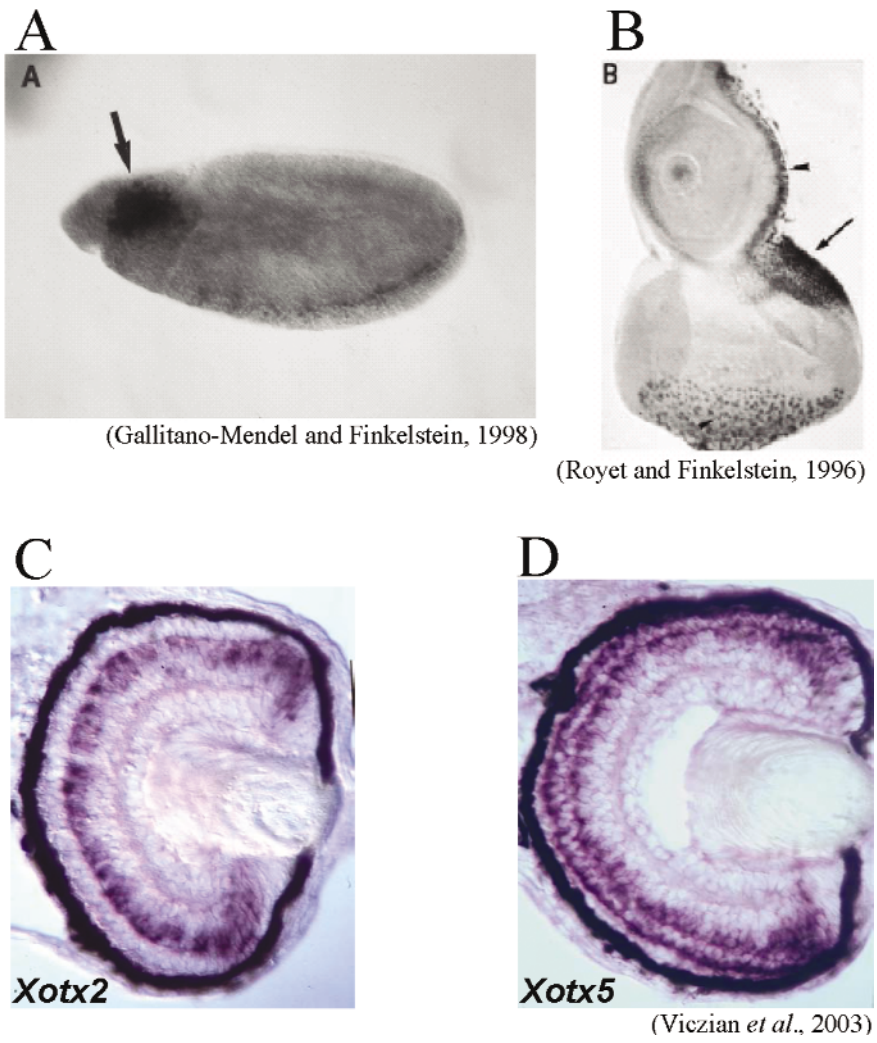


Fig. 6 - Expression of *otd/otx* genes in *Drosophila* and vertebrates. A) Germ-band-extended *Drosophila* embryo showing otd protein in procephalic head region (arrow) and at the ventral midline (from Gallitano-Mendel and Finkelstein, 1998). B) Third-instar eye-antennal imaginal disc showing otd protein in the head vertex primordium (arrow), in the first antennal segment (large arrowhead) and in the subretinal cells of the compound eye (small arrowhead) (from Royet and Finkelstein, 1996). C) Stage 42 *Xenopus* retina showing *Xotx2* expression in the inner nuclear layer. D) Stage 42 *Xenopus* retina showing *Xotx5* expression in the photoreceptor and inner nuclear layers (from Viczian *et al*., 2003).

isms as distant as *Drosophila* and mouse (Acampora *et al*., 1998, 2001; Leuzinger *et al*., 1998; Nagao *et al*., 1998; Pilo-Boyl *et al*., 2001). However, within this general role the specific *otx* genes show both conserved and divergent features (Acampora *et al*., 1996, 1998, 1999; Andreazzoli *et al*., 1997). This also applies to eye development. As already remarked, *otx2* plays an early role by providing the right competence to form eyes in the anterior neural plate (see Fig. 4); furthermore, *otx/otd* genes and their cognates also have specific functions in later stages of eye development, such as in cell type specification and/or maintenance within the retina (Fig. 6B-D). The first *otx*-like gene for which a role in specific retinal neurons was shown is the human *CRX*. Mutations in *CRX* were found in cone-rod dystrophy and Leber's congenital amaurosis, important retinopathies described in humans (Freund *et al*., 1997; Swain *et al*., 1997; Swaroop *et al*., 1999). Inactivation of murine *Crx* by homologous recombination leads to loss of the outer segment of photoreceptors, the only retinal cell type where *Crx* is normally expressed. Because earlier stages of photoreceptor development are not disturbed it was concluded that *Crx* is required for the maintenance but not the specification of photoreceptors in mammals (Furukawa *et al*., 1997; Swain *et al*., 1997). Also *Otx1* and *Otx2* are expressed in the eye during its formation. *Otx2* is expressed in both bipolar layer and pigmented epithelium cells in the mouse, while *Otx1* is expressed in the pigmented epithelium. These two genes seem to play a role in identifying the neural retina fate with respect to the pigmented epithelium: genotypes that are $Otx1^{-/-}$ and $Otx2^{+/-}$ show transformation of the pigmented epithelium into neural retina, and this may depend on dosage effects (Martinez-Morales *et al*., 2001). Evidence for a more specific role in cell fate determination within the retina has been reached in the frog, *Xenopus*. While *Xotx2* is expressed almost exclusively in bipolar cells, *Xotx5b* is expressed in both bipolar and photoreceptor cells (Fig. 6C, D; Viczian *et al*., 2003). Moreover, in vivo transfection of retinal cell precursors has shown that while *Xotx2* promotes the bipolar cell fate, *Xotx5b* biases retinal precursors to the photoreceptor cell fate; conversely, *Xotx2* and *Xotx5b engrailed*-repressor fusion constructs act as antimorphs, either suppressing bipolar cell fate or photoreceptor cell fate, respectively (Viczian *et al*., 2003). The different cell fate effects of these two very similar proteins are due to specific properties of their C-terminals, suggesting different in vivo biochemical properties of the two proteins, at least in the eye. This in turn may be also witnessed by the apparently dominant role of *Xotx2* over *Xotx5b* in a fraction of the bipolar cell population where both genes are expressed, thereby preventing these cells to become photoreceptors and pushing them instead towards a bipolar fate (Viczian *et al*., 2003).

The evolutionary relationship between *Drosophila otd* and vertebrate *otx* genes raises the question of whether *otd*, besides sharing functional aspects with *otx2*, may also share functional features with *Xotx5b* or *Crx*. Recent work in *Drosophila* has shown that *otd* also plays a role in controlling aspects of photoreceptor differentiation in the compound eyes of the fly (Fig. 6B). In particular, *otd* is involved in

regulating specific rhodopsin (*rh*) genes that are differentially expressed in the eight photoreceptors (R1-R8) of each ommatidium. The lateral eyes of *Drosophila* are made up of three types of ommatidia: all of them express *rh1* in the outer photoreceptors (R1 to R6), but can be distinguished on the basis of the pigment present in the inner photoreceptors (R7 and R8). In particular, in the dorsal rim area R7 and R8 express *rh3*, while in the rest of the retina two other types of ommatidia are intermingled: p ommatidia express *rh3* in R7 and *rh5* in R8, while y ommatidia express *rh4* in R7 and *rh6* in R8 (Cook and Desplan, 2001; Tahayato *et al*., 2003). *otd* is involved in the activation of *rh3* and *rh5* genes in p-type ommatidia, as well as in the repression of *rh6* gene expression. While *otd* is required, it seems not sufficient to activate *rh3* and *rh5*; in fact, *otd* is normally expressed in all photoreceptors; besides, when its is expressed, under a heat shock promoter, in all photoreceptors, it does not lead to general activation of *rh3* and *rh5*, or repression of *rh6*. This suggests that *otd* works together with cofactors for its various functions in the eye (Tahayato *et al*., 2003). Interestingly, *Crx*, one of the vertebrate homologs of *otd* involved in the differentiation of photoreceptors in the retina, interacts with the leucine zipper NRL to activate the opsin promoter (Chau *et al*., 2000) and it is possible that the different effects of *Xotx2* and *Xotx5b* in regulating different cell fates in the *Xenopus* retina may also depend on interactions with differential molecular partners via the C-terminal part of the two Xotx proteins (Viczian *et al*., 2003). Therefore, it appears that some molecular aspects of *otd/otx* gene function are paralleled in both *Drosophila* and vertebrate eye development, with *otd* involved in regulation of *rh* genes in specific ommatidia subtypes, and *otx* genes playing a role in regulation of specific cell fates within the retina.

The case of Retinal Ganglion Cells as the evolutionary counterparts to invertebrate rhabdomeric photoreceptors

As mentioned above, Gerhing and Ikeo (1999) proposed that the actual bilaterian eyes might have evolved monophiletically from a simple two-celled precursor, the prototypic eye as conceived by Darwin (1859) (Fig. 1A). Arendt and Wittbrodt (2001) have hypothesized that a prototype eye, endowed with rhabdomeric photoreceptors employing r-opsin, might have been present in the larvae of Urbilateria. This prototype eye is today found in the trocophora larvae (Arendt and Wittbrodt, 2001) and planarians (Gehring and Ikeo, 1999; Salò *et al*., 2002), while it differs from the chordate cerebral eyes, which are endowed with ciliary photoreceptors. This seems in contradiction with the notion that also the chordate eye may be directly derived from the ancestral bilaterian eye. With respect to this issue, Arendt and Wittbrodt (2001) propose that primary ciliary larvae (like trocophora and tornaria) were present in the ancestral chordates, whose descendants might have lost the primary larvae but not their eye structure. The rhabdomeric photoreceptors might have later been complemented with, and progressively replaced by, a population of ciliary photoreceptors, thus giving rise to the vertebrate eye.

Using the polichaete *Platinereis dumerilii* as a model system for the ancestral eye, it was found that not only *pax6*, but also *six1/2* and *athonal* homologs are involved in the development of its cerebral eyes: this result supports the view that the trocophora larval eyes were already present in the Urbilateria (Arendt *et al.*, 2002). The cell type where the *six* genes are expressed in invertebrates and vertebrates, respectively, is particularly significant. In *Drosophila* (Serikaku and O'Tousa, 1994), planarians (Pineda *et al.*, 2000) and *Platinereis* (Arendt *et al.*, 2002) *six1/2* are expressed in the rhabdomeric photoreceptors while in vertebrates *six2* is expressed in the differentiated retinal ganglion cells (RGCs; Ghanbari *et al.*, 2001). According to this observation, RGCs can be viewed as remnants, in the vertebrate retina, of the rhabdomeric photoreceptors. Since all *Drosophila* photoreceptors are rhabdomeric, RGCs might correspond to all the *Drosophila* photoreceptors; however, a particular correlation between RGCs and R8 photoreceptor cells of *Drosophila* has to be pointed out. The first correlative evidence is based upon temporal and morphological criteria: both types of neurons are the first to be born and send their axons directly to the brain. Moreover, both R8 cells in *Drosophila* (Hsiung and Moses, 2002; Ohnuma *et al.,* 2002) and RGCs in vertebrates (Austin *et al.*, 1995) are selected amongst a group of competent precursors by the action of Notch signaling. Another similarity is that both types of cells express homologous opsin molecules. r-opsin (see Arendt and Wittbrodt, 2001), employed by rhabdomeric photoreceptors, has indeed an homolog in the melanopsin, expressed by ganglion cells (Provencio *et al.*, 1998). Invertebrate rhabdomeric photoreceptors differentiate from *atonal*-positive precursors, as in insects (Jarman *et al.*, 1994) and polichaetes (Arendt *et al.*, 2002). Vertebrate ganglion cells derive from *ath* positive precursors in mouse (Brown *et al.*, 1998, 2001), frog (Kanekar *et al.*, 1997) and fish (Kay *et al.*, 2001). In all cases studied so far, the vertebrate *atonal* homologs are necessary for the initial determination step of ganglion cell fate and for their subsequent differentiation. However, cross-species comparison shows that, unlike the *pax6/eyeless* case, the *atonal* homologs are not always functionally conserved (Sun *et al.*, 2003). Indeed, the *Xenopus Xath5* is able to rescue the *Drosophila ato* mutant phenotype at an extent comparable with that of *atonal*; in contrast, the murine counterpart *Math5* is not able to rescue efficiently ommatidial formation and, moreover, the induced ommatidia lack R8 cells (Sun *et al.*, 2003). At the same time, overexpression of *atonal* in the *Xenopus* eye leads to ectopic differentiation of ganglion cells, as overexpression of *Xath5* does (Kanekar *et al.*, 1997; Sun *et al.*, 2003). Thus, it is clear that in spite of the high degree of conservation, additional species-specific factors may be required for the correct differentiation of a specific retinal cell type.

Conclusions

The kind of molecular studies I summarized here points to the idea that not only individual genes, but also «cassettes» of interacting genes, may have been conserved for eye development through evolution, even though different developmental requirements led to altered relationships between the members of the network. In addition, the occurrence of multi-gene families in Vertebrates has allowed the evolving of similar – but not identical – cassettes, each comprising different members of each family and each devolved to development of a different organ or structure. For example, *pax3*, *six1*, *eya2* and *dach2* (i.e., paralogous genes to those controlling eye development) act synergistically in driving the myogenesis process (Heanue *et al*., 1999). Thus, the conclusion should not be drawn that homologous gene networks necessarily support development of homologous structures. In fact continuity of the genetic information may regulate the development of similar but non-homologous structures, as exemplified by development of appendages (Tabin *et al*., 1999). Also, similar cassette gene networks may support development of different organs or tissues (e.g., eye or muscles). Orthologous genes may be conserved as for their similar expression and – at least in part – function during retina cell differentiation. This is the case of the *Drosophila otd* and the vertebrates *otx* genes, which are expressed and play a role in photoreceptor cells both in the ommatidia and in the neural retina, respectively. Again, however, by itself gene conservation may not be taken to imply homology of structures such as the compound eye of *Drosophila* (of ectodermic origin) and the neural retina of Vertebrates. A deeper, still unresolved question, question is: given the similarity of the genetic regulatory circuits, what are the developmental programs that make the eyes so different?

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