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Pax genes in eye development and evolution

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Animal eyes with widely different anatomical designs have long been thought to arise independently, multiple times during evolution. This view was challenged about a decade ago by the landmark discoveries that Pax6, a highly conserved transcription factor, plays a key role in eye morphogenesis in both flies and mammals. Since then, more evidence has emerged in favour of the redeployment of *Pax6* and some other developmental control genes within the genetic program underlying eye formation throughout the animal kingdom. Recent work has indicated that other members of the Pax gene family play a pivotal role in eye morphogenesis. The *Eye gone* gene regulates eye growth in *Drosophila*, whereas the *PaxB* gene is implicated in visual system development in jellyfish, the most basal organism possessing eyes.

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Introduction

The evolution of eyes remains a controversial topic. The obvious morphological diversity in addition to the different embryological origins of various types of eyes makes it hard to accept the view that there exists some common underlying genetic mechanism [1]. Moreover, two non-homologous signaling cascades for phototransduction can be found in bilateria (see Glossary). Both of these cascades use seven-transmembrane receptors as part of their photopigments. However, c-opsins, which are used in ciliary photoreceptors, and r-opsins, which are used in rhabdomeric photoreceptors, can be clearly categorized according to certain molecular characters. The downstream effector molecules are distinct for each cascade, as well. Rhabdomeric photoreceptors employ the phospholipase C cascade, whereas ciliary receptors use a phosphodiesterase system [2••,3]. The two photoreceptive systems coexist throughout the animal taxa; however, the rhabdomeric photoreceptors are much more common, especially among invertebrates. Vertebrates and jellyfish represent relatively rare cases of utilization of ciliary receptors [1].

Despite these variations, genetic studies have indicated that all eyes might share a similar developmental cascade of transcription factors, suggesting that eyes have had a common evolutionary origin. In particular, an almost universal use of the *Pax6* gene for eye morphogenesis in most species represents a powerful argument for a monophyletic origin of eyes, and a central role for the *Pax6* gene in visual system development [4]. *Pax6* is a member of the Pax gene family, which encodes transcription factors that are highly evolutionarily conserved among Metazoans [5,6], and seem to play fundamental roles in a wide variety of developmental processes [7,8].

The focus of this review is a small group of Pax proteins, Pax6, Eyeless (Ey), Twin of Eyeless (Toy), Eye gone (Eyg), Twin of Eyegone (Toe), Pax2 and PaxB, which are implicated in visual system development in a variety of species:. Here, I initially discuss the molecular properties of Pax transcription factors, and then describe some new findings regarding Pax function during eye development. For the purpose of this review, I define an eye as an organ of spatial vision with a minimum requirement of a single pigment cell and two photoreceptor cells [1]. Although most of our understanding about the genetic program underlying eye formation derives from vertebrates and flies, valuable new information about eye evolution has recently been obtained by studies of cnidaria and lophotrochozoa (see Glossary). Finally, I propose a tentative model to explain the universal use of Pax genes in eyes with fundamentally different building plans.

The structure of Pax transcription factors

Pax transcription factors are defined by the presence of a highly conserved 128 amino acid DNA binding domain, the paired domain [9], as proposed elsewhere [5]. The paired domain is a bipartite domain consisting of two independent subdomains: the amino-terminal PAI domain and the carboxy-terminal RED domain (Figure 1) [10,11]. The bipartite paired domain recognizes a bipartite binding site of about 17 nucleotides [10,12]. The PAI domain is generally more conserved than and seems to be dominant over the RED domain in the intact protein, which might explain why all Pax proteins seem to interact with similar target sequences. Nevertheless, some differences in specificity have been noticed. 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. The amino acids IQN at these

Glossary

Ascidians: Sea squirts; solitary or colonial marine invertebrates; free-swimming larvae exhibiting basic chordate body plan.

Bilateria: Sometimes called 'higher' animals, these are a group of animals representing the majority of phyla, with the notable exception of sponges, ctenophores and cnidaria. The two main lineages of Bilateria are protostomes and deuterostomes. Bilateria are characterized by bilateral symmetry, and are also called triploblasts because their bodies develop from three different germ layers (endoderm, mesoderm and ectoderm).

Cnidaria: A very ancient and highly diverse group of animals comprising jellyfish, corals and sea anemones in addition to common laboratory *Hydra*. Cnidarians are diploblasts, having their bodies constructed from only two germ layers, endoderm and ectoderm, separated by acellular mesoglea. Cnidarians possess radial body symmetry and they represent a natural animal outgroup to Bilateria for comparative studies.

Intercalary evolution: A likely scenario for the evolution of a morphogenetic pathway. Initially, a prototypic structure, such as an eye, is formed using key regulatory genes (such as *Pax6*) and structural genes (such as opsins). In the course of evolution, additional genes are intercalated (co-opted) between the top (*Pax6*) and bottom (opsins) of the developmental cascade.

Lophotrochozoa: One of the two major groups of protostomes, including animals such as molluscs, segmented worms and brachiopods. Sometimes, flatworms are included within this group.

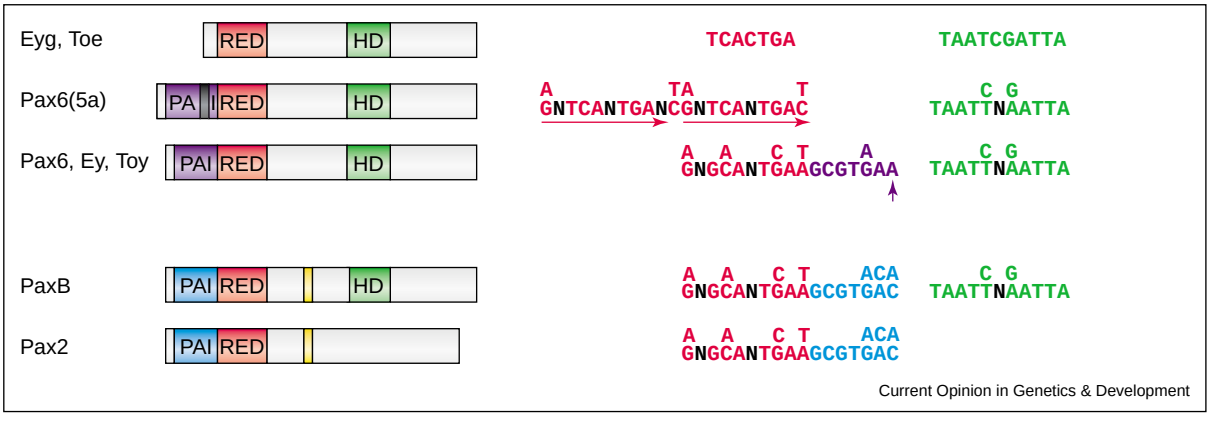
Planarians: Common name for several species of free-living flatworms characterized by very simple bilaterian body architecture.

positions specify the Pax6 class of transcription factors, whereas amino acids QRH determine Pax2/5/8 specificity [13]. Alternative splicing of the *Pax6* gene in mice and humans inactivates the PAI domain by virtue of an insertion of exon 5a (14 amino acids), thus generating the Pax6(5a) protein. As a result, an independent DNA binding capacity of the RED domain is fully uncovered, and the Pax6(5a) protein is targeted towards a tandem repeat of the RED consensus binding site [14,15]. *Drosophila Pax6* orthologues, *ey* and *toy*, do not undergo such a splicing event and, thus, produce solely the canonical Pax6 protein. Interestingly, two unusual Pax proteins, Eyg and Toe, are produced by the two paralogous genes, *eyg* and *toe*. Eyg and Toe lack the amino-terminal PAI domain, and rely only on the RED and HD domains for their binding capacity. The sequence specificity of Eyg has been studied and found to be very similar to that of Pax6(5a), although a single RED consensus site appears sufficient for Eyg binding [16]. Apparently, two alternative mechanisms have evolved for generating both a PAIRED-HD transactivator in addition to a RED-HD transactivator: alternative splicing in mammals and gene duplication and diversification in *Drosophila*. In addition to a paired domain, some Pax proteins (such as Pax6 or Eyg) contain a second DNA binding domain, a homeodomain (HD). The homeodomain in Pax proteins is always characterized by serine at the crucial position 50, which is known to determine homeodomain sequence-recognition specificity. The Pax homeodomain interacts with palindromic TAAT-like target sequences [17], either as a homodimer or as a heterodimer with another paired-type homeodomain. In summary, Pax proteins might regulate an unusually broad spectrum of target genes as a result of the interaction of individual DNA binding domains or cooperation between the domains [18].

Pax genes and eye organogenesis

The observations that mutations in a highly conserved transcription factor, Pax6, disrupt eye development in

Figure 1



Structure (left) and DNA binding specificity (right) of selected Pax proteins. The bipartite PAIRED domain (PD) recognizes a bipartite consensus and binds to DNA as a monomer [10,12]. PAI and RED recognition sequences within the bipartite consensus are shown in blue/violet and red, respectively. The Pax6 class has a strong preference for A over C in position 17 of the bipartite consensus (violet arrow) [13]. The RED domain of Eyg is capable of independent binding to a single 7 base pair consensus [16], whereas the Pax6(5a) protein prefers a 'tetrameric' binding site [14,15]. The red arrows indicate the two potential target sites of Pax6(5a) on the upper strand of DNA; the unique nature of the 'tetrameric' consensus enables two additional binding sites to be present on the lower DNA strand [15]. The paired-type homeodomain (HD; green box) binds preferentially as a dimer to palindromic TAAT-like sequences (consensus shown in green) [17]. Pax proteins are likely to interact with a wide variety of sites, either through a single DNA binding domain (PAI, RED or HD) or through cooperation of individual domains [18]. A conserved octapeptide motif present in Pax2/PaxB is shown as a yellow box.

both mammals [19] and insects [20], and that *Pax6* misexpression is able to induce ectopic eyes [21,22,23••] led to the proposal of *Pax6* being a 'master control gene' [4], although the term 'eye selector gene' seems more appropriate and is generally accepted [24••]. The term 'master control gene' implies that the Pax6 transcription factor is located at the top of a gene cascade and that it initiates eye development in almost any tissue in which it is ectopically expressed; however, neither scenario seems to be the case. For instance, eye development in the mouse proceeds by a series of inductive interactions between neuroectoderm (developing retina) and a surface ectoderm (developing lens), in which Pax6 is an essential early determinant in both compartments [25–27]. Nevertheless, in the absence of Pax6, presumptive retina can develop up to the optic cup stage, albeit abnormally [25]. Moreover, within the lens placode, *Pax6* expression is under the control of the Meis1 and Meis2 transcription factors [28], vertebrate homologues of the *Drosophila* protein Homothorax [29].

In *Drosophila*, the ability of the two *Pax6* paralogues, *ey* [22] and *toy* [30], to induce ectopic eyes is restricted both spatially and temporally. These limitations suggest that ey and toy modify an existing program of sensory organ development rather than initiate the entire eye morphogenesis. In accordance with this interpretation, the gene *atonal*, which encodes a transcription factor of the basic helix–loop–helix family, is required for a generic developmental program that controls formation of three adult sensory organs in *Drosophila* (eye, Johnston's and chordotonal organs) [31••]. Furthermore, *toy* controls more than just eye morphogenesis, because a loss-of-function mutation produces flies missing an entire head [32]. The two *Drosophila Pax6* paralogues have partially redundant functions [32], yet they have also functionally diverged [30,33].

Apart from Pax6, additional Pax proteins are essential for normal eye development in *Drosophila* and mice (Table 1, Figure 2). Two *Drosophila Pax6*-like genes, *eyg* and *toe*, might act in parallel to *ey* during eye formation [34•]. Recent results suggest distinct but coordinated roles for *ey* and *eyg*. In the current view, *ey* provides eye specification whereas *eyg*, being genetically downstream of Notch signalling [35••], a known regulator of eye growth [24••], regulates proliferation. Remarkably, Pax6(5a) protein, although playing a minor role in vertebrate eye development [36], can mimic Eyg in promoting tissue growth [35••], 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* [37] and mouse [38] eye development (Table 1). Some similarities in nested expression patterns of *Pax6* and *Pax2* in developing eye discs of fly and vertebrate eyes have been noticed [8]; 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 whereas *Pax6* is required for the development of the optic cup [39]. Both genes seem to have partially redundant functions in the retinal pigment epithelium [40•].

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 [41]. Of particular interest here is its eye-specific *spa* function [42], because the *sv* function is deployed for proper development of all bristles in the fly [43]. It has been suggested that cone cells, in a similar fashion to glial cells, might be considered as neuronal support cells of photoreceptor cells, a hypothesis additionally supported by the fact that *Pax2* is also expressed in glial cells of the developing peripheral nervous system of *Drosophila* [37]. Accordingly, expression of *Pax2* in *Drosophila* cone cells and glial cells of developing vertebrate eye might originate from the function of an ancestral *Pax2* gene in photosensory organ of an animal that lived before the separation of protostomes and deuterostomes [37].

Pax genes and the evolution of the eye: the Paxcentric view

The origin of Pax genes predates the origin of eyes and the nervous system. The Pax gene closest to the ancestral

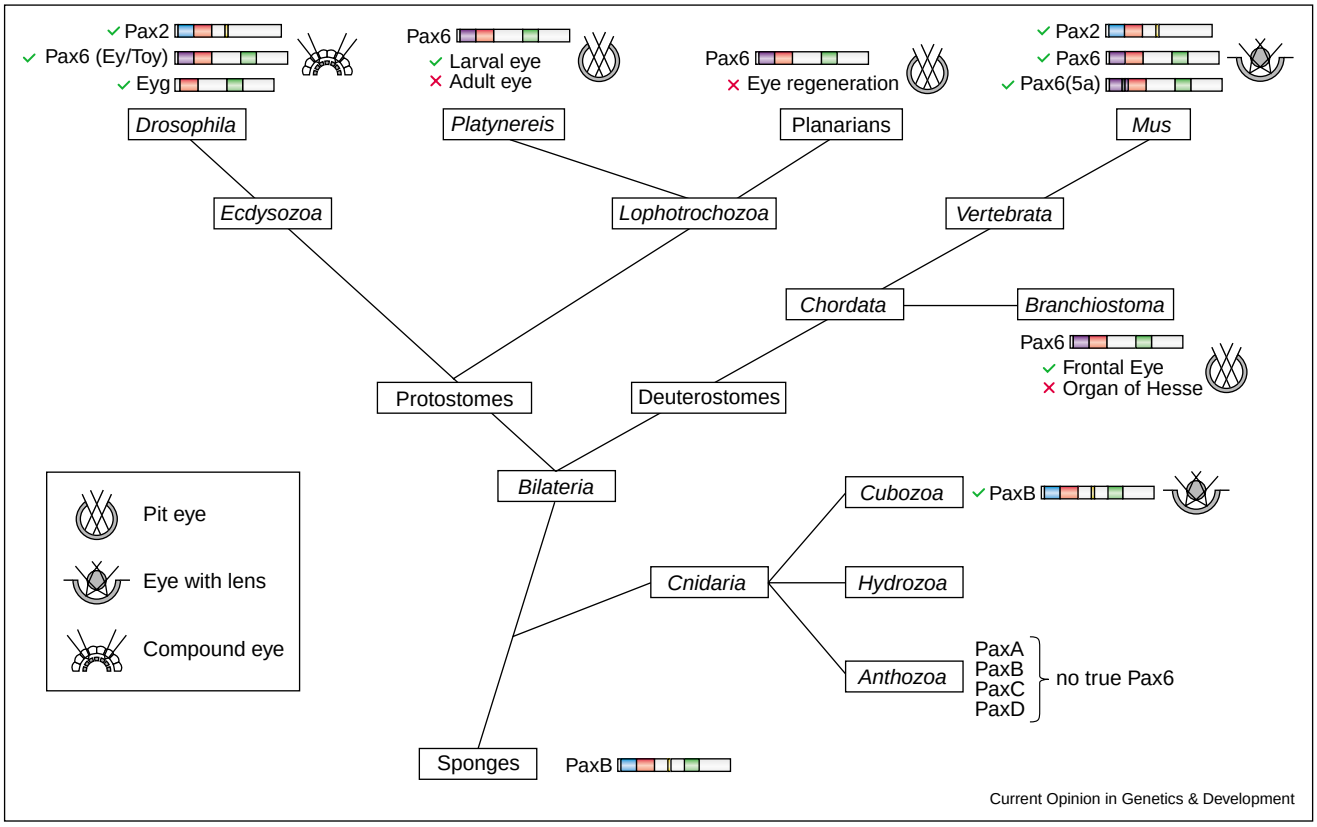
Table 1

Pax transcription factors implicated in eye development in mouse and *Drosophila*.

Pax structure	Mouse protein	Loss-of-function eye phenotype	*Drosophila* protein	Loss-of-function eye phenotype
PD-HD	Pax6	No eyes	Ey Toy	No eyes No head[a]
RED-HD	Pax6(5a)	Iris hypoplasia	Eyg Toe	No eyes ?
PD	Pax2	Agenesis of optic chiasma; retinal ganglion cells project ipsilaterally; retinal coloboma	Pax2	Abnormal cone and pigment cell development

[a] This phenotype is temperature-sensitive [32]. ?, phenotype not determined. For references, see text.

Figure 2

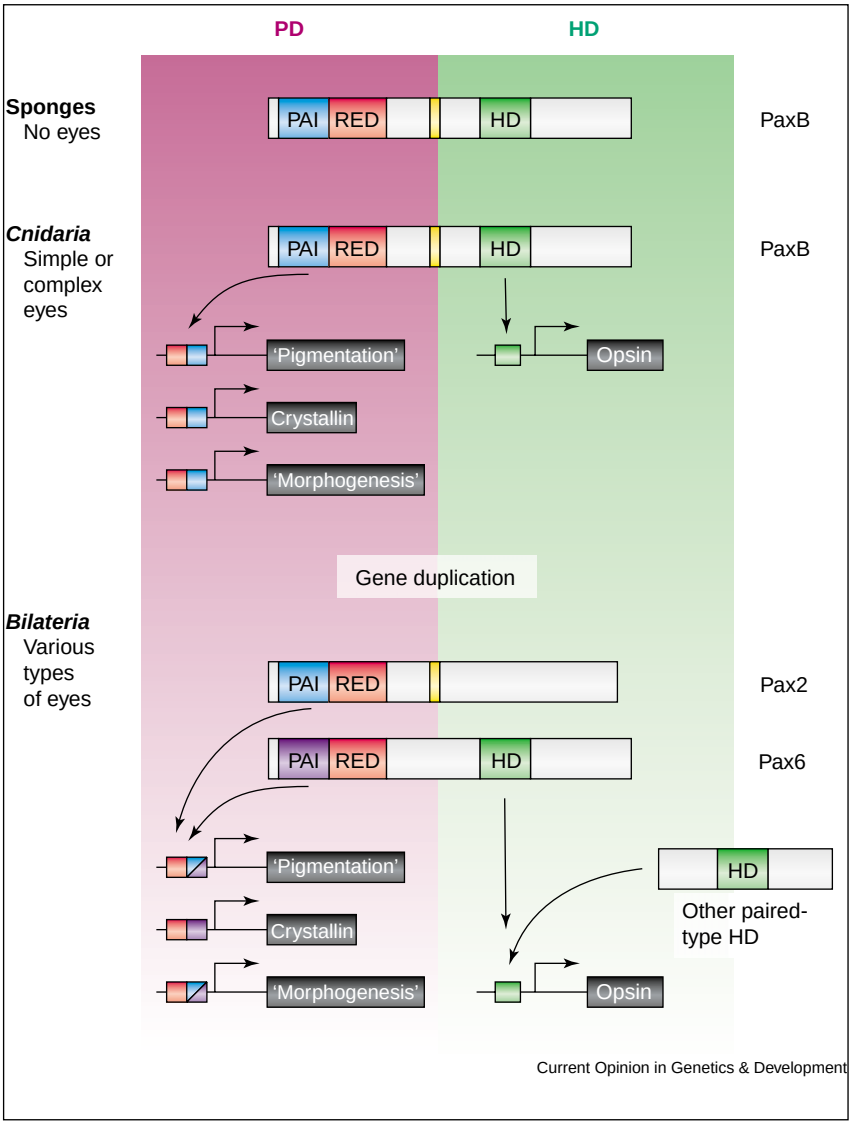


Pax proteins with known or suggested function in eye development in different animal groups. The widespread use of Pax6 for eye organogenesis among bilaterians represents a compelling argument for monophyletic origins of eyes [4]. However, if we define an eye as an organ with spatial vision with a minimum requirement of a single pigment cell and two photoreceptors [1], then we know of cases where such an eye develops in the absence of *Pax6*. For instance, development of the Hesse organ (eyecup) in the chordate amphioxus (*Branchiostoma*) is Pax6-independent [61]. Likewise, planarian eye regeneration does not require *Pax6* function and depends on genes of the *Six/sine oculis* family [49]. Finally, the *Pax6* gene in the polychaete *Platynereis* is only expressed in the larval but not in the developing adult eye, which again suggests that organogenesis of an adult eye is Pax6-independent [58]. Cubozoan jellyfish, the most basal animals with sophisticated eyes can form their eyes in the absence of a true *Pax6* gene [47••]. It is noteworthy that a similar set of Pax transcription factors with almost identical biochemical properties (Pax6, Pax6(5a)/Eyg and Pax2) is used for the development of the compound eye in *Drosophila* as well as for the morphogenesis of a camera-type eye in vertebrates.

Pax gene, a *PaxB*-like gene belonging to the *Pax2/5/8* subfamily, was identified in the sponge, which has neither eyes nor a nervous system (Figure 3) [44]. Cnidaria are the most basal animals that posses either simple or complex (lens-containing) eyes in addition to Pax genes [45,46,47••,48•]. Pax genes clearly have an ancient and fundamental role in visual system development. The question than remains: 'What is so special about Pax?'. In the following sections, I argue that the biochemical nature of Pax transactivators might have been the reason for selecting them initially to generate a prototypical eye structure as a photoreceptor/pigment cell combination.

As described above, Pax transcription factors represent regulatory proteins with an unusually broad spectrum of target sequences as a result of the interaction of independent DNA binding domains or the cooperation between the domains. Thus, they are capable of coordinated regulation of a large number of genes organized into networks or developmental programs. Separate, yet interdependent biological program(s) can be regulated by the paired domain and homeodomain, respectively. I propose (Figure 3) that two independent DNA binding domains within a single Pax transcription factor have been co-opted for two essential features of the proto-eye: production of a dark pigment (the 'pigmentation' program; paired domain-driven) and production of a photopigment (the 'opsin' program; HD-driven). Given that the two programs were driven by two independent DNA binding domains within a single transcription factor, they became inseparable. There is, indeed, evidence for such an evolutionary scenario (i.e. in favour of a role for Pax genes in the regulation of *opsin* as well as in pigment cell fate).

Figure 3



Pax gene evolution and 'the Paxcentric (PD-HD) model', suggesting specific roles for paired domain and homeodomain during eye evolution. 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 [47••]. The data indicate that modern *Pax2* and *Pax6* genes in bilateria evolved from a cnidarian *PaxB*-like ancestor by duplication and diversification. 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 (shown by violet color of PAI domain and the target sequence). 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. The genes under the 'pigmentation' program might represent components of the melanogenic pathway (i.e. enzymes such as tyrosinase) as well as key transcriptional regulators (Mitf). 'Morphogenesis' genes are the ones required for eye formation in any given animal. The role of the PD and HD was modified in the course of animal evolution by recruitment of other transcriptional regulators. For instance, the early Pax HD function in *opsin* gene regulation has been modified in some bilateria by co-option of other paired-type HD proteins (such as Otd or Crx [53••]). Alternatively, Otd/Crx might represent derivatives of the initial Pax family, which lost their PD. Note that *opsin* genes are homologous across phylogeny, whereas the highly abundant lens crystallins are encoded by structurally unrelated genes in different species. For simplicity, cooperative DNA binding activities (i.e. PD-HD, RED-HD and PAI-HD) and their possible roles in gene expression programs are omitted.

The *Pax6* gene is expressed in the pigment cells of the prototypic planarian eye [49]. Pax2 is required for the development of pigment cells in the *Drosophila* eye [37]. Moreover, Pax genes were found to specify mouse retinal pigment epithelium (*Pax6*, *Pax2*), neural crest-derived melanocytes (*Pax3*) and ascidian (see Glossary) sensory pigment cells (*Pax6*, *Pax3/7*) [50•]. Another argument derives from studies of a microphthalmia-associated

transcription factor, Mitf, that has a conserved and fundamental function in the development of melanin producing cells [50•]. 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. In addition, Mitf directly regulates melanogenic enzymes. Various Pax genes activate the *Mitf* gene promoter [40•,51]. Furthermore, Pax6 directly interacts through its paired domain with Mitf protein and, hence, is able to modify Mitf function through protein–protein interaction, thus adding another level of complexity [50•].

It was shown previously [52] that *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 is, thus, no longer used for activation of *opsins* promoters. Remarkably, *Pax6* expression remains in vertebrate retinal ganglion cells, which are considered to be a cell-type homologous to the ancestral rhabdomeric photoreceptor cell [2••]. 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 [53••]. In accordance with this scenario, an artificial reporter gene containing paired-type homeodomain binding sites (P3) is active in the photoreceptors of transgenic planarians (see Glossary) [54], being activated either by Pax6 or by any other paired-type homeodomain activator protein expressed in the photoreceptors.

Finally, at least on the basis of genetic data in vertebrates and flies, the *Pax6* paired domain seems to be more important than is the homeodomain for eye morphogenesis. Primarily, missense mutations in patients with aniridia and *Small eye* mice (both *Pax6* heterozygote conditions) occur predominantly in the paired domain. In addition, it is the mutual interaction of Pax2 and Pax6 that generates a complex structure of the mammalian eye [39]. Likewise, the *Pax6* paired domain but not homeodomain is essential for *Drosophila* eye development [55]. Thus, it appears reasonable to suggest that the paired domain played a more prominent role in eye morphogenesis in the animal kingdom by re-inventing various types of eyes through intercalary evolution (see Glossary) [4]. A prime example of the paired domain being recruited for modification of an eye design by intercalary evolution is the recruitment of the paired domain for the regulation of lens crystallins, non-homologous genes among animals, encoding proteins responsible for the refractive property of the lens. A *PaxB* gene regulates crystallin genes in jellyfish [47••], whereas *Pax6* has been extensively used for the same function in lenses of various vertebrates [56] (Figure 3). It remains to be seen if any of the regulatory relationships described above reflect an ancestral state or, rather, represent a much later co-option of Pax6.

The fascinating feature of the proposed model is that the morphological unity found in the eye, a photoreceptor linked to the shading pigment, is mirrored on the molecular level, by uniting two independent DNA-binding domains in one regulatory protein.

Conclusions

The theory of *Pax6* as 'master control gene' for eye development [4] is complicated by the fact that genes for several other transcription factors (*sine oculis*, *optix*, *eyes absent*, *dachshund*, *eye gone* and *teashirt*) also induce ectopic eyes [57]. Some of these genes, in particular *sine oculis* homologues, undoubtedly have an ancient and fundamental role in visual organ development in different animal groups [58]. One has to keep in mind, however, that many of the members of the eye developmental cascade of transcription factors, including Pax6, are also used for the development of other tissues. This is perhaps best documented by the *eyes absent* gene, which encodes a protein phosphatase, and which functions in a complex with transcription factors of the *sine oculis*/*Six* gene family [57]. Eyes absent phosphatase activity is required for eye development in *Drosophila*, yet the same phosphatase activity has already been found in a plant orthologue. Remarkably, the entire regulatory circuits can, thus, be co-opted for development of a new cell type, tissue or even an organ. These issues raise concerns connected with the idea of homology as a result of common developmental pathways. It has recently been shown that vertebrates and cnidaria share many more genes than was previously anticipated [59•]. This means that both animal groups use more or less the same set of genes to generate their significantly different body plans. Therefore, it is likely that changes in gene regulation, rather then 'new' genes, are the driving force behind the different eye designs found among animal eyes. Regulatory mutations can even result in the re-invention of eyes during the course of evolution. A 'minor' change in the regulatory region of a gene encoding a crucial transcription factor, such as *Pax*6, can lead to its misexpression. If misexpression happens in the 'competent tissue', the original developmental program of that tissue can be subverted to a new fate. Such an evolutionary scenario is, in fact, nothing else than the ectopic eye experiment in *Drosophila*, performed not by scientists but Nature. However, most of these experiments performed by Nature were probably deleterious and produced, as shown elegantly in the case of early ectopic expression in eye discs, headless flies [60].

The genetic basis for fundamentally different building plans that exist among animal eyes will remain a scientific challenge in the years to come. It has become clear that an approach combining anatomy, molecular and cell biology

in addition to paleontology needs to be used to obtain a clearer picture.

Update

Recent work has provided another piece of evidence supporting fundamental role of Pax genes in pigmentation programs [62•].

Acknowledgements

I regret that space constraints make it impossible to cite all relevant primary work. I apologize to those of my colleagues whose work could not be cited. I am grateful to Markus Noll, Jessica Treisman, Detlev Arendt and Ondrej Machon for valuable comments on the manuscript. I would like to thank Jan Paces for help in preparation of figures. Work in Kozmik's laboratory is supported by the Department of Education (1M6837805002) and the Grant Agency of Czech Republic (204/04/1358).

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- •• of outstanding interest

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