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Cubozoan jellyfish: an Evo/Devo model for eyes and other sensory systems

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ABSTRACT Cnidaria are the most basal phylum containing a well-developed visual system located on specialized sensory structures (rhopalia) with eyes and statocyts. We have been exploring the cubozoan jellyfish, *Tripedalia cystophora*. In addition to containing simple photoreceptive ocelli, each rhopalium in *Tridedalia* has a large and small complex, camera-type eye with a cellular lens containing three distinct families of crystallins which apparently serve non-lenticular functions. Thus, *Tridpedalia* recruited crystallins by a gene sharing strategy as have mollusks and vertebrates. *Tripedalia* has a single *Pax* gene, *PaxB*, which encodes a structural and functional Pax 2/5/8-like paired domain as well as an octapeptide and Pax6-like homeodomain. PaxB binds to and activates *Tripedalia* crystallin promoters (especially *J3-crystallin*) and the *Drosophila rhodopsin rh6* gene in transfection tests and induces ectopic eyes in *Drosophila*. *In situ* hybridization showed that *PaxB* and crystallin genes are expressed in the lens, retina and statocysts. We suggest from these results that an ancestral *PaxB* gene was a primordial gene in eye evolution and that eyes and ears (mechanoreceptors) may have had a common evolutionary origin. Thus, the numerous structural and molecular features of *Tridpalia* rhopalia indicate that ancient cubozoan jellyfish are fascinating models for evo/devo insights into eyes and other sensory systems.

KEY WORDS: *Cnidaria, rhopalia, eyes/ocelli, mechanoreceptors/ears, PaxB, evolution*

Introduction

Eyes come in many forms and shapes in the animal kingdom (Land and Nilsson, 2002, Tomarev and Piatigorsky, 1996). These include the organelle eyes of some unicellular organisms, the simple eye spots of flatworms, the pin-hole eyes of some invertebrates, the varied compound eyes of insects, the mirror eyes of scallops and the complex, camera-type (lens-containing) eyes sprinkled throughout the animal kingdom. Even complex eyes can be varied and show anatomical differences. For example, the lens-containing eyes of vertebrates have inverted retinas in which the ciliated photoreceptors lie behind the ganglion cells with respect to the light path, while those of cephalopods (squids and octopus) have (generally) rhabdomeric (microvillar) photoreceptors that are placed in front of the ganglion cells and are the first retinal cells to receive light (Arendt and Wittbrodt, 2001). The development and morphology of the lens and cornea also show differences between the complex eyes of vertebrates and cephalopods (Packard, 1972, Tomarev *et al.,* 1997, West *et al.,* 1995, West *et al.,* 1994).

Despite these variations, recent studies have indicated that all eyes may share a similar developmental cascade of transcription factors, suggesting that eyes have had a common evolutionary ancestor (Gehring and Ikeo, 1999). The initial molecular finding for the hypothesis of monophyletic eye evolution was that the gene for the *eyeless* (*ey*) mutation in *Drosophila* is *Pax6* (Quiring *et al.,* 1994), the very gene responsible for the *Aniridia* mutation in humans (Ton *et al.,* 1991) and the *Small eye* mutation in mice (Hill *et al.,* 1991). Subsequently, it was shown that misexpression of *ey* or of mouse *Pax6* in leg, wing or antennae imaginal discs of *Drosophila* induces supernumerary ectopic eyes in the corresponding adult structures (Halder *et al.,* 1995). These and other experiments showing that *Pax6* from many species [i.e., squid (Tomarev *et al.,* 1997), ascidian (Glardon *et al.,* 1997), cephalochordate (Glardon *et al.,* 1998), planarian (Callaerts *et al.,* 1999) and ribbon worm (Loosli *et al.,* 1996)] induce ectopic eyes in the fly have led to the idea that this transcription factor is encoded by a 'master control' gene for eye development (Gehring, 2002, Gehring and Ikeo, 1999). While the *Pax6* master control gene for eye development has much to offer and has generated a flurry of

Abbreviations used in this paper: ey, eyeless gene.

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activity in 'evo-devo' studies of the eye, it is complicated by the fact that genes for a number of other transcription factors (*twin of eyeless*, *sine oculis*, *optix*, *eyes absent*, *dachshund*, *eye gone* and *teashirt*) also induce ectopic eyes (Kador *et al.,* 1979, Kumar and Moses, 2001b, Pichaud *et al.,* 2001). In addition, eye specification occurs later than thought previously (Baker, 2001, Kumar and Moses, 2001a). Moreover, many of the members of the eye developmental cascade of transcription factors, including Pax6, are also used for the development of other tissues (Hanson, 2001). An example is the use of *daschund*, *eyes absent*, *six* and *Pax* family members for both eye and skeletal muscle development (Heanue *et al.,* 1999, Relaix and Buckingham, 1999). These issues raise concerns connected with the idea of homology due to common developmental pathways (Conway Morris, 2000, Simpson and Price, 2002, van Heyningen and Williamson, 2002). Indeed, eyes from different species may be partially homologous (Gehring and Ikeo, 1999) or show different levels of homology (Land and Nilsson, 2002). A recent study using a host of eye-field transcription factors suggested progressive tissue specification by a self-regulating feedback network, consistent with a partial evolutionary conservation of eye formation (Zuber *et al.,* 2003). A detailed review of the complexity of convergent versus divergent eye evolution at the anatomical level can be found elsewhere (Arendt and Wittbrodt, 2001).

Cubozoan eyes with special reference to *Tripedalia*

Cnidaria are the most basal animal phylum (Fig. 1) containing a well-developed visual system. In general among Cnidarians, it is the Cubozoa (known as 'box jellyfish' due to their square shape) that have lens-containing eyes (Coates, 2003, Piatigorsky, 2003b), although a few Hydozoa do as well (for example, *Cladonema radiatum*) (Weber, 1981) (Fig. 2). Photoreceptive organs in Cnidaria have diverse structures, not only between species but within the same species. The cubozoan that we have been investigating, *Tridpedalia cystophora*, has four equally spaced sensory structures (called rhopalia) dangling from a stalk and situated within open cavities surrounding the bell (Conant 1897, Laska and Hundgen, 1982, Pearse and Pearse, 1978, Piatigorsky *et al.,* 1989, Yamasu and Yoshida, 1976) (Fig. 3). Each rhopalium has six separate eyes. There are two complex, lens-containing eyes, one larger than the other, situated at right angles to each other and two pairs (one pit-shaped, one slit-shaped) of simple ocelli comprising photoreceptors on either side of the complex eyes (Coates, 2003). This eye diversity within *Tripedalia* may provide new insights into the mechanisms used in evolution for achieving greater anatomical complexity of eyes. In addition to the eyes, each rhopalium of *Tripedalia* has a prominent statocyst described below. The function of the diverse eyes of *Tripedalia* is still elusive. Recent studies indicate that the camera-type eye has the ability to form a low resolution image on the photoreceptors despite the proximity of the lens to the retina (Laska and Hundgen, 1982, Pearse and Pearse, 1978, Piatigorsky *et al.,* 1989). The behavioral role of the jellyfish eyes is under investigation (Coates, 2003). It seems likely that investigations of eye development in Cnidaria are directly relevant to eye development in other animal groups. The complex eyes of jellyfish show striking similarities in overall structure with the camera-type eye of vertebrates even though they differ in numerous anatomical details. In addition to a cellular lens and cornea, adult cubomedusan jellyfish eyes have ciliated photoreceptors, as do vertebrate eyes, rather than the rhabdomeric (microvillar) photoreceptors generally populating invertebrates (Eakin, 1962, Yamasu and Yoshida, 1976). Eakin believed from electron microscopic evidence that there was "a common ancestry of the taxa bearing light-sensitive cilia" (Eakin, 1979); however, close examination of diverse groups shows that the presence of ciliary or rhabdomeric photoreceptors is not neatly divided among species (Arendt and Wittbrodt, 2001). That Cnidarians have many genes believed previously to have arisen with the vertebrates (Ball *et al.,* 2002) provides additional support for the possibility that eye development in jellyfish shares many common features with that of more recently evolved triploblastic metazoans. Indeed, studies have revealed considerable conservation of regulatory genes between the diploblastic cnidarians and chordates (Galliot and Schmid, 2002, Hayward *et al.,* 2002), increasing the likelihood that there is overlap in the mechanisms of eye development between jellyfish and vertebrates. In addition, an investigation of the early embryogenesis of the marine hydrozoan jellyfish, *Podocoryne carnea*, suggested that the nervous system developed from anterior to posterior in serially repeated patterns, characteristic of bilaterally symmetrical metazoans (Groger and Schmid, 2001). Since jellyfish differ sufficiently from vertebrates, we anticipate that detailed studies of their eye development should provide new insights into whether eyes are monophyletic, convergent, or a combination of both as well as provide new information on eye development in general. There is one caveat concerning the evolutionary aspects of jellyfish eyes that seems appropriate to keep in mind. Although Cnidaria are ancient and predate the Cambrian explosion, the time at which jellyfish evolved eyes is not known. It has been predicted that eyes may develop relatively rapidly during evolution (Nilsson and Pelger, 1994) and it remains possible that jellyfish eyes are relatively recent acquisitions.

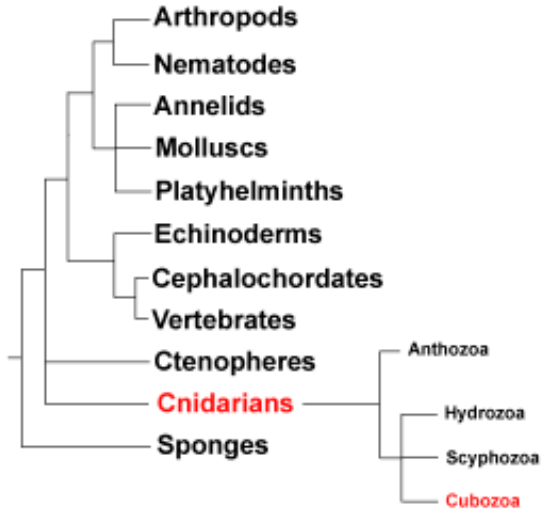

Fig. 1. Phylogenetic relationships among various animal groups. *For simplicity, some animal groups have been omitted.*

Obtaining and culturing *Tripedalia* for studies on eye development

One of the difficulties of using *Tridpedalia* for experiments is obtaining the adult medusae, rearing the embryos through meta-

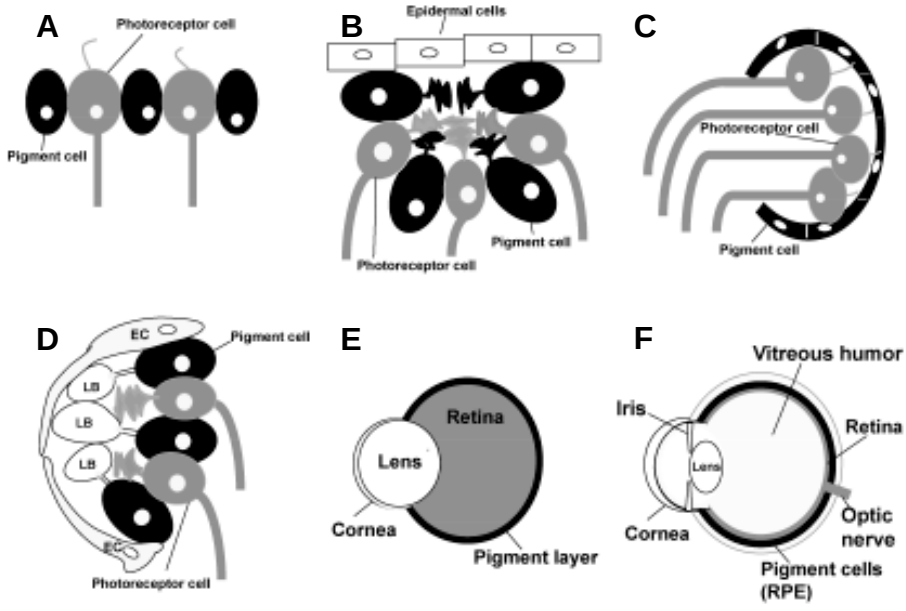

Fig. 2. Various types of eyes are found in Cnidaria, ranging from simple eye spots of hydrozoans (A) to complex eyes of cubozoan jellyfish (E). *Photoreceptor cells are shown in grey, pigment cells are black. EC, epithelial cells; LB, lens bodies.* **(A)** *Primitive eye spot as found in the hydrozoan,* Leuckartiara. **(B)** *Everted optic cup closed by a layer of epidermal cells found in hydrozoans* Polyorchis *and* Bougainvillia. **(C)** *Inverted pigment cup of scyphozomedusae,* Aurelia. **(D)** Cladonema ocellus *with lens bodies formed as distal cytoplasmatic processes of individual pigment cells. Adjacent epithelial cells extend over the lens bodies to form a primitive "cornea".* **(E)** *Complex camera-type eye of the cubomedusan,* Tripedalia, *containing retina, lens and cornea.* **(F)** *Vertebrate eye. (A, B) redrawn after Singla (Singla, 1974); (C) after Hyman (Hyman, 1940); (D) after Weber (Weber, 1981); (E) after Piatigorsky* et al., *(Piatigorsky* et al., *1989).*

morphosis and culturing the immature medusae to adulthood. We have captured adult *Tripedalia* medusae during the summer months swimming along the surface in the mangroves of La Parguera, Puerto Rico, where there is a Marine Station (University of PR). Sunbeams penetrate the foliage and water surface and reflect from the tentacles of the jellyfish. The live animals are caught with a dipnet from a small boat, placed in a bucket containing sea water and taken to the laboratory.

Tripedalia, as other jellyfish, undergo an alternation of generations between sessile, non-sexual polyps and swimming, sexually dimorphic medusae. Their life cycle and successful cultivation have been described (Kostrouch *et al.,* 1998, Werner, 1971). Fertilization is internal and the planulae larvae develop in the gastral pocket of the females; the swimming larvae are released within 2 days into the sea water, settle on the bottom and become pyriform shaped polyps. By the time they form 5 or 6 tentacles near the mouth the primary polyps bud secondary polyps that are eventually released. The primary or secondary polyps grow to approximately 1 mm in size by 8-10 weeks and contain a whorl of 7-9 tentacles. Interestingly, the cubozoan polyp of *Tridpedalia* differs from scypho- and hydrozoan polyps in having a nerve ring before the medusan stage (Werner, 1976). Full-grown polyps metamorphose into swimming ephydrae (immature medusae) within 4 or 5 days. Early studies indicated that the *Tridpedalia* polyps are fully transformed into the tissues of the medusae (Werner, 1971). This includes the four rhopalia developing at the base of the resorbing polyp tentacles, which group first into a tetraradiate pattern. A new, primary tentacle forms between the rhopalia in the young medusa and within a day two new tentacles form next to each primary tentacle (Fig. 4). It would be of great interest to examine this transformation of polyp tissue into rhopalia with ocelli at the molecular level, another fascinating aspect of the jellyfish model of eye development.

Jellyfish crystallins and gene sharing

The abundant, water-soluble crystallins, responsible for the optical properties of the transparent lens, have been considered historically among the most characteristic and specialized proteins of the complex eye. In striking contrast to the conservation of opsins in the retina as the visual pigments, the lens crystallins are diverse, multifunctional proteins that are often taxon-specific: the crystallins are heterogeneous and many are used selectively in different species (de Jong *et al.,* 1989, Piatigorsky and Wistow, 1989, Wistow and Piatigorsky, 1988). Thus, while there is phyloge-

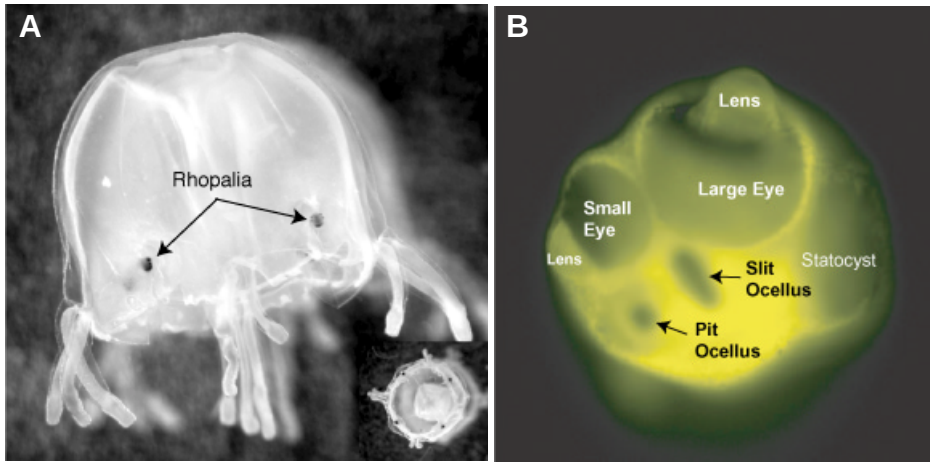

Fig. 3. *Tripedalia cystophora* medusae (A) and rhopalium (B). *Large panel in* **(A)** *shows a side view of adult medusae, while the inset represents the bottom view. Rhopalium in* **(B)** *has been stained with a fluorescent DNA dye (Hoechst 33342) and pseudocolored in yellow.*

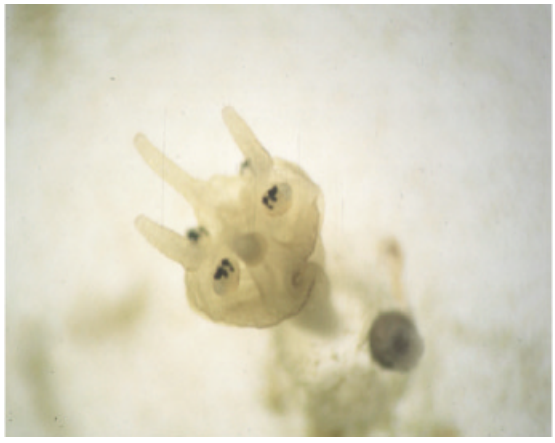

Fig. 4. Newly metamorphosed *Tripedalia* medusa with the rhopalia situated behind the primary tentacles. *See text for further details. Photograph taken by Dr. Zdenek Kostrouch (Institute of Inherited Metabolic Disorders, Charles University, First Faculty of Medicine, Prague, Czech Republic).*

netic inheritance of crystallins, such as for example the δ-crystallins in birds and reptiles (Piatigorsky, 1984), the precise crystallin composition within the lens is not diagnostic for evolutionary relationships. Although the protein(s) used as lens crystallins often differ among species, many if not all are related or identical to common, ubiquitously expressed metabolic enzymes or physiological stress proteins. Of the crystallins present in all vertebrate lenses, the α-crystallins are small heat shock proteins (de Jong *et al.,* 1993, Ingolia and Craig, 1982) and the β/γ-crystallins are related to microbial stress proteins (D'Alessio, 2002, Wistow, 1990). In addition to its optical role as a crystallin, αB-crystallin remains as a stress-inducible, widely expressed small heat shock protein (Klemenz *et al.,* 1991); both the sibling, lens-specialized α A-crystallins and the small heat shock protein/αB-crystallin are effective chaperones that protect partially denatured proteins from aggregation in the lens (Horwitz, 1992). This is an important function that retards cataract formation during aging. Most of the taxon-specific crystallins are derived from or are active metabolic enzymes (Piatigorsky, 1992, Tomarev and Piatigorsky, 1996, Wistow and Piatigorsky, 1988). An exception to the rule of taxon-specific crystallins being enzyme-crystallins is ι-crystallin in the gecko, which is cellular retinol-binding protein type 1 (Werten *et al.,* 2000). Although not an enzyme, ι-crystallin has been co-opted from another function for its optical role in the lens. This crystallin hallmark of being recruited from proteins with ubiquitous metabolic functions has led to the idea that crystallins are unified less by their protein phenotype than by their mode of high, lens-preferred gene expression (Carosa *et al.,* 2002, Piatigorsky, 1993, Piatigorsky and Wistow, 1991). We have called the dual use of a single protein encoded in one gene, 'gene sharing' (Piatigorsky *et al.,* 1988, Piatigorsky and Wistow, 1989). An important implication of gene sharing illustrated by the lens crystallins is that a protein can evolve a new role, without losing its original function, by a change in gene expression in the absence of gene duplication (Piatigorsky, 2003a, Piatigorsky and Wistow, 1991). As in other species, cubomedusan jellyfish crystallins are also taxon-specific, borrowed proteins that appear to have non-optical functions. There are three distinct crystallins (J1-, J2- and J3-crystallin) in *Tripedalia* lenses (Piatigorsky *et al.,* 1989). Interestingly, the larger, 35 kD J1-crystallins are present in the lenses of both the large and small eyes of the jellyfish rhopalia, while the 20 kD J2- and 19 kD J3-crystallins are confined to the lenses of the larger eyes. None of the *Tripedalia* crystallins have a sequence relationship to other crystallins in any species. J1-crystallins comprise three distinct polypeptides (J1A, J1B and J1C), each encoded in different, extremely similar genes (Piatigorsky *et al.,* 1993). J1-crystallins shows sequence similarity to ADP-ribosylglycohydrolases (Z. Kozmik and J. Piatigorsky, unpublished data). J2-crystallin does not appear to be related to J1- or J3-crystallins (Piatigorsky *et al.,* 1989), but it has not been cloned yet. More is known about J3-crystallin than about the other crystallins of *Tripedalia* (Piatigorsky *et al.,* 2001). This protein, encoded in a single-copy gene, is similar in sequence to the conserved saposins, which are multifunctional proteins that bridge lysosomal hydrolases to lipids and activate enzyme activity. Peptides derived from saposins have been also associated with nerve cell survival (Piatigorsky *et al.,* 2001). Interestingly, saposin motifs are present in acid sphingomyelinase and in acyloxyacyl hydrolase in humans, where they participate in regulating enzyme activity. Conceptually, then, the use of saposin sequences as lens crystallins in the jellyfish is akin to having a hybrid molecule displaying characteristics of both chaperones (like the vertebrate α-crystallins) and enzymes (like the taxon-specific enzyme-crystallins) of vertebrate lenses. As will be described below, this is not unlike the situation where PaxB, a protein with combined functions of Pax2 and Pax6, is employed for eye development in *Tripedalia* (Kozmik *et al.,* 2003). In addition to the sequence similarities between J3-crystallin and the multifunctional saposins, *in situ* tests suggest that the J1- and J3-crystallins have non-optical functions in the jellyfish. Both J1A- and J3-crystallin mRNAs have been detected in the outer lumen of the statocyst (Piatigorsky *et al.,* 2001). Moreover, J3-crystallin RNA is present in curious, alternating bands radiating from the pigmented region of the retina as well as at the tips of the tentacles. Further research is necessary to confirm that these crystallin RNAs are producing their respective crystallin proteins and to discover what non-crystallin function these proteins are serving.

Jellyfish PaxB: an ancestral transcription factor with functional properties of both Pax6 and Pax2/5/8

Pax proteins are a family of transcriptional regulators characterized by the presence of an evolutionarily conserved DNA binding domain, the paired domain (Bopp *et al.,* 1986, Treisman *et al.,* 1991). The paired domain is a bipartite DNA-binding sequence composed of two helix-turn-helix motifs, the PAI and RED domains (Czerny *et al.,* 1993, Xu *et al.,* 1999, Xu *et al.,* 1995). Based on sequence similarities, Pax proteins can be grouped into four subfamilies, two of which contain a second DNA-binding domain, the paired-type homeodomain (Noll, 1993). *Pax* genes are involved in many developmental processes in all higher eukaryotes. In particular, *Pax* genes have been associated with a number of developmental defects in *Drosophila*, mouse and human (Chi and Epstein, 2002). The role of the paired- and homeodomain-containing Pax6 during eye development has been characterized extensively (Gehring and Ikeo, 1999). Heterozygote mutations in the human *PAX6* gene results in *aniridia* (Glaser

et al., 1992, Jordan *et al.,* 1992, Ton *et al.,* 1991) and ocular structures are virtually absent in *Pax6* homozygote mice (Hill *et al.,* 1991). The observation that mutations in the *Drosophila* homologue of *Pax6* result in the *eyeless* (*ey*) phenotype (Quiring *et al.,* 1994), along with the facts that misexpression of *ey* (Halder *et al.,* 1995), *toy* (the second *Pax6* gene in *Drosophila;* Czerny *et al.,* 1999) or *Pax6* from many other species induces ectopic eyes has led to the proposal of *Pax6* being a universal master control gene for eye morphogenesis (Gehring, 2002, Gehring and Ikeo, 1999). Despite the importance of *Pax6* in eye development and the presence of eyes in *Tripedalia*, we have been able to clone only *PaxB*, not *Pax6*, from this species (Kozmik *et al.,* 2003). Cnidarian *PaxB* genes cluster with the *Pax2/5/8* subfamily in a phylogenetic tree analysis (Miller *et al.,* 2000). This cluster most likely represents an ancient group of genes within the *Pax* family since its members were found in sponges (Hoshiyama *et al.,* 1998). Jellyfish *PaxB* thus corresponds to an ancestral *Pax* gene encoding a paired domain, homeodomain and an octapeptide between the two domains (Balczarek *et al.,* 1997).

The *Pax2/5/8* subfamily consists of a single *Drosophila* member, *D-Pax2* (Czerny *et al.,* 1997, Fu and Noll, 1997) and three mammalian genes, *Pax2*, *Pax5* and *Pax8*, which arose by gene duplication at the onset of vertebrate lineage (Pfeffer *et al.,* 1998) and cnidarian *PaxB* genes(Groger *et al.,* 2000, Kozmik *et al.,* 2003, Sun *et al.,* 2001, Sun *et al.,* 1997). *D-Pax2* is required for development of ommatidial cone and pigment cells (*sparkling*) (Fu and Noll 1997) as well as mechanosensory bristles (*shaven*) (Fu *et al.,* 1998, Kavaler *et al.,* 1999). In mammals, *Pax5* is essential for B-lymphopoiesis (Nutt *et al.,* 1999, Rolink *et al.,* 1999). Loss of *Pax2* in mice results in severe kidney, eye and inner ear defects (Torres *et al.,* 1996). In addition, *Pax2* collaborates with *Pax5* in midbrain and cerebellum development (Schwarz *et al.,* 1997, Urbanek *et al.,* 1997). *Pax8* deficient mice display thyroid gland dysgenesis (Mansouri *et al.,* 1998). *Pax2* and *Pax8* are required for the specification of the nephric lineage, as mouse embryos lacking both *Pax2* and *Pax8* are unable to form the pronephros (Bouchard *et al.,* 2002). Thus, *Pax2*, which is closely related to *PaxB* of *Tripedalia*, plays developmental roles in many tissues as well as in the eye in higher metazoans.

Much less is known about the function of cnidarian *PaxB* genes. The expression of *PaxB* has been studied in the hydrozoan *Podocoryne carnea* (Groger *et al.,* 2000), which does not have eyes. The *Podocoryne PaxB* gene is expressed in the eggs, the ectodermal layer of larva and endoderm of the developing and adult medusa. Based on the *in vitro* transdifferentiation assays, it has been argued that *PaxB* is involved in nerve cell differentiation (Groger *et al.,* 2000).

The *Tripedalia PaxB* gene is expressed in swimming larvae as well as in the rhopalia of adult jellyfish (Kozmik *et al.,* 2003). Within the rhopalium *PaxB* expression occurs both in the eye (lens and retina) and in the statocyst (Fig. 5A). Interestingly, *PaxB* expression shows resemblances to that of vertebrate *Pax6* in the eye and *Pax2/5/8* family members in the eye and inner ear. We have performed a detailed structure-function study of PaxB protein and have shown that this ancient transcriptional activator represents a functional hybrid of Pax2/5/8 and Pax6 subfamilies (Kozmik *et al.,* 2003). PaxB has a number of Pax2/5/8 features. In addition to having the paired domain DNA-binding specificity of the Pax2/5/8 subfamily, PaxB also has a functional transactivation and inhibitory domain characteristic of the Pax2/5/8 class. It has been shown that the C-terminus of all Pax proteins studied so far carries the transactivation function (Czerny and Busslinger, 1995, Dorfler and Busslinger, 1996, Lechner and Dressler, 1996, Nornes *et al.,* 1996, Tang *et al.,* 1998). Pax6 contains a relatively long transactivation domain composed of shorter regions that act synergistically with each other (Tang *et al.,* 1998). In contrast, Pax2/5/8 proteins have an inhibitory domain in close proximity to a strong, short transactivation domain (Dorfler and Busslinger, 1996, Kreslova *et al.,* 2002). The most prominent Pax6-like feature of PaxB is a functional homeodomain as a second DNA-binding domain. It was shown previously, that *Drosophila Pax6* (*ey*) directly activates expression of *rhodopsin* genes through homeodomain binding sites in the proximal region of the promoters(Papatsenko *et al.,* 2001, Sheng *et al.,* 1997). We have shown that *PaxB* is able to activate the *Drosophila rhodopsin* rh6

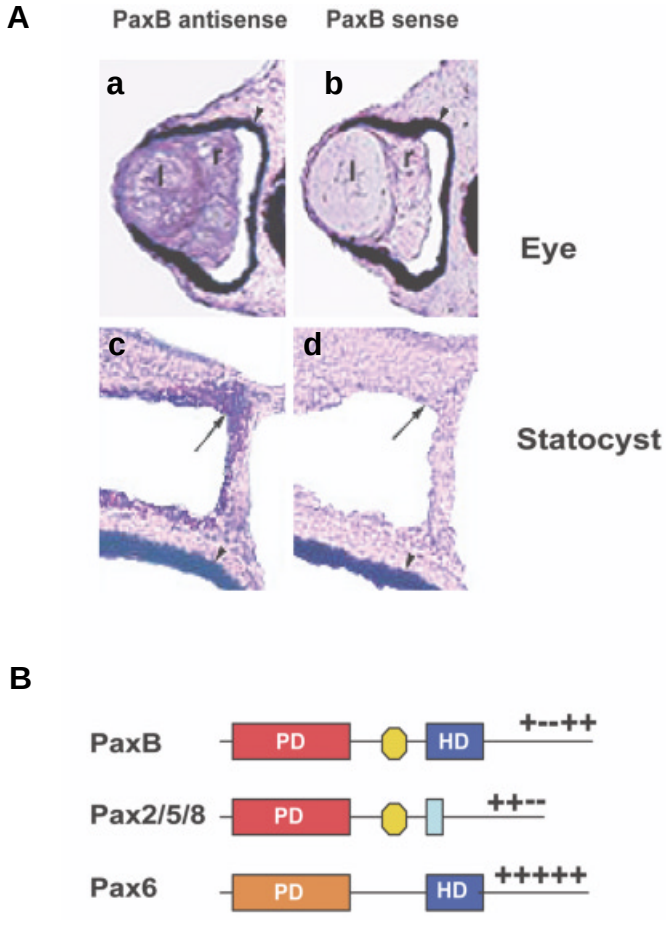

Fig. 5. *In situ* hybridization and diagrammatic structure of *PaxB* mRNA. (A) In situ *hybridization of* PaxB *in the lens and retina of the big eye (a,b) and statocyst (c,d) within a* Tripedalia *rhopalium with* PaxB *antisense and sense (negative control) probe. Methods are described elsewhere (Kozmik et al., 2003).* **(B)** *Schematic drawing of the structure of PaxB, Pax6 and Pax2/5/8 transcription factors. Paired domain (PD, red/brown), octapeptide (yellow) and homeodomain (HD, blue) is indicated. The C-terminus of Pax proteins harbors transactivation (+) and inhibitory (-) domains.*

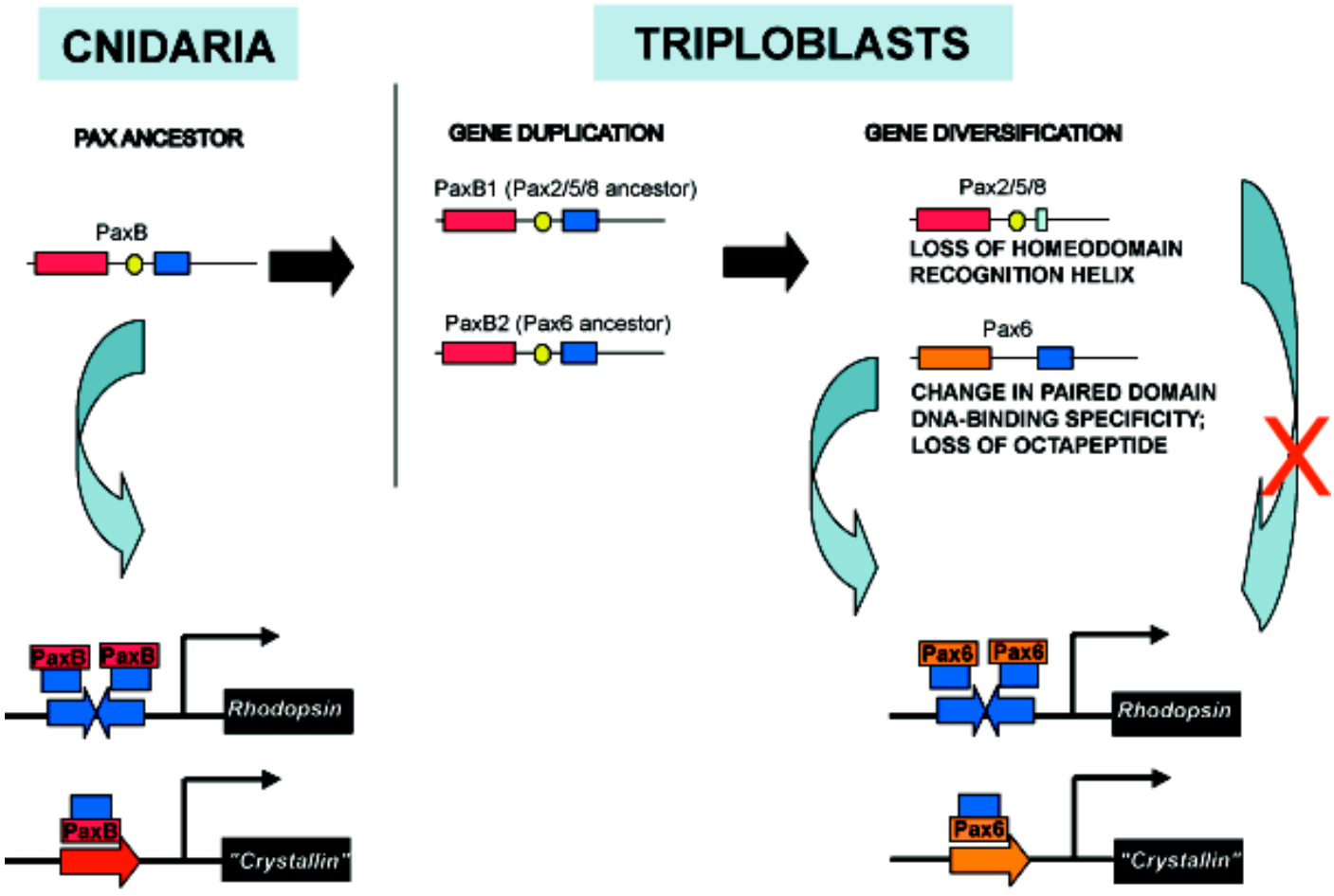

Fig. 6. Hypothesis suggesting that a *PaxB*-like gene, rather than a modern-like *Pax6* gene, was the primordial *Pax* gene involved in eye formation. *The model suggests that a PaxB-like protein was an initial regulator of eye development, of lens crystallin gene expression and of rhodopsin gene expression. In triploblasts, the* PaxB *gene duplicated, giving rise to early* Pax2/5/8 *and* Pax6 *genes. At least in some animal species,* Pax6 *has been recruited for the regulation of crystallin and rhodopsin genes.*

promoter via its homeodomain in transient transfection assays (Kozmik *et al.,* 2003). Finally, *in vivo* data using *Drosophila* as the test system indicated that *PaxB* is a multifunctional hybrid protein. It rescues the *spa* mutation (*D-Pax2* deficiency) and, when over expressed, induces ectopic eyes on *Drosophila* legs (as does *Pax6*), although with lower efficiency than *Pax6* (Kozmik *et al.,* 2003).

A *PaxB*-like gene was an ancestral regulator of lens crystallin genes

Pax6 is involved in the regulation of lens crystallin genes in mouse, chicken and guinea pig (Cvekl and Piatigorsky, 1996, Duncan *et al.,* 2004, Duncan *et al.,* 1998, Gopal-Srivastava *et al.,* 1996, Kamachi *et al.,* 2001, Kralova *et al.,* 2002) and, possibly, scallop (Carosa *et al.,* 2002). We have previously identified three major lens crystallins, J1-, J2- and J3-crystallin, in the cellular lenses of *Tripedalia* (Piatigorsky *et al.,* 1989, Piatigorsky *et al.,* 1993, Piatigorsky *et al.,* 2001). The J3-crystallin gene, expressed primarily in the jellyfish lens and statocyst, appears to be a natural target gene of *PaxB* (Kozmik *et al.,* 2003). Two paired domain binding sites that fit well with the Pax2/5/8 paired domain consensus (Czerny *et al.,* 1993) were identified within the –66/-30 region of the TATA box containing promoter of J3-crystallin gene. *PaxB* activated expression of the J3-reporter gene construct but not the reporter gene construct in which PaxB binding sites were mutated. Remarkably, only *PaxB* (or *Pax2*) but not vertebrate *Pax6*, *Pax1* or *Pax3* activated the J3-reporter gene in transfection tests (Kozmik *et al.,* 2003). *J3-crystallin* gene activation in jellyfish thus seems to be restricted to PaxB/Pax2/5/8 class of transcription factors.

Mutagenesis tests reinforced the requirement for the Pax2/5/8-like paired domain for activation of jellyfish crystallin promoters. Three amino acids (at positions 42, 44 and 47) within the paired domain are responsible for the difference in the DNA-binding specificity between Pax2/5/8 and Pax6. The amino acids IQN at these positions specify the Pax6 class of transcription factors whereas amino acids QRH determine Pax2/5/8 specificity (Czerny and Busslinger, 1995). It is also known that Pax2/5/8 DNA-binding specificity can be generated by converting residues IQN into QRH in positions 42, 44 and 47 of the Pax6 paired domain (Czerny and Busslinger, 1995). In accordance with this data, transfection tests using a *PaxB(IQN)* cDNA encoding a PaxB with a Pax6-like DNA-binding specificity did not activate the *J3-crystallin* promoter

(Kozmik *et al.,* 2003). We conclude that the *Tripedalia J3-crystallin* promoter sequences are optimized for PaxB/Pax2/5/8 and not Pax6 (Kozmik *et al.,* 2003).

Cnidarians suggest that *PaxB*, not *Pax6*, was the primordial gene during evolution of complex eyes

A gene with a typical *Pax6*-like structure has not been detected in the cubozoan, *Tripedalia cystophora* (Kozmik *et al.,* 2003) or in the hydrozoans, *Podocoryne carnea* (Groger *et al.,* 2000) and *Cladonema californicum* (Sun *et al.,* 2001), despite that *Tripedalia* and *Cladonema* have complex eyes. Even if *Pax6* does exist in *Tripedalia* but has escaped our efforts to detect its gene or cDNA, our co-transfection tests suggest that the jellyfish Pax6 protein would neither bind nor activate the *J3-crystallin* promoter, as does PaxB (Kozmik *et al.,* 2003). Four *Pax* genes (*PaxA*, *PaxB*, *PaxC* and *PaxD*) have been found in corals (also cnidarians)(Miller *et al.,* 2000). However, none represents a true *Pax6* ancestor and none has the three characteristic amino acids (IQN) at positions 42, 44 and 47 of the Pax6 paired domain (Kozmik *et al.,* 2003). Analyses of transgenic flies carrying chimeric *Pax* transgenes derived from the coral, *Acropora millepora*, are not consistent with the presence of a classical *Pax6* gene in cnidarians (Plaza *et al.,* 2003). Thus, although negative results do not establish the absence of *Pax6*, the data suggest that the *Pax6* gene originated after the separation of Cnidaria from Bilateria. This implies that development of jellyfish eyes were dependent on a PaxB/Pax2/5/8-like and not a Pax6-like protein.

The data indicate that modern *Pax2* and *Pax6* genes evolved from a *PaxB* ancestor by duplication and diversification in higher metazoans (Fig. 6). Pax2 lost most of its homeodomain while Pax6 lost the octapeptide and changed the DNA-binding specificity of the paired domain by acquiring amino acids I42, Q44 and N47, respectively. It follows that since Pax2 does not have a functional homeodomain it could no longer regulate expression of rhodopsin genes through homeodomain binding sites, as PaxB appears to be able to do in *Tripedalia* (Kozmik *et al.,* 2003) (Fig. 6). Consequently, either Pax6 or an unrelated paired-type homeodomain protein took over this function for *rhodopsin* gene expression in triploblastic metazoans. While *Pax6* homologs *ey* and/or *toy* are direct regulators of *Drosophila rhodopsin* genes *rh1*, *rh3*, *rh5* and *rh6* (Papatsenko *et al.,* 2001, Sheng *et al.,* 1997), the connection between *Pax6* and *rhodopsin* gene activation is not universal. Remarkably, *Pax6* has again been recruited for regulation of crystallin genes in vertebrates (Cvekl and Piatigorsky, 1996, Duncan *et al.,* 2004). Since the diverse crystallin genes in different species are structurally unrelated, the recruitment of *Pax6* for crystallin gene expression in higher metazoa represents convergent evolution.

The Jellyfish eye and other sensory systems and the putative eye/ "ear" connection

Study of the jellyfish eye has implications for the evolution of other sensory systems. Gehring has reviewed the possibility that the jellyfish eye may have preceded brain evolution (Gehring, 2002). He supports this view by noting that unicellular algae (i.e. *Chlamydomonas*) or dinoflagellates (i.e. *Erythropsis*) have eye organelles and no brain. Jellyfish do, however, have a number of specialized ganglia associated with the rhopalia as well as an interconnected nerve ring which may, arguably, be a type of brain for a radially symmetrical animal (Coates, 2003). An attractive feature of eye before brain is that it places sense reception before information processing. Examples of ancestral photoreception preceding a central nervous system are fascinating. One is *Chlamydomonas reihhardtii* (Roberts *et al.,* 2001). The eyespot of this unicellular alga orchestrates a positive phototaxis in low intensity light and a negative phototaxis in high intensity light by directly affecting the beating pattern of the two attached flagellae. Surprisingly, the *Chlamydomonas eye2-1* mutant revealed that formation of the eyespot requires a member of the thioredoxin protein family and that this developmental role does not depend on the catalytic redox capability of the thioredoxin protein. The sponge larva, *Reneira* sp., provides another example of coordinated phototaxis in a multicellular organism that lacks nerve cells altogether (Leys and Degnan, 2001). A posterior ring of columnar epithelial cells containing a cilia and pigmented-filled protrusions respond directly to light, leading to negative photaxis and directed swimming behavior. Increased light intensity makes the cilia rigid and subsequently bend, shielding the pigmented vesicles; decreased light intensity reverses the process. The resulting negative phototaxis is similar to the shadow response of tunicates and the unicellular *Euglena*. Spectral sensitivity tests suggest that the photoreceptive pigment in the sponge larva may be a flavin or carotenoid (Leys *et al.,* 2002). This would make the sponge larva the first metazoan not using a rhodopsin-like protein as the primary photoreceptive pigment. It is not known yet whether expression of *PaxB*, which is present in sponges (Hoshiyama *et al.,* 1998), is associated with the photoreceptive cilia in the sponge larva. Clearly, detailed studies on ancestral eyes and photoresponses are a rich source of new and unexpected insights.

Planula larvae of *Tripedalia* also have a photoreceptive system that appears to be directly connected to cilia for steering towards particular light conditions. A series of single-cell, pigment cup ocelli, lacking neural connections, surround the posterior half of the larval ectoderm (Nordstrom *et al.,* 2003) (see also Gehring in this issue). The positions of these ocelli vary in different species of cubozoan larvae. These light sensors apparently have photosensitive microvilli and a motor-cilium. The cilium responds directly to light and may act as a rudder to steer the larva. Thus, while ciliated and rhabdomeric photoreceptors are occasionally found in the same species, this is the first instance of the latter being reported in cnidarians.

Early developmental studies on embryonic induction have delineated the complex developmental relationships between the presumptive ear, nose and lens fields (Jacobson, 1963a, Jacobson, 1963b, Jacobson, 1963c). The primary role of *Pax6* in eye development (see above) links the visual system with other sensory systems, since Pax6 and other transcription factors that shape the eye are widely expressed in the nervous system (Simpson and Price, 2002, van Heyningen and Williamson, 2002). Interestingly, Pax6 is also expressed in non-neural cells of the visual (i.e. lens, cornea) and olfactory (i.e. sustentacular cells, basal cells and Bowman's glands) systems (Davis and Reed, 1996, Tomarev *et al.,* 1997), linking these distinct sensory modalities.

Our studies on *Tridpedalia* have revealed an intriguing, putative relationship between evolution of the eye and the inner "ear" (Kozmik *et al.,* 2003, Piatigorsky, 2003b). Like eyes, "ears" (i.e. mechanoreceptors) of invertebrates and vertebrates are believed to be evolu-

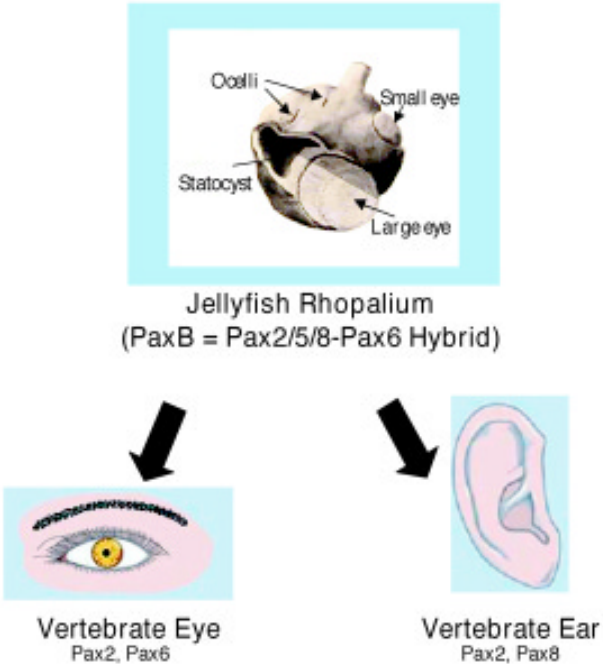

Fig. 7. A scheme suggesting that the eye and the ear may have had a common origin in evolution based on the expression pattern of Pax family members.

tionarily related (Fritzsch and Beisel, 2001, Fritzsch and Beisel, 2003, Jarman, 2002). The rhopalia containing the jellyfish eyes also include a statocyst (see Fig. 3). In general, cnidarian statocysts are associated with mechanoreceptor-like cilia that have been considered as evolutionary ancestors to vertebrate inner ears (Piatigorsky, 2003b, Singla, 1975), with the latter evolving through the stages of sensing water vibrations and then gravity (Horridge, 1969). Despite that mechanoreceptor-like cilia have not been observed directly in the statocysts of *Tripedalia* and that statocyst function has not been established in this species (Coates, 2003), it seems likely that the *Tripedalia* statocysts are related to other cnidarian statocysts that play a role in the "righting response". The relevance of this evolutionary relationship is that *J1-* and *J3-crystallin* genes (Piatigorsky *et al.,* 2001) as well as *PaxB* mRNA (Kozmik *et al.,* 2003), which appears critical for eye development, are expressed in *Tripedalia* statocysts, suggesting a connection between eyes and mechanoreceptors (future ears) in the ancient cnidarians. A relationship between *PaxB* expression and sensory nerve cells has also been noted in the hydrozoan jellyfish, *Podocoryne carnea*, where *PaxB* is expressed in the developing tentacle bulbs and edge of the manubrium, sites common to eyes and statocysts of other jellyfish (Groger *et al.,* 2000). Expression of *Pax2/5/8*, a *PaxB* derivative, in mechanosensory cells is conserved throughout evolution, including mammals, lampreys, chordates, ascidians, *Haliotis asinine* (gastropod mollusk; an abalone), *Drosophila*, *Caenorhabditis elegans* (nematode) and other species (O'Brien and Degnan, 2002a, O'Brien and Degnan, 2002b, O'Brien and Degnan, 2003). Perhaps the examples given above of photoreception leading to coordinated behavior by directly controlling ciliary motion in the unicellular alga, as well as the larval sponge and cubozoan jellyfish, represent primordial eye/mechanoreceptors before each sensory system diverged to elaborate either vision (eyes) or mechanoreception (ears).

The hypothesis of an eye/ear evolutionary relationship is reinforced by the fact that PaxB is a hybrid Pax protein, containing a Pax2-like paired domain and octapeptide, but has a Pax6-like homeodomain (Groger *et al.,* 2000, Kozmik *et al.,* 2003, Miller *et al.,* 2000, Sun *et al.,* 2001, Sun *et al.,* 1997) (see Fig. 6). In mammals, ear development depends on Pax2 and eye development depends on both Pax2 and Pax6 (Baker and Bronner-Fraser, 2001, Baker, 2001, Pichaud and Desplan, 2002) (Fig. 7). A number of other transcription factors from the same family (i.e. atonal/Math1, class IV POU, dachschund, six) are used for the development of eyes and ears and discussions can be found elsewhere (Fritzsch and Beisel, 2003, Jarman *et al.,* 1995, Pichaud and Desplan, 2002, van Heyningen and Williamson, 2002). Recent experiments with *Drosophila* have provided evidence implicating *atonal* for the formation of segment-specific sensory organs (Niwa *et al.,* 2004). These authors suggest that various sensory organs, including eyes and mechanoreceptors, evolved from an *atonal*-dependent protosensory organ.

The linkage between eyes and ears has clinical overtones. A functionally important protein overlapping vision and hearing/balance is class III myosin (Dose *et al.,* 2003, Walsh *et al.,* 2002). In *Drosophila*, the homologous protein, called NINAC, interacts with multiple components to organize the phototransduction machinery into a signaling complex (Wes *et al.,* 1999); NINAC is also responsible for a recessive retinal degeneration in the fly. The human homolog of NINAC, myosin IIIA, is expressed most highly in the retina and ear (cochlea) and recessive loss-of-function mutations of myosin IIIA are associated with hearing loss. A scaffolding protein, harmonin, connected with Usher's syndrome, affecting both vision and hearing, is another clinically intriguing association between these two sensory modalities (Montell, 2000, Verpy *et al.,* 2000). The *choroideremia* gene also bridges eye and ear diseases (Starr *et al.,* 2004). An example is the human *Choroideremia* gene, which is responsible for a slow degeneration of rod photoreceptors and retinal pigment cells. *Choroideremia* encodes Rab escort protein 1 which is essential for prenylation of Rabs. Zebrafish carrying *ru848*, the recessive homologue of *Choroideremia*, are unresponsive to acoustic stimuli and lack balance. The recent finding that Norrie disease and familial exudative vitreoretinopathy both implicate a Norrin-Frizzled-4 (a presumptive Wnt receptor) signaling system provide another link between eye and ear pathology in humans (Xu *et al.,* 2004).

Taken together, the jellyfish rhopalia, with their integrated ocelli and statocysts, have provided tantalizing new bits of information relating vision with other sensory modalities that warrant further study. We believe that the sophisticated sensory rhopalia containing anatomically diverse eyes and a statocyst make cubozoan jellyfish, such as *Tripedalia*, an advantageous model for eye and sensory research.

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