Conservation of the Retinal Determination Gene Cascade in the jellyfish *Cladonema radiatum*

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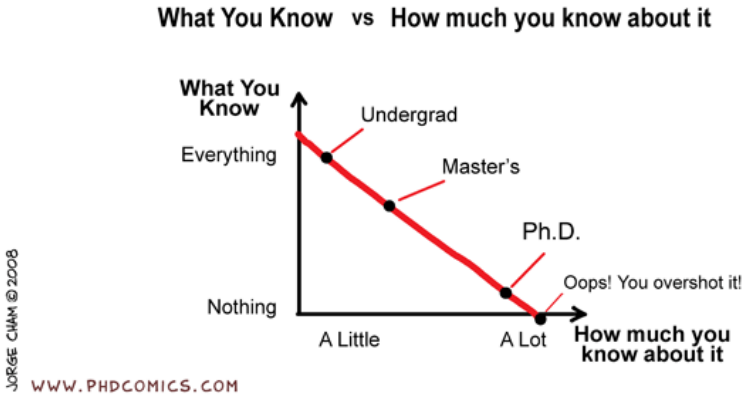
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List of abbreviations

AP	Anterior- Posterior
cDNA	complementary DNA
CNS	central nervous system
c-opsin	ciliary-type opsin
dac	dachshund (*drosophila*)
Dach	dachshund (vertebrate)
dpp	decapentaplegic
DNA	deoxyribonucleic acid
DV	Dorso- Ventral
EF1α	Elongation Factor 1α
evo-devo	evolutionary developmental biology
ey	eyeless
eya	Eyes absent
eyg	eyegone
HD	Homeodomain
mRNA	messenger RNA
ORF	open reading frame
PD	Paired Domain
PFA	paraformaldehyde
PIP2	phosphatidyl inositol diphosphate
PLC	phospholipase C enzyme
PCR	Polymerase Chain Reaction
P/S/T	Proline/Serine/Threonine
RACE	rapid amplification of cDNA ends
RDGC	Retinal Determination Gene Cascade
RD	Retinal Determination
RNA	ribonucleic acid
RNAi	RNA-mediated gene interference
r-opsin	rhabdomeric-type opsin
RTPCR	Reverse Transcription PCR
SD	Six Domain
so	sine oculis
TGFß	Transforming Growth Factor ß
TB	Tentacle Bulb
TBE	Tentacle Bulb Ectoderm
Toy	Twin of eyeless

Abstract

The species *Cladonema radiatum* belongs to the Cnidaria, a basal animal phylum which represents the closest sister group to bilateria. Despite their low position in the metazoan phylogenetic tree, Cnidaria are the only non-bilaterian animals with a defined body axis, a nervous system, sensory organs of great complexity such as photoreceptors and statocysts, and a remarkable regeneration capacity. Therefore cnidarians, for their basal position and with their surprising level of complexity, have become in the last years the organism of choice for evolutionary developmental studies, representing the appropriate outgroup necessary to understand the ancestral bilaterian condition.

The jellyfish of *C. radiatum* bears eight to twelve lens eyes at the bell margin, on the tentacle bulb. Each eye displays a cornea, a lens, pigmented cells and a retina. This species appears therefore suitable for studying the conservation of an important gene network, the Retinal Determination Gene Cascade (RDGC), that has been demonstrated to be responsible for the eye development in species as diverse as *Drosophila* and mice. This network is made up of four gene families: Pax, Eya, Six and Dac.

The full length sequence of a *Pax* gene from *C. radiatum* (*CrPaxA*) was already known. During my Ph.D. studies, I was able to isolate, by means of degenerate PCR, two more members of the Pax family (*CrPaxB* and *CrPaxE*) and one member of the Eya family (*CrEya*), described for the first time in Cnidaria. I then characterized the expression patterns of these genes by *in situ* hybridization, and analyzed by Real Time PCR their expression in the different tissues during the development of the jellyfish and at the different stages of the life cycle. *CrPaxA* is expressed in the retina and in nematocytes precursor cells in the tentacle bulb, whereas both *CrPaxB* and *CrPaxE* are expressed in the manubrium, the feeding and reproductive organ of the jellyfish where the gonads develop. In particular it was possible to detect the signal for *CrPaxB* in the maturing oocytes. *CrEya* is expressed at the same time in the retina and in the manubrium where it shows the same pattern at the level of the oocytes as *CrPaxB*.

Taking advantage of the capability of the jellyfish to regenerate the eye once it has been micro-surgically removed, I was able to investigate the involvement of these genes in the development of the eye. Surprisingly none of them seems to be clearly up-regulated during

the eye regeneration. This could indicate that *CrPaxA* and *CrEya* are involved in the maintenance of the adult eye.

To gain further insights on the role of the isolated genes in the eye determination we used targeted gene expression in *Drosophila*. Taking advantage of the UAS/GAL4 system, we mis-expressed the jellyfish genes in the imaginal discs of the fly and analysed the adults for ectopic eyes induction. At the same time we examined the capability of these genes to rescue *Drosophila* mutant phenotypes. Indeed UAS-*CrPaxA* was able to induce ectopic eyes, and both UAS-*CrPaxA* and UAS-*CrPaxB* were able to rescue the *Drosophila Pax2* mutant *sparkling*.

The expression of *CrPaxA* and *CrEya* in the retina taken together with the functional assays carried out in *Drosophila* argue for a conserved role of this gene network in the jellyfish eye. This result is also supported by data from a previous report, showing the expression of two members of the *Six* genes family in the eye of *Cladonema*. These results overall indicate a high structural conservation of the members of the RDGC between Cnidaria and Bilateria, and are in agreement with the theory of the monophyletic origin of the eye. The evidence for conservation is further strengthened by the expression of *CrPaxB*, *CrEya* and a third *Six* gene *CrSix4/5* in the oocytes, suggesting a possible preservation of the interactions among the members of the network and its redeployment to a different context. Changes in the temporal and spatial pattern of genes expression are one of the main mechanisms by which the phenotypic diversity arises, the redeployment of the RDGC in *Cladonema radiatum* might offer an example of this process.

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2 Introduction

2.1 Evolutionary developmental biology

The publication of the book "The Origin of Species" by Charles Darwin in 1859 was one of the most important advances in biology, having a great impact not only on scientists but also on the general public for its philosophical and religious implications. In his book Darwin presented convincing evidences that life forms do change through time, undergoing evolution. Darwin was the first to realize that evolution is the result of heritable changes in living organisms, among which natural selection will favour only the best adapted to their environment. The evolution of one species into another involves, together with morphological changes, also modifications of the developmental processes behind them. After Darwin's evolutionary theory, several biologist in the nineteenth century started to realize the importance of development in the understanding of morphological evolution. A classic example is given by the theory of recapitulation proposed by Ernst Haeckel. According to Haeckel's theory, each stage in the development of an organism corresponds to an ancestral stage in the evolutionary history of that species (Haeckel 1866). More recently, Gould proposed that recapitulation is the result when evolution proceeds by what he called "terminal addition", which means that evolution proceeds by adding new stages to the end of development (Gould 1977). These concepts as such are not totally accepted anymore because they are considered too simplistic, but they had the great merit of having renewed the interest in the evolution of development and having introduced some basic ideas in this field, such as the importance of two evolutionary developmental mechanisms: heterochrony and heterotopy, the changes in the timing or positioning of an aspect of development in a descendant relative to an ancestor, respectively (Haeckel, 1866; Gould, 1977).

For a long time, the nature of the heritable changes described by Darwin was not clear. When Mendel's work was finally rediscovered by de Vries, Correns and Tschermak at the beginning of the 20th century, it was possible to understand the mechanisms of heredity (de Vries 1889; Correns 1900; Tschermak 1900). Afterwards, Boveri and Sutton postulated the chromosomal theory of inheritance, proposing that the Mendelian laws could be explained by the behaviour of chromosomes (Boveri 1903; Sutton 1903; Boveri 1904). When later on the experiments conducted by Morgan on the fruit fly *Drosophila melanogaster* proved that the genes responsible for the heritable characters were located on the chromosomes (Morgan 1915),

genetics started to play an important role in the understanding of the basis for the variations that drive the evolution of a species. The importance of genetic mutations in the variation of a population lead to the creation of the "population genetics" field. The merging of the pre-existing evolutionary theories with the new experimental knowledge in population genetics gave rise to the "modern synthesis", a modern theory of evolution which takes into account and unifies all these new ideas.

During the last two decades of the 20th century, thanks to the advances in molecular biology and the availability of genomic sequences from a variety of different organisms, genetics went a step forward. The study of the role of genes during the development of the organisms, and in particular the discovery of the homeotic mutations by Lewis, opened the way to developmental genetics (Lewis 1978). One of the most surprising findings in this field came from the comparison of the genomes from several species: the high conservation and widespread use of the *Hox* gene complexes among distantly related organisms was clearly shown (McGinnis et al. 1984a). This discovery had a great impact on the evolutionary views of biologists and, as a consequence, the field of developmental genetics and evolution were brought together giving rise to the so called "evolutionary developmental biology" or "evo-devo" (For a review, see De Robertis 2008). More recently, it has become clear that the conservation of Hox genes can be extended also to other genes such as the juxtacrine factor Notch or the paracrine factor Wingless (Artavanis-Tsakonas et al. 1999; Sharpe et al. 2001). Similarly, it was also shown that the *Drosophila eyeless* (*ey*) gene, which if mutated causes a reduction or a loss of the eye in the fly, is the homologue of the mouse *Pax6* gene, whose mutation cause the small eye phenotype (Quiring et al. 1994). A finding which shows that, behind the high morphological diversity of animal structures, lies instead a common developmental program that is fulfilled by a set of genes shared among different species. This genes are therefore collectively named "developmental genetic toolkit" (Carroll 2001).

If the genes of the developmental genetic toolkit are so similar, how can such a wide variety of animal forms develop? It has been suggested that the phenotypic diversity derives from differences in the spatial pattern and timing of gene expression (Jacob 1977). Differences in the expression of both control and target genes are thought to be mainly due to changes that have occurred in their control regions (Carroll 2001). "It seems that when a useful developmental mechanism evolved, it was retained and redeployed in very different organisms, and at different times and places in the same organism" (Wolpert 2006).

Classic taxonomy has classified life forms according to morphological criteria, grouping them on the basis of similar features. After the emergence of the evolutionary theory, it became

clear that in order to understand the present diversity, it is also important to take into consideration the phylogenetic relationship among the different groups. Since evolution occurs by gradual accumulation of mutations in the genome, the comparison of genomic data from two different organisms can inform us on how closely related they are. Two species that have diverged more recently should be more similar, in terms of DNA sequences, than two genomes whose common ancestor is more ancient. This is the reason why sequence analysis has become the major method currently used for establishing evolutionary relationships. Evolutionary developmental biology takes into account all these different inputs, giving a wide look on life history. It explores the ancestral relationships among the organisms, the way the developmental processes evolved and the genetic basis by which these dynamics determine the morphological variations that lead to evolution. In order to understand these issues, evo-devo uses multiple research approaches (Muller 2007). A first approach is more morphological, by the comparison of anatomical details from fossils with the extant species in order to characterize "large-scale patterns of phenotypic evolution" (Muller 2007). An epigenetic approach which tries to prove the influence of the environment on evolution. And the most successful approach, the evolutionary developmental genetic one, which has proved to be the most productive area of evo-devo (Muller 2007). It focuses on the evolution of the genetic machinery of development through the comparison of the developmental genetic toolkit of organisms with completely different body plans, looking for the mutation, duplication or divergence of these genes and the differences in their regulatory networks and signalling pathways, in order to determine how the enormous diversity of animals has been achieved (Muller 2007). For these reasons, evo-devo needs to choose model systems for their phylogenetic informativeness, so that they might serve as a complement to existing model systems in reconstructing evolutionary history (Darling et al. 2005). In this seek for new model organisms, uncommon animals have been brought to the attention of the scientific community, among them Cnidaria have recently gained increasingly importance.

2.2 Cnidaria

The higher Metazoa are traditionally grouped apart from lower, simpler animals according to a number of distinctive features. For example, the presence of two body axes, an anterior-posterior (AP) axis and a dorsal-ventral (DV) axis, characterizes almost all the animal phyla, which, being bilaterally symmetrical, have been grouped together in the so called "Bilateria".

Another important characteristic in common to all the higher Metazoa is the presence of three germ layers, ectoderm, endoderm and mesoderm, which defines them as triploblasts.

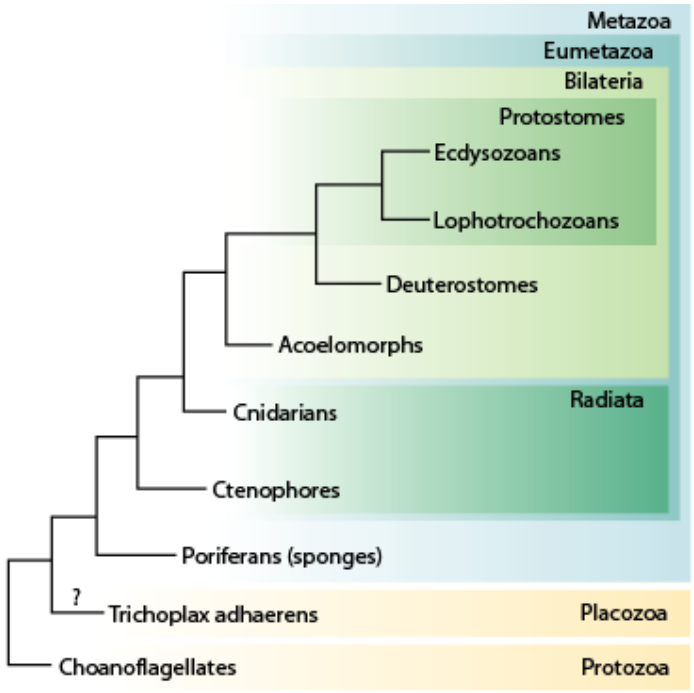

Fig 2.1 Metazoan phylogeny All animals showing a bilateral symmetry are collectively called Bilateria, out of this major group are the so called "lower metazoa": Cnidaria, Ctenophora, Porifera and Placozoa (from Martindale 2005).

The Bilateria subregnum comprises almost all the animal phyla. The only exceptions are four groups of lower Metazoa: Cnidaria (corals and jellyfish), Ctenophora (comb jellyfish), Porifera (sponges) and Placozoa (Fig. 2.1). In order to understand the evolution of the developmental process that underlie the diversity of the Bilateria, it is important to understand the ancestral bilaterian condition and this, in turn, requires an appropriate outgroup (Darling et al. 2005). Ctenophora and Cnidaria are the only two basal phyla showing a complex body organisation with a defined body axis, a nervous system, sensory organs of great complexity such as photoreceptors and statocysts, and a remarkable regeneration capacity. However the relative position of the four clades within the lower Metazoa is still controversial. In some cases, molecular data argue that cnidarians are the sister group to bilateria (Martindale 2005), but a more recent phylogenetic analysis strongly supports the idea that Ctenophores instead are in this position (Dunn et al. 2008). Regardless of this debate, Cnidaria are still basal organisms, which show however a certain level of complexity and therefore represent an

interesting outgroup to Bilateria. For these reasons, species belonging to this phyla have become the organism of choice for evolutionary developmental studies in the last years.

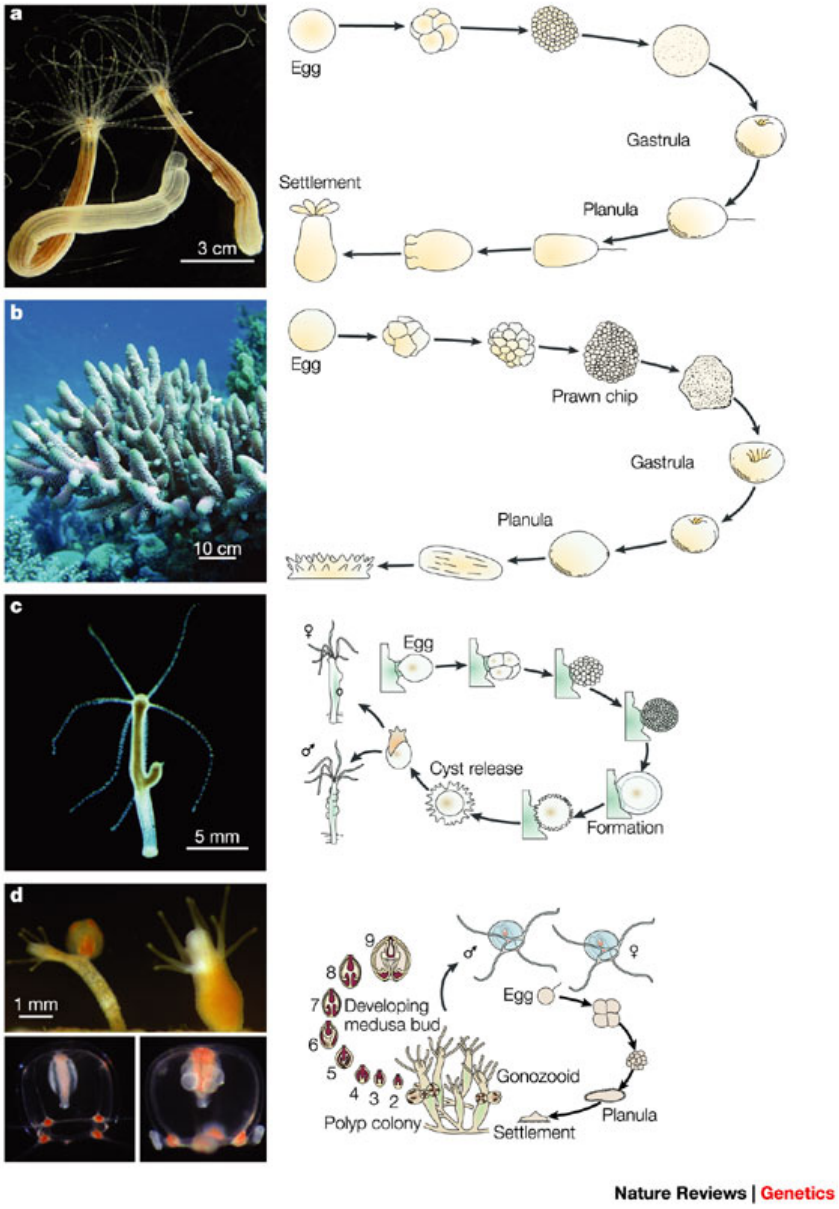

Fig 2.2 Cnidarian life cycles The different cnidarian classes have a wide variety of life cycles, here are described the major cnidarian model organisms; **(**a) *Nematostella vectensis* (Anthozoa); (b) *Acropora millepora* (Anthozoa); (c) *Hydra* (Hydrozoa); (d) *Podocoryne carnea* (Hydrozoa) (Ball et al. 2004).

Cnidaria are subdivided in five classes: the Anthozoa, to which the sea anemone *Nematostella vectensis* and the coral *Acropora millepora* belong, the Cubozoa or box jellyfish including

Tripedalia cystophora, the Scyphozoa, the Hydrozoa, to which *Hydra* and *Podocoryne carnea* belong and the Staurozoa.

The last four classes are further grouped together in the Medusozoa, being all characterized by an alternation of two different morphological life stages: a sessile sexually immature stage, the polyp, and an adult free swimming stage, the medusa, which develops the gonads and is responsible for the sexual reproduction. The four classes that belong to the Medusozoa differ in the way the jellyfish develops from the polyp. The Anthozoa instead have only the polyp stage that corresponds to the adult phase, in which the sexual reproduction takes place (Fig. 2.2).

2.2.1 Tissue organization

Cnidaria have been historically described as radially symmetric and, as they lack the mesoderm, have been considered diploblast. In general Cnidarian larvae and polyps are composed of two epithelial cell layers, the ectoderm and the endoderm, separated by a thin acellular extracellular matrix (ECM) (Fig 2.3b) (Grassé 1993; Seipel and Schmid 2005), which is made up of collagen-like molecules (type IV), laminin, heparane sulphate proteoglycan core protein and fibronectin (Sarras et al. 1991a; Sarras et al. 1991b; Fowler et al. 2000). The endoderm lines a blind gastrovascular cavity, which displays only one opening utilized for food intake and ejection of indigestible material. Several different types of cells can be found interspersed in the two epithelial layers, such as epitheliomuscular cells, gland cells, interstitial cells, sensory nerve cells, cnidocytes or nematocytes (Fig 2.3c). The epitheliomuscular cells are epidermal and gastrodermal cells that display a basal extension, in which longitudinal and circular muscle fibers are located. The contraction of these fibers allows for the movements of the polyp.

The nematocytes are the distinctive, unique cells of the phylum, it is from these cells that the name Cnidaria is derived. A nematocyte contains a surface modified cilium, the cnidocil, and a nematocyst, the actual stinging element. Nematocytes are typically located between or embedded within the surface epidermal cells, and they are especially prevalent on the tentacles where they are used for the capture of the prey. They are considered to be mechanoreceptor cells, able to sense the prey via the cnidocil and to respond in a few millisecond by the discharge of the nematocyst content (for a more detailed discussion on nematocytes, see paragraph 2.2.2).

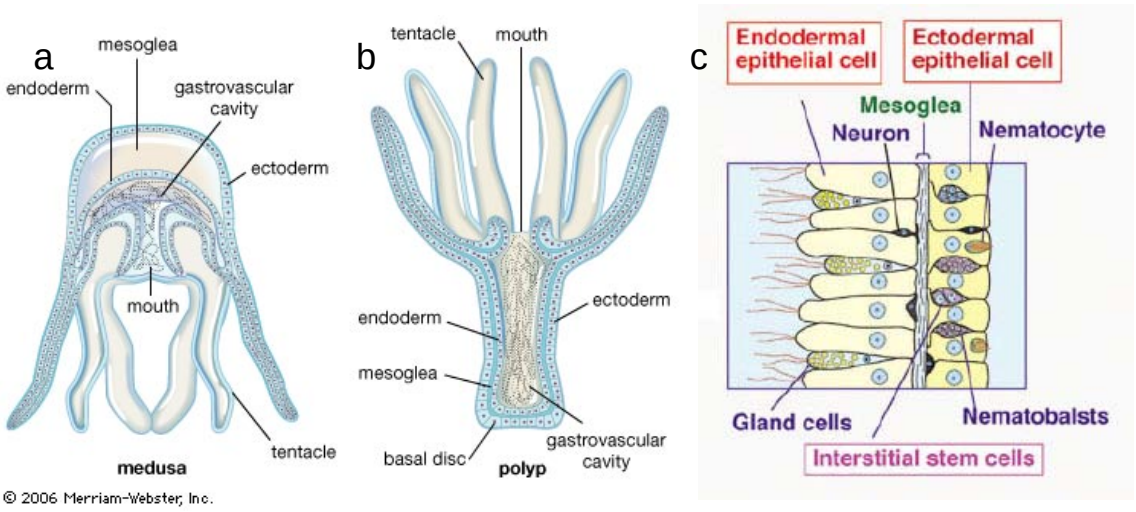

Fig. 2.3 Cnidarian Structure Two types of cnidarian body form are shown, the medusoid body form (a), and the polypoid body form (b). Body wall of *Hydra*, longitudinal section (c). (From Encyclopædia Britannica Online. www.britannica.com/ebc/art-66029.)

The medusa stage shows a more complex anatomy and the architecture of its tissues is more elaborate than that of the polyp (Fig. 2.3a). The mesoglea is thicker and can be with or without cells, where cells are present, they are derived from the epidermal layer (Dorit R. L. 1991). Besides the two epithelial cell layers, and contrary to the common text book knowledge, it has been shown that jellyfish have a well developed layer of mononucleated striated muscle cells, which is entirely or partially covered by an epidermal layer. In most hydromedusae, the striated muscles derive from the entocodon, a third layer which develops between the ecto- and the endoderm (reviewed in Seipel and Schmid 2005; Seipel and Schmid 2006). More recently, a wealth of molecular data has been reported indicating the presence and the expression, during the muscle development of jellyfish, of genes that are related to those involved in mesoderm and muscle specification genes in triploblastic animals (Spring et al. 2000; Spring et al. 2002; Muller et al. 2003). This molecular evidence, together with the ultrastructural data, is challenging the historical concept of cnidarians being diploblast animals. It also implies that the classical distinction between diploblast and triploblast is arbitrary, as the molecular mechanisms that underlie mesoderm specification might pre-date the origins of the Cnidaria (Ball et al. 2004).

Also the issue of the radiality of cnidarians has lately become matter of debate. The *Hox* genes have been extensively studied in bilaterians for their role in patterning the AP axis, and thus considered as a defining characteristic of bilaterian animals. The finding that cnidarians have *Hox*-like genes has therefore raised evident interest. The expression of several *Hox*-like

genes in distinct domains along the primary body axis during the development of *Nematostella vectensis* would argue for a rudimentary *Hox* code in cnidaria (Ryan et al. 2007), but whether these genes can be considered as true *Hox* genes is still controversial (for a review, see Martindale 2005). Furthermore some of these *Hox*-like genes, together with members of the TGFß family, are expressed asymmetrically along the directive axis of *Nematostella*, which indicates a homology between this axis and the bilaterian D-V axis (Finnerty et al. 2004). Further molecular markers are needed to understand the axial properties of cnidarians.

However, all these evidences show how the historical features that distinguish bilaterians from cnidarians might be regarded as an oversimplification, and how the knowledge acquired through the evolutionary developmental studies on Cnidarians, is slowly making the boundary between "higher" and "lower" metazoans less sharp.

2.2.2 Nervous system and sensory organs

The nervous system of the polyp is usually very simple, being constituted by nerve cells located at the base of the epidermis and gastrodermis and arranged as a nerve net. In *Hydra* the nervous system is constituted by sensory-motoneurons, ganglia neurons and mechanoreceptor cells, the nematocytes.

In *Hydra* polyps, the nematogenesis process (differentiation of nematocytes) begins with the proliferation of early committed interstitial stem cells (I-cells) scattered in the body ectoderm. These I-cells form nematoblasts that undergo nuclear morphology changes and start to divide synchronously (Holstein 1981; Bode 1988; Campbell 1988). After this division step, the primordium of the nematocyst capsule develops from the Golgi apparatus. The tubule that will give rise to the thread develops at the apical part of the capsule, in the cytoplasm, from the fusion of vesicles deriving as well from the Golgi. Once the tubule is completely formed, it will invaginate into the capsule. At this point the formation of the cnidocil apparatus starts. In the last steps, the nematocyst capsule will develop the high inner pressure that allows the fast discharge of the thread. The mature nematocyte will then migrate to its final destination, the tentacles (Holstein 1981; Bode 1988; Campbell 1988). In hydrozoan medusae, the site of nematocytes differentiation is more localised than in *Hydra* and does not coincide with the point where the stinging cells will exert their function. The nematocytes of the manubrium differentiate at its base and then move to the mouth, while the tentacle nematocytes differentiate in the tentacle bulbs and then migrate to the tentacles (Carré 1974a; Carré 1974b;

Carré 1974c; Bouillon 1995). Recently the progression of nematogenesis in tentacles bulbs of an hydrozoan jellyfish, *Clytia hemisphaerica* has been studied in great detail (Denker et al. 2008). The authors used a combined morphological and molecular approach to describe the nematogenesis in the tentacle bulb ectoderm (TBE). Making use of microscopy and known nematogenesis-associated genes, they could describe a polarized process, with undifferentiated cells at the proximal side of the tentacle bulb ectoderm, going through maturation as they approach the more distal part of the TBE, where the tentacle inserts.

As might be expected, medusae have a more sophisticated nervous system than the sessile polyps do. In many groups, especially in the hydromedusae, the nerve cells are organized in two nerve rings at the bell margin. These nerve rings connect with fibers innervating the tentacles, muscles and sense organs (Brusca and Brusca 1990).

Most of the neurons are sensory-motor interneurons, these neurons and the synapses among them are non polar, therefore impulses can travel in either direction along the cell or across the synapse. Only a few nerve cells and synapses are polarized (bipolar) and allow for transmission in only one direction.

The nervous system of cnidaria is mainly peptidergic, most of cnidarian neuropeptides have been classified according to their C- terminal aminoacid sequence which is identical among members of the same family (e.g. RFamides, RPamides, GLWamides) (Grimmelikhuijzen et al. 1996; Kass-Simon and Pierobon 2007).

Many jellyfish do not only show an organized nervous system but also have complex sensory organs such as photoreceptors and statocysts. The photoreceptors include simple eyespots, pigment cups, complex pigment cups with lenses and camera eyes (for a review, see Martin 2002).

The photoreceptors of cnidarians resemble those of vertebrates in that they are of the ciliary type, unlike those of most invertebrates which are rhabdomeric, and that they hyperpolarize in response to light (Fig 2.4).

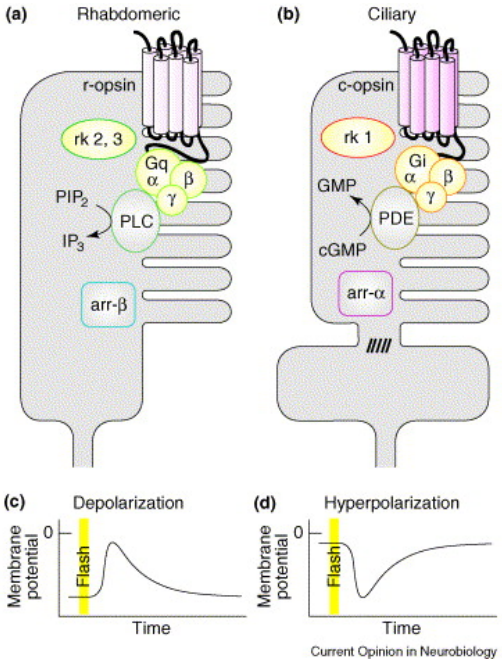

Fig 2.4 Photoreceptor cell type In rhabdomeric photoreceptors (a) the r-opsin activates a G-protein coupled with a phospholipase which finally leads to a depolarization of the cell. In ciliary photoreceptors (b) the cascade activated by c-opsin leads to the activation of a phosphodiesterase with the hyperpolarization of the cell. (Nilsson 2004)

The most sophisticated sensory structures can be found in the Cubomedusae and Schyphomedusae. The bell margins of these jellyfish bear club-shaped structures, called rhopalia, which are sensory centers each containing a concentration of sensory neurons, a pair of ocelli, a statocyst and often one or more complex eyes (Brusca and Brusca 1990).

In the last years the rhopalia of the Cubomedusa *Tripedalia cystophora* have been studied in detail, revealing a surprising complexity for organisms that have been so far considered to be "basal" (Nilsson et al. 2005; Parkefelt et al. 2005). Each *Tripedalia* rhopalium has a total of 6 eyes: 2 more complex lens eyes, an upper smaller one and a lower bigger one (Fig. 2.5a, and Fig. 2.6a,b), and 4 simpler eyes, two slit eyes (Fig 2.6a) and two pit eyes (Fig 2.6b). These simpler eyes are mainly constituted by pigmented photoreceptor cells. Whereas the upper and lower lens eye display all the major components of camera-type eye: a cornea, a lens, a retina, a pigment layer and an iris. The iris of the lower eye is mobile and can close or open according to the light intensity (Fig 2.5b,c). Nilsson and co-workers have studied the optics of the lens eyes, showing that the lenses contain a finely tuned refractive index gradient producing nearly aberration free images. However due to the inappropriate eye geometry, this sharp image falls below the retina, in this way the precise focus generated is wasted. To explain this contradiction, the authors argue that an interpretation might be that the eyes are purposely under-focused in order to remove unnecessary fine details from the retinal image.

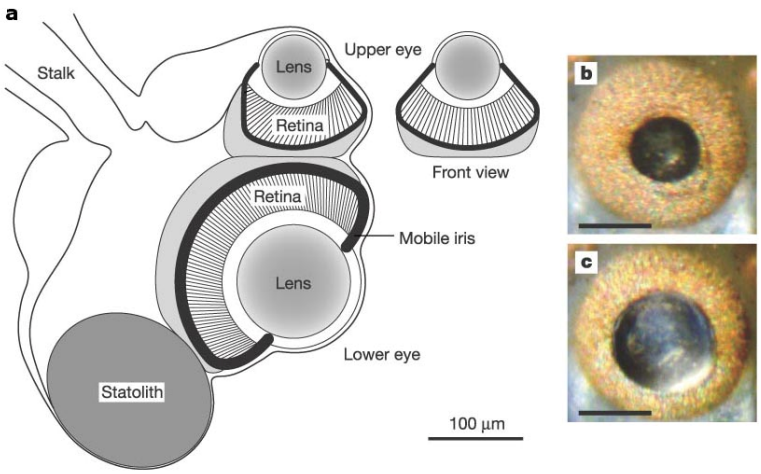

Fig. 2.5 The eyes of *Tripedalia cystophora* Schematic drawing of the rhopalium showing the upper eye, the lower eye and the statolith (a). The mobile iris of the lower eye responds to changes in the light intensity by closing (b) and opening again (c) (after Nilsson et al. 2005).

Moreover, it has been recently shown by Parkefelt and co-authors that the neurons present in each rhopalium have a bilaterally symmetric organization, with three commissures connecting the two sides (Fig 2.6) (Parkefelt et al. 2005). It has been speculated that this neuronal system integrates the visual signals and directly signals to the swimming pace-maker located in the rhopalia. In any case these results highlight the importance of cnidarians for understanding the evolution of sensory organs and of nervous systems.

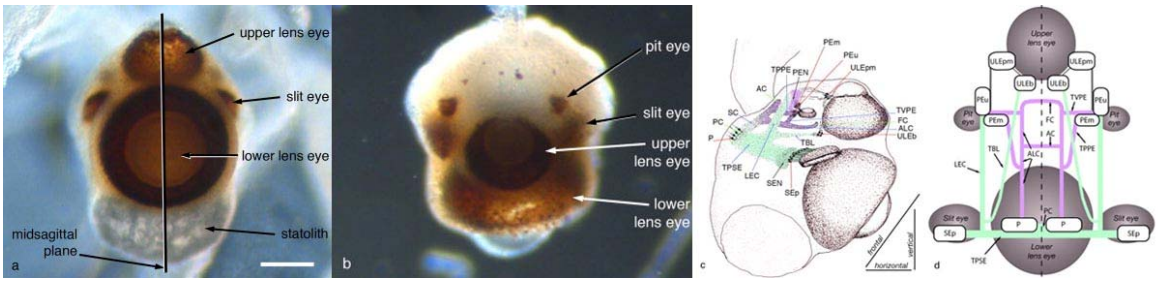

Fig. 2.6 Bilateral symmetry in the rhopalium of *T. cystophora* (a) Frontal view and (b) top view; (c) schematic drawing showing a lateral view of the rhopalium and of the PCNAir neurons; (d) schematic overview of the bilaterally symmetric organization of the neurons. AC, anterior commissure; ALC, apical lateral connective; FC, frontal commissure; LEC, lateral eye connective; P, posterior nucleus; PC, posterior commissure; PEm, medial nucleus of the pit eye; PEN, pit eye neuropil; PEu, upper nucleus of the pit eye; SC, stalk connective; SEN, slit eye neuropil; SEp, posterior nucleus of the slit eye; TBL, basal lateral tract; TPPE, posterior pit eye tract; TPSE, posterior slit eye tract; TVPE, vertical pit eye tract; ULEb, basal nucleus of the upper lens eye; ULEpm, posteromedial nucleus of the upper lens eye. Scale bar is 100 µm (applies to a,b) (after Parkefelt et al. 2005).

2.2.3 Germ cells and development

In Hydra polyps, the germ line cells derive form a subpopulation of interstitial cells committed to egg and sperm production (Littlefield 1985; Littlefield 1991). In the case of hydromedusae, the origin of the germ cells is more controversial. Some authors have shown evidence of the derivation of germ cells from somatic cells that undergo transdifferentiation (Seipel et al. 2004; Torras et al. 2004). The authors followed the expression pattern of two germ line specific genes, during the development of *Podocoryne carnea*: *Cniwi*, the cnidarian homologue of *Piwi,* needed in *Drosophila* for self-renewing of germ stem cells (Cox et al. 1998), and *nanos* which in *Drosophila* participates to the differentiation of primordial germ cells (Kobayashi et al. 1996; Forbes and Lehmann 1998). In both cases it was not possible to detect any expression in the polyp, but the genes started to be expressed only in medusa buds and later in the gonads of adult jellyfish. This indicates that, conversely to *Hydra*, hydrozoan jellyfish such as *Podocoryne*, lack a subpopulation of stem cells set aside early in the development. More data from different cnidaria species are needed to reach a better understanding of this process.
However, in hydromedusae the germ cells that form in the budding area migrate to the manubrium during early development, regardless of their origin. Later they migrate from the manubrial endoderm to the ectoderm (Boelsterli 1977), where they form temporary gonadal masses on the surface of the manubrium, beneath the radial canals or on subumbrellar surface (Brusca and Brusca 1990). Hydromedusae are usually gonochoristic, the male and female reproductive organs being on different individuals, with the gametes released into the water, where the fertilization occurs. In schyphomedusae, the gonads always develop on the gastrodermis and gametes are generally released through the mouth (Brusca and Brusca 1990).

The pattern of embryonic development among the different classes of cnidarian is variable, but most cnidarians have a ciliated, lecitotrophic planula larva. The larvae freely swim for some time until they find the appropriate substrate to settle. During the metamorphosis there is an inversion of the AP polarity of the larva, with the anterior part of the planula becoming the aboral end of the polyp. Despite the fact that generally the larvae are very simple, bilayered organisms, Nordström and colleagues recently reported a visual system in the larva of *Tripedalia cystophora* (Nordstrom et al. 2003). The planulae display in the posterior

ectoderm the presence of 10-15 single cell, pigment cup ocelli that, having photosensory microvilli, are considered to be of the rhabdomeric type, contrary to the ciliary photoreceptors found in the rest of cnidaria (Nordstrom et al. 2003). Since the ocelli also posses a cilium, it has been hypothesized by the authors that they have both sensory and effectory properties. The cilium bends in response to light and in doing so it influences the swimming direction given by the movement of the epidermal cilia. Despite bearing this visual structures, the larvae of *Tripedalia* do not display the presence of any nerve cells, although a simple nervous system has been already described in larvae of other cnidarians (Martin 2000; Hayward et al. 2001). Therefore the ocelli of the larvae of *Tripedalia* may represent the simplest visual system described in multicellular organisms.

2.2.4 The model organism of choice: *Cladonema radiatum*

The species *Cladonema radiatum* belongs to the Hydrozoa class. It displays the typical metagenetic life cycle characterized by the alternation of the sessile polyp and of the free swimming medusa (Fig. 2.7,b).

This species can be found in the North Atlantic Ocean, the North Sea, the English Channel, the Mediterranean Sea and is also present in Japan. The colonies used in our laboratory were collected from the Tyrrhenian Sea.

The polyps are 0,5 mm high, have four capitate tentacles surrounding the mouth and four filiformous tentacles in the lower part of the body. The medusa buds form in the region in between these two sets of tentacles. Usually, on a polyp not more than two medusae buds form at the same time. The polyp form branching colonies that can grow up in the water column for one or two centimetres.

The adult jellyfish usually have a bell (also called umbrella) diameter of 2-3 mm. The feeding organ, the manubrium, dangles from the centre of the sub-umbrellar cavity, at its distal side the mouth opens in the gastrovascular cavity, from which eight prolongation, the radial channels, descend to connect with the circular channel, at the bell margin. On the proximal side of the manubrium the gonads develop. The lower part of the umbrella is almost completely closed by a thin layer of ectoderm, called velum; eight tentacle bulbs are present on the bell margin, from each of which overhang a regular tentacle and an adhesive tentacle. The nematocytes in the tentacle are of two types: stenoteles and desmonemes.

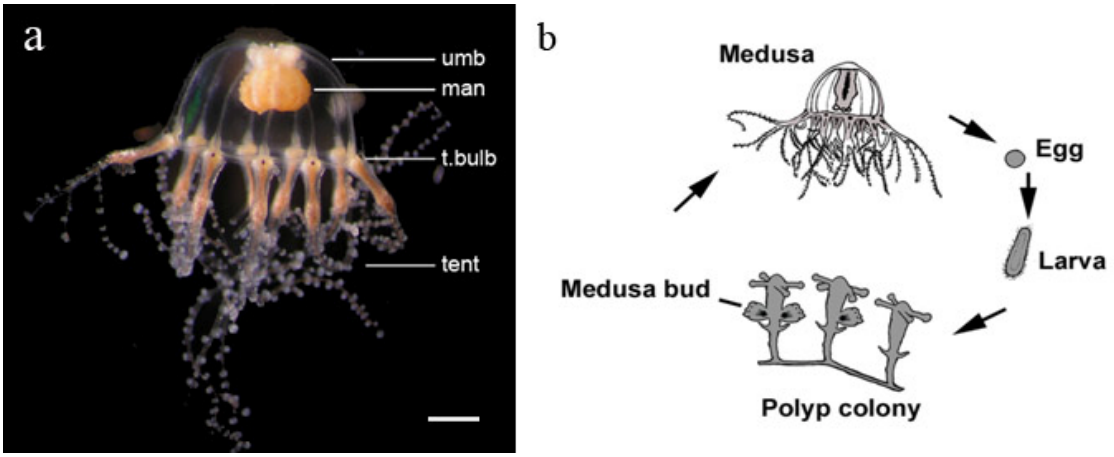

Fig 2.7 ***Cladonema radiatum*** Morphology (a) and development (b) of *Cladonema radiatum*; umb: umbrella; man: manubrium; t.bulb: tentacle bulb; tent: tentacle, (panel (b) from Stierwald et al. 2004). Scale bar in (a) is 1mm.

Each tentacle bulb also presents an ocellus, approximately 45µm wide and 55µm deep, that is separated from the endoderm by an acellular layer of mesoglea (Weber 1981b). The ocellus is made up of a retina constituted by ciliary type photoreceptors, melanin-containing pigment cells, a biconvex lens and a transparent cornea, formed by the intensively vaculated distal processes of the adjoining epithelial cells (Fig. 2.8). The lens is composed by a few subunits, each of which is the distal cytoplasmic portion of a pigment cell. Beneath the retina, touching the mesoglea, there are the basal cells that might represent early stages of differentiating pigment cells.

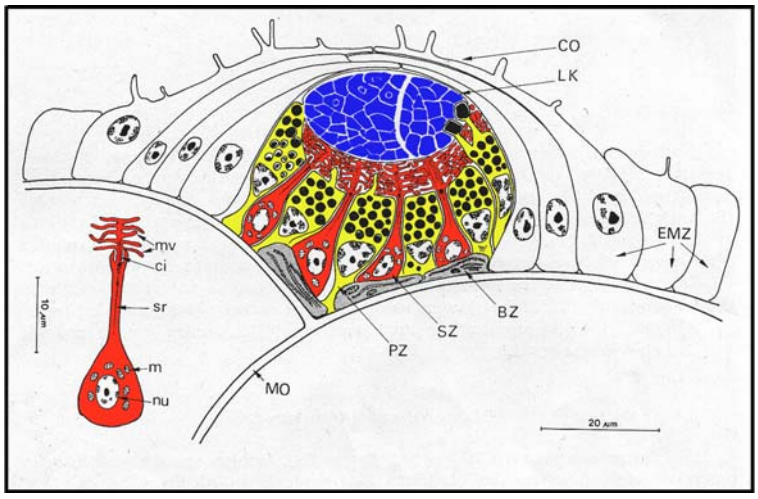

Fig 2.8 Schematic drawing of a cross-section of *Cladonema* eye In red are displayed the photoreceptor cells, in yellow the pigment cells and in blue the lens. BZ, basal cells; ci, cilium; CO, cornea; EMZ, epithelial muscle cell; LK, lens; m, mitochondria; MO, mesogloea; mv, microvilli; Nu, nucleus; PZ, pigment cell; sr, striated root; SZ, photoreceptor cell (Michael Stierwald, adapted from Weber 1978).

2.2.5 Eye development and regeneration in Cladonema radiatum

The histogenesis of the eye of *Cladonema* follows the same course during both ontogeny and regeneration (Weber 1981b). The whole process of the development of the medusa bud takes approximately ten days, the first primordium of the eye appears in five days-old medusa buds. In eight days-old medusa buds the ocellus is clearly visible (Weber 1981b). The eye entirely derives from ectodermal cells which in 5-day-old medusa buds will start to differentiate in the three types of cells: pigment, sensory and cornea cells. The lens and the cornea start forming in 6-day-old medusa buds. In 8-day-old jellyfish, all the elements of the eye are clearly visible, and they will still grow for two more days.

Weber also described the regeneration of the eye after removal (Weber 1981b). The wound healing starts after a few minutes, by the movement of the surrounding epithelial cells. After one day, the epithelial cells at the side of the regeneration area develop vacuolated distal processes that grow over the regeneration area and form a cornea. Three to six days after removal, the pigment and sensory cells have begun to differentiate, some of the pigment cells have started to form the distal portion that will give rise to the lens. After ten to 15 days the differentiation of the different elements that constitute the eye has reached a point where it looks just the same as the ontogenetically developed (Fig. 2.9).

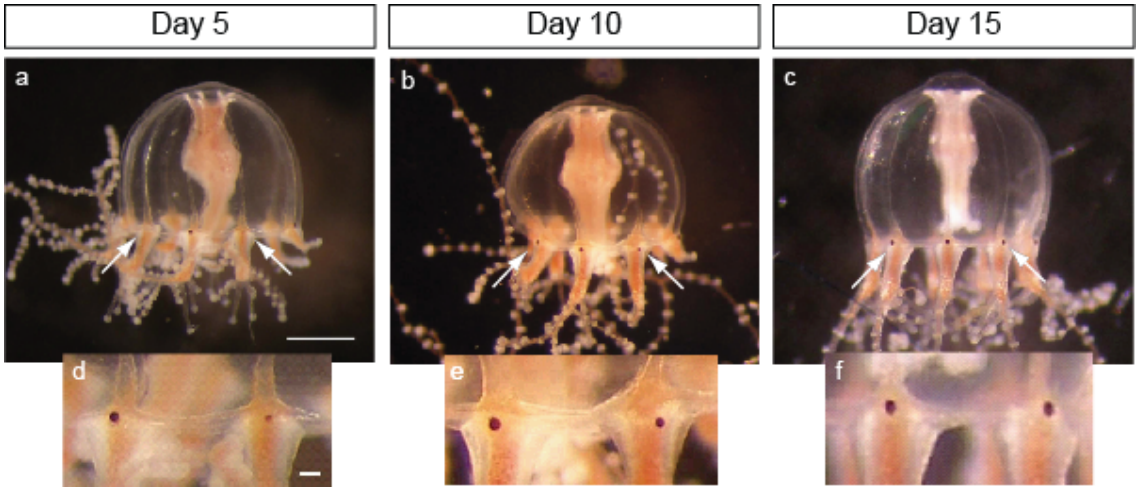

Fig 2.9 Eye regeneration in *Cladonema* Time course of the regeneration of the eye, for an easier comparison only every second eye was removed (white arrows), after five days the pigment of the regenerating eye starts to be visible, after ten days the regenerating eye is still clearly smaller than the regular eye, after 15 days there is no evident difference anymore between the removed and not removed eyes. Scale bar are: 1mm in (a), (b), (c) and 400µm in (d), (e), (f).

2.3 Retinal Determination Gene Cascade (RDGC)

The development of the eye in *Drosophila* is specified by seven nuclear factors, which for their role have been collectively named Retinal Determination Gene Cascade (RDGC): *twin of eyeless* (*toy*), *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*), *dachshund* (*dac*), *eye gone* (*eyg*) and *optix*. These genes interact with each other via direct transcriptional regulation and via the formation of biochemical complexes (Bonini et al. 1993; Mardon et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994; Hazelett et al. 1998; Czerny et al. 1999; Seimiya and Gehring 2000). The understanding of the eye development in *Drosophila* is further complicated by the presence of additional factors such as *homothorax* (*hth*), *extradenticle* (*exd*), *teashirt* (*tsh*) and patterning genes such as *hedgehog* (*hh*) and *decapentaplegic* (*dpp*) (Pan and Rubin 1998; Chen et al. 1999; Curtiss and Mlodzik 2000; Jaw et al. 2000). The members of the RDGC in *Drosophila* are identified by four criteria: 1) Loss of function mutations lead to a small or no eye phenotype; 2) mis-expression of these genes in other imaginal discs induces the development of ectopic eyes; 3) their expression is restricted to the eye field in the eye-antennal disc; 4) the encoded proteins are nuclear, in most cases transcription factors (Pappu and Mardon 2004).

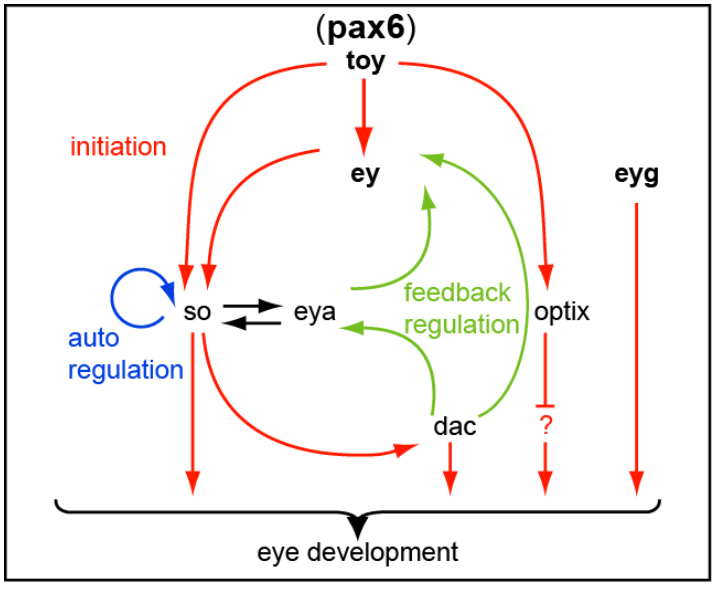

Fig 2.10 Genetic control of the eye specification A set of seven genes takes part to the cascade of interactions which in the end leads to the eye development. toy: twin of eyeless; ey: eyeless; so: sine oculis; eya: eyes absent; dac: dachshund; eyg: eye gone. (Wehner and Gehring 2007).

These principles, however, cannot be considered absolute, as some of the genes that are defined as RDGC members do not fulfil every requirements. For example *so* is not able to induce ectopic eyes when mis-expressed on its own, but it does so when mis-expressed in combination with *eya*. Genetic analysis of mutants of all these genes, together with expression analysis and mis-expression data, helped to unravel the relationship among all the members of this pathway and thus to put *toy* and *ey* on top of the genetic hierarchy controlling eye development. These conclusions were deduced from the following evidences: *toy* and *ey* are the first genes to be expressed in the eye-antennal imaginal disc, the expression of *toy* and *ey* is unaffected in all the other mutants and the mis-expression of *ey* can induce the expression of all the other genes. Furthermore it was possible to understand that *toy* acts upstream of *ey*, because ectopic expression of *toy* can only induce *ey*, and the expression of *ey* is reduced or lost in *toy* mutants, whereas the expression of *toy* in *ey* mutants is unaffected. *ey* itself is able to activate *so* independently of *eya*, and *eya* independently of *so*, but it requires *eya* and not *so* in order to activate *dac*, therefore *eya* seems to act between *ey* and *dac*, but no binding sites for *ey* have been found on *eya*, suggesting that the regulation of *eya* by *ey* may be indirect. However this linear pathway is contradicted by the fact that the ectopic expression of either *eya* or *dac* can induce expression of *ey*, and ectopic *dac* can induce expression of *eya* and *so* (For a review on the above mentioned interactions, see Pappu and Mardon 2004). Taken together, these data suggest that the RDGC, rather than being a linear pathway, is a complex network of mutual interaction and feedback loops (Fig. 2.10). In parallel to this pathway also two other genes, *optix* and *eyg*, are able to induce ectopic eyes in an *ey* mutant background, and therefore to specify eye development independently of *ey*, although *optix* is activated by *toy*.

The surprising finding that *ey* is the homolog of mammalian *Pax6* (Quiring et al. 1994), a gene known to be affected in the mouse *Small eye* mutant and in human *Aniridia* patients, strengthened the importance of this gene in the eye specification. The discovery that *Pax6* homologs are essential for eye morphogenesis in insects and vertebrates lead to the idea that *Pax6* might be the universal "master control gene" for eye evolution and development (Quiring et al. 1994; Gehring and Ikeo 1999). The classical polyphyletic view on eye evolution was motivated by the morphological observation of the tremendous diversity on animal eyes, which lead Salvini-Plawen and Mayr to propose that the eye evolved 40 to 65 times during animal evolution (Salvini-Plawen and Mayr 1977). This theory is contradicted by the isolation of *Pax6* homologs from several animal phyla. So far, true *Pax6* homologs have been isolated from mammals, amphibians, fish, amphioxus, sea squirts, sea urchins,

squid, ribbonworms, nematodes and planarians. Not only *Pax6* from mammals can induce ectopic eyes when mis-expressed in *Drosophila*, but also the homologs from the ascidian *Phallusia mammillata* and from squid are capable to do the same. Furthermore, almost all genes isolated so far are expressed in the developing eyes, which would indicate that *Pax6* was already used for the eye development in the common ancestor of all bilateria. Transcription factors can regulate any target gene which has the appropriate cis-regulatory elements, there are no functional constraints. Therefore, the conservation among different organisms of the target genes activated by a transcription factor is due to historical evolutionary reasons. All these observations indicate that the bilaterian eye has a monophyletic origin (Gehring and Ikeo 1999). This idea is further strengthened by the fact that besides *eyeless* almost all the RDGC genes have been isolated from several phyla. The comparison of their amino acid sequences allowed for the identification of conserved domains with important functions. In general, these domains are responsible for DNA binding or protein-protein interactions, but might also have some transactivation or enzymatic functions (Fig. 2.11).

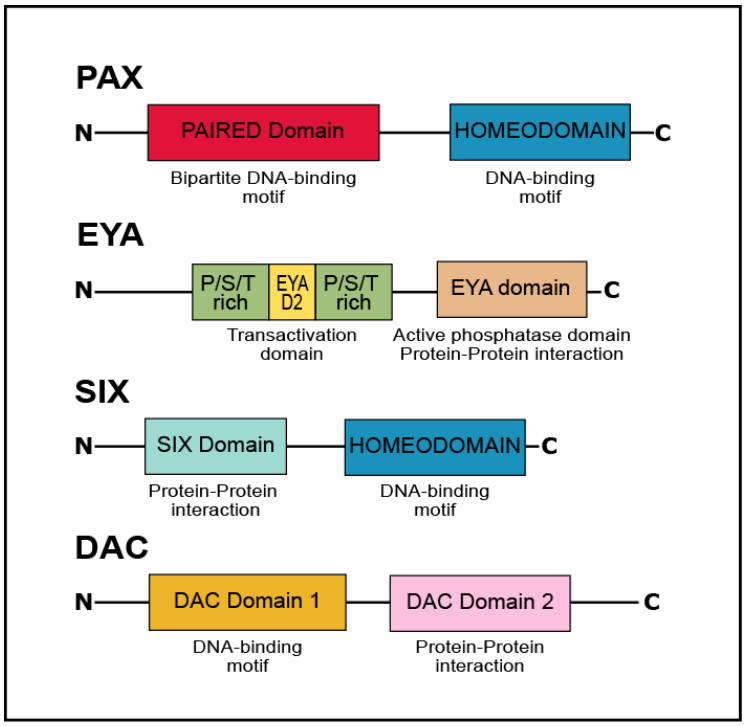

Fig 2.11 Domain structures of RDGC members Representative members of the different family of genes making up the RDGC are displayed. The structure of EY is shown for the PAX family, the EYA family, of SO for the SIX family and of the DAC family(adapted from Silver and Rebay 2005).

2.3.1 Pax family proteins

The RDGC members Ey-Toy/Pax6, and Eyg belong to the Pax family proteins, characterized by the presence of two DNA binding motifs: the Paired Domain (PD) and the Homeodomain (HD), separated by a linker region where a conserved eight-amino acid domain, called octapeptide (OCT) can be present (Fig. 2.11) (Treisman et al. 1991). This family of proteins is made up of nine members (Pax1-9) in vertebrates and 10 in flies, divided into four groups or subfamilies according to the presence or absence of the PD, OCT and HD and to their sequence similarity (see Fig. 2.12). The PD is a 128-amino-acid bipartite DNA binding domain, constituted of two subdomains, the N-terminal PAI subdomain and the C-terminal RED subdomain (Xu et al. 1999). It is named after the first protein in which it has been described, the *Drosophila* Paired (Prd) protein (Bopp et al. 1986; Frigerio et al. 1986). The PAI and RED subdomains recognize a bipartite DNA binding site of approximately 17 nucleotides, however the PAI domain has a dominant effect on DNA binding (Treisman et al. 1991; Epstein et al. 1994).

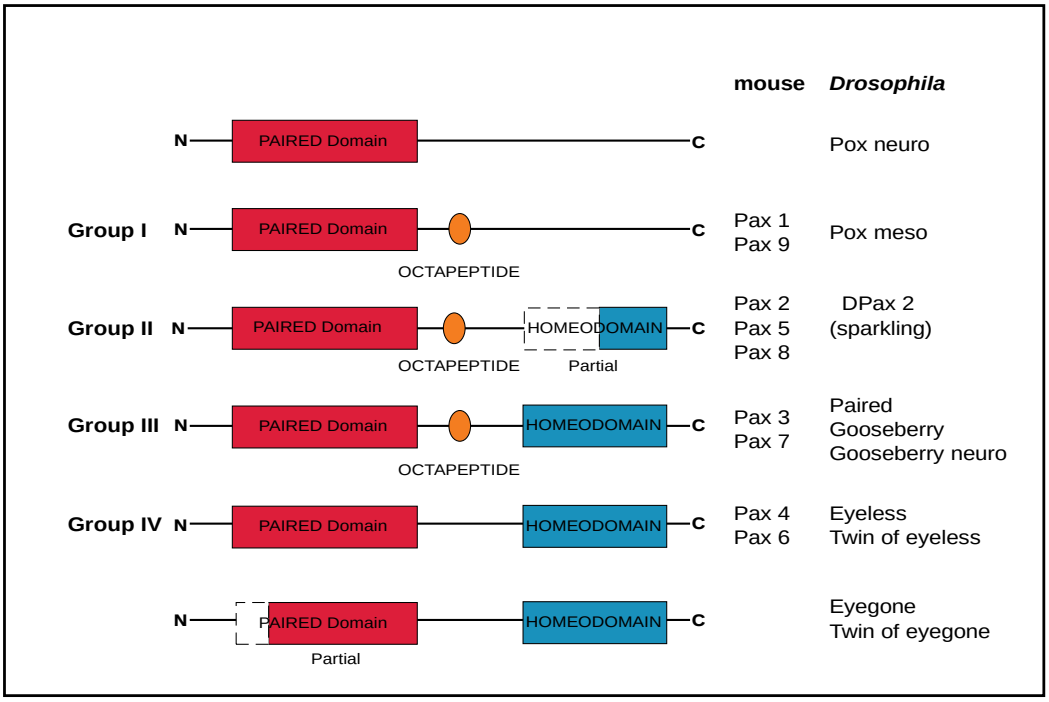

Fig 2.12 Domain structures of the different subfamilies of the Pax family proteins In red is displayed the Paired Domain, in orange the Octapeptide and in blue the Homeodomain. On the left are indicated the groups to which the different Pax proteins belong, and on the right the correspondent known mouse and *Drosophila* members for each group.

The difference in binding specificity for the Pax2/5/8 proteins and Pax6 is given by a difference in three amino acids in the PAI domain (Czerny and Busslinger 1995). The second DNA binding domain, the HD, is composed of 60 amino acids and has been first described in the *Drosophila* homeotic gene *Antennapedia* (McGinnis et al. 1984b). The HD is encoded by a 180 nucleotides sequence, called the *homeobox*, which characterizes the *homeobox* genes superfamily, whose most famous members are the *Hox* genes, well known for the essential role they play in early embryonic development of both vertebrates and invertebrates (Gehring 1993). The third conserved domain of the Pax proteins, the OCT, has homology with the Engrailed repression domain and functions as a transcriptional inhibitory motif, through the binding to the Groucho family of co-repressors (Eberhard et al. 2000).

The RDGC members Ey-Toy/Pax6 and belong to group IV of Pax proteins together with mouse Pax4, they are characterized by the presence of the PD and of the HD, but they lack the OCT. Besides the already mentioned expression in the developing eye, *Pax6* is also expressed during nasal placode development, in the central nervous system and together with *Pax4*, in the pancreatic endocrine cells (for a review, see Chi and Epstein 2002)

Eyg is another paired class Homeodomain protein. It has been so far identified only in *Drosophila* and does not have any known homolog in vertebrates, although it resembles the Pax6 splicing variant, Pax6(5a), in that it has a truncated PD (Jang et al. 2003), lacking a functional PAI domain.

Also another Pax protein, Pox neuro, has been hitherto only isolated in *Drosophila*. Pox neuro has not been included in any group since it differs from the other Pax proteins in that it has only the PD, lacking both the OCT and the HD. *pox neuro* in *Drosophila* is involved in specifying the difference between monoinnervated and polyinnervated sensory organs and it also involved in the development of chemosensory bristles and male courtship behaviour (Dambly-Chaudiere et al. 1992; Awasaki and Kimura 1997; Boll and Noll 2002).

Besides Ey-Toy/Pax6 and Eyg, another Pax protein belonging to group II, Pax2, is expressed in the eye of both flies and mammals. Group II Pax proteins possesses a PD, an OCT and an incomplete HD.

In mouse, *Pax2* is expressed during early morphogenesis in the ventral part of the optic vesicle and becomes later confined to the optic stalk. In *Drosophila*, DPax2 is required for proper specification and differentiation of cone and primary pigment cells. A mutation in an enhancer of *DPax2* causes the *sparkling* mutant phenotype (spa^{pol}), which is characterized by a rough eye (Fu and Noll 1997). *Pax2* is also essential for kidney development: loss of *Pax2* in mice results in severe urogenital defects, including kidney absence (Torres et al. 1995). It

has been shown that a Pax2/Six/Eya network, similar to the already described retinal determination cascade, operates during kidney development (Brodbeck and Englert 2004). Pax2 has also been shown to be involved in the mouse inner ear development, since *Pax2* mutants show cochlear and sensory tissues defects. Interestingly, this system provides another example of interaction among Pax, Six and Eya, since it has been recently reported that Pax2 may synergize with Eya1 and Six1, during the inner ear development (Zou et al. 2006).

The other two members of Group II are *Pax5* and *Pax8*.

Pax5 is expressed by lymphoid precursors committed to B-cell development and it has been demonstrated to be essential for regulating the transcription of several B-cell specific genes (Urbanek et al. 1994). *Pax8* is essential for the development of the endodermally derived follicular cells of the thyroid gland and directly regulates thyroid specific genes (Pasca di Magliano et al. 2000).

Pax3 and Pax7 belong to Group III and display all the three domains. The respective genes are expressed in somites and skeletal muscles and function upstream of the myogenic regulator *MyoD* (for a review, see Buckingham and Relaix 2007). Again, the muscle development offers another striking example of interaction among the family proteins of the RDGC. It has been proved that Pax3, Dach2, Eya2 and Six1 are all expressed during skeletal muscle development, and show a synergy in the muscle specification analogous to that in the eye, ear and kidney determination (see, Silver and Rebay 2005). Pax3 and Pax7 play also an important role for neural crest cells (NCCs) development. NCCs migrate trough the mouse embryo, differentiating into many cell types and contributing to development of several organs. Among these different cell types, the role of Pax3 has been shown to be particularly important for the melanocyte stem cell differentiation, as mice and humans with mutation in the *Pax3* gene show lack of hair and skin pigmentation (Lang et al. 2007).

Pax1 and Pax9 belong to Group I, they have a PD and OCT, but lack the PD. The genes are both expressed in the developing vertebral column of the mouse embryo, in the limb buds and in the embryonic and adult thymus (Chi and Epstein 2002).

There is increasing evidence that *Pax* genes do not only play important roles in embryonic development and organogenesis but are also essential for stem cell maintenance. Furthermore it is clear that dysregulation or activation of *Pax* genes is involved in the progression of cancers in those organs that are specified by *Pax* genes (Robson et al. 2006). Therefore, Pax genes exert a diversity of activity at the cellular level that strengthens the increasing importance of these family of proteins.

2.3.2 Eyes absent family proteins

Eya was first characterized as a novel nuclear protein important for *Drosophila* eye development (Bonini et al. 1993). *eya* loss of function leads to the programmed cell death of progenitor cells anterior to the morphogenetic furrow (Bonini et al. 1993). Eya is a transcriptional coactivator that lacks a DNA binding motif, but that exerts its transcriptional regulative role via interaction with both Six and Dac. The proteins of this family are characterized by a conserved C-terminal domain, called EYA DOMAIN 1 (ED1) and by a less conserved domain in the N-terminal part, called EYA DOMAIN 2 (ED2), which is surrounded by a region rich in Proline/Serine/Threonine (Fig. 2.11). It has been shown that the P/S/T rich region is important for the transactivation activity of Eya, whereas the ED2 domain embedded in this region is not essential for this function (for a review, see Jemc and Rebay 2007). The ED1 was initially described for its activity in protein-protein interaction with the fly RDGC members, where Eya can form a complex with both So (Pignoni et al. 1997) and Dac (Chen et al. 1997).

More recently a novel and interesting function of the ED1 has been described. This domain has been identified as a catalytic motif belonging to the phosphatase subgroup of the haloacid dehalogenase enzyme superfamily (Li et al. 2003; Rayapureddi et al. 2003; Tootle et al. 2003). Only two substrates have been show to be dephosphorylated *in vitro* by Eya: Eya itself and RNA polymerase II. Therefore Eya displays a dual function, regulating the phosphorlation state of either itself or of its transcriptional cofactors via its protein tyrosine, and serine/threonine phosphatase activity, and affecting in this way the transcriptional output (Jemc and Rebay 2007).

Eya homologs have been identified in several animals. Mammals have four paralogs (*Eya1-4*), whereas *Drosophila* has a single gene, present in two isoforms that derive from alternative splicing (Jemc and Rebay 2007). An *Eya* homolog has also been isolated from the plant *Arabidopsis thaliana* (Takeda et al. 1999).

Eya genes are expressed in both *Drosophila* and vertebrates in regions of the embryo that will give rise to the visual system (for a review, see Jemc and Rebay 2007), only the mouse *Eya4* gene has a different pattern, being expressed in the craniofacial mesenchyme, in the dermamyotome and in the limb (Borsani et al. 1999). In humans, mutations in the *eya1* gene are responsible for the brachio-oto-renal (BOR) syndrome and for the brachio-oto (BO) syndrome. On the other hand, eye defects are absent in these diseases and are also absent in

Eya1 knockout mice. However, this might be due to a possible redundancy of the vertebrate genes.

The functional conservation of these genes is also demonstrated by the fact that the *Drosophila* eye mutant phenotype caused by mutations in the fly gene can be rescued by the expression of mouse *Eya1*, *Eya2* or *Eya3* (Bonini et al. 1997; Bui et al. 2000).

Eya genes, besides their involvement in eye development, have been reported to be important for cell survival and cell differentiation in several other tissues (Xu et al. 1997a; Xu et al. 1997b).

2.3.3 Six family proteins

The RDGC members *so* and *optix* belong to the *Six* family gene. The first gene of this family to be identified was *Drosophila sine oculis* (*so*). Loss of function in this gene leads to the development of adult flies with reduced or no eyes and no ocelli, due to apoptosis anterior to the morphogenetic furrow (Cheyette et al. 1994; Serikaku and O'Tousa 1994).

By sequence homology to *Drosophila so*, *Six* family genes have been isolated from many vertebrate and invertebrate taxa (see Kawakami et al. 2000).

All the family members are characterized by two conserved domains (Fig. 2.11): the SIX domain (SD) and the DNA binding Homeodomain (HD). The SD is 110-115 aa long domain, required for protein-protein interaction with Eya and other proteins.

The members of this family have been divided into three groups according to the sequence conservation of their SD and HD: *Six1/2/so*, *Six4/5/Dsix4* and *Six3/6/optix*. Each group comprises two vertebrate members (the first two genes in each group) and a single representative from *Drosophila* (the last gene in each group).

The genes of this family of transcription factors are implicated in the development of many tissues and play important roles in regulating cell proliferation. *Six1*, for example, has been reported to be involved in malignancy-associated overproliferation (Yu et al. 2004).

Interestingly, members of the same subfamily do not necessarily show the same expression pattern across different species. For example, *Dso* is expressed in early eye development, whereas mouse *Six1* is involved in the differentiation of the otic vescicle and nasal placode and *Six2* is expressed in the head mesoderm and limb mesenchyme (for a review, see Kawakami et al. 2000).

In addition, it has been shown that the Six1/2/So and Six4/5/Dsix4 groups are able to interact with Eya through their SD, whereas the Six3/6/Optix proteins do not interact with Eya and

might act as transcriptional repressors through interaction with the Groucho family of corepressors.

2.3.4 Dac family proteins

The Dac family proteins has been first defined in *Drosophila* with the isolation of the *dac* gene (Mardon et al. 1994), and later with the isolation of two homologs from vertebrates, *Dach1* and *Dach2* (Davis et al. 1999; Davis et al. 2001). The proteins of this family are characterized by two conserved domains: the amino-terminal DachBox-N/DD1 and the carboxy-terminal DachBox-C/DD2. Both these domains show sequence homology to the *Ski/Sno* proto-oncogenes. The DD1 has been demonstrated to be a DNA-binding motif, although specific binding sites still have to be identified (Ikeda et al. 2002). The DD2 is instead involved in protein-protein interactions, and is thought to interact with the ED of the Eya proteins (Chen et al. 1997), though direct binding has not yet been shown *in vivo*. It has been proved in *Drosophila*, that the DD1 is the domain essential for *dac* function, whereas the DD2 only seems to assist (Tavsanli et al. 2004), therefore the importance of the interaction between Dac and Eya remains unclear. In addition, in mammals Dac can act both as a transcriptional activator or repressor (Li et al. 2003).
In *Drosophila*, *dac* is expressed anterior to the Morphogenetic Furrow (MF) and *dac* mutants have reduced or roughened eyes (Mardon et al. 1994). In vertebrates, *Dach1* and *Dach2* are expressed in the developing eyes, brain and limbs (Heanue et al. 2002; Davis et al. 2006). However, *Dach1/2* double knockout mice do not show gross defects in these organs (Davis et al. 2008). This suggests that vertebrate *Dach* play a minor role in eye development.
Thus, even if the involvement of *dac* in the RDGC network has been shown to be important, the molecular mechanisms of its function still have to be completely understood.

2.4 Members of the Retinal Determination Gene Cascade known in Cnidaria

Several members of the RDGC have been described so far in Cnidaria. In the last years the attention of the scientific community has focused mostly on the conservation of the *Pax* family in lower Metazoa. This has led to the isolation of a wealth of *Pax* genes from the different classes of Cnidaria. A total of four *Pax* subfamilies are known in Cnidaria: *PaxA*, *PaxB*, *PaxC* and *PaxD* (Fig. 2.13). The whole complement of the four *Pax* classes has been described only in the Anthozoans *Acropora millepora* (Catmull et al. 1998; Miller et al.

2000), and *Nematostella vectensis* (Matus et al. 2007), whereas Medusozoa seem to lack the *PaxD* and *PaxC* class. In *Nematostella*, the expression of the isolated *Pax* genes has been described during embryogenesis, showing extremely diverse localisation (Matus et al. 2007). However, the expression pattern suggests a role of these genes in patterning components of the ectodermal nerve net (Matus et al. 2007).

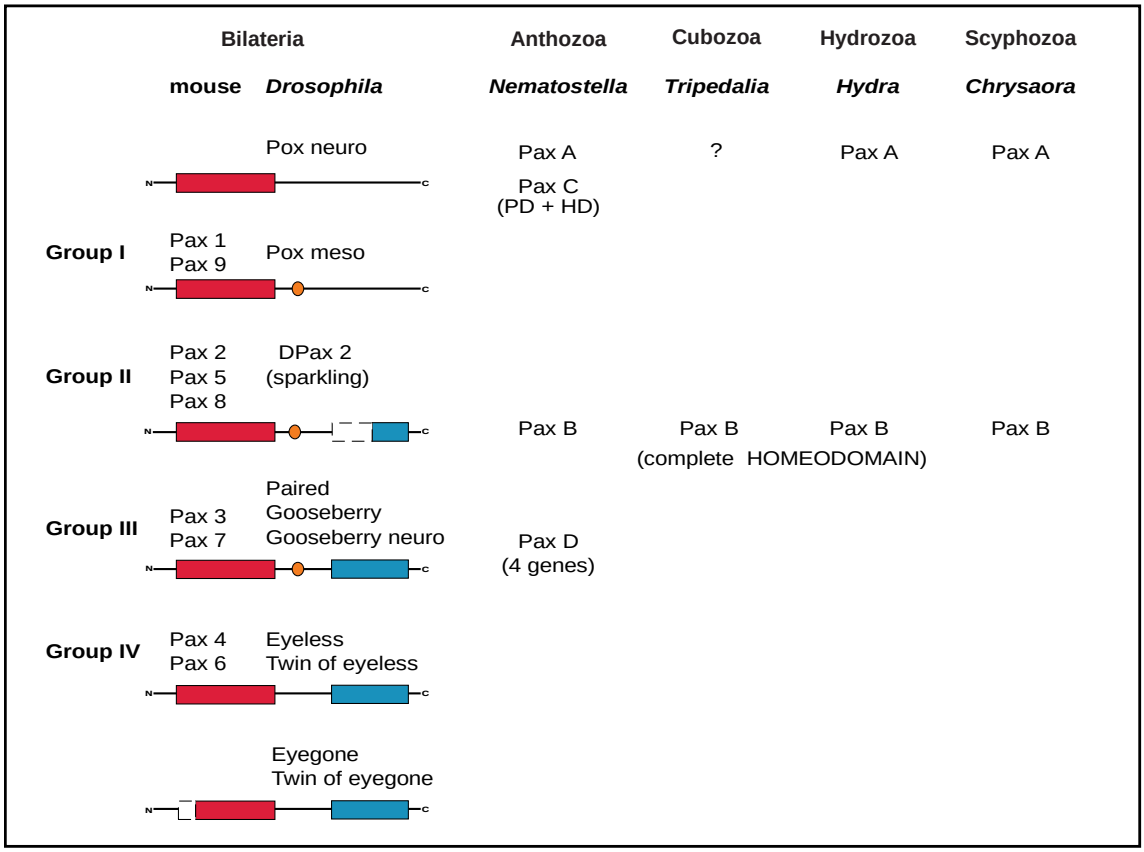

Fig 2.13 Pax proteins subfamilies, in Bilateria and Cnidaria The subfamilies of *Pax* genes are shown in Bilateria (mouse and *Drosophila*) on the left and in the four cnidarian classes (Anthozoa, Cubozoa, Hydrozoa, Scyphozoa) on the right. Below each bilaterian subfamilies the conserved domains are exemplified (red: Paired Domain, orange: Octapeptide, blue: Homeodomain). For each Cnidarian class only representative species are indicated, (for detailed references see text). Complete genome sequences are available for *Nematostella vectensis* and *Hydra magnipapillata*.

In Hydrozoa, *PaxB* genes have been isolated from *Podocoryne carnea* (Groger et al. 2000), *Hydra magnipapillata* (Hoshiyama et al. 1998), *Hydra littoralis* (Sun et al. 1997), and from *Cladonema californicum* (Sun et al. 2001), a close relative of *C. radiatum*, whereas *PaxA* genes have been found only in *Hydra sp.* (Hoshiyama et al. 1998; Sun et al. 2001). Only the expression pattern of *PaxB* from *P. carnea* has been analysed in this class: the gene seems to be ectodermally expressed in unfertilized eggs, in the planula larva and in scattered cells

below the hypostome of the polyp; instead in developing and adult medusa the expression is at the level of the endoderm, particularly in the tentacle bulbs.
In cubozoa, a *PaxB* gene has been reported from *Trypedalia cystophora* (Kozmik et al. 2003), and *Carybdea marsupialis* (Groger et al. 2000). The expression of *TcPaxB* has been localised at the level of the retina and lens of the eye and in the statocysts. In addition, the role of this gene has been studied in *Drosophila* by targeted gene expression, displaying the capability of the jellyfish gene to induce ectopic eyes and to rescue the *sparkling* mutant phenotype (Kozmik et al. 2003).
In the Scyphozoa *Chrysaora quinquecirrha*, *PaxA* and *PaxB* have been found (Sun et al. 1997).

The cnidarian Pax classes have been grouped together with the genes from Bilateria according to their sequence similarity and phylogenetic analysis, forming the following major clusters:

- *PaxA/PaxC/pox neuro*: *PaxA* from cnidaria resembles *pox neuro* from *Drosophila* in that it does not have a HD and an OCT.
- *PaxB/Pax2/Pax5/Pax8/DPax2*, *PaxB* clusters with the *Pax2/5/8* family, but in contrast to the Bilaterian proteins, PaxB displays a complete HD
- *PaxD/Pax3/Pax7/paired/gooseberry*.

No true *Pax6* homolog has been hitherto described in Cnidaria.

In *Cladonema radiatum* three *Six* genes (*six1/2*, *six3/6* and *six4/5*) have been recently cloned and their expression analysed during the development of the jellyfish, showing that two of these genes (*Six1/2* and *Six3/6*) are involved in eye development, and the third one is expressed in the oocytes (Stierwald et al. 2004). Members of the *Six1/2/so* subfamily have also been described in the other cnidarian classes (Bebenek et al. 2004).
To date, no sequences of *eya* or *dac* genes in Cnidaria have been reported.

Aim of the Ph.D. project

The finding that *eyeless* is the fly homolog of vertebrate *Pax6* and that both genes are involved in the eye development has lead to the idea that *Pax6/eyeless* is the universal "master control gene" for eye evolution and development. The isolation of *Pax6* and other members of the RDGC from several bilaterian phyla and the demonstration of their widespread involvement in eye development, has challenged the classical view of the polyphyletic origin of the eye. This has shown that, despite the morphological differences, the specification of this organ is driven by the same molecular mechanisms even in distantly related species.

All these findings indicate that the bilaterian eye has a monophyletic origin. But in order to shed light on the mechanisms involved in eye development in the last common ancestor of all Metazoa, it is necessary to have an outgroup to Bilateria.

For their phylogenetic position as the sister group to Bilateria, Cnidaria represent the most suitable organisms to study the evolution of the eye.

Members of the RDGC are already known in several Cnidaria, but the isolation of all the different family genes composing the network has never been undertaken in a single species. Instead different species have been investigated for single gene families. Furthermore the expression data for the known genes, are dispersed and very limited.

For these reasons, the first aim of my Ph.D. project was to isolate all the members of each RDGC family from a jellyfish bearing eyes, *Cladonema radiatum*. Already three members of the *six* family were known in this species, and their expression in the eye described. Furthermore, this species displays the interesting capacity to regenerate the eyes, which offers a reliable method to study the role of the RDGC genes.

Therefore the second aim of my Ph.D. project was to study the expression and the role of the isolated genes during eye development and regeneration in the medusa. In order to do so, we made use of both Real Time PCR and *in situ* hybridization approaches.

Finally, to gain more insights about the functional conservation of Cnidarian RDGC, we also studied the capability of the isolated genes to interact with bilaterian members of the RDGC, by targeted gene expression in *Drosophila*.

3 Results

3.1 Isolation of members of the Pax gene family in *Cladonema radiatum*

3.1.1 Isolation of *CrPaxA*

The first member of the *Pax* gene family identified in *Cladonema radiatum*, *CrPaxA*, was isolated by Michael Stierwald (Stierwald 2004, unpublished data). Subsequently Patrick Tschopp (Tschopp 2005, unpublished data) obtained the full length sequence by RACE and inverse PCR. *CrPaxA* resembles *pox neuro* from *Drosophila* in that it has only the paired domain (PD) but it lacks the homeodomain (HD) (Fig. 3.1). We also found for the first time an octapeptide-like motif in the *pox neuro/PaxA* family (for a detailed descritpion, see Suga et al. submitted). When the full length deduced protein sequence of CrPaxA is blasted, it shows the highest homology to PaxA from *Hydra littoralis* (60%). When only the PD of CrPaxA is considered, *Hydra magnipapillata* PaxA is the closest relative (100%). Interestingly, when compared to higher metazoans, the PD of CrPaxA is more closely related to the PD of mammalian Pax2 (79% amino acid identity to human and mouse) than to *Drosophila* Poxn (74%) and to *eyeless* (69%). In phylogenetic trees *CrPaxA* clusters together with other *PaxA* genes from cnidarians and with *pox neuro*.

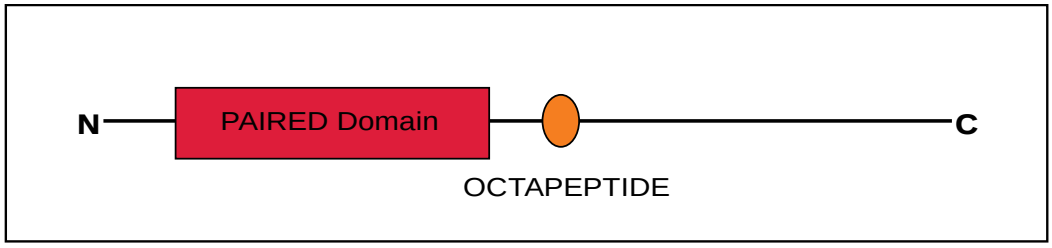

Fig 3.1 CrPaxA Schematic drawing of the conserved protein domains of CrPaxA: Paired Domain (red box) Octapeptide (orange oval).

3.1.2 Designing of degenerate primers for the isolation of *PaxB* and *PaxE*

Knowing that *C. radiatum* possesses a *PaxA* gene, we were then interested in investigating the *Pax* genes complement of this species. In order to identify the most conserved regions across different species, we aligned the amino acid sequences of several Pax proteins from various organisms (Fig. 3.2). The degenerate primers were then designed from these conserved regions and used in a degenerate PCR approach.

```
 1. ==INV   1 D84254   9902 Halocynthia roretzi Pax-37
 2. =p2  A43698 9303  BSH9 protein - fruit fly (Drosophila melanogaster)
 3. =p2  B43698 9303  BSH4 protein - fruit fly (Drosophila melanogaster)
 4. ==INV   1 M14548   9508 Drosophila melanogaster prd
 5. ==ROD   1 MMPAX3R     X59358   9111 Mouse Pax-3 gene for a DNA binding p
 6. ==HUM   2 X96744   9901 Homo sapiens PAX7
 7. ==INV   5 CEF27E5     Z48582   0001 Caenorhabditis elegans cosmid F27E5.
 8. ==INV   1 M86927   9304 Drosophila melanogaster pox neuro protein
 9. ==INV   1 U96193   9705 Hydra littoralis Pax-A
10. ==INV   1 U96195   9705 Chrysaora quinquecirrha Pax-A1
11. = AF053458  1 Acropora millepora transcription factor Pax-A (PaxA) mRNA, com
12. = CrPaxA                                                              fra
13. ==INV   1 AB006675 9806 Halocynthia roretzi HrPax-258
14. ==INV   1 AF053762 9904 Branchiostoma floridae paired box protein Pax-2
15. ==INV   1 AF053763 9904 Branchiostoma floridae paired box protein Pax-2
16. ==PRI   1 HUMBSAP     M96944   9412 Human B-cell specific transcription
17. ==HUM   1 U45255   9810 Homo sapiens paired-box protein PAX2
18. ==PRI   1 HUMPAX8A    L19606   9312 Human paired box homeotic protein (P
19. ==INV   1 AF016884 9709 Paracentrotus lividus paired box protein
20. ==INV   1 AF010256 9711 Drosophila melanogaster sparkling protein
21. = AF241310  1 Acropora millepora transcription factor PaxB (PaxB) mRNA, comp
22. ==INV   1 AY280703    AY280703 0311 Tripedalia cystophora paired and hom
23. ==INV   1 U96197   9705 Chrysaora quinquecirrha Pax-B
24. ==INV   1 U96194   9705 Hydra littoralis Pax-B
25. ==INV   1 PCA249563   AJ249563 9911 Podocoryne carnea mRNA for paired bo
26. ==INV   1 AB007462 9902 Ephydatia fluviatilis Pax-2/5/8
27. = Cladonema cal? PaxB
28. = AF053459 AF053460  1 Acropora millepora PaxC transcription factor (PaxC) m
29. = Nematostella PaxC
30. = Carybdea PaxC
31. ==ROD   1 AB010557 9912 Mus musculus Pax4
32. ==ROD   1 AF053100 9807 Rattus norvegicus Pax4a
33. ==HUM   1 AB008913 9801 Homo sapiens Pax-4
34. ==INV   1 U31537   9512 Caenorhabditis elegans variable abnormal-3
35. ==INV   1 AB017632 9903 Dugesia japonica DjPax-6
36. ==INV   1 AJ010498 9901 Girardia tigrina DtPax-6 protein
37. ==INV   1 Y09975   9702 Phallusia mammilata Pax-6
38. ==INV   1 AF134350    AF134350 0001 Drosophila melanogaster transcriptio
39. ==INV   1 U14621   9509 Paracentrotus lividus Pax-6
40. ==VRT   1 D88737   9902 Xenopus laevis Xenopus Pax-6 long
41. ==INV   1 X79493   9710 Drosophila melanogaster transcription factor
42. ==PRI   1 HUMPAX6AN   M93650   9302 Human paired box gene (PAX6) homolog
43. ==INV   1 X95594   9607 Lineus sanguineus Pax6-like protein
44. ==INV   1 LOU59830    U59830   9912 Loligo opalescens Pax-6 mRNA, comple
45. ==INV   1 AF002702 9807 Caenorhabditis elegans PAX protein
46. ==INV   5 U50072   9604 Caenorhabditis elegans K06B9.5
47. ==INV   1 AF016885 9709 Paracentrotus lividus paired box protein
48. ==INV   1 AF016886 9709 Strongylocentrotus purpuratus paired box protein
49. = AF241311  1 Acropora millepora transcription factor PaxD (PaxD) mRNA, comp
50. ==INV   8 U53336   9905 Caenorhabditis elegans K07C11.1
51. =x|D.melanogaster CG9610 pox meso X16992
52. ==INV   1 U20167   9511 Branchiostoma floridae AmphiPax-1
53. ==INV   1 AJ238974 9905 Branchiostoma lanceolatum amphipax1 protein
54. ==HUM   3 AL035562 9907 Homo sapiens dJ1065O2.3 (paired box gene 1
55. ==HUM   1 AJ238381 9904 Homo sapiens pax9 protein
56. ==INV   1 AB020763 9907 Ptychodera flava Pax19
57. ==INV   1 AB021143 9907 Halocynthia roretzi Pax19 related gene
58. ==INV   1 AB020762 9907 Ciona intestinalis Pax19
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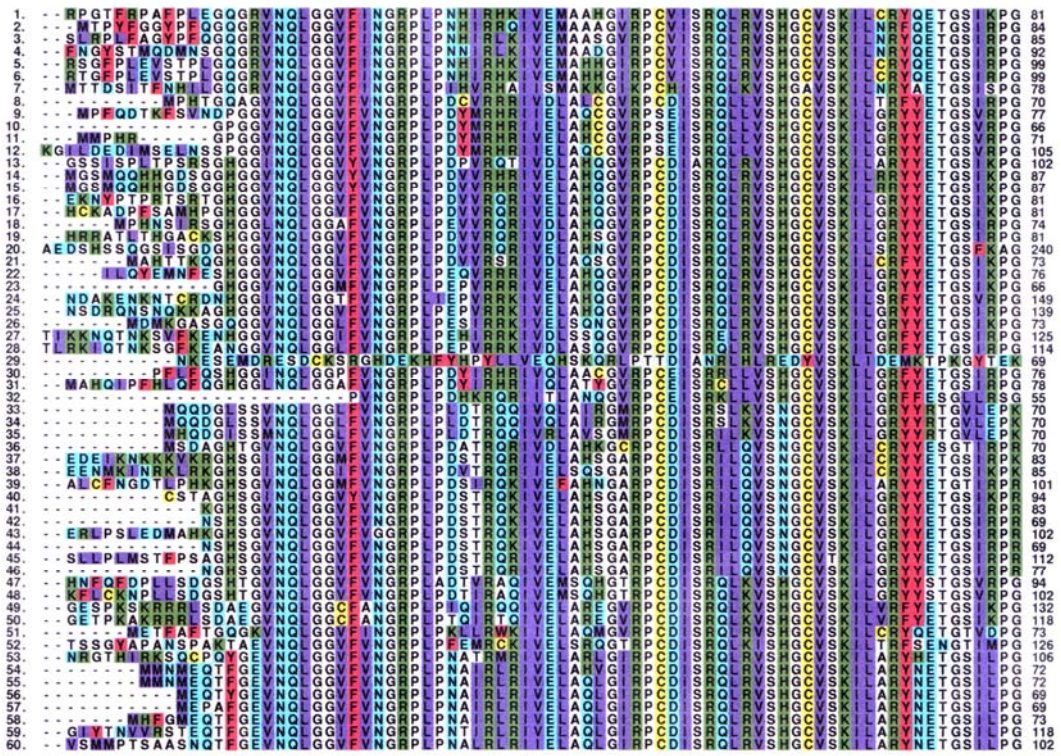

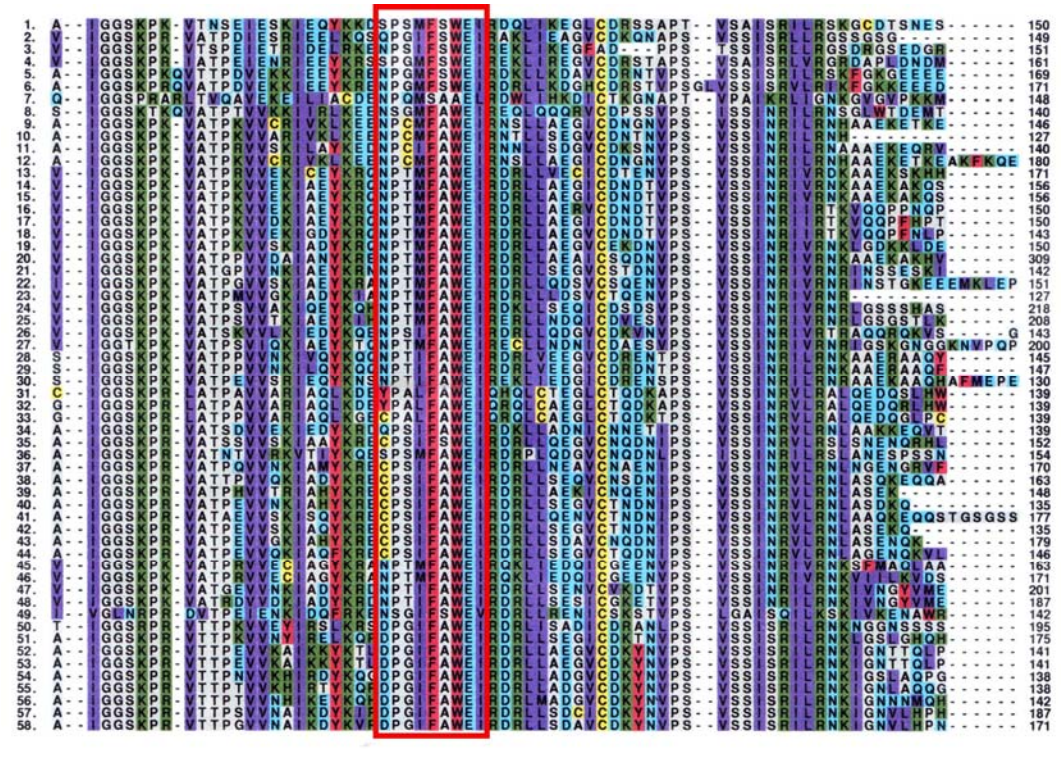

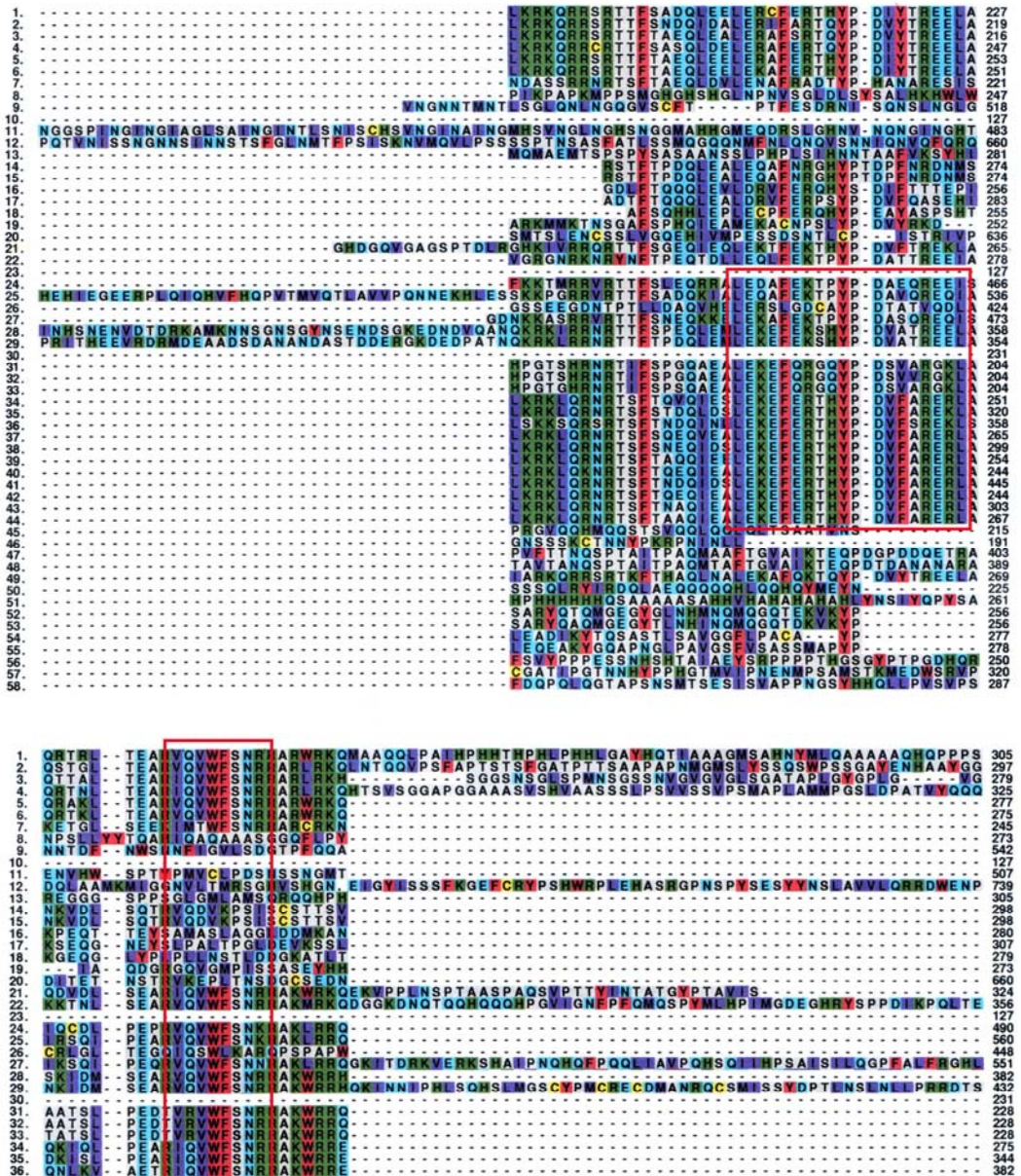

Fig. 3.2 Pax proteins sequence alignment To localise the most conserved regions within the Pax protein subfamilies, we aligned the amino acid sequences of Pax proteins from different species. The species and the respective accession numbers are indicated in the first panel, except for: *Carybdea marsupialis* PaxC is an unpublished sequence isolated by Michael Stierwald (Stierwald 2004, unpublished data), CrPaxA is AB379656, *Cladonema californicum* PaxB is AF260128, *Nematostella vectensis* PaxC is AY730691. Red boxes indicate the conserved regions chosen to design the degenerate primers. The first box is at the level of the PD, and the other two boxes are in the HD (for detailed primers sequence information, see paragraph 5.3.1)

By aligning sequences of different Pax groups, we were able to discriminate among subfamily-specific conserved regions. Since the sequence of *CrPaxA* was already identified, in order to exclude this gene from the amplification, primers using HD sequences were designed (see paragraph 5.3.1.1 for the sequences).

3.1.3 Isolation of CrPaxB

By means of homology PCR, we amplified a partial cDNA fragment of *PaxB*. The full length sequence was obtained by 5' and 3' RACE.

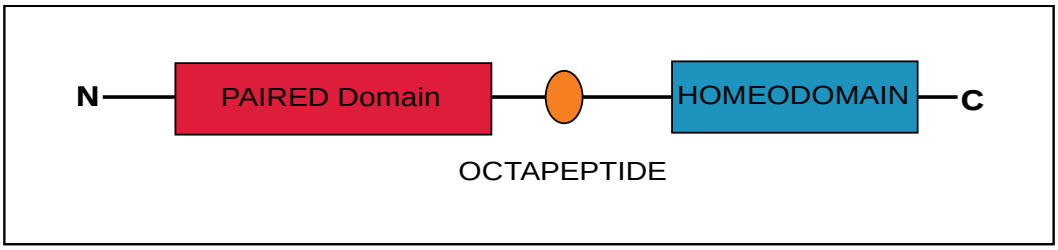

Fig. 3.3 CrPaxB Schematic drawing showing the conserved protein domains of CrPaxB: Paired Domain (in red), Octapeptide (orange) and Homeodomain (blue).

CrPaxB encodes for a protein containing all the three characteristic domains of Pax proteins: the Paired domain (PD), the Octapeptide (OCT) and a complete Homeodomain (HD) (Fig. 3.3 and Fig. 3.4).

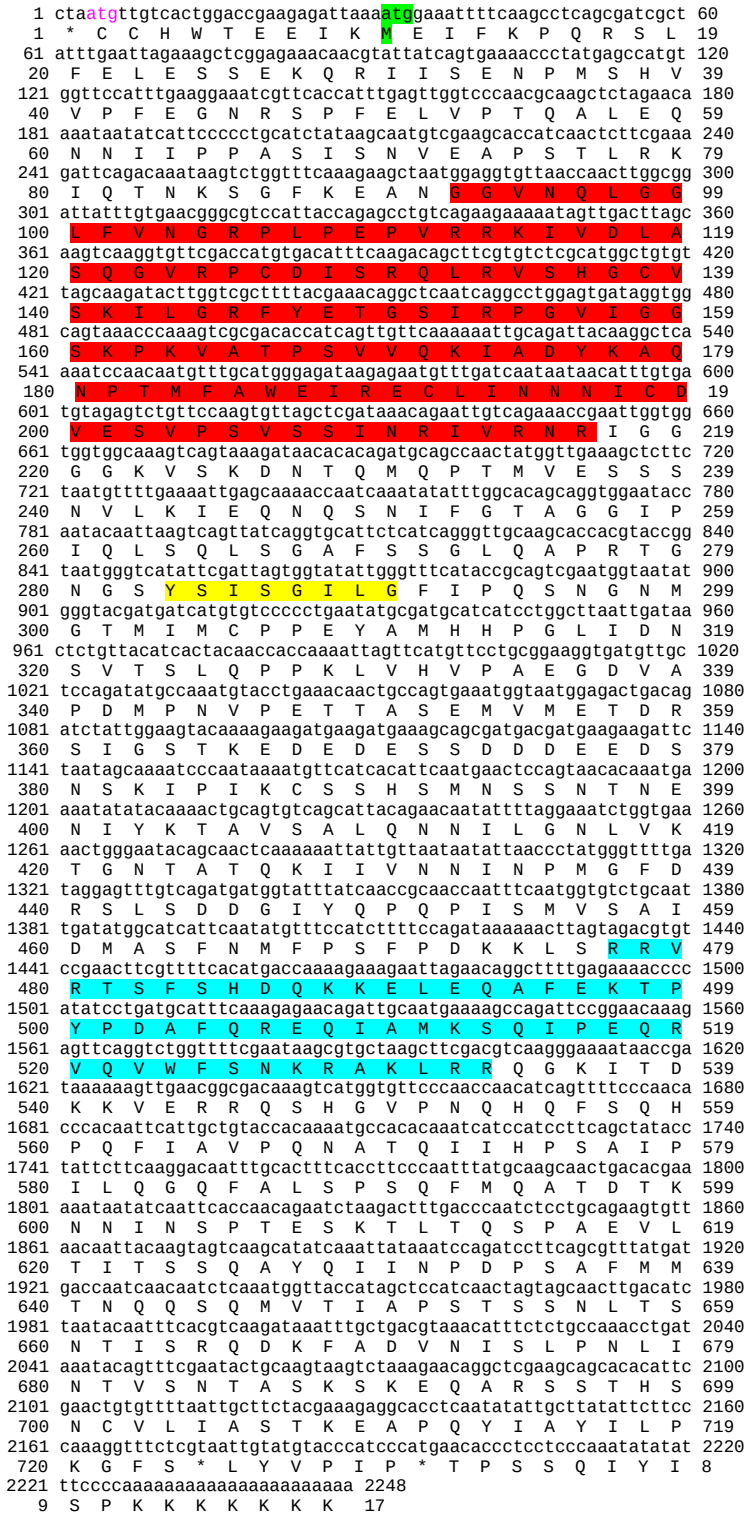

Fig. 3.4 *CrPaxB* Full length cDNA sequence of *CrPaxB* and deduced amino acid sequence. In red is highlighted the Paired Domain, in yellow the Octapeptide and in blue the Homeodomain. The starting methionine is in green, a triplett coding for a methionine not in frame is in pink (see paragraph 3.3.3)

The full length protein sequence, deduced from the cDNA sequence of *CrPaxB*, shows 66% amino acid identity to PaxB of *Cladonema californicum*. The PD can be assigned to the Pax2/5/8 subfamily, showing high sequence identity to mammalian PD (80% to Pax8 and Pax5, and 79% to Pax2). The identity to eyeless is however 67%. Moreover the three amino acids at the positions critical for DNA binding specificity of the PD (Czerny and Busslinger 1995; Kozmik et al. 2003), differ from those present in Pax6 (IQN), and are identical to those characteristic of Pax2/5/8 (QRH) (Fig.3.5, red arrowheads).

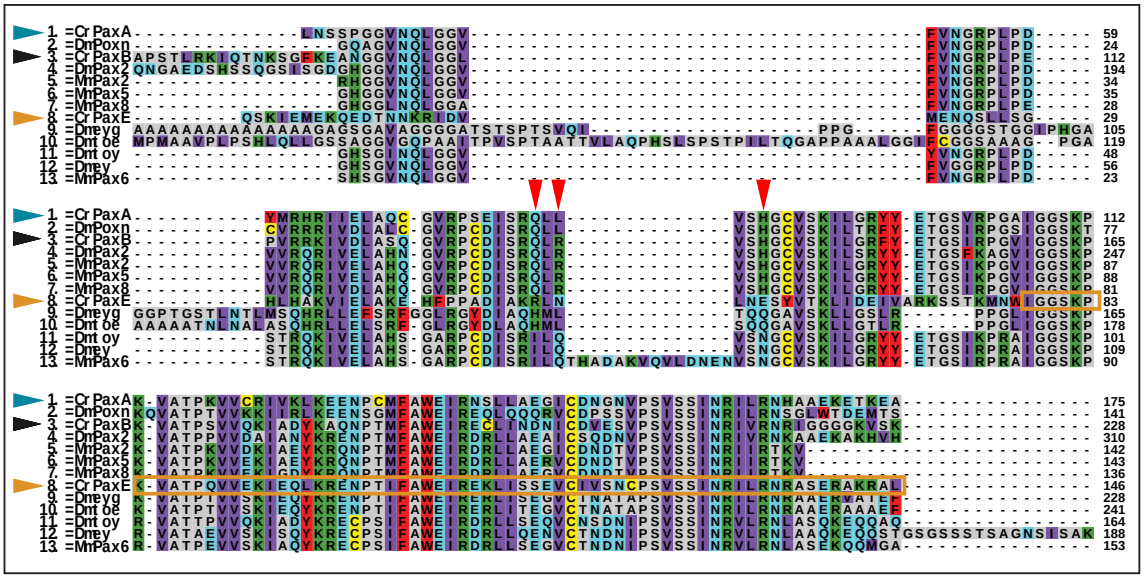

Fig. 3.5 Amino acid sequence alignment of different PD The amino acid sequence of the PD of CrPaxA **(**blue arrowheads), CrPaxB (black arrowheads) and CrPaxE (golden arrowheads) are aligned to the PD of Pax proteins from other organisms. Red arrowheads point to the three amino acids crucial for DNA binding properties of the PD. The golden boxed sequence indicates the region of higher conservation in the RED subdomain of CrPaxE, whereas the PAI N-terminal domain is more divergent. Dm: *Drosophila melanogaster*; Mm: *mus musculus*; Cr: *Cladonema radiatum*.

CrPaxB differs from other members of the Pax2/5/8 subfamily in that it has a complete HD (see Fig. 3.6), which shows the highest level of amino acid identity to other cnidarian PaxB (*C. californicum*, 87%) and to the *Drosophila* eyeless Homeodomain (59%). The identity to the Pax2/5/8 subfamily is very weak, as expected from the fact that this subfamily has a partial HD.

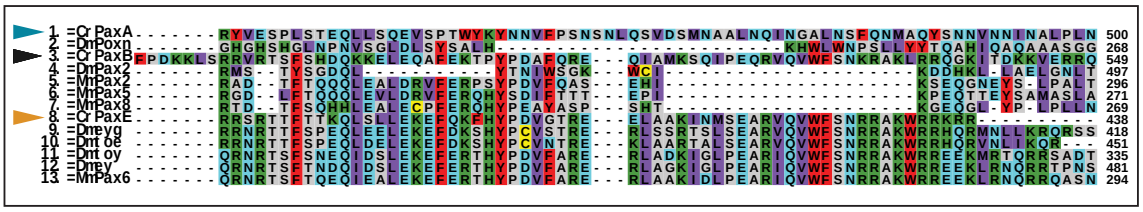

Fig. 3.6 Amino acid sequence alignment of different HD. The amino acid sequence of the HD of CrPaxA **(**blue arrowheads), CrPaxB (black arrowheads) and CrPaxE (golden arrowheads) are aligned to the HD of Pax proteins from other organisms. CrPaxB, contrary to the other members of the Pax2/5/8 subfamily, displays a complete HD. Dm: *Drosophila melanogaster*; Mm: *mus musculus*; Cr: *Cladonema radiatum*.

Therefore CrPaxB displays a structure that partially resembles both the Pax2/5/8 subfamily and the Pax4/6, as it was already shown for PaxB of *Podocoryne carnea* another hydrozoan and of *Tripedalia cystophora*, a cubozoan jellyfish (Groger et al. 2000; Kozmik et al. 2003) and as it is the case for all the *PaxB* genes so far isolated in cnidarians.

The phylogenetic analysis, based on the PD, clearly shows that *CrPaxB* clusters with *PaxB* genes from other Cnidarians and with the *Pax2/5/8* subfamily (Fig. 3.7). This is in agreement with the domain structure comparison carried out for the PD, showing the highest identity to the Pax2/5/8 subfamily.

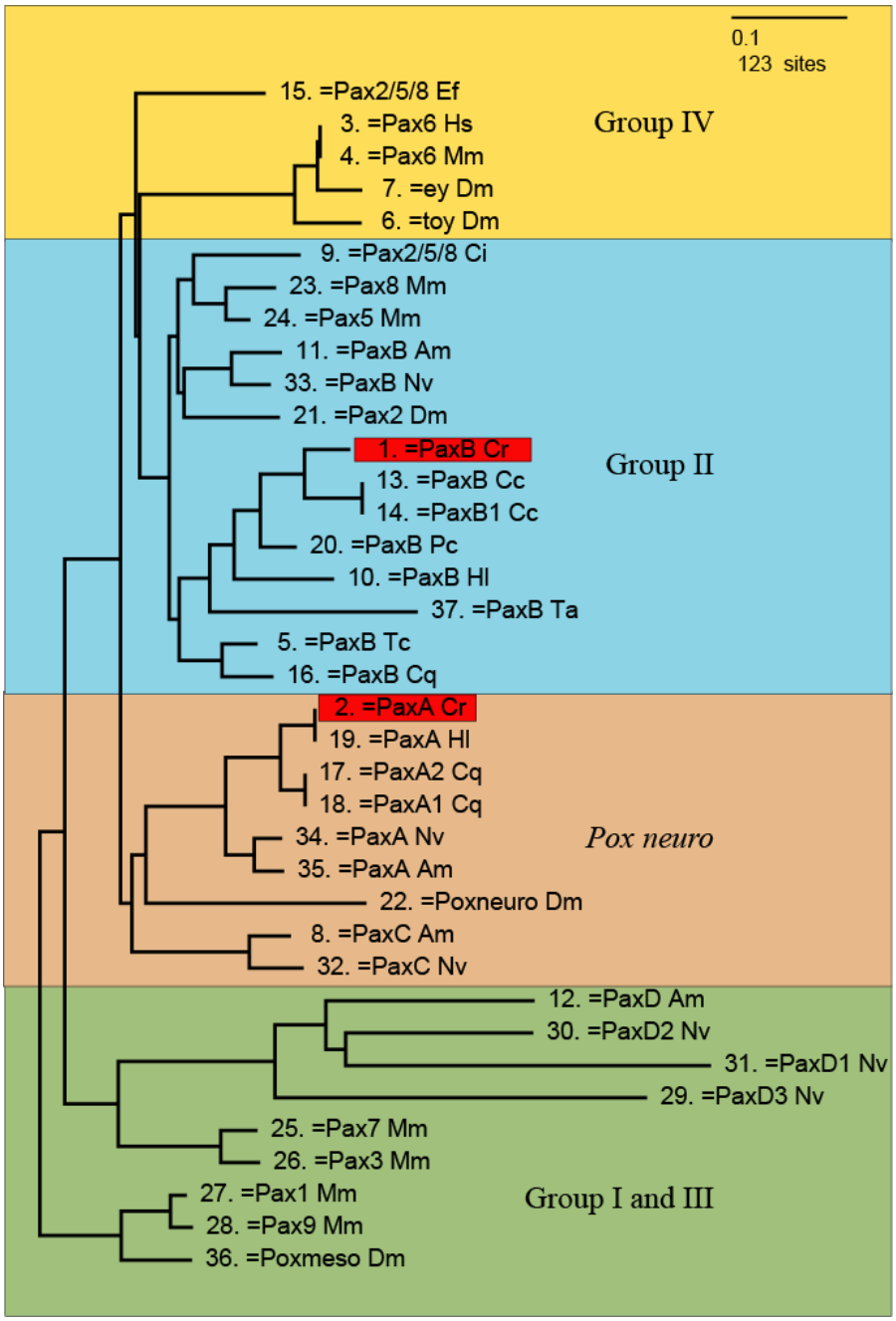

Fig. 3.7 *Pax* genes phylogenetic tree. The tree is inferred by Neighbour-Joining method, with the distances calculated by the Maximum likelihood method (ML), and is based on the comparison of 107 sites in the PD. The clusters of genes are boxed in different colours according to the group to which they belong. In red are highlighted the *Cladonema radiatum* genes. Am*: Acropora millepora*; Cc: *Cladonema californicum*; Ci: *Ciona intestinalis*; Cq: *Chrysaora quinquecirrha*; Cr: *Cladonema radiatum*; Dm: *Drosophila melanogaster*; Ef: *Ephydatia fluviatilis*; Hl: *Hydra littoralis*; Hs: *Homo sapiens*; Mm: *Mus musculus*; Nv: *Nematostella vectensis*; Pc: *Podocoryne carnea*; Ta: *Trichoplax adhaerens*; Tc: *Tripedalia cystophora*.

3.1.4 Isolation of *CrPaxE*

Using again a degenerate PCR approach followed by 5' and 3' RACE we were able to amplify the full length cDNA of another *Pax* gene, which we called *CrPaxE*.

CrPaxE displays an incomplete PD and a complete HD (Fig. 3.8 and 3.9). The PD is highly divergent in its amino terminal part (called the PAI subdomain) (see Fig. 3.5, the conserved part of the PD is highlighted by a golden box, and Fig. 3.9), therefore the PD is incomplete. This feature has not been found in any other Pax gene so far isolated from Cnidaria, but it can be observed in the *Drosophila eyegone* gene (Jun and Desplan 1996).

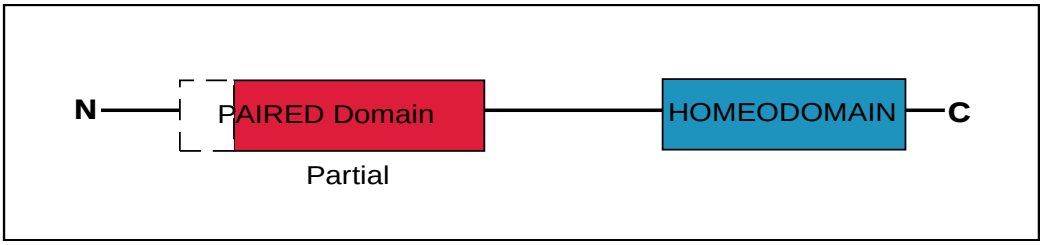

Fig. 3.8 CrPaxE Schematic drawing of the conserved protein domains of CrPaxE, the partial Paired Domain (red) and the complete Homeodomain (blue) are displayed.

The full length protein sequence of *CrPaxE* shows 61% identity to *Drosophila eyegone* and only 43% and 33% to *PaxC* from *Acropora millepora* and *Nematostella vectensis* respectively.

The whole PD of CrPaxE is closely related to the PD of PaxB of *Podocoryne carnea* (50%). Interestingly the level of identity to Pax2/5/8 proteins from vertebrates is the same (50%). A high identity (49%) is also shown to *Drosophila* sparkling PD. The three amino acids essential for DNA binding specificity lie in the missing PAI subdomain, therefore they can not be used for discrimination (Fig.3.5, red arrowheads).

The HD of CrPaxE mostly resembles that of cnidarian PaxC (77% identity to the HD of *Nematostella vectensis* and 76% to the HD of *Acropora millepora*). In addition it also shows high identity to *Drosophila* eyegone (69%) and Retinal homeobox (67%), and to mouse Pax6 (66%).

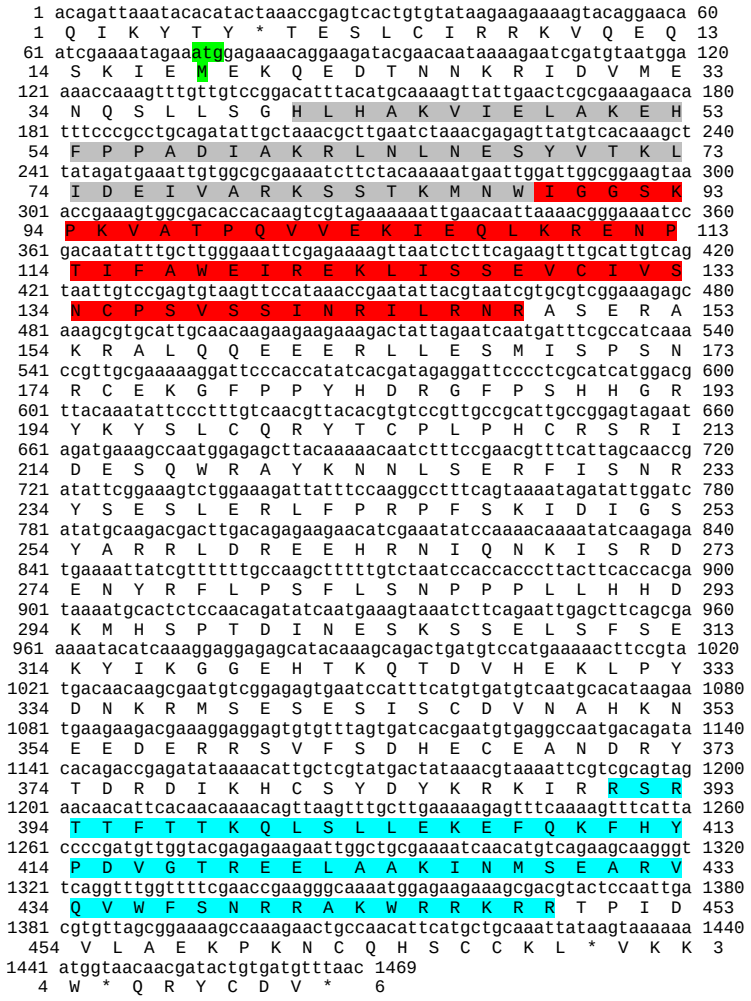

Fig. 3.9 *CrPaxE* Full length cDNA sequence of *CrPaxE* and deduced amino acid sequence. In red is highlighted the C-terminal subdomain (RED) of the PD, that is conserved between PaxE and the other Pax proteins, whereas in grey is highlighted the part of the PD that does not show conservation. In blue is the HD.

It is therefore not easy to assign *CrPaxE* to a specific subfamily of *Pax* genes. The phylogenetic analysis of these gene is not simple because of the presence of a partial PD. If *CrPaxE* is included in the phylogenetic tree in Fig. 3.7, only the RED subdomain would have to be taken into consideration for the analysis, and therefore the number of sites for the comparison would decrease. Furthermore, when included in the phylogenetic analysis, *CrPaxE* undergoes long branch attraction and artificially clusters together with the *PaxC* subfamily (which normally would cluster with the *PaxA* subfamily) and the *Pax4/6* subfamily (Fig. 3.10).

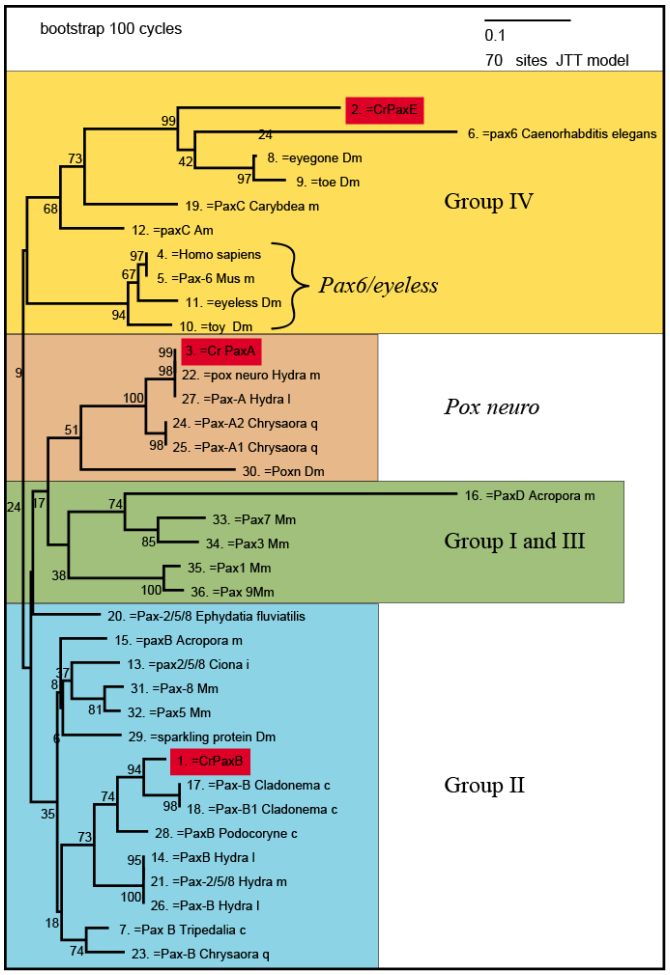

Fig. 3.10 *Pax* genes phylogenetic tree displaying the long branch attraction The tree shows the consequences of taking into consideration *CrPaxE* in the phylogenetic analysis. The number of sites is reduced to only 70 (compared to 123 of Fig. 3.7) and the long branch attraction cause the artificial clustering of *CrPaxE* and the *eyegone* genes with the *Pax4/6* subfamily and with *PaxC* from other cnidarians.

For these reasons the phylogenetic analysis of *CrPaxE* has to be carried out separately.

Considering the lack of the PAI subdomain and the high identity of the HD it is possible to assume an overall closer relationship of *CrPaxE* to *eyegone*. This hypothesis is confirmed by the phylogenetic analysis (Fig 3.11), which shows that *CrPaxE* clusters with *eyg* and *toe* from *Drosophila*, and with other *eyg*-like genes from other species. This indicates that this cluster might likely correspond to a novel subfamily (eyg subfamily).

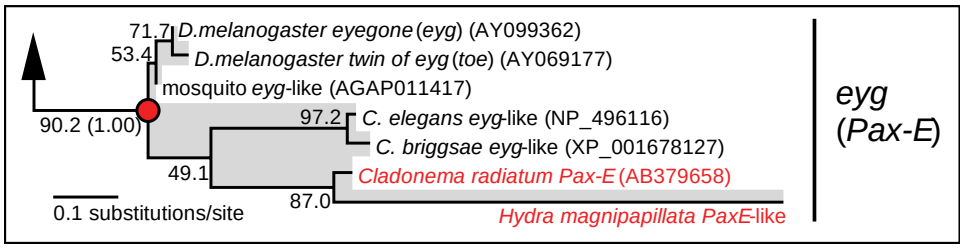

Fig. 3.11 *Eyg* subtree The subtree is inferred by Neighbour-Joining method, with the distances calculated by the Maximum likelihood method (ML), and is based on the comparison of 57 sites in the RED subdomain (from Suga et al. submitted).

3.1.5 Isolation of *CrEya*

In order to design the degenerate primers to isolate *Eya*, we aligned several protein sequences from different species. To identify the most conserved sequences, we spanned across a wide variety of organisms (vertebrates, a worm, a tunicate, two plants and two cnidarians) (Fig. 3.12).

```
 1. ==HUM    1 BC041063     BC041063 0608 Homo sapiens eyes absent homolog 4 (Dros
 2. ==ROD    1 BC120898     BC120898 0610 Mus musculus eyes absent 4 homolog (Dros
 3. ==HUM    3 AL450270     AL450270 0505 Human DNA sequence from clone RP11-704J1
 4. ==HUM    1 BC121799     BC121799 0608 Homo sapiens eyes absent homolog 1 (Dros
 5. ==VRT    1 BC125672     BC125672 0610 Xenopus tropicalis cDNA clone IMAGE:7674
 6. ==ROD    1 BC060260     BC060260 0607 Mus musculus eyes absent 1 homolog (Dros
 7. ==VRT    1 AF118106     AF118106 0001 Danio rerio eyes absent homolog 1 (eya1)
 8. ==VRT    1 BC078315     BC078315 0411 Danio rerio eyes absent homolog 1, mRNA
 9. ==VRT    1 AF352028     AF352028 0106 Xenopus laevis eyes absent-1 alpha mRNA,
10. ==VRT    1 AF031484     AF031484 9812 Gallus gallus eyes absent 2 (Eya2) mRNA,
11. ==ROD    1 AY212511     AY212511 0301 Mus musculus strain 129.AKR EYA2 alpha (
12. ==ROD    1 BC003755     BC003755 0607 Mus musculus eyes absent 2 homolog (Dros
13. ==HUM    1 BC008803     BC008803 0607 Homo sapiens eyes absent homolog 2 (Dros
14. ==HUM    1 Y10261       Y10261   0003 H.sapiens EYA2 gene.
15. ==MAM    1 BT021681     BT021681 0503 Bos taurus eyes absent homolog 2 (Drosop
16. ==ROD    1 AY366465     AY366465 0309 Rattus norvegicus Drosophila-type eyes a
17. ==VRT    1 BC081636     BC081636 0607 Danio rerio zgc:92279, mRNA (cDNA clone
18. ==VRT    1 BC125841     BC125841 0610 Danio rerio cDNA clone MGC:152966 IMAGE:
19. ==VRT    1 BC090243     BC090243 0611 Xenopus laevis hypothetical protein MGC8
20. ==HUM    8 AL512288     AL512288 0505 Human DNA sequence from clone RP11-460I1
21. ==ROD    1 MMU81604     U81604   0112 Mus musculus Eya3 homolog (Eya3) mRNA, c
22. ==INV    1 AJ557023     AJ557023 0404 Dugesia japonica eya gene for eyes absen
23. ==ISC    1 DROABSNTI    L08502   0510 Drosophila melanogaster developmental pr
24. ==INV    1 DQ011272     DQ011272 0509 Oikopleura dioica eyes absent (eya) gene
25. =[nvc] scaffold_142
26. = Hydra magnipapillata
27. ==PLN   55 AP008212     AP008212 0608 Oryza sativa (japonica cultivar-group) g
28. ==PLN    1 AY054555     AY054555 0109 Arabidopsis thaliana EYA-like protein (A
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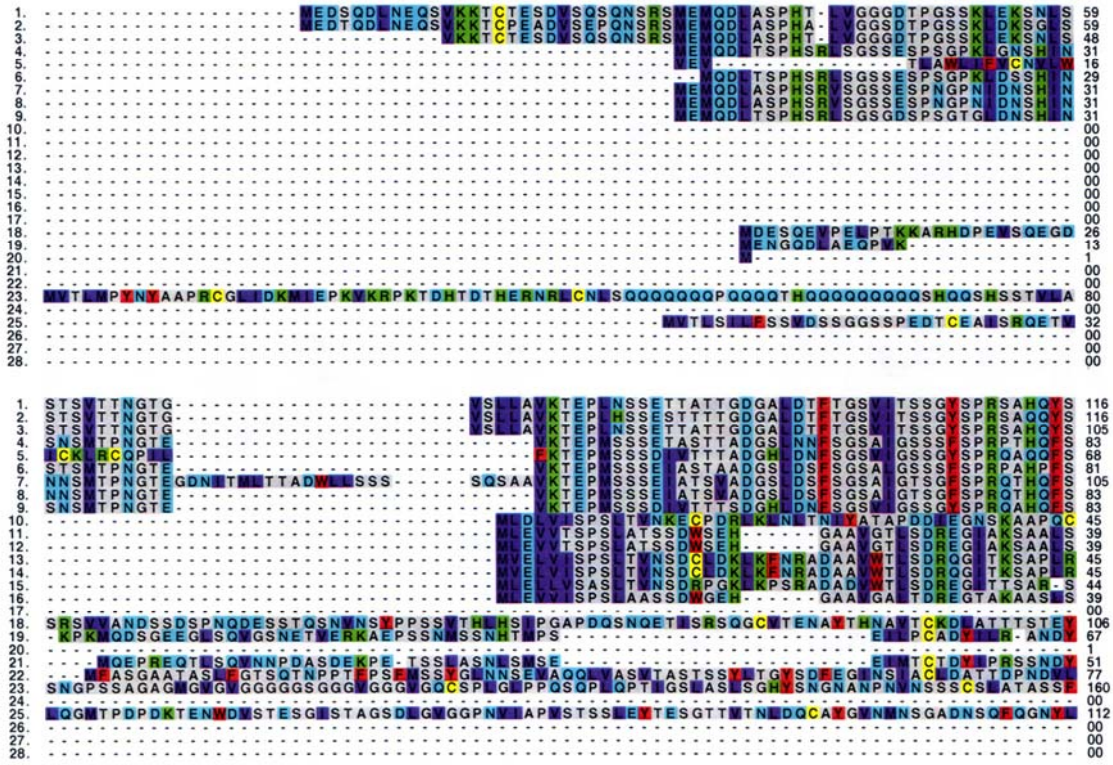

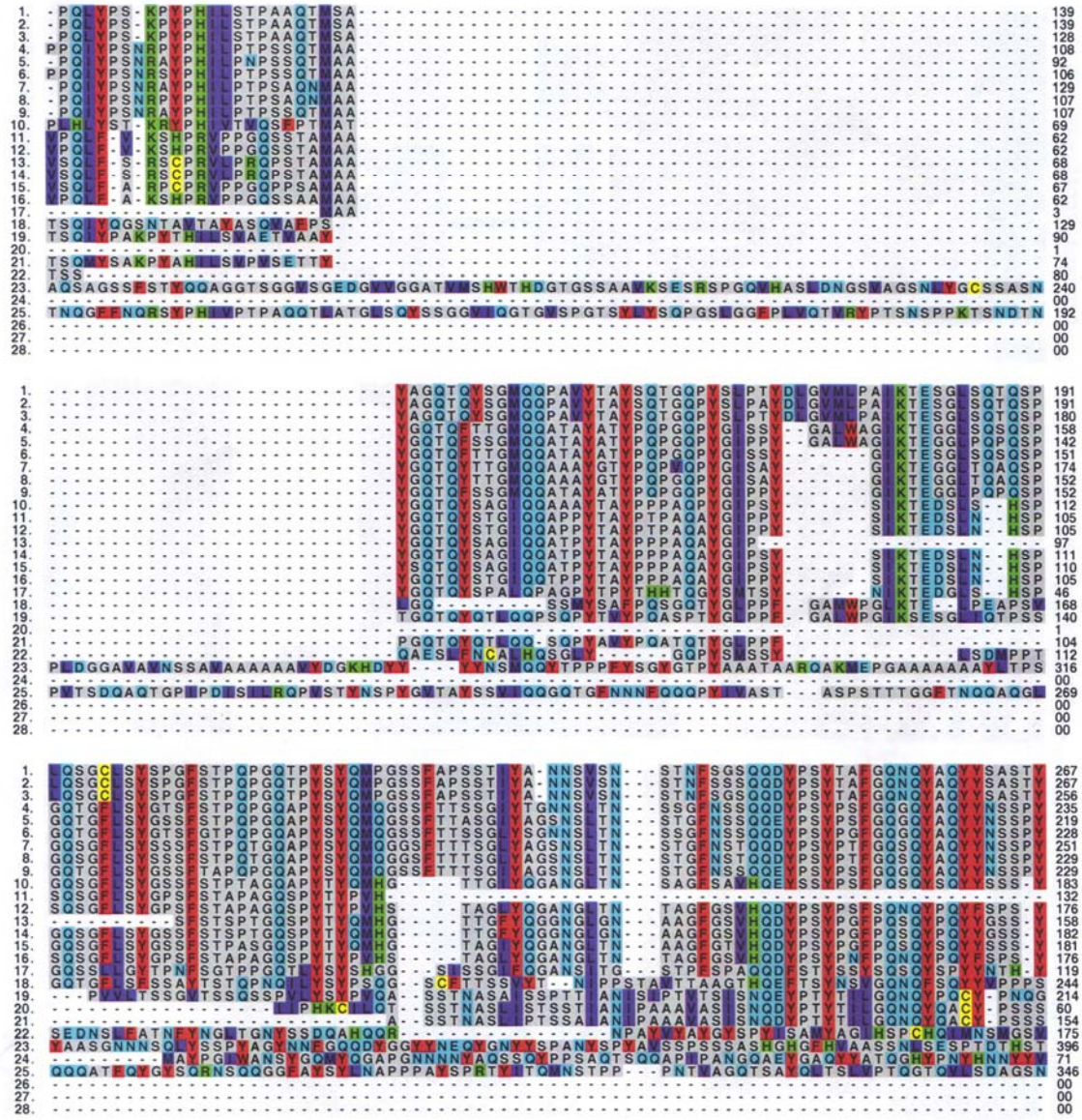

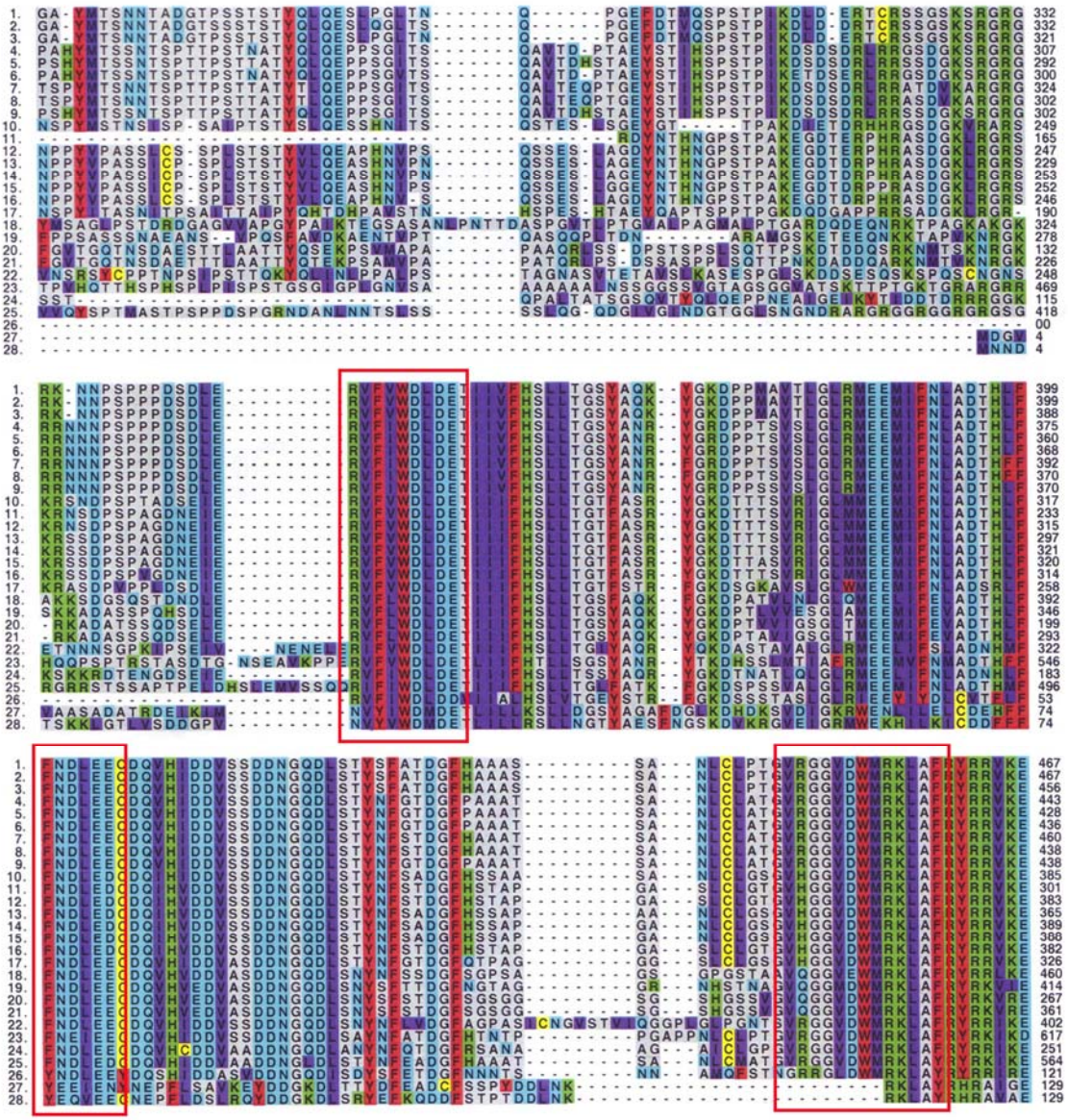

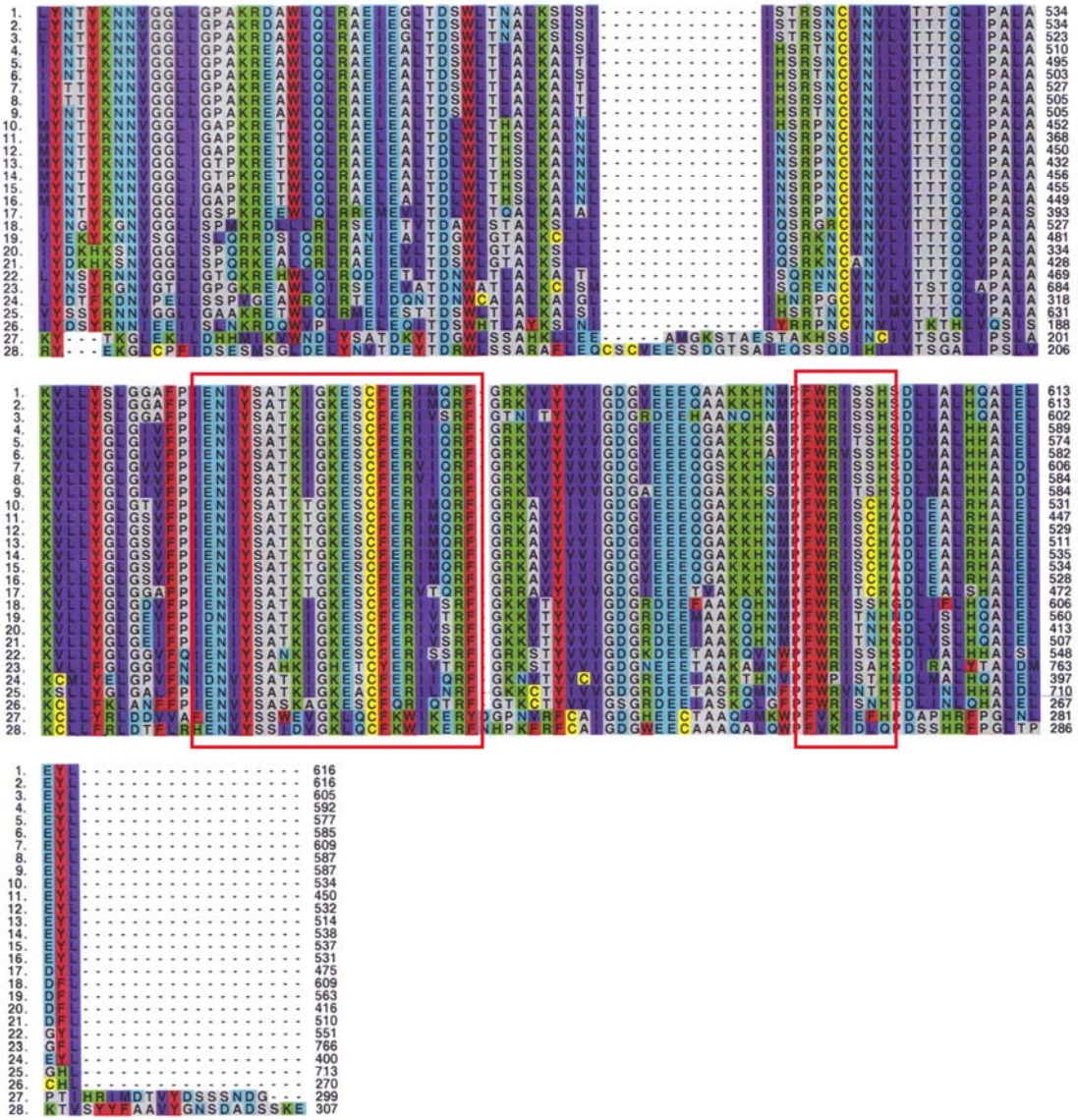

Fig. 3.12 Eya proteins sequence alignment We aligned different Eya proteins sequences from various organisms in order to localise the most conserved domains, the degenerate primers were designed within these conserved regions (red boxes), (for detailed sequences information see paragraph 5.3.1). The species used are listed in the first panel, the corresponding accession numbers are indicated (nvc: *Nematostella vectensis*). Both the sequences of *Nematostella* and of *Hydra* Eya are unpublished and have been found in the present work by blasting the genome of the two species available on line at: http://genome.jgi-psf.org/Nemve1/Nemve1.home.html for *Nematostella* and http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/ for *Hydra*.

The degenerate primers designed form the alignment allowed us to isolate a fragment of cDNA from *Cladonema radiatum*, which we designated *CrEya*.

The jellyfish gene encodes for a protein containing a conserved EYA DOMAIN1 (ED1) in the C-terminal part (269 aa), whereas in the amino terminal region it is not possible to localise the EYA DOMAIN 2. However this region still displays the presence of Proline/Serine/Threonine (P/S/T) repeats (Fig.3.13), typically found in the vertebrate genes (see paragraph 2.3.2, and

Fig. 2.11) (for a complete alignment of the amino acid sequence of CrEya with Eya from other organisms see Appendix, Fig. 7.1). We were also able to amplify a second *CrEya* transcript, a splicing variant, named *CrEya-alt*. This isoform lacks nine nucleotides in the ED, causing an early truncation in the predicted protein sequence.

When compared with other proteins, CrEya shows a 63% amino acid identity at the level of the ED1 with mouse Eya4. Interestingly, when CrEya is compared to a *Nematostella* Eya sequence (predicted protein: GENE ID: 5508888 NEMVEDRAFT_v1g116873) the percentage of identity is the same.

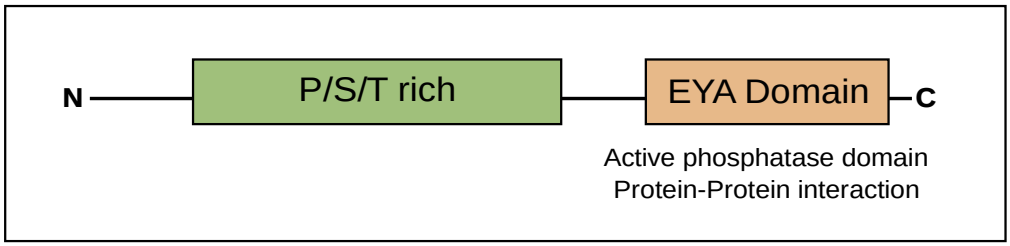

Fig. 3.13 CrEya Schematic drawing of CrEya protein displaying the conserved domains. In pink is the Eya domain1 (ED1) at the carboxy-terminal portion of the protein. The amino-terminal region shows no conservation of the ED2 domain, but like the human and *Drosophila* proteins it displays a P/S/T rich stretch of amino acids.

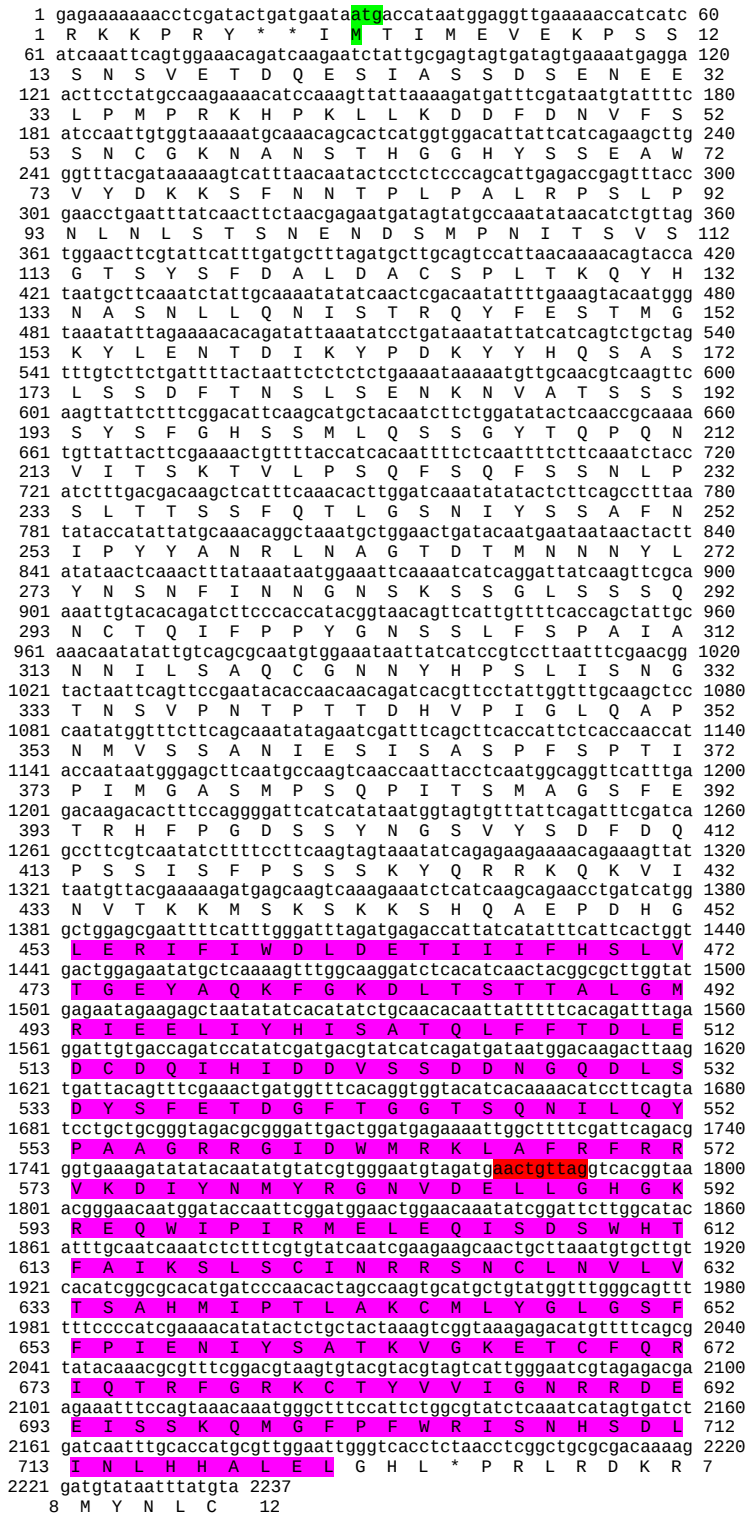

Fig. 3.14 ***CrEya*** Full length cDNA sequence of *CrEya* and deduced amino acid sequence. In pink is highlighted the EYA domain (ED) (the P/S/T rich domain in the N- terminal region is not highlighted in the figure).In red is highlighted the sequence missing in the splicing variant.

3.1.6 Attempts to isolate a *Dac* homolog from C. radiatum

With the exception of the *Dac* family, homologs of all the other members of the RDGC have been isolated in Cnidaria. In order to have a complete picture of the conservation of the RDGC in the lower Metazoa, we were greatly interested in the identification of a *Dac* homolog in *C. radiatum*. We therefore used, also in this case, an homology PCR approach. We screened the two cnidarians genomes available on line (*Hydra and Nematostella*) looking for *Dac* homologs. We could only identify a *Dac* homolog in the genome of *Nematostella*, but not in the genome of *Hydra*. We used the *Nematostella* predicted protein sequence to build an alignment together with *Drosophila* and several vertebrate genes. On the conserved regions thus isolated, we designed the degenerate primers (Fig. 3.15, red boxes). The presence of the cnidarian sequence in the alignment helped us to design less degenerate primers. Despite extensive efforts, using different PCR conditions and different primers combinations, we were not able to isolate a *Dac* homolog. A possible interpretation is that Cnidaria originally had a *Dac* gene, as suggested by the *Nematostella* sequence, and subsequently hydrozoans lost it. However it can not be excluded that *Cladonema* has a *Dac* homolog that has not yet been isolated. The future identification of other cnidarian *Dac* genes will help to design more specific and efficient primers.

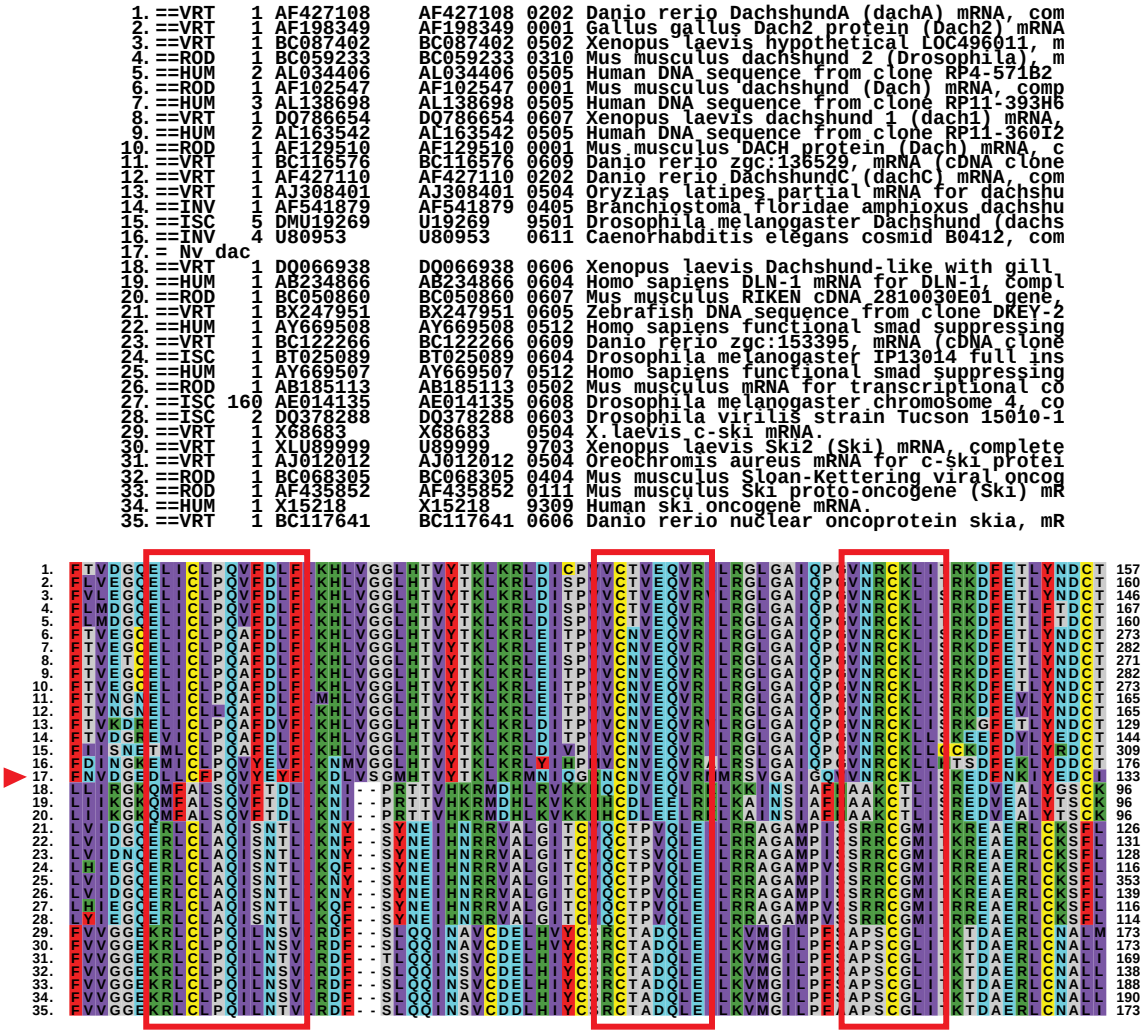

Fig. 3.15 Dac proteins sequence alignment We aligned different Dac proteins sequences from various organisms in order to localise the most conserved domains, the degenerate primers were designed within these conserved regions (red boxes), (for detailed sequences information see paragraph 5.3.1). The species used are listed in the first panel, the corresponding accession numbers are as indicated (Nv: *Nematostella vectensis*). The sequence of *Nematostella* Dac is unpublished and has been found in the present work by blasting the genome available on line at: http://genome.jgi-psf.org/Nemve1/Nemve1.home.html against *dachshund* from *Drosophila*.

3.2 Characterization of the expression pattern of the RDGC members in *C. radiatum*

3.2.1 Definition of medusae growth stages

In the following sections the terms “young” and “adult” jellyfish will be very often used to describe the specimens used in the experiments. Generally, it was considered “adult” a

jellyfish that has reached sexual maturity and whose gonads were well developed and visible on the manubrium. It usually takes more than three weeks to reach the reproductive maturity. A jellyfish that had detached from the polyp body not more than five or six days before, was considered "young". It is not simple to give a precise temporal frame to these definitions because the size of the jellyfish drastically depends on their feeding regime. Since the jellyfish are kept in big aquaria, it is not possible to uniformly feed them. Therefore the jellyfish that eat more will also grow faster. For this reason is preferable to determine the growth stage of the jellyfish according to the size and to the sexual maturity. In Fig. 3.16 examples of an adult and of a young jellyfish are displayed. A young jellyfish usually has a bell of 1mm in diameter and an adult jellyfish of 2-3mm. When kept in good conditions *Cladonema* jellyfish can live up to two months.

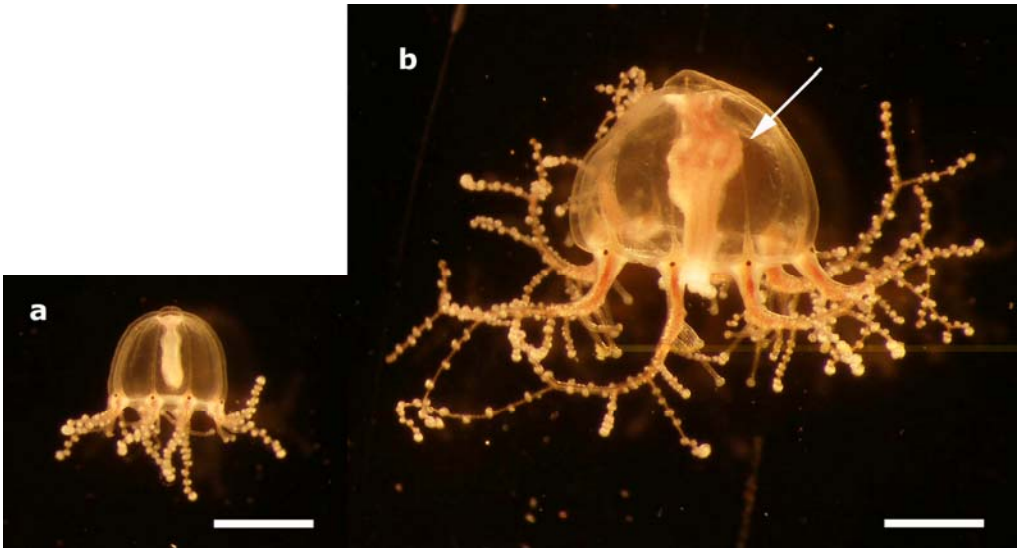

Fig 3.16 Young and adult jellyfish (a) a young jellyfish, (b) an adult jellyfish which has reached the sexual maturity, the well developed gonads are visible on the manubrium (white arrow) (scale bar is 1mm).

3.2.2 *CrPaxA*

In a previous report the expression of *CrPaxA* was mainly detected in the eyes of the jellyfish, both by Real Time PCR and *in situ* hybridization. In addition an up-regulation during eye regeneration was described (Stierwald 2004, unpublished data). In order to reproduce these data we analyzed again the expression pattern of *CrPaxA* using the same experimental approach.

3.2.2.1 Real Time PCR expression data

We first used a Real Time PCR approach to investigate the spatial expression of *CrPaxA*.
We dissected the medusa in four different body parts: the umbrella or bell, the manubrium (the feeding and reproductive organ), the tentacles (containing the stinging nematocytes) and the tentacle bulbs (where the eyes are located). The expression analysis was repeated at least three times on each cDNA set (consisting of the four tissues), independently generated from different jellyfish. The mean for the three values was calculated. The error bars in Fig. 3.17 indicate the standard deviation from the mean value. The expression levels are presented relative to *Elongation Factor 1α* (*EF1α*). For both young and adult jellyfish the expression pattern remains the same throughout the growth of the jellyfish, showing a significantly higher level of expression in the tentacle bulbs (Fig. 3.17), than in the other tissues. This pattern is in agreement with the previous results (Stierwald 2004, unpublished data).

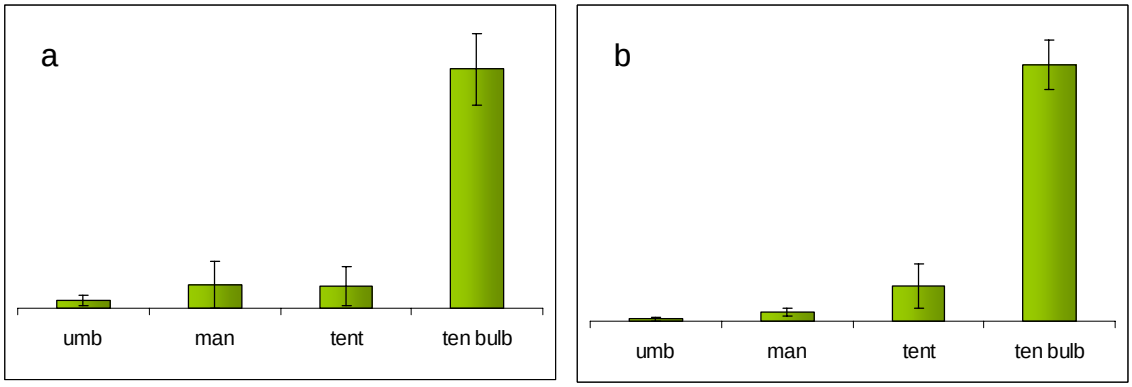

Fig. 3.17 Real Time PCR expression data for *CrPaxA* in young and adult jellyfish Expression levels for *CrPaxA* detected by RT PCR in young jellyfish (a) and in adult jellyfish(b). In both cases the highest level of expression was detected in the tentacle bulbs. The expression levels are presented relative to the housekeeping gene *Elongation Factor 1α* (*EF1α*). The experiments were repeated at least three times on independent samples, the mean values were calculated, error bars indicate standard deviation. (umb: umbrella; man: manubrium; tent: tentacles; ten bulb: tentacle bulbs)

The y-axes in the two graphs of Fig. 3.17 are arbitrary, as they represent the expression in the different body parts in relation to the whole body which is considered equivalent to 1. Therefore, the levels of expression displayed in the graphs referring to young and adult specimens are not directly comparable. The expression levels of young and adult medusae have to be referred to the same y-axe, in order to allow for comparison. Furthermore, the detection of the expression of the young and adult samples was carried out in the same Real Time PCR run. This allows to calculate the concentration of the samples with reference to the

same standard dilution, and to have comparable reaction efficiency for all the samples. Once identified the body part where the gene is mainly expressed, we could directly compare the levels of expression in this body part between young and adult jellyfish. The comparison in the tentacle bulbs for expression levels of *CrPaxA* in young and adult jellyfish is shown in Fig. 3.18.

The direct comparison showed that the expression levels in tentacle bulbs of young and adult medusae are very similar and no significant changes seem to occur during the growth of the jellyfish.

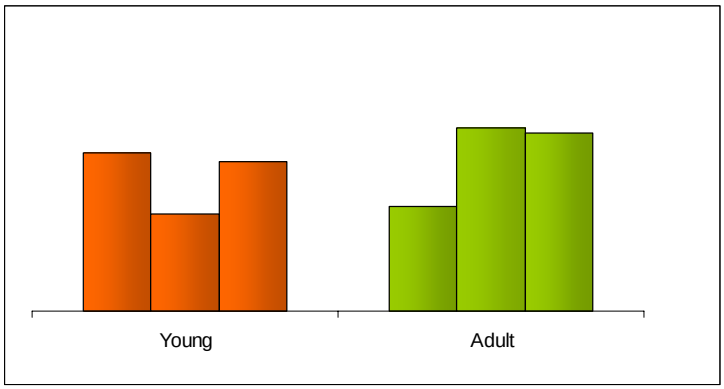

Fig. 3.18 Direct comparison of the expression of *CrPaxA* detected by Real Time PCR in tentacle bulbs of young and adult jellyfish The columns displaying the level of expression in the tentacle bulbs for the three young jellyfish (orange) are grouped together and shown next to the three columns for the adult jellyfish (green). In both cases the levels are similar and the difference is not significant (t-test, p=0,5). All data are relative to *EF1α*.

3.2.2.2 *In situ* Hybridization expression data

We then analyzed the expression pattern of *CrPaxA* in the tentacle bulbs by whole mount *in situ* hybridization. *CrPaxA* was previously reported to be expressed exclusively in the pigment and photoreceptor cells of the eye (Stierwald 2004, unpublished data). We could reproduce this result (asterisk in Fig. 3.19 and Fig. 3.20). In addition to the eye, we could observe a strong expression also in the outer part of the tentacle bulb (TB) (black arrows).

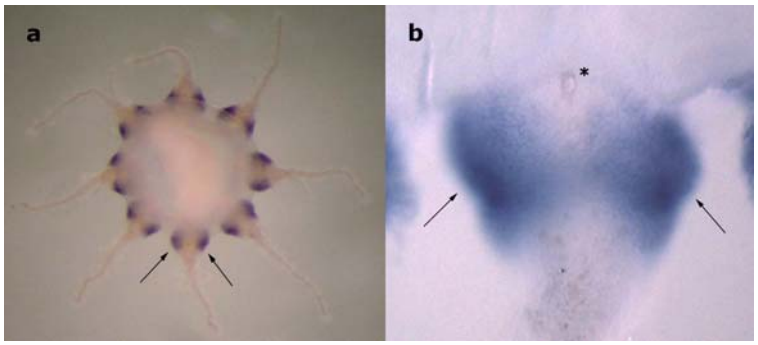

Fig 3.19 Whole mount in situ hybridization for CrPaxA in young jellyfish In (a) is shown a top view of a young jellyfish displaying the signal for CrPaxA localised mainly at the side of the tentacle bulb (black arrows). (b) Higher magnification of a single tentacle bulb, frontal view. A weak signal is also detectable in the eye (asterisk). (Data provided by Hiroshi Suga)

The expression seems to slightly vary according to age of the jellyfish: the signal in the TB margin is stronger in young jellyfish (Fig. 3.19) than in the adult (Fig. 3.20), whereas the expression in the eye seems to be more intense in adult jellyfish.

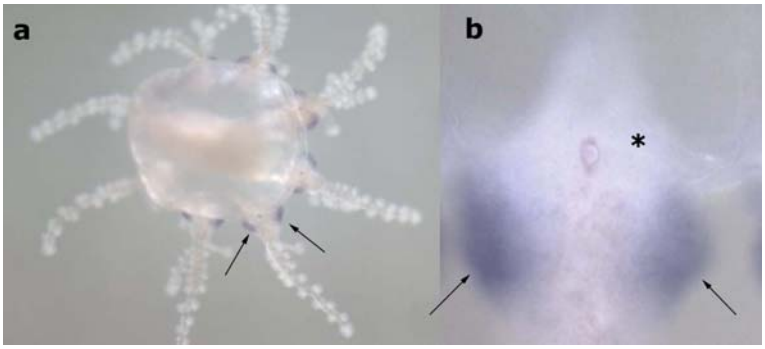

Fig 3.20 Whole mount in situ hybridization for CrPaxA in old jellyfish In (a) is shown a top view of an adult jellyfish. The signal for CrPaxA is still mainly localised at the side of the tentacle bulb (black arrows), though it is weaker than when compared to Fig. 3.19 (b). Higher magnification of a single tentacle bulb, frontal view, the signal in the eye (asterisk) is stronger than in younger jellyfish (Fig. 3.19) . (Data provided by Hiroshi Suga)

3.2.2.3 Expression data during eye regeneration

In order to find out whether *CrPaxA* is involved in the development of the eye, we tested its possible up-regulation during the process of regeneration upon eye removal (Weber 1981b). For this purpose the eyes were sucked off using a thin glass needle connected to a vacuum pump. The process of complete regeneration takes approximately 10-15 days from the excision. During eye regeneration, the tentacle bulbs of the operated jellyfish were dissected, at regular intervals, and used for mRNA extraction and cDNA synthesis. The cDNA obtained

at the different time points during the regeneration, was then analyzed by Real Time PCR, to look for a possible increase in the expression levels of *CrPaxA*.

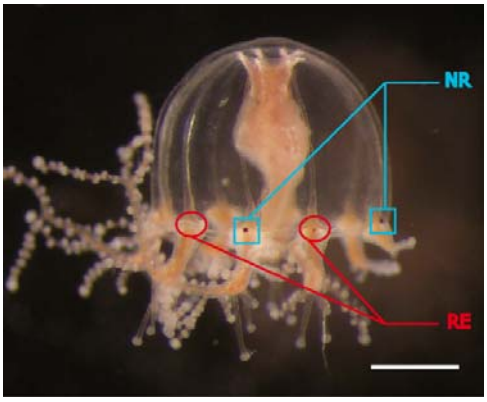

Fig. 3. 21 Alternate eye removal A jellyfish whose eyes have been removed in an alternate fashion is shown. Red ellipses indicate the removed eyes (RE) and blue squares indicate the eyes not removed (NR).

The regeneration process was initially studied in jellyfish to which all the eyes were removed (defined as "all removed"). Jellyfish that did not undergo eye removal ("all not removed") were used as a control. Later, in order to have an internal control, we took a different eye removal approach: we removed the eye in an alternate fashion. *Cladonema* medusae usually have eight eyes. The four tentacle bulbs from which the eyes were removed (defined as "alternate removed") were used to analyse *CrPaxA* expression during regeneration. The four intact tentacle bulbs were instead used as a control for expression in not removed eyes ("alternate not removed"), (Fig. 3.21).

We first analyzed the expression of *CrPaxA* in young jellyfish, at three time points after the excision: day 0 (three hours after the eye removal), day 4 and day 13 (Fig. 3.22).

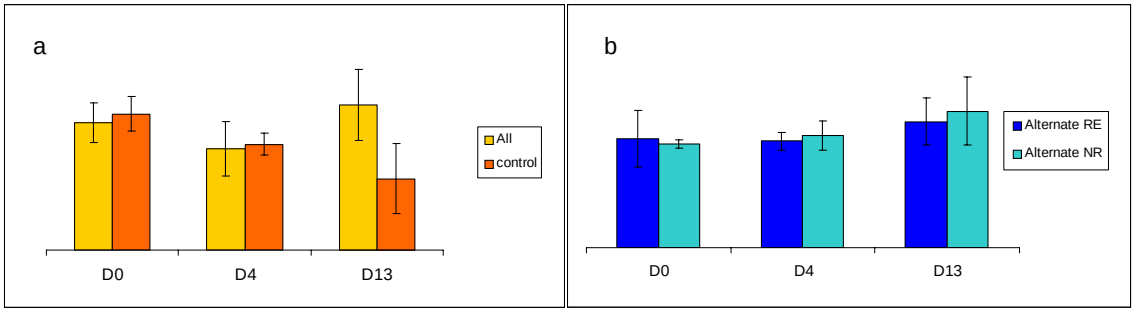

Fig. 3.22 Expression pattern of *CrPaxA* during eye regeneration in young jellyfish analyzed by Real Time PCR (a) Analysis of *CrPaxA* expression in jellyfish to which all the eyes were removed ("All", yellow columns), compared to untreated jellyfish ("control", orange columns). (b) *CrPaxA* expression in tentacle bulbs where the eyes were removed ("Alternate RE", dark blue columns), compared to the untouched tentacle bulbs ("Alternate NR", light blue columns). X-axes indicate the day after eye removal: D0 is three hours after excision, D4 is four days after excision and D13 is 13 days after excision. All data are relative to *EF1α*. The expression analysis was repeated at least three times on independent samples, the mean values were calculated, error bars indicate standard deviation. (Data provided by Hiroshi Suga)

The analysis was carried out using tentacle bulbs cDNA from “all removed” jellyfish (including the control “all not removed”), and on “alternate removed” (including the control “alternate not removed”). In both cases, no up-regulation or any significant change in the expression for *CrPaxA* was detected at the different time points during the regeneration process. In addition, the treated animals did not show a significant difference compared to the controls.

Considering the strong expression of *CrPaxA* in the TB margin, detected by *in situ* hybridization, we wanted to exclude the possibility that this expression could interfere with the detection of the possible increase in *CrPaxA* expression during eye regeneration. Ideally the quantification should be carried out only on the eye tissues, separately from the TB. This is technically not possible, due to the minute size of the eye. We therefore tried to overcome this problem in a different way. We noticed that the intensity of the signal in the TB margin decreases in adult jellyfish, for this reason we tried the same experiment in very old jellyfish (at least 3mm in diameter). By doing this, we were expecting that the weaker signal at the margin would minimize the disturbance in quantifying the signal restricted to the eye, and that the sensitivity of the Real Time PCR would detect for any increase of *CrPaxA* expression during eye regeneration. Also in this case we performed the experiment in the “all” and “alternate” fashion. Furthermore, we monitored the trend of the expression for a longer period, extending the time course of the documented regeneration period to 20 days post-removal.

The whole experiment was hampered by the age of the jellyfish employed. It was necessary to have medusae at least four weeks old. At this age the jellyfish display only limited regeneration capabilities compared to younger specimens. Therefore, the stressful procedure of eye removal often did not result in recovery, but in the death of the medusae. Furthermore, the 20 days required for the whole regeneration time course, in same cases, exceeded the regular life span of the jellyfish, which thus underwent natural senescence and death before reaching the desired time point.

The new approach employed, however, did not lead to a different result. No up-regulation in the expression during the regeneration period was detected (t-test for “All”: “D0 All” to “D5 All”, p= 0.4; “D5 All” to “D10 All”, p= 0.4; “D10 All” to “D15 All”, p= 0.6; “D15 All” to D20 All”, p= 0.6; “D20 All to D0 All”, p= 0.9. t-test for “Alternate” “D0 all” to “D5 Alt”, p= 0.3; “D5 Alt” to “D10 Alt”, p= 0.1; “D10 Alt” to “D15 Alt”, p= 0.09; “D15 Alt” to D20 Alt”, p= 0.03; “D20 Alt to D0 Alt”, p= 0.1). In addition, no significant difference between the

treated animals and the control was observed, independently of the eye-removal method employed ("all" or "alternate", Fig. 3.23), (t-test for "All": "D0 All" to "D0 control", p= 0.9; "D5 All" to "D5 control", p= 0.02; "D10 All" to "D10 control", p= 0.9; "D15 All" to "D15 control", p= 0.8; "D20 All" to "D20 control", p= 0.2. t-test for "Alt": "D0 Alt" to "D0 control", p= 0.4; "D5 Alt" to "D5 control", p= 0.6; "D10 Alt" to "D10 control", p= 0.7; "D15 Alt" to "D15 control", p= 0.7; ; "D20 Alt" to "D20 control", p= 0.05).

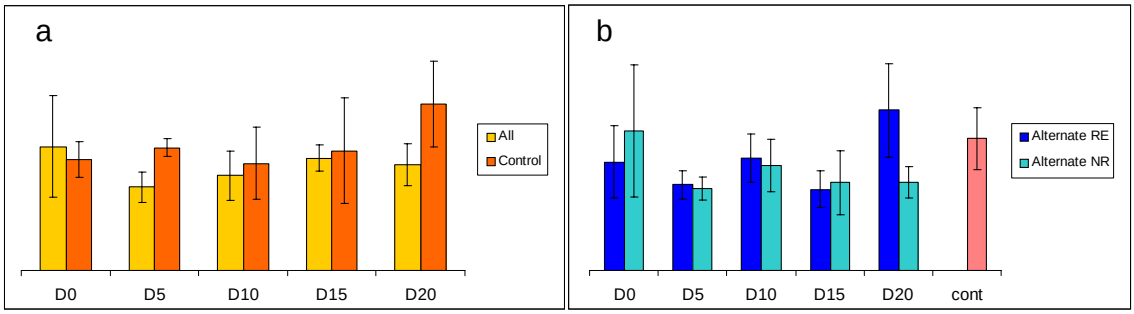

Fig. 3.23 Expression pattern of *CrPaxA* during eye regeneration in adult jellyfish analyzed by Real Time PCR (a) No specific increase of signal during the regeneration was detected in **"**All removed" TB (yellow columns) and also no significant difference to the control (orange columns) was observed. (b)The expression pattern of "alternate removed" (dark blue columns) did not exhibit any significant difference compared to the control "alternate not removed" (light blue columns). In this case a further external control, represented by untreated jellyfish, has been added (pink column). All data are relative to *EF1α*. The expression analysis was repeated at least three times on independent samples, the mean values were calculated, error bars indicate standard deviation.

The same experimental method was used to confirm the Real Time PCR data by whole mount *in situ* hybridization.

The *in situ* hybridization data are in agreement with the results obtained by Real Time PCR, for both "all removed" and "alternate removed".

At day 5, the pigment and photoreceptor cells have started to differentiate and the dark pigment of the eye is already visible (see paragraph 2.2.5). Therefore, if *CrPaxA* was involved in the eye regeneration process, its expression should be detectable already at this point. But no staining is observed by *in situ* hybridization at day 5 (Fig. 3.24). *CrPaxA* expression becomes visible approximately 15 days after eye removal, when the regeneration of the eye is almost complete. Furthermore, we could observe that the time course of eye regeneration, similarly to the growth of the entire jellyfish, is drastically influenced by the feeding regime. The duration of the regeneration process can therefore be longer than what expected from previous studies (Weber 1981a; Weber 1981b) (see also paragraph 2.2.5), varying between 10 and 20 days. For this reason, in some cases the staining for CrPaxA was detectable only after 20 days from eye removal.

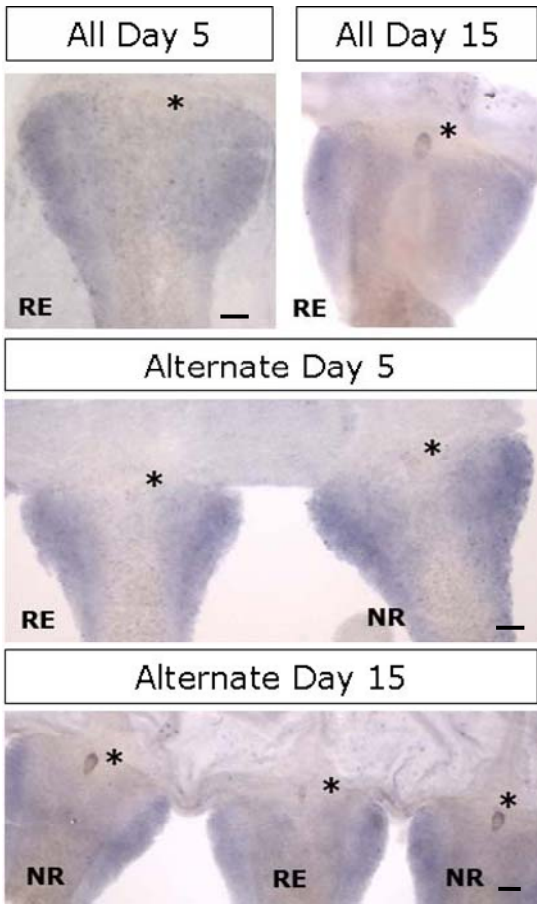

Fig. 3.24 In situ hybridization for *CrPaxA* on adult jellyfish during eye regeneration Tentacle bulbs, frontal view. Only representative time points are shown (namely Day 5 and Day 15) for both "all removed" and "alternate removed". Asterisks indicate the position of the eyes; RE: Removed Eyes, NR: Not Removed eyes. (Scale bar is 60 µm)

The Real Time PCR as well as the *in situ* hybridization results did not show up-regulation of *CrPaxA* expression during eye regeneration. Therefore, our data raise the possibility that *CrPaxA* is not directly involved in eye development. However, the constant presence of *CrPaxA* expression in the intact eye of young and adult jellyfish, suggests that this gene could play an important role in eye maintenance.

After the detailed study of the expression of *CrPaxA* in the eye, we focused on the analysis of its expression detected by *in situ* hybridization at the TB margin (See Fig. 2.19 and Fig. 2.20). It is known that in hydromedusae the TB is the site where the nematocyte precursor cells, the nematoblasts, differentiate (Carré 1974a; Carré 1974b; Carré 1974c; Bouillon 1995; Denker et al. 2008). In transversal sections, the tentacle bulb ectoderm (TBE) appears densely packed with nematoblast (Denker et al. 2008). Cryosections of tentacle bulbs in which *CrPaxA* expression was detected by *in situ* hybridization, showed that the signal is localised in the TBE, at the level of the differentiation area, and specifically in the numerous nematoblasts at various maturation stages (Fig. 3.25). Therefore, it is tempting to speculate that the expression of *CrPaxA* in the TBE indicates an involvement of this gene in the differentiation of the nematoblast.

In order to further prove the involvement of *CrPaxA* in nematoblast differentiation we analyzed by Real Time PCR its possible up-regulation during tentacles regeneration.

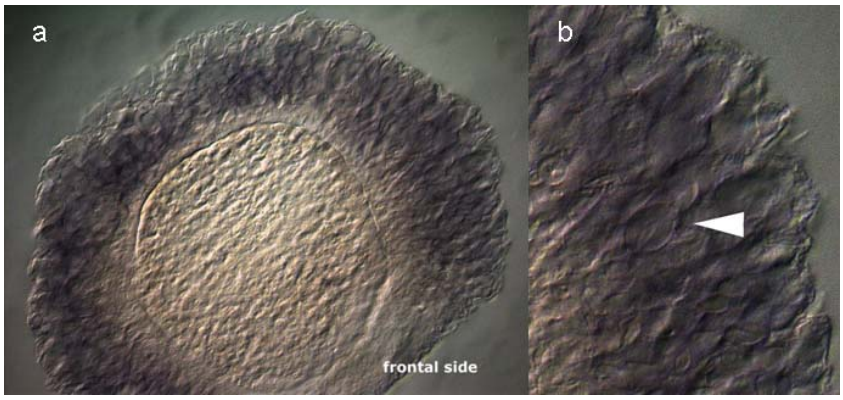

Fig. 3.25 *CrPaxA in situ* hybridization, cryosection of tentacle bulb The whole transversal cryosection of a tentacle bulb is shown (a). The staining is localised in the ectoderm, within the nematogenesis zone. Higher magnification of the nematogenesis area (b), displaying the high density of nematoblasts at various stage of differentiation. The white arrowhead points to a nematoblast containing a maturing nematocyst. (data provided by Hiroshi Suga)

We observed that the process of tentacle regeneration, after excision at the base of the TB, is accomplished in 10 to 12 days. During this period, the growing tip of the tentacle has to be newly equipped with stocks of mature nematocytes. Therefore, we expected that the increased need of nematocytes, would cause in turn a peak in the nematoblast differentiation rate in the TBE. This would lead to the up-regulation of the expression of the genes involved in this process. For this reason, we analyzed by Real Time PCR the expression of *CrPaxA* in the TB during tentacle regeneration. The analysis was carried out every three days, from the tentacle excision (day 0), until the complete regeneration (12 days), (Fig 2.26, a). However, no increase of *CrPaxA* expression during tentacle regeneration was observed. An explanation for this result might be that the up-regulation of the genes involved in the nematoblasts differentiation is a process limited to a few hours after amputation. For this reason, we did not repeated this experiment on three independent sample. Instead, we tested again the expression of *CrPaxA* by Real Time PCR, focusing on a shorter time course, ranging from 30 minutes until 12 hours after excision (Fig 2.26 b). However, this different approach did not lead to the detection of an up-regulation in the expression of *CrPaxA* during the considered time course of tentacle regeneration. The process of tentacle regeneration, and the dynamics of nematoblasts differentiation during this process are not known in hydromedusae. Therefore we do not know whether the obtained results are due to an incorrect evaluation of the length of the process of nematoblast differentiation. Or whether instead, the increase in the mature nematocytes required in the distal part of the tentacle, does not directly affect the nematoblast differentiation rate. A further investigation would require the characterization of the precise

progression of tentacles regeneration and the development of specific nematoblast differentiation markers, which was beyond the aim of the present work.

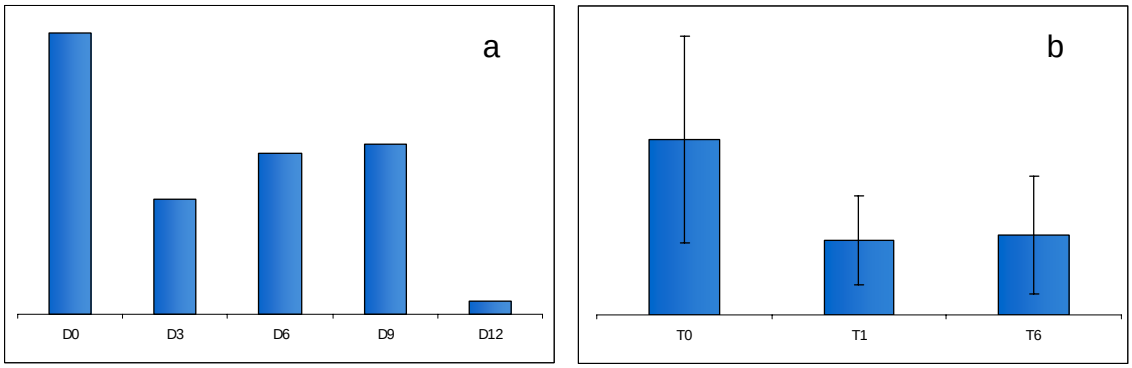

Fig. 3.26 Expression pattern of *CrPaxA* during tentacle regeneration, analyzed by Real Time PCR The expression levels of *CrPaxA* in the tentacle bulbs were analysed every three days for a total of 12 days (a). This experiment was performed a single time. The expression levels of *CrPaxA* during tentacle regeneration were also studied on a shorter time course (b), T0= 30min after tentacle excision, T1= 1h, T6= 6h. This experiment was performed three times on independent samples, the mean values were calculated, error bars indicate the standard deviation. All data are relative to *EF1α*.

3.2.3 CrPaxB

In order to analyse the expression pattern of *CrPaxB* we used, as described for *CrPaxA*, a combined approach. We first identified by Real Time PCR the body part where the gene is mainly expressed, and then studied the expression in the tissues by *in situ* hybridization.

3.2.3.1 Real Time PCR expression data

The Real Time PCR data clearly show a pattern that varies according to the age of the jellyfish. In young specimens *CrPaxB* expression is found in every body part, particularly in the umbrella (Fig. 3.27, a). In adults, *CrPaxB* is almost exclusively expressed in the manubrium and at lower levels in the tentacle bulbs. Tentacle and umbrella show almost no signal (Fig. 3.27, b).

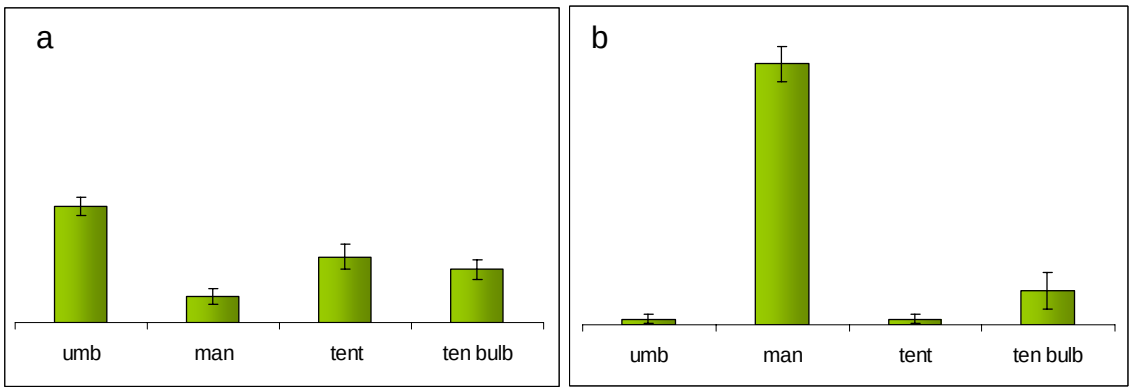

Fig. 3.27 Real Time PCR expression data for *CrPaxB* in young and adult jellyfish Young jellyfish show almost uniform expression levels of *CrPaxB*, the expression is slightly higher in the umbrella (a). Adult jellyfish instead display *CrPaxB* expression mainly in the manubrium (b), a weaker but still detectable expression is also observed in tentacle bulbs. All data are relative to *EF1α*. The experiments were repeated at least three times on independent samples, the mean values were calculated, error bars indicate the standard deviation are shown. (umb: umbrella; man: manubrium; tent: tentacles; ten bulb: tentacle bulbs)

To further validate the age related pattern, we examined and directly compared the expression levels of *CrPaxB* in the manubrium of young and adult jellyfish (Fig. 3.28). The analysis was carried out in the same run of Real Time PCR. The obtained results are in agreement with the previous data. Despite a certain variability in signal intensity, adult jellyfish (in green, Fig. 3.28) clearly show a stronger expression compared to young jellyfish (in orange, Fig. 3.28).

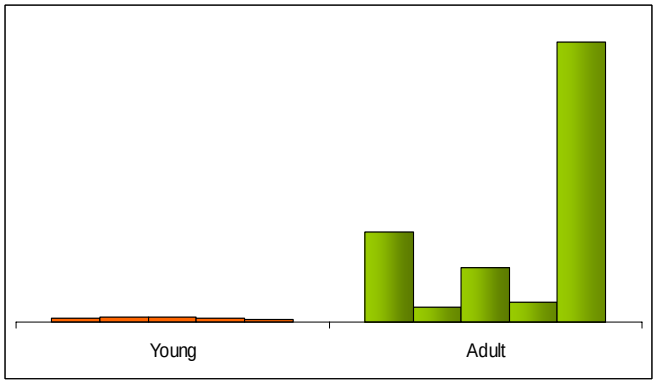

Fig. 3.28 Direct comparison of the expression of *CrPaxB* detected by Real Time PCR in tentacle bulbs of young and adult jellyfish The expression levels of *CrPaxB* in the manubrium of three young jellyfish (orange) are shown next to the expression levels in adult jellyfish (green). Clearly, the levels of expression are higher in adult jellyfish, despite the apparent variation displayed in this case (t-test, p= 0,1). The expression levels are relative to *EF1α*.

To test whether the weak expression of *CrPaxB* detected in TB by Real Time PCR (Fig. 3.27, b) had any relevance during the eye regeneration process, we analysed, by Real Time PCR, the expression of *CrPaxB* during this process. We employed a less complicated experimental approach than that described for *CrPaxA*. We analyzed the possible up-regulation during 15

days of eye regeneration. Only the "All" eye removal method was employed. The levels of expression in the treated animals were compared to a single control. However, also in this case, no up-regulation of *CrPaxB* expression levels during eye regeneration was observed (Fig. 2.29), (t-test. Day0 to control: p=0.6; day5 to control: p= 0.02; day10 to control: p= 0.3; day15 to control: p= 0.06)

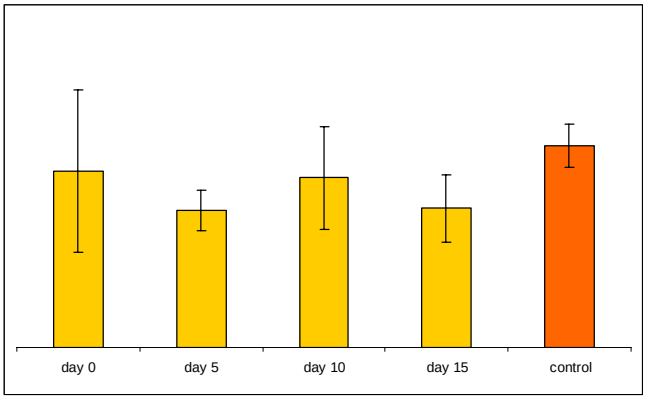

Fig. 3.29 Expression pattern of *CrPaxB* during eye regeneration in adult jellyfish analyzed by Real Time PCR Only the "All" eye removal method was employed. On the X-axe the days from excision are indicated. A single external control, represented by untreated jellyfish, has been added (orange column). All data are relative to *EF1α*. The expression analysis was repeated three times on independent samples, the mean values were calculated, error bars indicate standard deviation.

3.2.3.2 *In situ* hybridization expression data

To further characterize the age related differences in the expression patter of *CrPaxB* detected by Real Time PCR, we carried out whole mount *in situ* hybridization on both young and adult specimens. We made use of an RNA antisense probe against the linker region between the PD and the HD of CrPaxB, in order to avoid possible crossreactions among the conserved domains of different *Pax* genes.

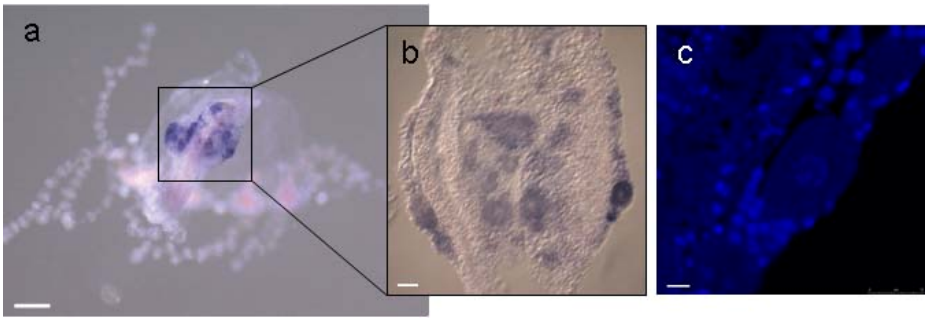

Fig 3.30 *In situ* hybridization for *CrPaxB* Whole mount *in situ* hybridization, the expression of *CrPaxB* is localized in the manubrium where the gonads are situated (a). Cryosection of a stained manubrium, the signal is restricted to the cytoplasm of oocytes at various stages of differentiation (b). DAPI staining of cryosections displays the localization of the nuclei of the oocytes (c). Scale bar are: (a) 500µm, (b) 100µm, (c) 10µm

The signal for CrPaxB was detected by whole mount *in situ* hybridization of adult, sexually mature jellyfish, in the upper part of the manubrium, where the gonads are localised (Fig.

3.30, a). In cryosections, the staining appeared to be confined to the oocytes at different maturation stages (Fig. 3.30, b). The oogenesis of *C. radiatum* has not been studied in details. By morphological analysis, no follicle or nurse cells were identified in another Hydromedusa, *Podocoryne carnea* (Boelsterli 1977). However, it is not known whether the oocytes of *C. radiatum* are surrounded by such cells. To exclude the possibility that the signal detected in the gonads would not localise in the oocytes but rather in neighbouring nurse cells, and to confirm the cytoplasmatic localization of the observed expression, we carried out DAPI staining on the cryosections. In this way we localised the nuclei of the oocytes (Fig. 3.30, c), identified the different cells and specifically assigned the signal of *CrPaxB* to the cytoplasm of the maturing oocytes. On the other hand, no signal was detected in the manubrium of young, not sexually mature jellyfish (Fig. 3.31, right panel). The specificity of the detected signal was further confirmed by performing *in situ* hybridization with a sense RNA probe for *CrPaxB* (Fig. 3.31).

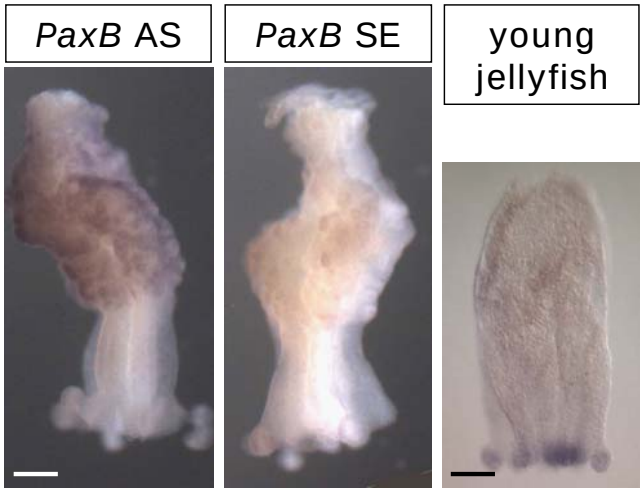

Fig 3.31 *CrPaxB* whole mount *in situ* hybridization for, dissected manubria Dissected manubria of sexually mature medusae, stained with *CrPaxB* antisense RNA probe (left panel) and *CrPaxB* sense probe (central panel) are shown. No specific signal was detected in the case of the sense probe. A dissected manubrium of a young jellyfish, not sexually mature, stained with CrPaxB antisense probe (right panel) is shown. Also in this case no specific signal was detected. (Scale bar is 250 μm in "PaxB AS" and "PaxB SE", and 125μm in "young jellyfish").

The *in situ* hybridization data confirm the expression pattern detected by Real Time PCR. The localization of the signal for *CrPaxB* in the oocytes explains the variability in the expression levels observed by Real Time PCR in the manubrium of adult jellyfish. The intensity of the signal depends on the number of maturing oocytes present in the gonads, since this number is highly variable among jellyfish, is not possible to obtain a constant signal by Real Time PCR analysis.

In order to find out whether *CrPaxB* is a maternally contributed factor, we also performed *in situ* hybridization on released unfertilized eggs. We kept sexually mature jellyfish in a small glass cup and collected the eggs overnight. However, no specific signal was detected in the released eggs (Fig. 3.32). This indicates that *CrPaxB* is not maternally contributed and that might be mainly required for oocytes maturation.

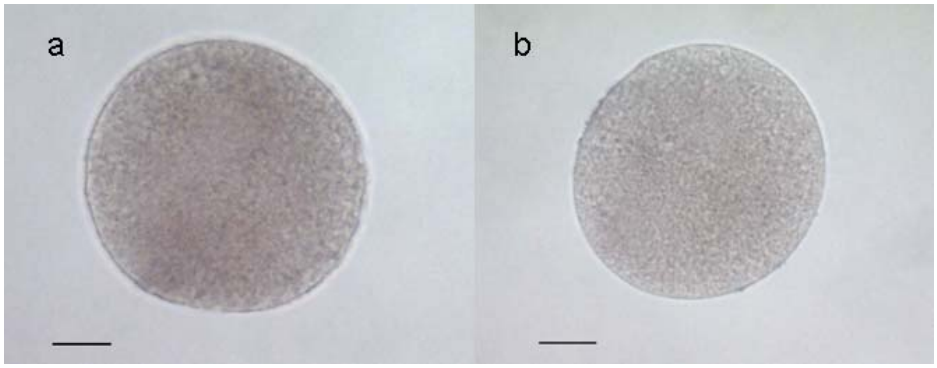

Fig 3.32 *CrPaxB in situ* hybridization on released eggs *In situ* hybridization on unfertilized eggs; no signal is detected in *CrPaxB* anti sense probe (a), as well as in *CrPaxB* sense probe (b). (Scale bar is 25µm).

3.2.4 *CrPaxE*

3.2.4.1 Real Time PCR expression data

Real Time PCR expression analysis of *CrPaxE* showed that, similarly to *CrPaxB*, the signal is mainly localised in the manubrium. However, while the expression pattern of *CrPaxB* displayed age related differences, *CrPaxE* expression remains constantly localised mostly in the manubrium of both young and adult jellyfish.

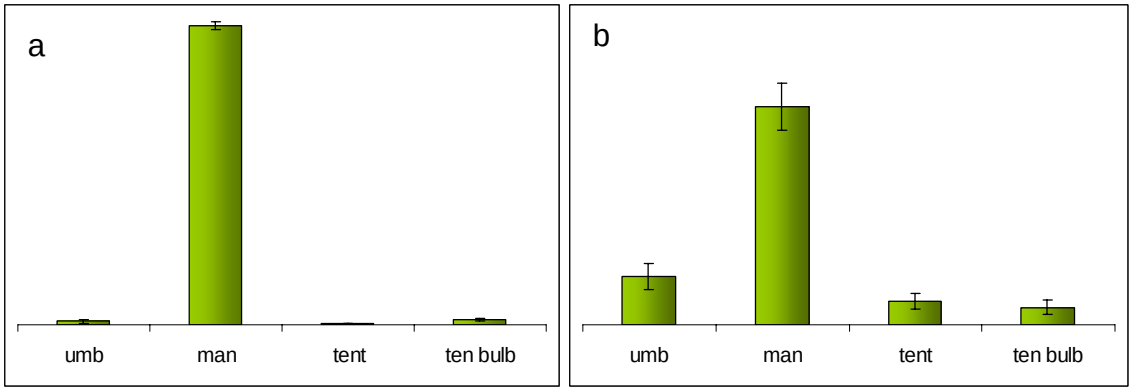

Fig 3.33 Real Time PCR expression data for *CrPaxE* in young and adult jellyfish In both young (a) and adult jellyfish (b) *CrPaxE* is mainly expressed in the manubrium. All data are relative to *EF1α*. The expression analysis was repeated at least three times on independent samples. The mean values were calculated. Error bars indicate standard deviation. (umb: umbrella; man: manubrium; tent: tentacles; ten bulb: tentacle bulbs).

The direct comparison of *CrPaxE* expression in young and adult jellyfish, shows a complementary pattern to *CrPaxB*: *CrPaxE* is more strongly expressed in the young specimens than in the adults. Therefore, *CrPaxE* might exerts a different functions at the level of the manubrium, probably not related to gonads maturation since it is preferably expressed in sexually immature jellyfish.

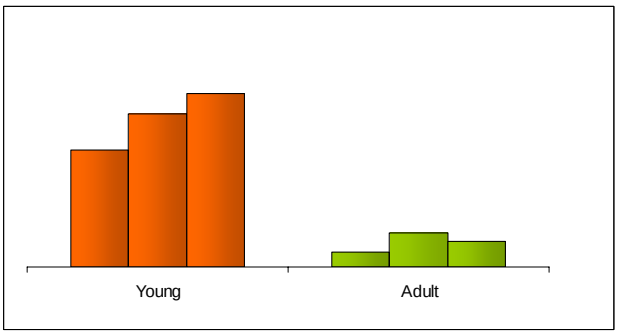

Fig. 3.34 Direct comparison of the expression of *CrPaxE* detected by Real Time PCR in manubrium of young and adult jellyfish The expression levels of *CrPaxB* in the manubrium of three young jellyfish (orange) are shown next to the expression levels in adult jellyfish (green). Clearly, the levels of expression are higher in young jellyfish than in adults (t-test, p= 0.01). The expression levels are relative to *EF1α*..

3.2.4.2 *In situ* hybridization expression data

Despite extensive attempts, we were not successful in detecting by *in situ* hybridization a specific expression pattern for *CrPaxE*. We synthesized three different RNA probes against different regions of the gene. To analyse the sensitivity of the different probes we took advantage of the transgenic fly lines generated for the targeted gene expression experiments (see below). We performed *in situ* hybridization with the different jellyfish RNA probes, using imaginal discs of flies mis-expressing UAS-*CrPaxE* under the control of the dpp-Gal4

driver. Thus, *CrPaxE* is expressed in a pattern resembling that of *dpp*. The RNA probes against the jellyfish gene, if functional, will bind to the mis-expressed *CrPaxE* and display a *dpp*-like staining.

The three probes tested, gave positive results when used in the transgenic imaginal discs (data not shown). For this reason we can exclude the possibility that the lack of staining detection in the jellyfish was due to inactivity of the probes. More likely, the levels of expression of *CrPaxE* in the manubrium of the jellyfish are very low, yet detectable by Real Time PCR, but too weak to be successfully detected with the *is situ* hybridization method.

3.2.5 *CrEya*

3.2.5.1 Real Time PCR expression data

The first indication of *CrEya* expression pattern given by Real Time PCR, showed that the gene is strongly, but not exclusively, expressed in the manubrium. Indeed, also the other body parts show a certain level of expression (Fig. 3.35). In addition, there are no significant age related differences in the expression pattern.

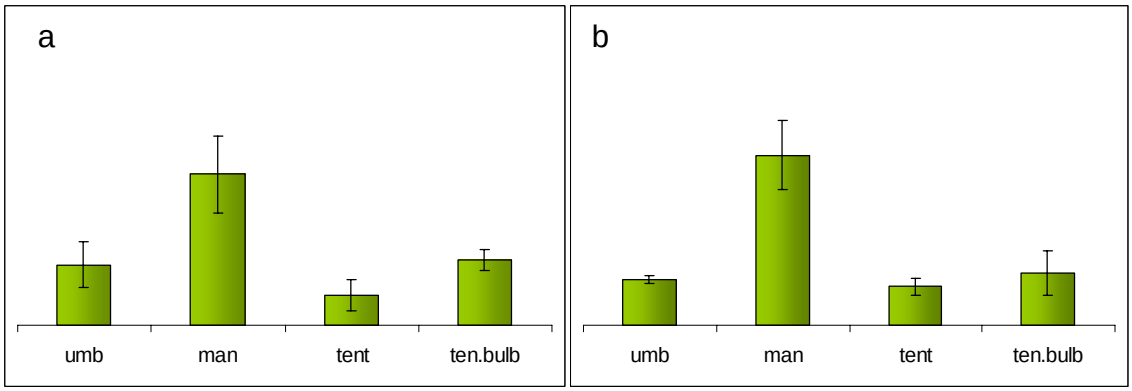

Fig 3.35 Real Time PCR expression data for *CrEya* in young and adult jellyfish In both young (a) and adult jellyfish (b) *CrEya* is strongly expressed in the manubrium, signal is anyway detectable also in the other body parts. All data are shown relative to the normalization with *EF1α*, and were repeated at least three times on independent samples, the mean values and error bars indicating standard deviation are shown. (umb: umbrella; man: manubrium; tent: tentacles; ten bulb: tentacle bulbs).

The comparison (performed on five different specimens) of the expression levels in young and adult jellyfish, did not show a significant difference (Fig. 2.36). It seems that the levels of expression at the different stages of development are very variable.

Therefore, despite being expressed in the same body part, namely the manubrium, *CrPaxA*, *CrPaxE* and *CrEya* show a different behavior, these data might reflect their different developmental roles.

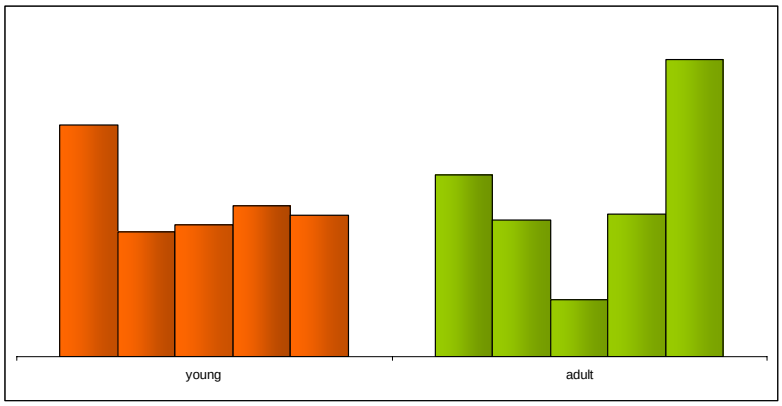

Fig. 3.36 Direct comparison of the expression of *CrEya* detected by Real Time PCR in tentacle bulbs of young and adult jellyfish The expression levels of *CrEya* in the manubrium of five young jellyfish (orange) are shown next to the expression levels in adult jellyfish (green). The levels of expression are not significantly different (t-test, p= 0.9). In adult jellyfish a certain level of variation is displayed. The expression levels are relative to *EF1α*.

The detection of *CrEya* expression in the tentacle bulbs prompted us to investigate whether this gene could play a role in eye regeneration. We employed the Real Time PCR approach already described for the expression analysis of *CrPaxB* during eye regeneration (see paragraph 3.2.3.1). We did not detect any significant difference in *CrEya* expression levels in the treated jellyfish (all eyes removed), compared to the basal expression in untreated jellyfish (Fig. 2.37) (t-test. Day0 to control: p= 0.1; day5 to control: p= 0.06; day10 to control: p= 0.4; day15 to control: p= 0.4). We can therefore conclude that *CrEya*, as well as *CrPaxA* and *CrPaxB*, is not directly involved in the development and regeneration of the eye.

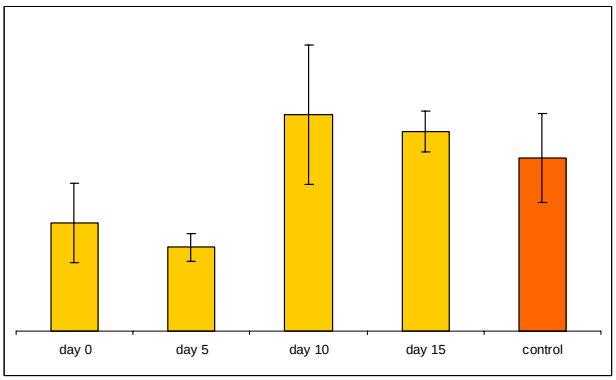

Fig. 3.37 Expression pattern of *CrEya* during eye regeneration in adult jellyfish analyzed by Real Time PCR Only the "All" eye removal method was employed. On the X-axe the days from excision are indicated. A single external control, represented by untreated jellyfish, has been added (orange column). All data are relative to *EF1α*. The expression analysis was repeated three times on independent samples, the mean values were calculated, error bars indicate standard deviation.

3.2.5.2 In situ hybridization expression data

In order to further analyse the expression pattern of *CrEya* in the manubrium we performed whole mount *in situ* hybridization experiments.

Surprisingly, also in the case of *CrEya*, as already shown for *CrPaxB*, the signal localises at the level of the oocytes in the gonads. In addition, *CrEya* expression was detected also in the eye. However, *CrEya* seems to display a stronger background in the manubrium when compared to *CrPaxB* (Fig. 3.39). *In situ* hybridizations for *CrPaxB* (Fig. 3.31), show a clear and distinct staining restricted only to the upper part of the manubrium, at the level of the gonads ectoderm, in the oocytes, whereas the lower part of the manubrium is completely free of staining. In contrast, the hybridization signal of *CrEya* is predominantly strong in the oocytes (Fig. 3.39, b), but in addition is present throughout the whole manubrium, including the lower part. Such a dispersed signal is also detected in young jellyfish (Fig. 3.39, c), but is absent in the hybridization with the sense probe. Therefore, it is likely that this signal does not reflect only background, but a specific staining. This would also explain the expression detected by Real Time PCR in young specimens.

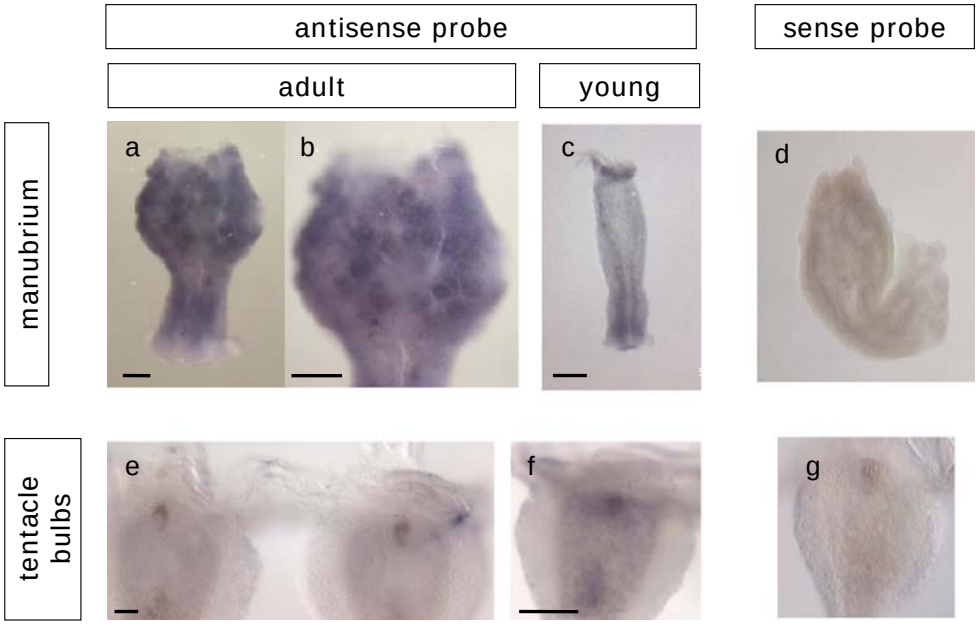

Fig 3.38 Whole mount *In situ* hybridization for *CrEya* In adult jellyfish *CrEya* is expressed in the oocytes (a and b), though the hybridization signal is detectable throughout the whole manubrium (a). Also in young jellyfish there is a basal level of staining in the entire manubrium (c). Both young (f) and adult (e) jellyfish show staining in the eye. Right panels, *in situ* hybridization with sense probe. (Scale bar is 500µm , except (c) and (f): 125µm; (e) and (g): 60µm).

The staining in the eye appears specific and persistent, being detected in both young (Fig. 3.39, f) and adult jellyfish (Fig. 3.39, e), in accordance with the Real Time PCR data.
The *in situ* hybridization on unfertilized eggs showed no staining (Fig. 3. 39). Therefore, also *CrEya* like *CrPaxB*, appears not be maternally contributed to the egg.

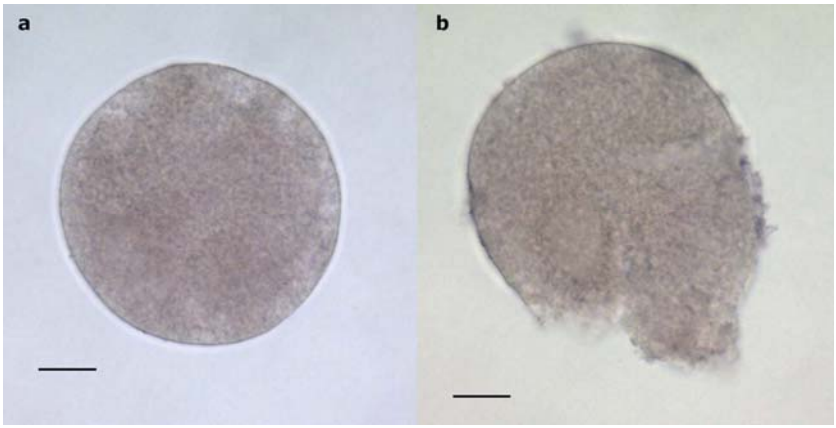

Fig 3.39 *CrEya* in situ hybridization on released eggs The *in situ* hybridization expression analysis does not display staining in unfertilized eggs; (a) *CrEya* anti sense probe; (b) *CrEya* sense probe. (Scale bar is 25μm).

3.3 Targeted gene expression in Drosophila

After studying the expression pattern of the isolated genes, the next step in order to unravel their role in the organism is to test their function. This is usually achieved by studying mutants or transgenic animals, or by knocking down the gene, e.g. by RNAi. Some of these methods are available in cnidarians. For example in *Hydra magnipapillata* some mutants are known, some transgenic lines have been generated (Wittlieb et al. 2006) and the RNAi gene silencing method has been established (Lohmann et al. 1999). Unfortunately, in *Cladonema radiatum* there are no functional assays available. During the present work we tried to establish an RNAi silencing method based on transfection, following a published protocol used with another hydrozoan, *Eleutheria dichotoma* (Jakob and Schierwater 2007). However, we were not successful in establishing the optimal protocol and, given the restricted number of animals available, we could not extensively try different factors.
For these reasons, in order to functionally characterize the cloned *C. radiatum* genes we made use of an heterologous organism and took advantage of the *Drosophila* genetics.

3.3.1 Generation of transgenic flies

We cloned the cDNAs corresponding to the different genes isolated from *Cladonema* in the pUAST vector. This vector allows for the targeted expression of genes upon induction of the yeast transcription factor Gal4. Gal4 binds to the Upstream Activating Sequences (UAS) carried from the vector in front of the insert (Brand and Perrimon 1993). Once generated, the transgenic flies containing the *C. radiatum* genes under the control of the UAS, we made use of a driver line carrying the Gal4 under the control of a *dpp* promoter. In this way the jellyfish genes are mis-expressed in the fly imaginal disk in a *dpp*-like pattern. By doing this we wanted to test whether the *Cladonema* genes had retained the capability to interact with the members of the Retinal Determination Gene Cascade. If this was the case, we would expect to observe the induction of ectopic eyes in the adult body parts corresponding to the imaginal disc where the genes were mis-expressed. Furthermore we also tested whether the *C. radiatum* genes were able to rescue eye mutant phenotypes caused by mutation in genes involved in eye development in *Drosophila*. In this sense it has been already reported that a *PaxB* gene from another cnidarian,the cubozoan jellyfish *Tripedalia cystophora*, is able to induce ectopic eyes in *Drosophila* and to rescue the *sparkling* phenotype (Kozmik et al. 2003).

3.3.2 *CrPaxA*

It has been previously found (Tschopp 2005, unpublished data), that *CrPaxA* is able to induce ectopic eyes in *Drosophila* in a small percentage of the progeny when mis-expressed under the control of the dpp^{blink}-Gal4 driver (Staehling-Hampton et al. 1994). The percentage of the offspring displaying ectopic eyes, was significantly increased when the level of Gal4 was amplified by using the dpp^{blink}-Gal4 driver in combination with an additional UAS-Gal4 construct (Tschopp 2005, unpublished data).

CrPaxA also displayed the capability to rescue the *Drosophila* spa^{pol} eye mutant (Tschopp 2005), caused by a mutation in the *Drosophila Pax2* gene (also called *sparkling*), when mis-expressed under the control of the eye specific enhancer *spa* of *DPax2* by the use of the *spa*-Gal4 driver (Fu and Noll 1997).

However, the jellyfish gene was not able to rescue the *eyeless* mutant phenotype (Tschopp 2005, unpublished data), when driven by the eye specific *ey*-Gal4 driver (Hazelett et al. 1998), it instead caused the development of headless flies.

3.3.3 *CrPaxB*

After injection we were able to recover eight different lines carrying the UAS-*CrPaxB* transgene.

All the lines were tested for ectopic eyes induction by crossing them separately with the *dpp*^{blink}-Gal4 driver line, as well as with the line carrying both *dpp*^{blink}-Gal4 and UAS-Gal4. In both cases we were not able to detect ectopic eyes (Fig. 3.40). This result is in contrast with what previously shown for *PaxB* from another cnidaria, the cubozoa *Tripedalia cystophora*, which was proved to be capable of ectopic eye induction (Kozmik et al. 2003). A mutated form of *CrPaxB*, changing 3 amino acids in the PD known to be essential for DNA binding capability (Czerny and Busslinger 1995), from those of the Pax2/5/8 subfamily to those of *Pax6*, surprisingly did not suffice to induce ectopic eyes either (Suga, pers. comm.).

Fig 3.40 *dpp*^{blink}-Gal4/UAS-*CrPaxB* flies Offspring of the cross of the flies carrying the UAS-*CrPaxB* transgene with the *dpp*^{blink}-Gal4 driver line. A female (on the left) and a male (on the right) adult flies are displayed. The progeny shows wild type phenotype and does not show any ectopic eye.

Considering the structural similarity between *CrPaxB* and the *Pax2/5/8* subfamily (see paragraph 3.1.3), we wanted to test whether it would be possible to rescue the *sparkling* mutant phenotype, caused by a mutation in the *Drosophila Pax2* gene, by using the jellyfish *CrPaxB* gene.

We therefore brought the UAS-*CrPaxB* lines in the *sparkling* background and then crossed these lines to the *spa*-Gal4 driver (Fu and Noll 1997). *CrPaxB* was not capable to rescue the *sparkling* mutant phenotype (Fig. 3.41). This is in contrast to what previously reported for *Tripedalia PaxB* (Kozmik et al. 2003), which was instead capable to substitute for *DPax2*.

Fig 3.41 *spa*-Gal4/UAS-*CrPaxB spa^{pol}* flies Offspring of the cross of the flies carrying the UAS-*CrPaxB* in the *spa^{pol}* background with the *spa*-Gal4 driver line. A female (on the left) and a male (on the right) adult flies are displayed. The progeny does not display rescue of the *sparkling* phenotype. (Picture courtesy of Hiroshi Suga).

In order to account for this unexpected incapability, the protein sequence of CrPaxB was carefully analyzed. Thus, a methionine not in frame, prior to the starting methionine (see Fig. 2.4, highlighted in pink) was identified. The presence of this methionine might interfere with the correct translation of the CrPaxB protein and might therefore be the reason for the displayed incapability to rescue the *sparkling* phenotype. For this reason the methionine was removed, and the construct containing the new sequence of *CrPaxB* used for generating transgenic flies (Suga et al. submitted). The fly lines thus obtained were tested for both ectopic eyes induction and *sparkling* eye mutant phenotype rescue. Also this construct did not show any ectopic eye induction, but it was capable to successfully rescue the *sparkling* mutant phenotype (Fig. 3.42) (Suga et al. submitted). Therefore, the presence of the extra methionine seemed to be the reason for the inability of the first construct to rescue the *sparkling* phenotype.

CrPaxB was also tested for the rescue of the *ey*$^{J5.71}$ mutant (Kammermeier et al. 2001; Punzo et al. 2001). No increase of the percentage of flies showing bigger or normal eyes compared to the mutant phenotype was observed (Suga, pers. comm.).

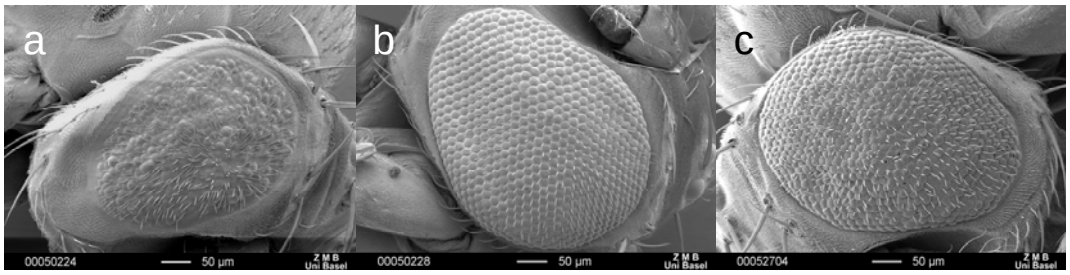

Fig 3.42 Scanning Electron Microscopy showing the results of *sparkling* rescue experiments by *CrPaxB* In (a) is displayed the eye of a *sparkling* mutant, in (b) the rescue of the *sparkling* phenotype by *DPax2* and in (c) a fly *spa*Gal4/UAS-*CrPaxB.* Although less efficiently than *DPax2*, *CrPaxB* is capable to rescue the *sparkling* phenotype (pictures courtesy of H. Suga).

3.3.4 *CrPaxE*

Similarly, we carried out the targeted gene expression in *Drosophila* also for *CrPaxE*. After injection we recovered seven different transgenic fly lines containing the UAS-*CrPaxE* construct.

We made use of the same experimental approach already described for *CrPaxB* (see paragraph 3.3.3). None of the fly lines analysed succeeded to induce ectopic eyes under the control of the dpp^{blink}-Gal4 or dpp^{blink}-Gal4/UAS-Gal4 driver lines (Fig. 3.43).

Fig 3.43 dpp^{blink}-Gal4/UAS-*CrPaxE* flies Offspring of the cross of the flies carrying the UAS-*CrPaxE* transgene with the dpp^{blink}-Gal4 driver line. A female (on the left) and a male (on the right) adult flies are displayed. The progeny results wild type and does not show any ectopic eye.

In addition, none of the lines was capable to rescue neither the *sparkling* (Fig. 3.44), nor the *ey*$^{J5.71}$ mutant phenotype

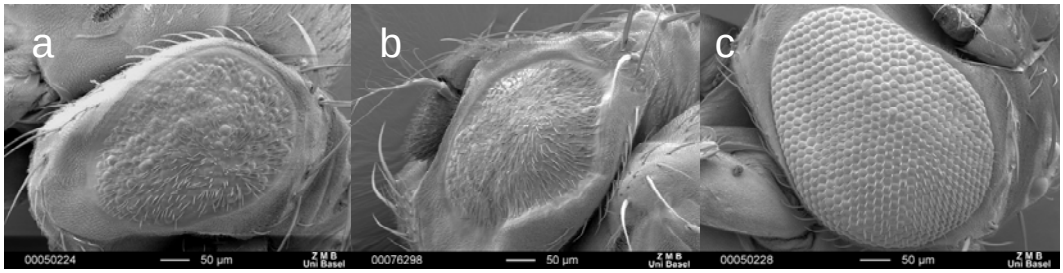

Fig 3.44 Scanning Electron Microscopy showing the results of the *sparkling* rescue experiments by *CrPaxE* In (a) the eye of a *sparkling* mutant is displayed, in (b) a fly *spa*Gal4/UAS-*CrPaxE* and in (c) the rescue of the *sparkling* phenotype by *DPax2.* Clearly *CrPaxE* does not display rescue.

3.3.5 *CrEya*

The targeted gene expression approach in *Drosophila* was employed also in the case of *CrEya*. We cloned both *CrEya* and the splicing variant, *CrEya-alt* (see paragraph 3.1.5), in the pUAST vector, and injected these constructs in *Drosophila* embryos. We recovered six transgenic lines for *CrEya* and four lines for *CrEya-alt*.

The capability of *Drosophila eya* to induce ectopic eyes when mis-expressed under the control of the *dpp*^{blink}-Gal4 driver was already described (Bonini et al. 1997). In particular, 10% of the flies showed ectopic eyes when UAS-*eya* and *dpp*^{blink}-Gal4 (Staehling-Hampton et al. 1994) were in single copy, and a considerably higher percentage (96%) when UAS-*eya* and *dpp*^{blink}-Gal4 were in two copies. However, it has also been shown that the efficiency of ectopic eyes induction by *Drosophila eya* drastically depends on the integration site of the construct in the chromosome. It has been reported that the percentage of offspring displaying ectopic eyes can decrease down to 2% (Pignoni et al. 1997).

All the lines for both *CrEya* and *CrEya-alt* did not induce ectopic eyes neither in heterozygosity nor in homozygosity when driven by *dpp*^{blink}-Gal4. In order to increase the level of the produced CrEya protein, we also crossed the *dpp*^{blink}-Gal4 driver to a line carrying multiple UAS-Gal4 insertions (Hassan et al. 2000). Also in this case we were not able to detect ectopic eyes.

Considering the different efficiency in ectopic eyes induction already reported in the literature (Pignoni et al. 1997) we did not consider this as a final result, and in order to be sure whether *CrEya* still retains the capability to interact with the RDGC, we tested the efficiency of *CrEya* to induce ectopic eyes in synergy with *Drosophila sine oculis* (*so*). It is known that the co-

expression of *so* and *eya* can greatly increase the percentage of flies showing ectopic eyes induction (Pignoni et al. 1997).

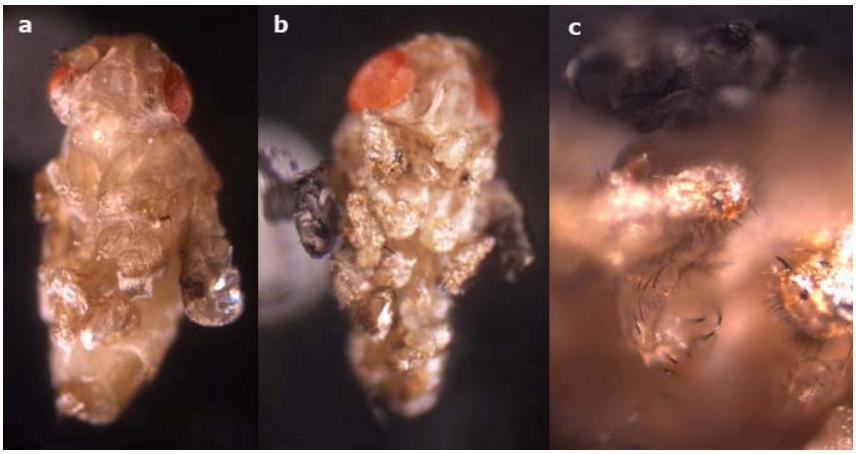

Fig 3.45 UAS-*CrEYA*; UAS-*so* synergistic ectopic eye induction In (a) is displayed a UAS-*CrEya*; UAS-*Dso*/ *dpp*^{blink}-Gal4 fly that clearly shows developmental defects but not ectopic eyes. In (b) UAS-*Deya* UAS-*Dso*; *dpp*^{blink}-Gal4, showing ectopic eyes on the legs. In (c) close up on the legs showing the ectopic red pigmented cells.

We therefore took advantage of the UAS-*Dso* lines available in our laboratory (kindly provided by Makiko Seimiya), and crossed them with our UAS-*CrEya* lines. Then we crossed the homozygous UAS-*CrEya*; UAS-*Dso* with the *dpp*^{blink}-Gal4 driver, and screened for ectopic eyes. However, although in synergy with *Drosophila so*, the *CrEya* and *CrEya-alt* lines tested still did not show the capability to induce ectopic (Fig. 3.45).

As most of the RDGC genes, also mutations in the *eya* gene give rise to eye mutant phenotypes (see paragraph 2.3.2).

The *eya*^{2} mutant flies are completely eyeless due to cell death of eye progenitor cells anterior to the morphogenetic furrow (Bonini et al. 1993). It has been shown that the mouse *eya2* gene is able to restore normal eye development in *eya*^{2} mutant flies, when expressed under the control of the *ey*-Gal4 driver (Pignoni et al. 1997). Therefore, we tested for the ability of the two isoforms from *C. radiatum* to rescue the *Drosophila eya*^{2} mutant phenotype. The UAS-*CrEya*, UAS-*CrEya-alt* lines and the *ey*-Gal4 driver were brought in the *eya*^{2} background. The UAS lines were then crossed with the driver line. *CrEya* and *CrEya-alt* did not display any capability to functionally substitute for the fly gene during eye development, failing to show any improvement of the mutant phenotype (Fig. 3.46). We can therefore conclude that both the isoforms of *CrEya* seem not to be able to interact with the fly RDGC.

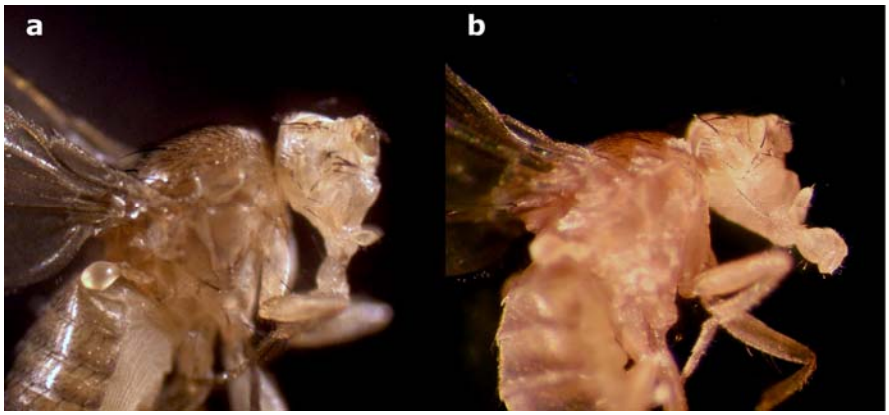

Fig 3.46 Rescue of the *eya*^{2} mutant phenotype In (a) an *eya*^{2} UAS-*CrEya*/*ey*-Gal4 fly is displayed, in (b) an *eya*^{2} mutant fly is shown. The *eya* mutant phenotype is not improved by the *C. radiatum* gene.

4 Discussion

4.1 RDGC in *C. radiatum*

Genes that belong to the families composing the Retinal Determination Gene Network (RDGC) have been isolated in several cnidarian species during the last years (see paragraph 2.4). However, to date a comprehensive approach for the identifications of members from all the families composing this network has not been undertaken.

Here, we report the first attempt to describe the RDGC complement in a single cnidarian species, *Cladonema radiatum*, a hydrozoan that displays a metagenetic life cycle, with the alternation of two morphologically different stages: a sessile polyp and a free swimming medusa. This characteristic of *Cladonema* is an advantage compared to *Hydra*, one of the best known hydrozoan model system, which displays only the polyp stage and has a basic nervous system, characterized by a diffuse nerve net. *Hydra*, conversely to most Cnidaria, is a freshwater organism. Usually organisms that live in a different environment show morphological and functional specialization in order to adapt to the different conditions, for this reason *Hydra* is very divergent compared to other members of this phylum, as a consequence in many instances it does not represent the appropriate cnidarian model.

Another important hydrozoan model system is *Podocoryne carnea*. The larval development of this species is well studied and thus offers a reliable embryological system. Moreover, the process of medusa bud formation has been studied in great details. However, for the investigation of the cnidarian photosensitive structures *Podocoryne* has the disadvantage of lacking sensory organs. *Cladonema* instead, carrying from 7 to 10 true lens eyes, appears to be the ideal hydrozoan organism to study the photoreceptors of Cnidaria and the conservation of the RDGC in the specification of the eyes. In addition to the development of the eye during the medusa bud formation, the eye regeneration capability of this species (see paragraph 2.2.5), which follows the same course as ontogeny (Weber 1981b), offered another method to study the role of the genes involved in eye determination.

All these aspects of *C. radiatum* raised the interest in the eye development of this species, leading to the isolation of three members of the *Six* family (Stierwald et al. 2004) and a *PaxA* gene (unpublished data, Stierwald 2004; Tschopp 2005).

Here, we have presented the characterization of two more members of the *Pax* family, *CrPaxB* and *CrPaxE*, the latter belonging to a novel cnidarian subfamily. We have also

described, for the first time in Cnidaria, an *eya* homolog, *CrEya*. Instead, despite extensive search, we did not isolate a *dac* homolog.

4.1.1 *Pax* genes repertoir in Cnidaria

In the last years, a wealth of *Pax* genes has been isolated from several cnidarian species. This has lead to the identification of four *Pax* classes in Cnidaria: *PaxA*, *PaxB*, *PaxC* and *PaxD* (Fig. 4.1). However, the relationships among these genes and the bilaterian classes are not simple, due to the high degree of divergence in their sequences.

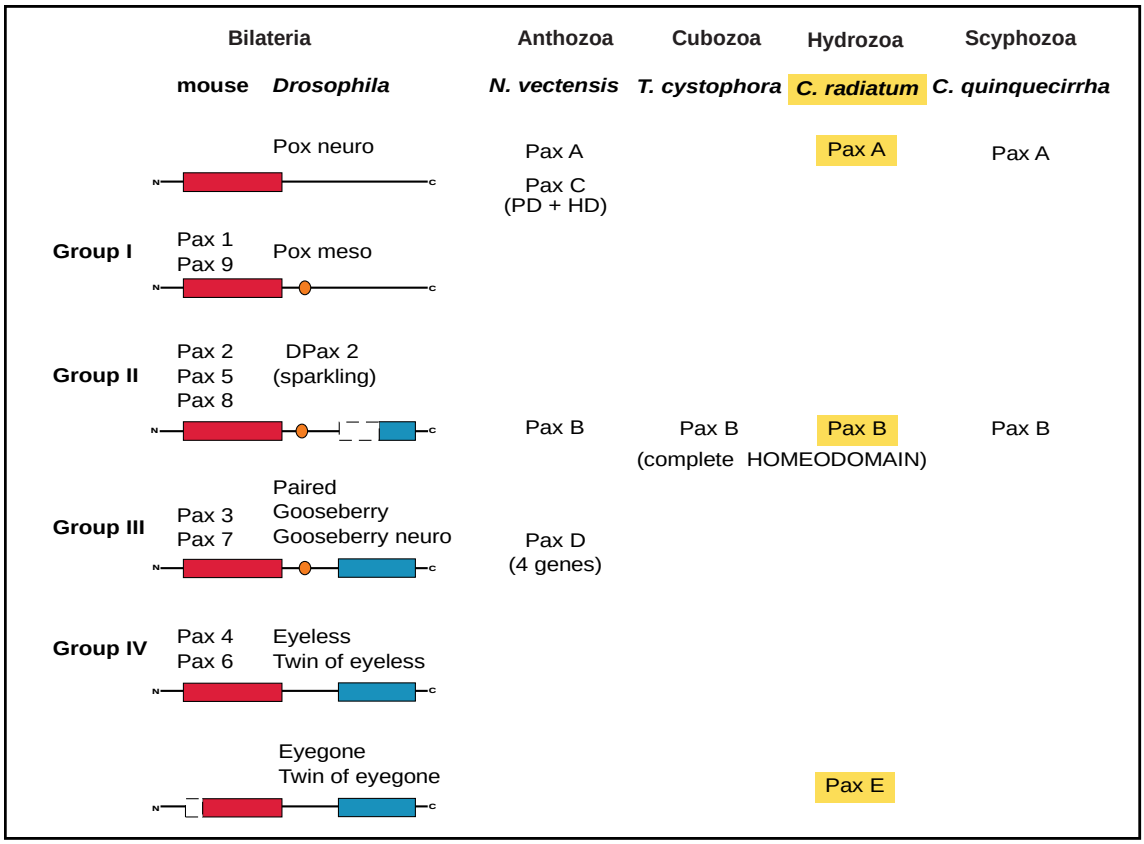

Fig 4.1 Pax proteins subfamilies, in Bilateria and Cnidaria The subfamilies of *Pax* genes are shown in Bilateria (mouse and *Drosophila*) on the left and in the four cnidarian classes (Anthozoa, Cubozoa, Hydrozoa, Scyphozoa) on the right. Only representative species for each class are shown. For *Nematostella vectensis*, complete genome sequences are available. Below each bilaterian subfamilies, the conserved domains are exemplified (red: Paired Domain, orange: Octapeptide, blue: Homeodomain). In yellow are highlighted the *Pax* genes identified in *Cladonema*, (see also paragraph 2.4 and Fig. 2.13).

The assignment of the cnidarian *Pax* genes to the bilaterian classes based on sequence comparison and phylogenetic analysis is summarized in Fig. 4.1.

Cnidarian *PaxA* is closely related to *pox neuro* from *Drosophila* and resembles the fly gene in lacking the HD.

PaxB genes cluster with the *Pax2/5/8/DPax2* group. However the main difference among the cnidarian and bilaterian genes resides in the HD. In the case of the bilaterian genes, the HD is partial, whereas in *PaxB* genes the HD is complete. As a result, PaxB HD shows homology to the HD of Pax3/7 or Pax4/6 classes.

Two more *Pax* classes have been found in Cnidaria: *PaxC* and *PaxD*. However, to date these genes have been isolated only from the Anthozoa and might have been lost in Medusozoa (Matus et al. 2007). The only putative *PaxC* gene in Medusozoa has been found in the Cubozoa *C. marsupialis* (Stierwald 2004, unpublished data), however the cDNA sequence is partial and needs to be confirmed.

PaxC genes in Cnidaria have both the PD and the HD, but phylogenetic analysis indicates that they are more closely related to cnidarian *PaxA* than to any other bilaterian group, suggesting that this subfamily might be cnidarian specific (Miller et al. 2000).

PaxD seems to be the least ambiguous of all the cnidarian *Pax* genes identified so far. It displays a PD and HD that can be clearly assigned to the *Pax3/7/Paired/gooseberry* subfamily (Miller et al. 2000). More recently in *Nematostella*, four different *PaxD* genes have been cloned, and it has been shown that three of them besides the PD and HD also possesses the OCT (Matus et al. 2007).

The complete genome sequences of *N. vectensis* and *Hydra* did not lead to the identification of new cnidarian *Pax* classes. Therefore, despite the study of *Pax* genes in Cnidaria was initially undertaken in order to search for a cnidarian *Pax6* gene (Sun et al. 1997), to date no true homologs of the *Pax4/6* subfamily have been isolated from Cnidaria.

However, in order to improve the phylogenetic analysis of the *Pax* genes in Cnidaria, it is necessary to have a broader taxon sampling. Here, we reported the sequences of two *Pax* genes from a hydrozoan species that has never been investigated before, *Cladonema radiatum*, and we discuss the possible function of a previously identified *Pax* gene, *CrPaxA* (Stierwald 2004; Tschopp 2005, unpublished data). This has surprisingly led to the identification of a new *Pax* class (see below).

By means of homology PCR we were able to isolate a *PaxA* and a *PaxB* gene, but we were not able to find a *PaxC* or a *PaxD* gene (see Fig. 4.1). Our findings are in agreement with the previously reported data from other Hydrozoa and with the data from the complete genome

sequences of *Hydra magnipapillata* (Sun et al. 1997; Groger et al. 2000; Sun et al. 2001), and support the hypothesis that these two genes have been lost in this class.

Our results present the two following novelties compared to what was already described in other Cnidaria:

1. For *CrPaxA*, in addition to the PD we were able to identify for the first time a conserved OCT motif (Suga et al. submitted).
2. The third gene isolated, *CrPaxE*, differs from all the other known cnidarian classes in that it has an incomplete PD (see Fig. 3.9), resembling in this feature *Drosophila eyegone*. The phylogenetic analysis agrees with the structural data: *CrPaxE* clusters together with *eyegone* and other *eyegone*-like genes (see Fig. 3.11), thus forming a possible new class: the *PaxE/eyegone* class (Fig. 4.1). *In silico* search on the genome sequences of *Hydra* allowed us to identify a *PaxE*-like gene (Suga et al. submitted), therefore bringing support for the presence of this subfamily at least in the Hydrozoa class (see 4.1.1.4).

The *Pax* genes isolated in *Cladonema* correspond to all the known genes present in *Hydra* genome sequences (Fig. 2.13), thus our homology PCR based approach very likely allowed us to isolate the whole *Pax* genes complement of *Cladonema*.

Two main theories for the evolution of *Pax* genes have been formulated so far.

A first hypothesis proposed by Galliot and Miller (Galliot and Miller 2000) suggests a scenario in which a *PaxA*-like ancestor, displaying the presence only of the PD, independently captured the OCT and the HD, giving rise to the different subfamilies.

This hypothesis however has been challenged by other authors that brought forward more convincing evidences (Breitling and Gerber 2000; Miller et al. 2000; Hadrys et al. 2005). Because of 3D structure similarity, it has been proposed that the PD derived from a proto-Pax-transposase, to which, by a single capturing event, both the OCT and the HD were added, giving rise to the ancestor of all metazoan *Pax* genes, the so called *Ur-Pax* (Breitling and Gerber 2000). The different *Pax* classes were then generated by duplication events that might have occurred already before the divergence of Porifera and Eumetazoa (Hoshiyama et al. 1998; Miller et al. 2000). Therefore, they propose that the domains missing in some of the *Pax* classes, such as the HD in *PaxA*, have been secondarily lost.

The present identification of the OCT in the *PaxA/pox neuro* class, demonstrates the presence of the OCT in representatives of all the cnidarian subfamily. This fact lends support to the hypothesis that in the ancestral *Pax* gene all the three motifs were already present and have been subsequently lost.

4.1.1.1 *Pax* genes in the eye of *C. radiatum*: the involvement of *PaxA*

Of the three *Pax* genes isolated, only *CrPaxA* is expressed in the eye, while *CrPaxB* and *CrPaxE* are expressed in the manubrium. This makes *CrPaxA* the most interesting *Pax* gene candidate for the exertion of a role in the eye. However, the expression analysis suggested that *CrPaxA* is not directly involved in the eye development and regeneration. The constant presence of *CrPaxA* expression in the adult eye rather indicates an important role for this gene in eye maintenance. This is consistent with data from the planarians *Dugesia japonica* and *Girardia tigrina*, where *Pax6* is expressed in the adult eyes but not during the eye development (Pineda et al. 2002). The continuous expression of *Pax6* has been shown also in the adult vertebrate retina in both mouse and humans (Koroma et al. 1997; Stanescu et al. 2007). These findings highlight the importance of Pax6 not only in the embryo but also in the adult eye. Therefore, although *CrPaxA* may not be directly involved in the eye development and regeneration, its importance at the level of the eye is underlined by the presence of expression in the eyes of adult jellyfish.

The significance of the expression of *CrPaxA* in the eye is also strengthened by the targeted gene expression in *Drosophila*: *CrPaxA* is the only *Pax* gene of *Cladonema* able to induce ectopic eyes (Tschopp 2005, unpublished data). This argues for the capability of this gene to interact with the fly members of the RDGC. This finding becomes even more interesting if we consider that *Drosophila pox neuro*, similarly to *Paired*, *gooseberry* and *Pox meso*, can not induce ectopic eyes (Kozmik et al. 2003). Therefore, despite of the high similarity between *CrPaxA* and *pox neuro*, the jellyfish gene retains a broader spectrum of functions compared to the more specialized fly gene.

This wider range of activities is further confirmed by the fact that *CrPaxA* is able to rescue the *Drosophila Pax2* mutant, *sparkling*, suggesting that this gene can also substitute for the function of *Pax2*. The same capability was also shown for *CrPaxB*, confirming its similarity to the *Pax2/5/8* subfamily.

This suggests a redundancy in the role of the *Pax* genes in the last common ancestor of Cnidaria and Bilateria and supports the origin of the current *Pax* genes via duplication followed by divergence (Gehring 2004). The ancient duplication, that likely occurred before the split of cnidaria from bilateria (Miller et al. 2000; Hoshiyama et al. 2007), or even before the split of sponges from eumetazoa (Hoshiyama et al. 1998), allowed the duplicated genes to start playing new roles, while maintaining some of their previous features (Ohno 1970). This scenario is in good agreement with the overlapping functions of several *Pax* genes and their

capability to substitute for each other. The redundancy of functions displayed by *Cladonema Pax* genes might therefore reflect the properties of the ancestral *Pax* genes.

4.1.1.2 *PaxA* in nematogenesis

The expression of *CrPaxA* in the tentacle bulb, besides the eye, is also strongly localised in the Tentacle Bulb Ectoderm (TBE) (see Fig. 3.19 and Fig. 3.20).

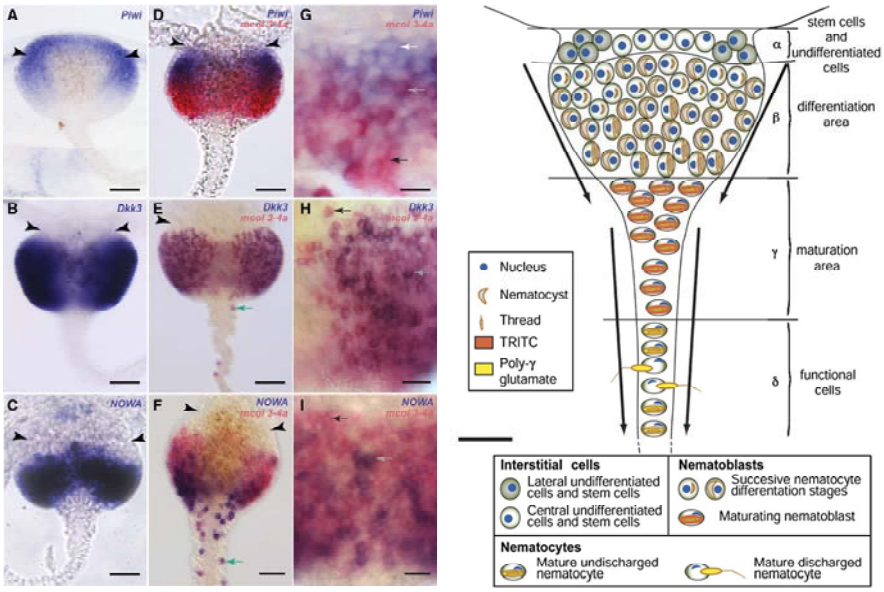

Fig 4.2 Nematogenesis in *Clytia hemisphaerica* In the left panel the *in situ* hybridizations for three genes involved in nematogenesis are displayed: *Piwi*, a stem cell marker, *Dkk3*, a late regulator, and the structural gene NOWA; the red staining shows the expression of the *mcol3-4a* gene. In the right panel, a model illustrating the proximo-distal distribution of nematogenesis stages in the TBE is shown (adapted from Denker et al. 2008).

Denker and co-authors showed in a recent paper (Denker et al. 2008) that in another hydrozoan jellyfish, *Clytia hemisphaerica*, the main nematogenesis area is represented by the TBE (Fig. 4.2). The authors isolated four genes already known to be involved in *Hydra* nematogenesis: *minicollagens*, encoding a protein which is the major component of the nematocyst wall (Kurz et al. 1991) and therefore used as a marker for the nematogenesis area; *Piwi*, a stem cell marker (Seipel et al. 2004); *Dkk3*, which is expressed in differentiating nematoblast (Fedders et al. 2004); *NOWA*, which is involved in minicollagen condensation (Engel et al. 2002). They performed single *in situ* hybridization for *Piwi*, *Dkk3* and *NOWA*. In addition they also performed double *in situ* hybridization with *minicollagen* (*mcol 3-4a*) for each of these genes. By doing this, they were able to identify the relative position of the expression of these genes

in the nematogenesis area (Fig. 4.2). Interestingly, they could show that these genes are expressed in a proximo-distal pattern, with *Piwi* being expressed more proximally, *Dkk3* more medially and *NOWA* more distally, therefore indicating a spatially and temporally ordered progression of the nematocyte differentiation along the tentacle bulb.

The expression pattern of *CrPaxA* at the level of the tentacle bulbs (see Fig. 3.19 and Fig. 3.20) is particularly similar to that of *Dkk3*, suggesting a role for *CrPaxA* in nematocytes differentiation. In addition, cryosections display that the *in situ* hybridization signal for *CrPaxA* is localised in the TBE (see Fig. 3.25), which is densely populated by nematoblasts at various stage of differentiation. Taking into consideration all these evidences, although more molecular markers would be required, we propose a role for *CrPaxA* in nematogenesis. Remarkably, nematocytes are usually regarded as mechanosensory cells, belonging to the nervous system of Cnidaria (Westfall 1966; Miljkovic-Licina et al. 2004) (see paragraph 2.2.2). Similarly, the fly homolog of *CrPaxA*, *pox neuro* is also involved in the differentiation of sensory organs and in the development of chemosensory bristles (Dambly-Chaudiere et al. 1992; Awasaki and Kimura 1997). Hence our data could argue for a conserved role of the *PaxA/pox neuro* family in the nervous system of both Cnidaria and flies.

4.1.1.3 Is *PaxB* involved in oocytes differentiation?

CrPaxB is specifically expressed in oocytes at various stages of development (see Fig. 3.30).

In accordance with this expression pattern, *CrPaxB* is not able to induce ectopic eyes. The expression of *CrPaxB* in the oocytes is detectable already at very early stages of maturation and it lasts until the oocytes reach the final differentiation state before the release. However, no expression was detected in released eggs. This argues for a possible function of *CrPaxB* in oocytes differentiation rather than a role as a maternally contributed factor.

Our knowledge about germ cells development in Cnidaria is very limited, and it is still matter of debate whether germ cells have a somatic origin or derive from an I-cell subpopulation responsible only for producing gametes. The investigation of germ cells differentiation is complicated by the fact that genes that are expressed during gametogenesis show often also a somatic expression. The stem-cell marker *Piwi* (*Cniwi*) and the gene *nanos*, expressed in *Drosophila* primordial germ cells, have been shown to be localized in *Podocoryne* and *Nematostella* not only in both male and female gonads, but also in different tissues throughout the whole development (Seipel et al. 2004; Torras et al. 2004; Extavour et al. 2005). Considering the exclusive oocytes expression of *CrPaxB* in the adult jellyfish, this gene may

represent a specific marker to follow the origin of germ cells during embryonic and larval development in *Cladonema*.

To date *CrPaxB* is the first cnidarian *Pax* gene to be related to oocytes development. *PaxB* genes from other cnidarian have been shown to be mostly involved in nervous system and sensory structure development. *PaxB* from *Podocoryne carnea* is expressed in the planula ectoderm, whereas in the adult jellyfish it localizes in the endoderm of the radial canals and of the tentacle bulbs (Groger et al. 2000). Histological analysis of the expression in the tentacle bulbs suggested that in *Podocoryne PaxB* is involved in nervous system development. In the cubozoan jellyfish *Tripedalia cystophora*, the expression of *PaxB* is reported in the sensory organs of the medusa, which is to say the retina, the lens and the statocysts (Kozmik et al. 2003). In *N. vectensis*, *PaxB* is expressed in the epidermis during the larval development, but at the stage of polyp formation it starts to be expressed in the endoderm in a ring around the mouth (Matus et al. 2007). Although the adult polyp of *Nematostella* is not comparable to adult medusa, it is interesting that for both *P. carnea* and *N. vectensis* the expression is mainly ectodermal during larval development and becomes endodermal during the adult stage. In addition, this expression is the only endodermal expression detected for *Pax* genes in *Nematostella* and might indicate the involvement in the patterning of the circum-oral nerve ring (Matus et al. 2007), in accordance with the data from *Podocoryne* and *Tripedalia*.

In summary, the new and peculiar expression pattern of *CrPaxB* confirms the variety of roles that *Pax* genes can exert in different cnidarian species. In addition, it highlights once again the importance to collect expression data from a wide range of cnidarians in order to better understand the evolution and the ancestral functions of *Pax* genes.

4.1.1.4 A novel *Pax* gene subfamily in Cnidaria: *PaxE*

CrPaxE differs from all the other cnidarian Pax proteins isolated to date in that it has an incomplete PD, which lacks the PAI subdomain. The residual RED subdomain shows high homology to the RED subdomain of PaxB, whereas the HD mostly resembles PaxC HD (see paragraph 3.1.4).

Two Pax proteins with an incomplete PD are know in Drosophila: Eyegone (Eyg) and Twin of eyegone (Toe) (Jun et al. 1998; Yao et al. 2008), which arose by gene duplication and diversification. They both lack the PAI subdomain and rely on the RED and HD for their DNA binding capability. *eyg* and *toe* are involved in eye development, but do not appear to play redundant roles (Yao et al. 2008). In mammals, alternative splicing of the exon 5a of the

Pax6 gene leads to the insertion of 14 amino acids in the PD and disrupts the PAI subdomain, the splicing variant thus obtained is called Pax6(5a) (Epstein et al. 1994). As a consequence, the PAI domain of Pa6(5a) is not functional and the DNA binding activity is exerted only by the RED subdomain (Epstein et al. 1994). *Pax6* and *Pax6(5a)* are both expressed in eye, brain, spinal chord and olfactory epithelium, but the 5a splicing variant is expressed at lower levels. Therefore, despite the fact that mammals do not have a specific gene that encodes a Pax protein lacking the PD, they make use of alternative splicing in order to generate proteins similar to Eyg and Toe.

Due to the different structure and to the lack of available sequences, *eyg* was not assigned to any *Pax* subfamily. The isolation of *CrPaxE* prompted us to search for similar genes in other species. We were able to successfully identify *eyg*-like genes from two species of nematodes, from mosquito and from *Hydra magnipapillata* (Suga et al. submitted). Phylogenetic analysis of these genes supported their grouping in a new *Pax* genes subfamily, the *PaxE/eyg* class. In addition, it strongly suggested that *CrPaxE* is the homolog of Drsophila *eyg* and *toe* and that the fly genes originated by a *PaxE*-like ancestor via gene duplication.

Previous studies have indicated that the cnidarian-bilaterian ancestor possessed at least three *Pax* genes, *PaxA/C/pox neuro*, *PaxB/2/5/8*, and *PaxD/3/7* (Matus et al. 2007). Our work suggests the presence of a fourth subfamily, *PaxE/eyegone* (Suga et al. submitted), in which the amino terminal part of the PD was lost after the separation from the other *Pax* subfamilies. However, more data from non-chordate animals, other cnidarian classes and other lower metazoa are needed in order to better understand the relationships among the *Drosophila*, mammalian and cnidarian genes. Specifically, the presence of a *PaxE/eyegone* subfamily has to be validated by more data from a wide range of taxa.

4.1.2 Identification of the first *eya* homolog in Cnidaria

We have described for the first time in Cnidaria an *eya* gene homolog, *CrEya*. Furthermore, screening the genome sequences of *H. magnipapillata* and *N. vectensis*, we could also find one homolog in both species.

CrEya displays high homology levels with the ED1 C-terminal domain of *Drosophila* and mammalian Eya, but unlike the bilaterian genes, the jellyfish homolog does not display conservation of the N-terminal ED2 domain, although this region of the protein still appears to be rich in P/S/T repeats.

In *Cladonema*, *CrEya* is expressed in both the eyes and oocytes. The presence of *CrEya* expression in the eye is in good agreement with what has been reported for mammals and *Drosophila*. However, *CrEya* did not seem to be up-regulated during eye regeneration and did not induce ectopic eyes in *Drosophila* neither on its own nor in combination with *so*. Furthermore, the jellyfish gene was not able to rescue the Drosophila *eya*^{2} mutant phenotype. These results are probably due to the low level of conservation in the N-terminal region between the jellyfish protein and the fly protein. Indeed, it has been shown in *Drosophila* that the ED2 domain and the P/S/T rich region are necessary for the transactivation activity of Eya and therefore for ectopic eye induction *in vivo* (Silver et al. 2003). This region is not required for Eya-So interaction, for which instead the ED1 domain accounts (Silver et al. 2003). Therefore, it is possible that the *Cladonema* Eya and So proteins are still able to interact with each other, and that the capability to form this complex dates back before the cnidarian-bilaterian divergence. All these lines of evidence taken together indicate an involvement of this gene in the eye maintenance rather than in the eye development, as already shown for *CrPaxA* (see paragraph 4.1.1.1).

The localisation of *CrEya* expression in the oocytes of Cladonema correlates with what has been already described for *eya* during *Drosophila* gametogenesis. In flies *eya* is expressed in the somatic cells that assist the germ line differentiation. *eya* seems to repress the polar cell specification during spermatogenesis (Bai and Montell 2002), whereas it promotes the somatic gonadal precursor cells differentiation during oogenesis (Boyle et al. 1997). Although detailed studies have not been undertaken, the presence of somatic nurse cells in *Cladonema* is not likely, since histological studies in *Podocoryne* did not reveal such cells (Boelsterli 1977). Moreover, we demonstrated that the expression of *CrEya* in *Cladonema* is specifically localised in the germ cells. It is tempting to speculate that genes that in Bilateria are involved in the differentiation of gonadal somatic cells, could instead be directly involved in the germ cells differentiation in Cnidaria, where the nurse somatic cells are missing. In the controversy whether germ cells originate in Cnidaria by transdifferentiation from somatic cells or via differentiation of I-cells, *Cladonema* would represent a suitable model to learn more about this process in Cnidaria. In this sense, it will be interesting in the future to compare the expression patterns of the *CrEya* with the *eya* homologs found in *Nematostella* and *Hydra*.

4.2 The RDGC in Cnidaria and its flexible deployment

4.2.1 Conservation of the RDGC in Cnidaria

We have demonstrated the conservation of the *Pax* and *Eya* gene families in the Hydrozoa *Cladonema radiatum*. The presence of the *Six* family genes has been previously reported (Stierwald et al. 2004). Our study allows for the first time the comparison of the expression pattern and of the putative functions of different members of the RDGC in a cnidarian species.

We have demonstrated that both *CrPaxA* and *CrEya* are expressed in the eye, where also two *Six* genes (*Six1/2-Cr* and *Six3/6-Cr*) are expressed (Stierwald et al. 2004).

Furthermore, we have shown that *CrPaxA* is able to induce ectopic eyes in *Drosophila* (Tschopp 2005, unpublished data), which plausibly indicates the conservation of the capability of this gene to interact with the members of the retinal specification network in the fly. In the case of CrEya, the low level of homology of the N-terminal region to the bilaterian proteins is likely to account for its incapability to induce ectopic eyes. However, the domain essential for the interaction with the Six proteins is instead highly conserved, suggesting a similar conservation for the RDGC itself.

Both *CrPaxA* and *CrEya* may not be directly involved in eye development and regeneration, but rather exert an important function in the eye maintenance. In contrast, *Six1/2-Cr* and *Six3/6-Cr*, which are up-regulated during eye regeneration (Stierwald et al. 2004) may be directly involved in eye specification. These findings resemble the situation in planarians. In *Girardia tigrina* it has been demonstrated that *Six-1* is essential for eye regeneration, whereas *Pax6* is not required (Pineda et al. 2000; Pineda et al. 2002). Thus, it is plausible that the genetic control of eye development in *Cladonema*, similarly to planarians, has undergone a peculiar adaptation and that other genes of the network compensate or substitute for the role of *Pax6*.

Nevertheless, our data prove the importance of *CrPaxA* and *CrEya* in eye maintenance. The future development of functional assays in *Cladonema*, such as RNAi, will help to identify the roles of these genes and to test the possible interaction among them at the level of the eye.

Despite extensive search, we were not able to isolate *dac* homologs from *Cladonema*. However, by *in silico* screening we could find a gene belonging to this family in the genome of *Nematostella* but not in the genome of *Hydra*. The absence of a homolog from *Hydra* has

to be carefully interpreted. It is expected that the gene complement of *Cladonema* is more similar to that of another hydrozoan like *Hydra*, than to that of an anthozoan such as *Nematostella*. But it is important not to underestimate the high divergency of *Hydra* compared to other cnidarians. Although the fact that both *Hydra* and *Cladonema* do not have a *dac* homolog makes it conceivable to assume that this class has lost the *dac* family, we cannot exclude the possibility that this gene has been lost only in *Hydra* but that it is still conserved in the genome of *Cladonema*. Only the final output of the ongoing genome project will help us validate our hypothesis.

In addition to what has been shown in the eye, some of the genes isolated displayed overlapping expression patterns also at the level of the oocytes. Both *CrPaxB* and *CrEya* are expressed in the oocytes, as it was already shown for *Six4/5-Cr* (Stierwald et al. 2004) (for a discussion see paragraph 4.2.3).

The data collected so far clearly show overlapping expression patterns for three RDGC families (*Pax*, *Eya* and *Six*) in two distinct context in the medusa of *Cladonema*: in the eye and in the oocytes. Although more functional data are necessary, these results suggest a possible conservation of the interaction of the RDGC members in these two different tissues.

4.2.2 Different cnidarian classes employ distinct *Pax* gene subfamilies in the eye

Kozmik and co-authors showed that the cubozoan jellyfish *Tripedalia cystophora* has a single *Pax* gene, *PaxB,* which binds to the promoter of both *Tripedalia crystallin* and *Drosophila rhodopsin rh6* gene. Furthermore, *TcPaxB* is able to induce ectopic eyes in *Drosophila* (Kozmik et al. 2003).

For these reasons, the authors propose that the ancestral *Pax* protein involved in eye development and evolution was a *PaxB*-like protein and that *Pax6* genes evolved independently in Bilateria after their separation from Cnidaria. They argue that *PaxB* was the ancestral gene regulating *rhodopsin* in Cnidaria, and that later this function was taken over by *Pax6* in Bilateria, which then expanded its role to the control of eye development. They suggest that the involvement of *Pax6* in eye specification has been acquired by this gene only after the separation from Cnidaria. For this reason, Kozmik and co-workers come to the conclusion that cnidarian and bilaterian eyes are not homologous and have evolved independently, therefore the eye would have a polyphyletic origin.

In the present study we investigated the role of three *Pax* genes in a hydrozoan jellyfish with lens eyes. We showed that in *Cladonema radiatum CrPaxA* is expressed in the eye and is able to induce ectopic eyes in both wild type and *eyeless* mutant flies (Suga et al. submitted). Furthermore, *CrPaxA* directly binds to the 5' upstream regions of two eye specific *Cladonema opsin* genes (Suga et al. submitted). The two other *Pax* genes isolated in *C. radiatum, CrPaxB*, and *CrPaxE* are expressed in the manubrium and are not able to induce ectopic eyes. In addition *CrPaxB* does not display the capability to bind to the 5' upstream regions of the eye specific *opsin* genes (Suga et al. submitted). Hence, in *Cladonema CrPaxA* is the gene exerting the most important role in the eye. These findings seem to be incompatible with the hypothesis proposed by Kozmik and co-workers of a *PaxB*-like gene being the primordial gene in eye evolution.

However, both our data and the data of Kozmik et al. do not contradict the hypothesis that after duplication different *Pax* genes were having overlapping functions in the last cnidarian-bilaterian common ancestor, e.g. in the eye, as attested by the similarity of roles for *CrPaxA* and *TcPaxB*. This theory is further supported by the rescue experiments of the *sparkling* mutant phenotype. Not only *TcPaxB* (Kozmik et al. 2003) was able to substitute for the function of *DPax2*, but also *CrPaxA*, *CrPaxB* and, although less efficiently, *ey* and *toy* (Kozmik et al. 2003) from *Drosophila*. This findings clearly indicate that *Pax* genes belonging to distinct subfamilies have conserved the capability to substitute for each other (Suga et al. submitted).

Our results indicate that both Cnidaria and Bilateria use *Pax* genes in their eyes, although different subfamilies are employed. Furthermore, in *Cladonema* other members of the RDGC, namely *Eya* and *Six* genes (Stierwald et al. 2004), are also conserved and are expressed in the eye. These data strongly suggest that Cnidaria use the same regulatory network used also in higher Metazoa at the level of the eye, and consistently argues for the monophyletic origin of the eye of all animals.

4.2.3 Possible redeployment of the RDGC in the oocytes

We showed that both *CrPaxB* and *CrEya* are expressed in the oocytes. Intriguingly it was already shown that another member of the RDGC of *Cladonema radiatum*, *Six4/5-Cr*, has the same expression pattern (Stierwald et al. 2004). These data suggest that the same regulatory network is employed in both the eye and in the oocytes differentiation of *Cladonema*.

In *Drosophila*, *eya* and *Six* family genes have been shown to be involved in gonads development. *eya*^{3cs} mutants display not only severely reduced eyes but also both male and female sterility (Fabrizio et al. 2003). It has been shown that two members of the network, *eya* and *so* act as a transcriptional complex in the differentiation of somatic cyst cells in *Drosophila* spermatocyte development (Fabrizio et al. 2003). Furthermore, *Six4* has been implicated in the migration of the primordial germ cells towards the somatic gonadal precursor (Clark et al. 2007).

In *C. elegans*, the *Pax2/5/8* homolog *egl 38* functions in both somatic gonad and vulval cells to coordinate the development of the egg-laying system (Rajakumar and Chamberlin 2007).

In summary, *Pax*, *Six* and *Eya* family genes are involved at diverse levels of gonads development in different organisms.

The redeployment of the RDGC in various context has been already well characterized at the level of kidney, muscle and inner ear development. *Pax2*, *Pax8*, *Six1*, *Six2* and *Eya1* have been shown to be expressed during mammalian kidney development and mutations in this genes are all associated with syndromes that include kidney malformations (for a review, see Brodbeck and Englert 2004). In early mouse skeletal muscle development, the expression of *Pax3* and *Dach2* overlaps and is mutually regulated by feedback loops (Heanue et al. 1999). When *Pax3* is mis-expressed, it can induce *Eya2* and *Six1* expression. Moreover, when both *Eya2* and *Six1* as well as *Eya2* and *Dach2* are mis-expressed, they can synergize to induce the expression of muscle markers (for a review, see Silver and Rebay 2005). Another striking example of redeployment of this network of genes is offered by the otic placode development. *Pax2, Pax8, Eya1* and *Six1* are all expressed during the early mouse placode differentiation. Mutations in *Eya1* and *Six1* cause inner ear defects in mouse and have been implicated in the brachio-oto-renal syndrome in humans (for a review, see Silver and Rebay 2005).

These lines of evidence indicate that the interactions among orthologous members of the gene families composing the RDGC are conserved in different organisms and act in multiple tissues. Given the presence of a similar cascade in *Drosophila* eye and gonads development and its conservation across flies and vertebrates for the specification of different tissues, it is tempting to speculate that an evolutionary conserved Pax/Eya/Six pathway exists in the oocytes differentiation of *Cladonema*. Furthermore this suggests that the redeployment of a whole network of genes is an evolutionary old mechanism.

It has been proposed that the evolution of the morphological diversity, is more due to changes that have occurred in the control regions of the genes that regulate the development of specific

features, which in turn affect the spatial pattern and timing of gene expression, than to mutations in their coding regions (Jacob 1977; Carroll 2001). The possible redeployment of the RDGC in *Cladonema radiatum* oocytes might offer an example of this process.

Future studies will help to understand the molecular mechanisms at the base of different developmental context, *Cladonema radiatum* and Cnidaria will offer a valid help to elucidate the conservation and evolution of signalling networks across the animal kingdom.

5 Materials and methods

Standard molecular methods such as PCR products purification, alkaline phosphatase treatment, digestion, ligation, , plasmid miniprep, agarose gel electrophoresis, preparation and transformation of competent cells were performed according to (Sambrook and Russell 2001) and according to manufacturer protocols.

5.1 Animal cultures

Cladonema radiatum Dujardin (Cnidaria, Hydrozoa, Anthomedusae) colonies were reared in artificial see water and kept in big aquaria at a constant temperature of either 14°C, 16°C. The jellyfish and the colonies were fed every second day with 2-day-old artemia.

5.2 mRNA extraction and cDNA synthesis

The mRNA extraction was carried out with the Dyna-beads® mRNA DIRECT Kit™ (DYNAL®) Biotech. The SuperScript™ III First-Strand Synthesis System for RT-PCR from Invitrogen was used for the cDNA synthesis, as described by the manufacturer.

5.3 PCR

5.3.1 Homology (degenerate) PCR

The homology PCRs with degenerate primers were always performed on cDNA for the first round PCR and on 1µl of the first round PCR product for the nested PCRs, for a final volume of 20µl in both cases. The first round PCR, conducted on cDNA, was always performed using a degenerate forward primer and the reverse AUAP primer (Invitrogen). The DNA polymerase employed was Expand High Fidelity (Roche).
The program used was:

Hot Start	80°C		
initial denaturation	94°C	4’	
denaturation	94°C	15”	x30 in the first round PCR x35 in the nested PCRs
annealing	X° C	1’	
elongation	72°C	Y	
final elongation	72°C	8’	
stop	12°C	∞	

The annealing temperature X varied according to the primers used. The elongation time Y was 1’ for 1.5 kb.

The PCR products obtained were analysed by agarose gel electrophoresis, the bands visualised were cut out separately and the DNA eluted from the agarose with the QIAEX gel extraction kit (Qiagen).

5.3.1.1 Degenerate primers

CrPaxB

PBdegF1 (NPTMFAWE) (forward) 5’-AATCCNACNATGTTYGCNTGGGA-3’

PBdegR1 (QVWFSNK) (reverse) 5’-CARGTNTGGTTYTCNAATAAGCG-3’

CrPaxE

PaxdegF1 (NPTIFAW) (forward) 5’-AATCCIACIATITTYGCNTGG-3’

PaxdegR1 (HYPDVGT) (reverse) 5’-CAYTAYCCNGAYGTNGGNCAN-3’

CrEya

EYAF1 [F(V/I/L)WDLDE] (forward) 5’-TTTITITGGGAYYTNGAYGA-3’

EYAF3 [F(F/Y)NDLE(E/D)] (nested forward) 5’-GTTAGAGGIGGIGTIGANTGGATG-3’

EYAR4 [CF(E/Q)RI(V/I/M/Q)(Q/N/T)RF] (reverse) 5-AAACGIKKIWIDATICKYTSRA ARCA-3’

EYAR5 [FWR(I/V)(S/N/T)(NT)H] (nested reverse) 5’-TGIKTIBWIAYICKCCAAAA-3’

Degenerate primers for *Dac*

DACF1 [EDL(I/L)C (L/F)PQ] (forward) 5’-GAWTTAHTITGYYTNCCNCA-3’

DACF2 [PQ(V/A)(F/Y)(D/E)(L/V/Y)F] (nested forward)	5’-CCACARGYITWYGANBW NTT-3’
DACF3 [NC(T/N)VEQ] (nested forward)	5’-AATTGYAMIGTIGARCA-3’
DACR1 [C(T/N)VEQVR] (reverse)	5’-CGTACYTGYTCIACIKTRCA-3’
DACR2 (VNRCKL) (nested reverse)	5’-TAATTTRCANCKRTTNAC-3’
DACR3 (NRCKLI) (nested reverse)	5’-TATAATTTRCANCKRTT-3’

5.3.2 RACE PCR

In order to obtain the full length sequences of the cloned genes we performed 5’ and 3’ RACEs (rapid amplification of cDNA ends). The RACE PCRs were carried out with the 3’ and 5’ RACE system for rapid amplification of cDNA ends (Invitrogen), according to the manufacturer’s protocol. For the 3’ RACE we always used the 3’ adapter primer (AP) (Invitrogen) for the first strand cDNA synthesis.

5.3.2.1 RACE primers

5’RACE *PaxB*

PBnR1 (reverse)	5’-GTCTGTTCCAAGTGTTAGCTC-3’
PBnR2 (nested reverse)	5’-GGAGGTGTTAACCAACTTGG-3’
PBnR3 (nested reverse)	5’-CCAAGTTGGTTAACACCTCC-3’

3’RACE *PaxB*

PBnF1 (forward)	5’-AGGCCTGGAGTGATAG-3’
PBnF2 (nested forward)	5’-GTAAAGATAACACACCGATGCA-3’

5’RACE *PaxE*

PCnR1 (reverse)	5’-CCATCAAACCGTTGCGAAA-3’
PCnR2 (nested reverse)	5’-CGAATATTACGTAATCGTGCG-3’
PCnR3 (nested reverse)	5’-GTCCGAGTGTAAGTTCCATAAA-3’

3’RACE *PaxE*

PCnF1 (forward)	5’-GGAGGAGTGTGTTTAGTGATCAC-3’
PCnF2 (nested forward)	5’-GGCCAATGACAGATACACAGACC-3’

PCnF3 (nested forward)	5’-GGAGAAGAAAGCGACGTACTC-3’

5’ RACE *Eya*

EYA5’R1 (reverse)	5’-CAATATGTATCGTGGGAATGTAG-3’
EYA5’R2 (nested reverse)	5’-GATATATACAATATGTATCGTGGG-3’
EYA 5’R3 (nested reverse)	5’-CAACTACGGCGCTTGGTATGAG-3’
EYA 5’R4 (nested reverse)	5’-CTCACATCAACTACGGCGCTTGG-3’
EYA5’R5 (nested reverse)	5’-GGCAAGGATCTCACATCAACTAC-3’
EYA5R6 (nested reverse)	5’-GGAAATAATTATCATCCGTCC-3’
EYA5R7 (nested reverse)	5’-GCAATGTGGAAATAATTATC-3’
EYA5R8 (nested reverse)	5’-CTAACGAGAATGATAGTATGCC-3’

3’ RACE *Eya*

EYA3’F1 (forward)	5’-CCCAACACTAGCCAAGTGCATGC-3’
EYA3’F2 (nested forward)	5’-CACTAGCCAAGTGCATGCTGTATGG-3’
EYA3’F3 (nested forward)	5’-GTGCATGCTGTATGGTTTGGG-3’

5.4 Cloning of PCR products

The purified PCR products were cloned in the pCR®II-TOPO® vector from the TOPO TA cloning® kit (Invitrogen). PCR products with the 3’ A-overhangs added by the Taq polymerase can easily be cloned into the pCR®IITOPO® vector, which contains a single 3’ T-overhang. As M13 Reverse and M13 Forward priming sites are surrounding the multiple cloning site of the pCR®II-TOPO® vector, we used M13 Reverse and M13 Forward primers or specific primers to sequence the insert.

5.5 DNA sequencing

We used the BigDye® Terminator reaction kit (PE Applied Biosystems) in order to label the DNA fragments to sequence. The PCR reactions were done according to the manufacturer. The sequencing analysis was done on an ABI PRISM 310 Genetic Analyzer.

5.6 Real Time PCR

Every PCR reaction consist of an initial exponential phase, when all the reaction components, fresh and in excess, are able to double the initial amount of the target at every cycle. The exponential phase is followed by a linear phase, during which the concentration of components of the reaction start to decrease, leading to a decrease also in the efficiency of the reaction. The stage at which the PCR reaction components are used up and there is no amplification anymore is called plateau phase, at this point the PCR products can even undergo degradation. Traditional PCR uses endpoint detection of amplification product by gel electrophoresis at the plateau phase, this method is not precise and sensitive, the concentration of the PCR product at this point does not reflect anymore the initial concentration of the target. Real Time PCR instead monitors the amplicon increase at every cycle, during the exponential phase when the reaction efficiency is maximal and directly reflects the initial amount of starting material. The real time detection is allowed by the use of DNA binding dyes that fluoresce upon association with double stranded DNA

We used the Light Cycler® Real Time PCR machine from Roche. The fluorescent dye used for the amplification detection was SYBR green provided in the QuantiFast™ SYBR® Green PCR kit (Qiagen).

The expression levels in the different parts were calculated by comparison to the expression levels in standard dilutions of whole medusa cDNA. The obtained values were normalized by the level of expression in the same tissues for the housekeeping gene *Elongation Factor 1α* (*EF1α*). *EF1α* should be stably expressed in all the tissues throughout the development of the organism. The choice of a housekeeping gene for the relative quantification of the gene of interest represents an endogenous control that corrects for sample-to-sample variations, caused by different amount of starting material (quantity of tissue), and variable efficiency in mRNA extraction and/or reverse transcription. The expression analysis was repeated at least three times on each cDNA set (consisting of the four tissues), independently generated from different jellyfish. The mean for the three values was calculated. The mRNA extraction was carried out with the Dyna-beads® mRNA DIRECT Kit™ (DYNAL®) Biotech.

The specific primers used for real-time PCR were designed with the help of a primer software available online at www.genscript.com/ssl-bin/app/primer.

The amplification protocol was:

pre-incubation	95°C	5’	
denaturation	95°C	10”	45x
combined annealing/extension	60°C	30”	
detection	60°C	single	

The specificity of the amplification was tested by performing a melting curve at the end of the amplification.
The reaction mix for each tube was:

2x QuantiFast SYBR® Green PCR Master mix (Qiagen)	5µl
Forward primer (10µM)	1µl
Reverse primer (10µM)	1µl
cDNA sample	0.7µl
add water to a final volume of 10µl	

5.6.1 Real Time PCR primers

CrPaxB

PBnHiFW1(forward)	5’AGGCCTGGAGTGATAG3’
PBnHiR1 (reverse)	5’GTCTGTTCCAAGTGTTAGCTC3’

CrPaxE

PCnexpR1 (forward)	5’TATGACAACAAGCGAATGTCG3’
PCnexpF1 (reverse)	5’GCACTCTCCAACAGATATCAATG3’

CrEya

EYALCFI (forward)	5’GCTGGAACAAATATCGGATTC3’
EYALCR1 (forward)	5’CCATACAGCATGCACTTGGC3’

5.7 *In situ* hybridization

5.7.1 Probe synthesis

For the probe synthesis we always first amplified by PCR with regular primers the target sequence from the cDNA, and then used the obtained PCR product for the amplification with

the primers containing the T7 RNA polymerase promoter sequence. For the synthesis of the antisense probe we used the regular forward primer in combination with the reverse primer containing the T7 promoter sequence, similarly for the synthesis of the sense probe we used the forward primer containing the T7 promoter in combination with the regular reverse primer. The PCR products thus obtained were purified with the QIAquick® PCR purification kit (Qiagen) and used for the in vitro transcription according to the following protocol:

1µg of DNA (purified PCR product with T7 promoter at the 3'end)
2µl of 10X transcription buffer (roche)
2µl of DIG-NTP labeling Mix (Roche)
0.5µl RNase inhibitor (Promega)
2µl of T7 RNA polymerase (Roche)
add dH2O to 20µl

- incubate 2 hours at 37°C
- Remove DNA template by adding 1 µl of RNAse free DNAse I and incubate for another 15 min at 37°C.
- Stop the reaction by adding 2µl 0,2 M EDTA pH 8.
- Adjust volume to 50 µl by adding DEPC treated H2O.
- Precipitate by adding 25µl 7.5 M NH4Ac (Sigma) and 150µl 100% EtOH.
- Incubate 30 min at -20°C
- Centrifuge 15 min at 4°C
- Wash with ice cold 80% EtOH
- Resuspend visible pellet in 25µl DEPC treated H2O, analyze 1 µl on agarose gel
- Dilute the probe to 50µg/ml with 50% formammide in DEPC water.

1µl of the probe thus prepared is used for 1ml of Hybridization buffer.

5.7.1.1 Probe amplification primers

CrPaxB

PBnHybF1 (forward) 5'GGCAAAGTCAGTAAAGATAACAC3'
PBnHybR1 (reverse) 5'CAGATGATGGTATTTATCAACCG3'
PBprobe2F1 (forward, T7promoter) 5'TGCATCTCTGTAATACGACTCACTATAGGGAG

AGGCAAAGTCAGTAAAGATAACAC3’

PBprobe2R1 (reverse, T7 promoter) 5’TGCATCTCTGTAATACGACTCACTATAGGGAG ACGGTTGATAAATACCATCATCTG3’

***CrPaxE* first probe**

PChybF1 (forward) 5’CCCACCATATCACGATAGAGG3’

PCnexpR1 (reverse) 5’TATGACAACAAGCGAATGTCG3’

PCprobe3F1T7 (forward, T7 promoter) 5’TGCATCTCTGTAATACGACTCACTATAGGG AGACCCACCATATCACGATAGAGG3’

PCprobe3R1T7 (reverse, T7 promoter) 5’TGCATCTCTGTAATACGACTCACTATAGGG AGACGACATTCGCTTGTTGTCATA3’

***CrPaxE* second probe**

PChyb2FW (forward) 5’GGAACAATCGAAAATAGAAATG3’

PChyb2RE (reverse) 5’ CCTCTATCGTGATATGGTGGG3’

PCT7FW1 (forward, T7 promoter) 5’TGCATCTCTGTAATACGACTCACTATAGGGAG AGGAACAATCGAAAATAGAAATG3’

PCT7RE1 (reverse, T7 promoter) 5’TGCATCTCTGTAATACGACTCACTATAGGGAGA CCTCTATCGTGATATGGTGGG3’

CrEya

EYAhybF1 (forward) 5’-CAACTACGGCGCTTGGTATGAG-3’

EYAhybR1 (reverse) 5’-CCATACAGCATGCACTTGGC-3’

EYAT7F1 (forward, T7 promoter) 5’-TGCATCTCTGTAATACGACTCACTATAGGGAGA CAACTACGGCGCTTGGTATGAG-3’

EYAT7R1 (reverse, T7 promoter) 5’-TGCATCTCTGTAATACGACTCACTATAGGGAGAC CATACAGCATGCACTTGGC-3’

5.7.2 *In situ* hybridization protocol

Fixation

1. Fix in PFA 4% (solution with filtered sea water) freshly made 30’at RT (within 2 weeks from preparation, kept at +4)

2. Fix in MeOH (solution with water), increase stepwise (use sol -20°C cold, and perform on ice)

50%	5’ at RT
75%	5’ at RT
2x 100%	5’ at RT

Store the fixed animals at -20°C for at least 16h.

Re-hydration and hybridization

3. Substitute MeOH with EtOH 100%	5’ at RT
4. Put Xylene/EtOH 50% (keep the solution in a glass container)	30’ at RT
5. Wash with EtOH 100%	2x 15’ at RT
6. Substitute EtOH with Water stepwise	75% 5’-15’ at RT
	50% 5’-15’ at RT
	25% 5’-15’ at RT
	Water 5’-15’ at RT
7. Substitute with 80% Aceton/Water (V/V) (kept at -20°C)	10’ at -20°C
8. Wash with DEPC Water	5’-10’ at RT
9. Wash with PBST	5’-10’ at RT
10. Postfix with PFA 4% freshly made (solution with PBS)	30’ at RT
11. Wash with PBST	2x 15’ at RT
12. Prehybridize in Prehybridization Buffer (PB)	1h at 50-55°C
13. Substitute PB with Hybridization Buffer (HB)	1-3h at 50-55°C
14. Prewarm the Probe in HB	10’ at 65°C
15. Substitute HB with pre-warmed HB+Probe	Overnight (18h) at 50-58°C
16. Wash with washing solution 1	3x 30’ at 50-58°C
(pre-warm solution at performing temperature)	
17. Wash with washing solution 1	30’ at 60-65°C
18. Wash with washing solution 2	2x 10’ at 60-65°C
	2x 15’ at 60-65°C
19. Wash with maleic acid buffer (MAB)	3x 10’ at RT
20. Block with blocking buffer	60’ at RT
21. Antibody	3h at RT or 4°C on
22. Wash with MAB	3x 60’ at RT
23. Wash with detection buffer	15’ at RT
24. Color detection in detection buffer + NBT + BCIP	1-5h at RT in the dark

(check every 15' for the first hour and then every 30')

25. Stop the reaction in PBST and keep in PBS + 0.1% Na azide at 4°C

26. Mount in PBST/Glycerol 50%

Solutions

- Paraformaldehyde (PFA) 4%

  0.4g PFA in 10 ml PBS or artificial sea water, dissolve by heating up and stirring

- PBST

  0.1% Tween 20 in 1x PBS

- Prehybridization Buffer

  50% Formamide
  100µg/ml tRNA
  5x SSC
  100 µg/ml tRNA
  100 µg/ml heparin
  0.1% Tween 20
  10mM DTT

- Hybridization Buffer (HB)

  50% Formamide
  100µg/ml tRNA
  5x SSC
  100 µg/ml tRNA
  100 µg/ml heparin
  0.1% Tween 20
  10 mM DTT
  10% Dextran Sulphate

- Probe 50ng/ml

  Stock solution is 50µg/ml (in 50% formamide, solution with water), take 1µl in 1ml of HB

- Washing Solution 1 (WS1)

  50% formamide
  5xSSC
  0.1% Tween

- Washing solution 2 (WS2)
  50% formamide
  2 xSSC
  0.1% Tween
- Maleic Acid Buffer (MAB)
  Maleic Acid 100mM
  NaCl 150mM
  Tween20 0.1%
- Blocking buffer 1%
  (Stock 10%) dilute the stock blocking reagent 10% (Roche) with MAB
- Antibody (1/2000)
  Anti –DIG-AP Fab fragments (Roche) diluted in Blocking buffer 1%
- detection buffer (Roche) Stock 10%
  dilute with water
- Color detection
  10ml detection buffer + 18µl NBT (50µg/µl) + 35µl BCIP (50µg/µl) (Sigma)

Cryosections

After whole mount in situ hybridization some of the specimens were cryosectioned. The jellyfish were transferred in a mould containing Sakura Tissue-Tek® O.C.T. compound (Miles laboratories) and frozen in liquid nitrogen. The embedded specimens were kept at -80°C until cryosectioning.
The sections were 10µm in thickness.

5.8 Targeted gene expression

Ectopic expression of *CrPaxB*, *CrPaxE* and *CrEya* in larval imaginal discs of *D.melanogaster* was performed using the UAS-Gal4 system (Brand and Perrimon 1993). Full-length *CrPaxB*, *CrPaxE* and *CrEya* cDNAs were inserted as EcoRI-XhoI fragments into the pUAST vector. For each construct several lines of transformant flies were recovered and used in the targeted gene expression experiments: eight lines for *CrPaxB*, seven lines for *CrPaxE*, six lines for *CrEya* and four lines for *CrEya-alt*. In order to test for the ectopic eye induction, the UAS transgenic lines were crossed to the dpp^{blink}-Gal4 driver line (Staehling-Hampton et al. 1994)

as well as to the line carrying both *dpp*^{blink}-Gal4 and UAS-Gal4 (Hassan et al. 2000). For the rescue of the *Drosophila ey*$^{J5.71}$ mutant phenotype (Kammermeier et al. 2001; Punzo et al. 2001) the UAS constructs for *CrPaxB* and *CrPaxE* were brought in the *eyeless* background and crossed to the *ey*-Gal4 driver (Hazelett et al. 1998). For the rescue of the *Drosophila spa*^{pol} mutant phenotype the UAS-*CrPaxB* and UAS-*CrPaxE* constructs were brought in the *sparkling* background and crossed to the *spa*-Gal4 driver (Fu and Noll 1997). For the synergistic induction of ectopic eyes, the UAS-*CrEya* and UAS-*CrEya-alt* lines were crossed to the UAS-*Dso* lines available in our laboratory (kindly provided by Makiko Seimiya) and their expression in the imaginal discs was driven by the *dpp*^{blink}-Gal4 driver line. For the rescue of the *eya*^{2} mutant phenotype (Bonini et al. 1993) the UAS-*CrEya* and UAS-*CrEya-alt* lines were crossed to the *ey*-Gal4 driver line (Pignoni et al. 1997).

5.8.1 Primers for the amplification of the full length cDNAs to clone in pUAST

CrPaxB

PBflF1 (forward)	5’TTGAATTCCTAATGTTGTCACTGGACGGAAG3’
PBflR1 (reverse)	5’GCCTCGAGGGGAGGAGGGTGTTCATGGG3’
PBflR2 (nested reverse)	5’GCCTCGAGGGAGGGTGTTCATGGGATGGG3’

CrPaxE

PCflF1 (forward)	5’TTGAATTCGGAACAATCGAAAATAGAAATG3’
PCflR1 (reverse)	5’GCCTCGAGGTTAAACATCACAGTATCC3’
PCflR2 (nested reverse)	5’GCCTCGAGGTTAAACATCACAGTATCCTTC3’

CrEya* and *CrEya -alt

EYAFLF1 (forward)	5’GATTAAATACACATACTAAACCGAGTCAC3’
EYAFLF2 (nested forward)	5’TTGAATTCGAGAAAAAAACCTCGATACTGATGAATA ATG3’
EYAFLR1(reverse)	5’CACTATATACAAATACATAAATTATACATCC3’
EYAFLR2 (nested reverse)	5’GCCTCGAGAGCGTAATCTGGAACATCGTATGGGTACA TAAATTATACATCCTTTTGTCGCG3’

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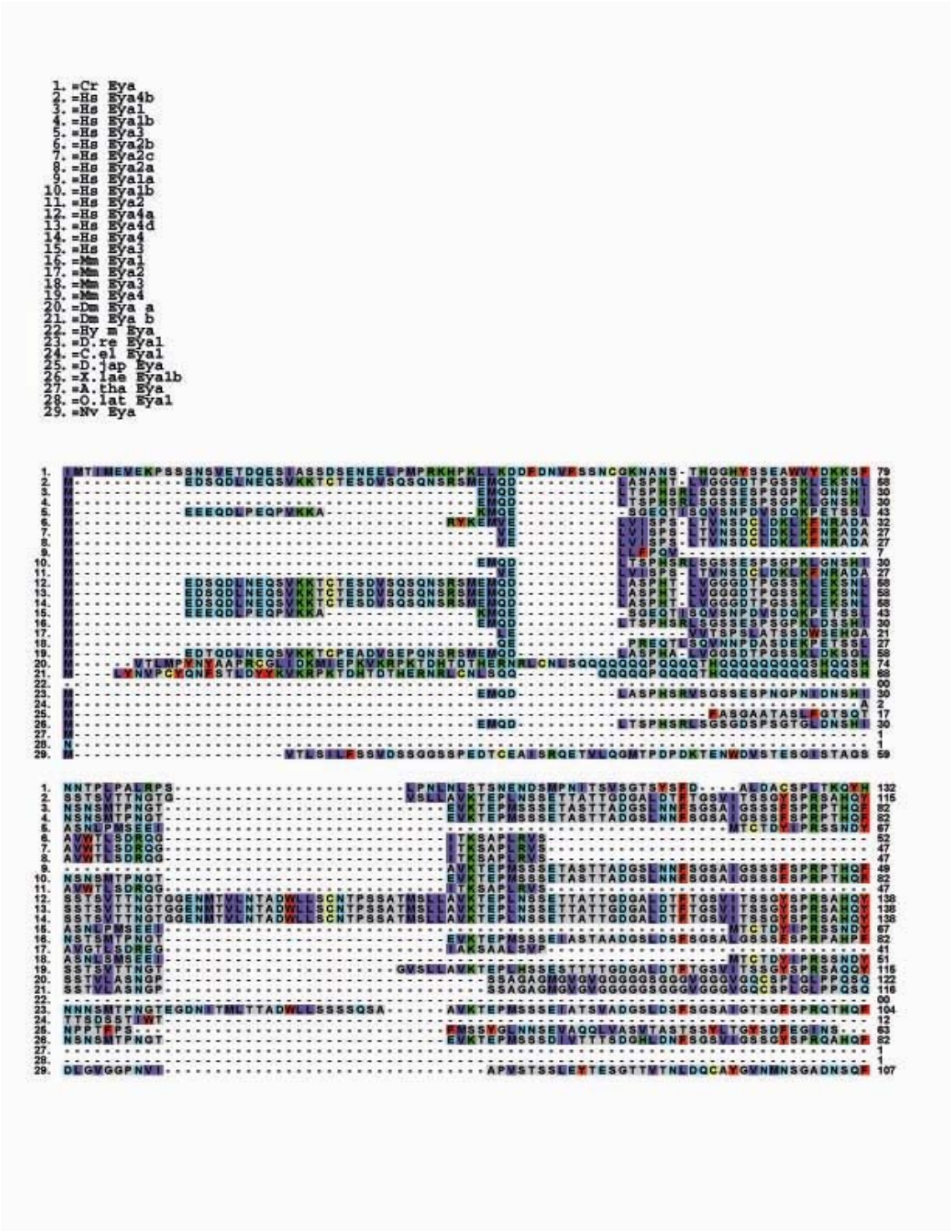
7 Appendix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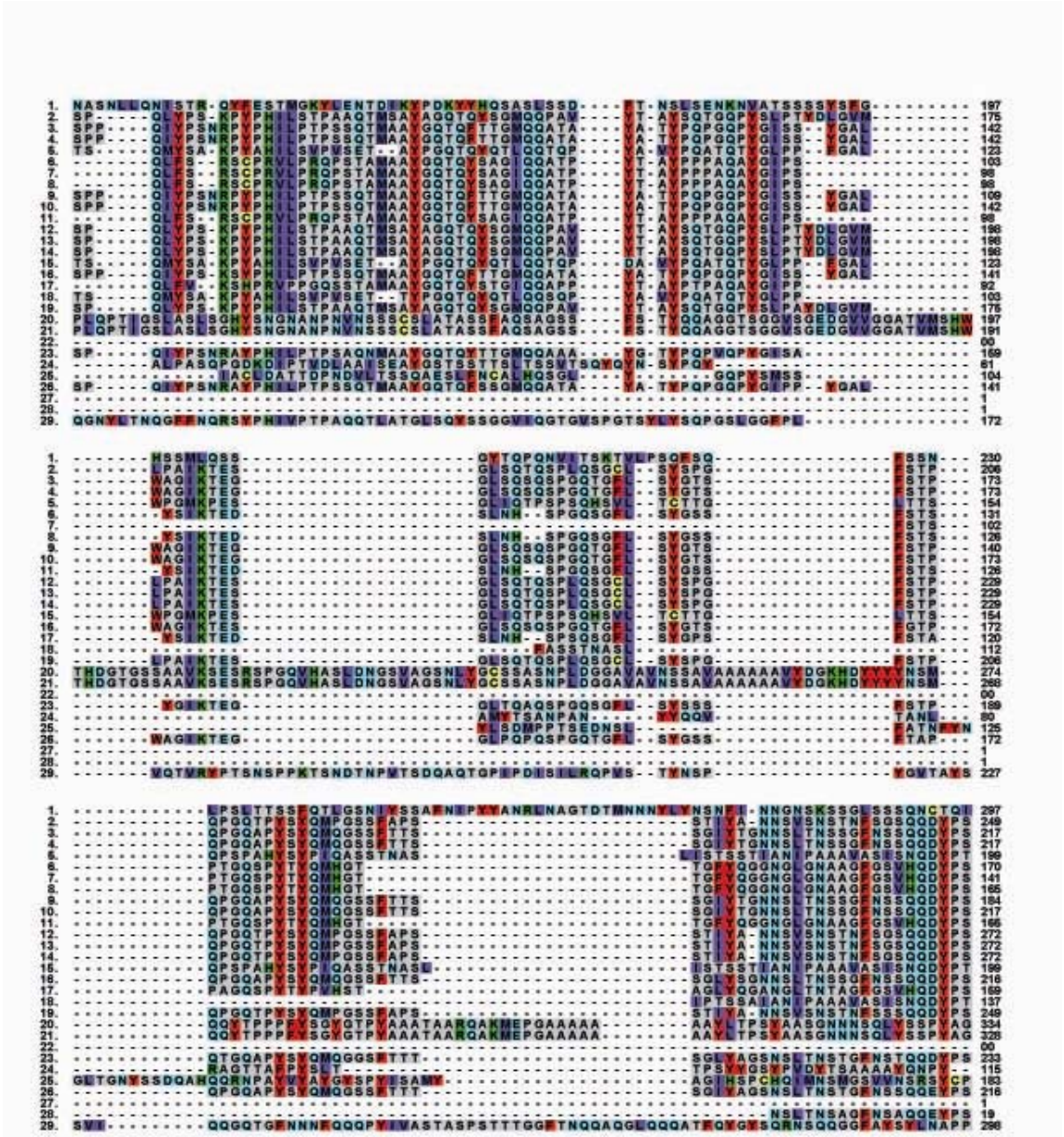

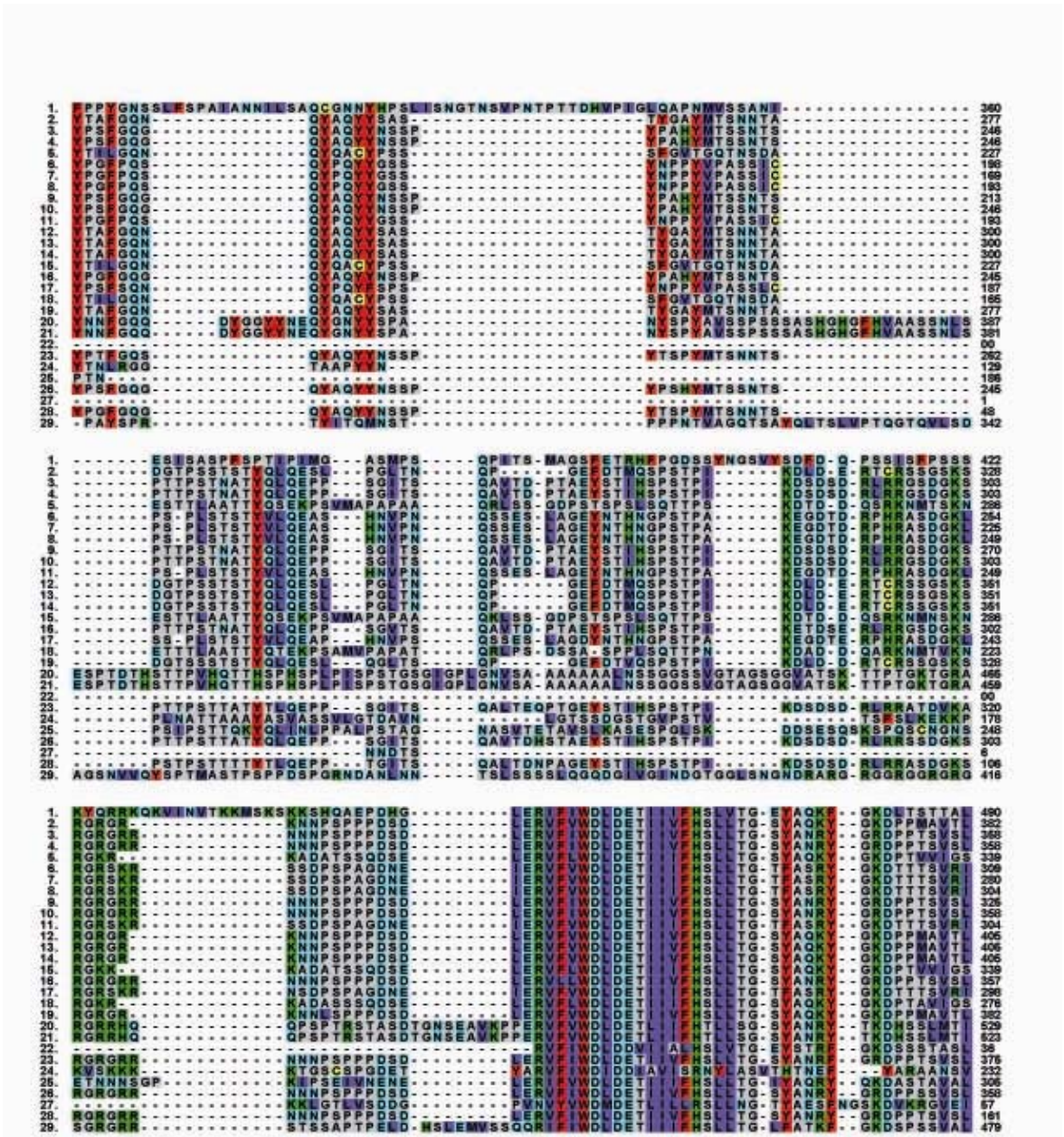

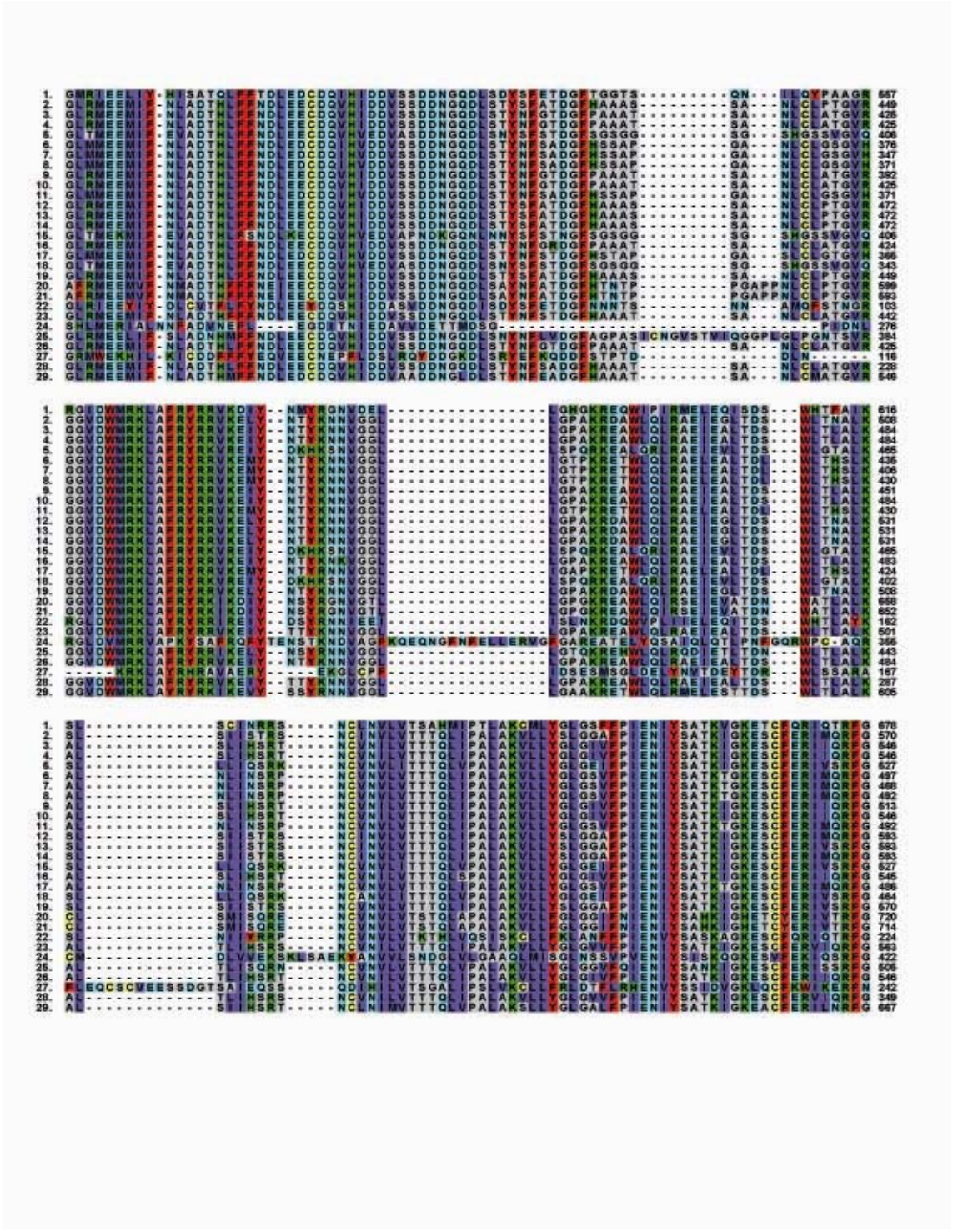

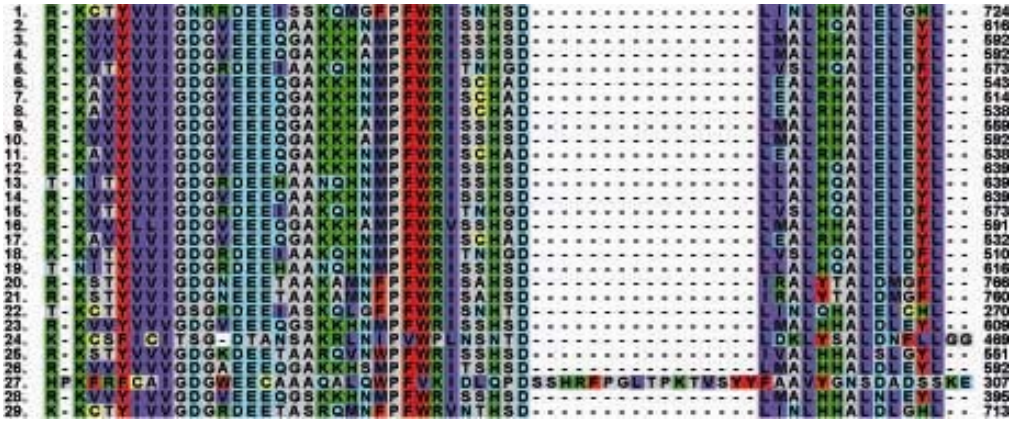

Fig. 7.1 Eya proteins alignment The predicted protein sequence of *Cladonema* CrEya (1) is aligned with Eya proteins from several organisms. The names, and corresponding numbers, of the species used in the alignment are displayed in the first panel. A. tha: *Arabidopsis thaliana*; C. el: *Caenorhabditis elegans*; Cr: *Cladonema radiatum*; D. jap: *Dugesia japonica*; D. re: *Danio rerio*; Hs: *Homo sapiens*; Hy m: *Hydra magnipapillata*; Mm: *Mus musculus*; Nv: *Nematostella vectensis*; O. lat: *Oryzias latipes*; X. lae: *Xenopus laevis*.

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Final Mark: 110/110 magna cum laude

2004 - to present **Ph.D. project**
Topic: Characterization of the Retinal Determination Gene Cascade in the jellyfish *Cladonema radiatum* (Cnidaria, Hydrozoa)

Supervisors:
- Prof. Walter J. Gehring - Biozentrum, Cell Biology Dept., University of Basel
- Prof. Stefano Piraino - Laboratory of Evolutionary Biology of Marine Invertebrates, University of Lecce

3/2006 – 4/2007 Collaboration with the University of Lecce, working on various molecular aspects of hydroids of the Mediterranean sea.

SKILLS

Languages Italian - mother tongue
English - fluent
German- basic

I.T. knowledge Microsoft Office (Word, Excel, Power Point), Adobe Photoshop, Adobe Illustrator

POSITIONS OF RESPONSIBILITY

4/2006 – 4/2007 Supervision and coordination of three master students on the following topics:

- Characterization of the expression of the genes *BMP 2/4* and *otx* during the reverse development of *Podocoryne carnea* (Cnidaria, Hydrozoa)
- Apoptosis and cell cycle regulation during the reverse development of *Turritopsis nutricula* (Cnidaria, Hydrozoa)
- Intra-specific variability in colonial hydroids of the Mediterranean sea

COURSES AND MEETINGS

2004 Experimental Biology course on marine invertebrates, Station biologique de Roscoff, France

" Developmental Biology course on marine organisms, Banyuls sur mer, France

2005 International workshop: Hydra and the molecular logic of regeneration, Tutzing, Germany, 19-22 September
Poster: Reshuffling the puzzle of the *Hox/ParaHox* homeobox genes evolution: the cnidarian inventory includes a group 3 *ParaHox* gene.

2007 68th Congress of the Italian Zoological Society
Poster: Isolation and characterization of RDGC genes in the jellyfish *Cladonema radiatum*.

2008 8th International meeting on zebrafish development & genetics, Madison, WI, USA

PUBLICATIONS

Induction of reverse development in two marine Hydrozoans. Schmich J, Kraus Y, De VitoD, Graziussi D, Boero F, Piraino S. Int J Dev Biol. 2007;51(1):45-56

Flexible recruitment of *Pax* genes in the eye during early animal evolution, demonstrated by studies in hydrozoan jellyfish. Suga H, Graziussi D, Tschopp P, Stierwald M, Schmid V, Gehring W J. Submitted.

REFERENCES

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