ON THE EVOLUTION OF CNIDARIAN EYES

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Dekan

“ la plus libre, la plus souple, la plus voluptueuse des danses possibles, m’apparut sur un écran où l’on montrait de grandes méduses: ce n’étaient point de femmes et elles ne dansaient pas.

Point des femmes, mais des êtres d’une substance incomparable, translucides et sensibles, chairs de verre follement irritables, dômes de soie flottante, couronnes hyalines, longues lanières vives toutes courues d’ondes rapides, franges et fronces qu’elles plissent, déplissent; cependant qu’elles se retournent, se déforment, s’envolent, aussi fluides que le fluide massif qui les presse, les épouse, les soutient de toutes parts, leur fait place à la moindre inflexion et les remplace dans leur forme. Là, dans la plénitude incompressible de l’eau qui semble ne leur opposer aucune résistance, ces créatures disposent de l’idéal de la mobilité, y détendent, y ramassent leur rayonnante symétrie. Point de sol, point de solide pour ces danseuses absolues; point de planches, mais un milieu où l’on s’appuie par tous les points qui cèdent vers où l’on veut. Point de solides, non plus, dans leur corps de cristal élastique, point d’os, point d’articulations, de liaisons invariables, de segments que l’on puisse compter…”

Paul Valéry
Degas, danse, dessin, 1936, in Oevres II, Pièces sur l’art

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SUMMARY

Cnidarians and their medusa stage are generally considered to be diploblasts and therefore ancestral to Bilaterians. They represent the most primitive phylum where striated muscle tissue, a complex system of nerve rings and different sense organs of high complexity, including eyes have evolved in the jellyfish stage.

We demonstrated that jellyfish and the triploblast Bilateria use homologous gene cascades and developmental pathways to build these muscle systems. The expression of *JellyD*, a derived jellyfish homolog of the master regulator of muscle tissue *MyoD*, is correlated with that of bilaterian muscle determination factors.

Furthermore, the eye determination genes of the *Pax* and *Six* families of cnidarians have bilaterian-like expression patterns. Although no *bona fide Pax6* homolog could be found, it can be shown that among the four *Pax* genes characterized, cnidarians do have a *Pax* gene (*PaxA-Cr*) that is exclusively expressed in the maintenance and regeneration of eye tissue. Additionally the hypothesis of a loss of *Pax* genes within the cnidarians can be rebut as well as the claim that cubozoans would possess only one *Pax* gene. *Cladonema* jellyfish have three cognate members of the *sine oculis/Six* class family of which *Six1/2-Cr* and *Six3/6-Cr* are upregulated during eye regeneration. Analysis of gene expression patterns during eye regeneration shows that the cnidarian *Pax* gene is upregulated before the *Six* genes, indicating a possible upstream position in the gene regulatory network.

The results are in agreement with monophyly of eye evolution and indicate that the common ancestor between Cnidaria and Bilateria had a more complex anatomy than commonly anticipated.

INTRODUCTION

Are Cnidaria diploblasts?

Evolution is an excruciatingly slow process and cannot be understood without understanding the evolution of development, and how the process of development itself constrains evolution. Mutation and selection are the basis to portray evolution and provide a steady supply for new genes. Developmental mechanisms control body shape, pattern, and therefore establish the field wherein mutations act. In other words, evolution roots in changes found in developmental mechanisms and evolution is primarily the evolution of genomes (Davidson, 2001).

The comparative study of the spatio-temporal expression patterns of developmental genes, mainly transcription factors, has been one principal focus in evolutionary developmental biology over the last years. Studies on the evolution of development yielded in the astonishing finding that shared regulatory genes have conserved roles in development across phyla and morphological diversity is often based on changes in the developmental roles of transcription factors and not necessarily in the appearance of completely new genes. Additionally it is true that the number of genes, or the size of the genome, is not correlated with an animal's complexity (Davidson, 2001). The discovery of the homeobox (McGinnis et al., 1984; Scott and Weiner, 1984) and its widespread phylogenetic conservation was one of the most important key events linking molecular data to body plan architecture and so helped much to explain the relationship within phyla.

Therefore the expression pattern of developmental genes of a species is useful to define the evolutionary position of its phylum. Today about 35 different animal phyla, each with visibly distinct body plans, are distinguished. Since fossil records demonstrated that most of them suddenly appeared during the Cambrian explosion (Carroll, 2001) it was suggested that this

great evolutionary diversification occurred before the onset of the Cambrian period (Chen et al., 2000). At the time a deep reorganization of the metazoan phylogenetic tree is taking place as a result of the availability and input of DNA sequence analysis (Adoutte et al., 2000) and the genesis of bilaterian complexity has to be reinterpreted.

The new rRNA-based phylogeny leaves diploblasts as the only sister group to Bilateria. According to the textbooks diploblasts are animals with a two-layered body structure. Ctenophores and Cnidaria, and according to some, Sponges and Placozoa constitute the diploblasts. A bilateral symmetry along an anterior-posterior axis, the presence of three germ layers, a coelom, a through gut, a central nervous system, and the principle of colinearity of Hox cluster gene expression are characteristics of bilateral animals. Cnidaria display also many bilaterian-like traits. In *Podocoryne carnea* (Cnidaria, Hydrozoa) the expression pattern of the homeobox gene Gsx-Pc (Yanze et al., 2001), the formation of a subset of nerve cells (Gröger and Schmid, 2001) and the expression of atonal in endodermal cells (Seipel et al., 2004) indicate the existence of an anterior-posterior polarity in axis formation in the development of the planula larva. During medusa development, the entocodon, a third ECM bordered (Bölsterli, 1977) cell layer is formed from the early bud ectoderm (Bouillon, 1994; Hyman, 1940; Kühn, 1910). Then the entocodon cavitates and the outer layer will differentiate striated and smooth muscle of the bell, the inner layer the smooth muscle of the manubrium. This cavity could represent a coelom-like structure which gives rise to the subumbrellar cavitiy of the bell, in which later (the adult medusa) the gametes are shed. Many molecular markers and regulatory proteins typical for the mesoderm and myogenic lineage in Bilateria were isolated from jellyfish (see chapter 3 and 4 of this thesis) (Schuchert et al., 1993; Gröger et al., 1999; Müller et al., 1999, 2003; Spring et al., 2000; 2002). Their expression patterns in the entocodon or its derived tissues strengthen the idea that the entocodon is a mesoderm-like structure. Even a jellyfish homolog of the master regulator of

muscle development MyoD (Davis et al., 1987) was characterized from *Podocoryne* (Müller et al., 2003). The high sequence conservation of cnidarian genes with insect and vertebrate homologues confirms again the hypothesis, that the striated muscle of jellyfish is related to the striated muscle of bilaterians. The argument that the lack of a through gut and a central anteriorized nervous system (brain) clearly separates Cnidaria from Bilateria has to be interpreted cautiousely. Another big phylum with radial symmetry, the echinoderms which are believed to be true bilaterians, have reduced the through gut in the Ophiuroidea and some asteroid species and have given up an anteriorized nervous system.

Evolution of eyes

A discussion about eye evolution leads to date ultimately into a discussion of a possible monophyletic, polyphyletic or perhaps biphyletic origin. The ubiquity and perplexing Pax6 gene expression in developing visual organs throughout the Bilateria and mutant results in mice and fruit flies provide a compelling case for a key position of this gene throughout phyla in the development of animal eyes and as a conclusion, seem to justify the claim of a monophyletic origin of eye evolution. Those overwhelming molecular data stay obviously in strong contrast to the classical morphological view of extreme polyphyly of eyes. Eyes and photoreceptive cells would have originated in at least 40 if not 65 or more different lines (Salvini-Plawen and Mayr, 1977). Salvini-Plawen and Mayr argue also, that earliest invertebrates (sessile carnivores like cnidarians) did not bear eyes. Eyes would have evolved late because early Bilateria would have lived interstitially or tunneled in the substrate.

From their anatomy the different eye types in the animal kingdom are fundamentally different. Eyes can be discriminated by their ontogenetic origin, structure of photoreceptor cells, position of receptor axons and the organisation of phototransduction.

Cnidaria are in a key position to unravel the enigma of the eye evolution representing the most basal phylum with a nervous system and eyes. Recent work showed that this basal phylum already contains a surprising diversity of transcription factors and metabolic enzymes previously assumed to be restricted to vertebrates (Kortschak et al., 2003). Genes formerly thought to be vertebrate inventions must have been present in the common metazoan ancestor. The results of this thesis confirm this hypothesis as is demonstrated by the identification of the Six class homeobox family genes (chapter 1) and members of the paired box family (chapter 2) display. Expression analysis and regeneration experiments demonstrate that *Cladonema* employs the same highly conserved eye-specification network as proposed for Bilateria. But the question remains: Does the mere ownership of a highly conserved gene-cascade (master control gene network) and its corresponding function legitimate the claim of a monophyletic eye evolution?

To answer this question we first have to define and focus on the prerequisite of vision. All animals (including the eyes of cnidarians, see below) use rhodopsin as a photoreceptor molecule. Opsins of all animals are probably homologous and when activated they all couple to a trimeric, GTP binding G-protein. A G-protein coupled receptor with the characteristic seven transmembrane helices is used as a light sensitive receptor even in bacteria. Bacteriorhodopsin is perhaps among the simplest known ion pumps which functions by converting light energy into an electrochemical gradient pumping protons out of the cytoplasm. The fact that already bacteria possess light sensitive receptors shows the importance of light as a selective force, probably the most profound selective force during evolution (Fernald, 2000). Light influences movement, photosynthesis, navigation, timing, vision and behavior. Hence we may assume that a unicellular ancestor of all Metazoa possessed some kind of a simple light perception unit consisting of receptor protein, protein cascade and corresponding nuclear reaction to that incoming stimulus. Although this

unicellular ancestor was in a way certainly influenced by light it did not bear eyes and we are far from organogenesis. Sooner or later evolution must have passed the status of a myosensory cell, a cell equipped with cilia or with a flagellum. This myosensory cell would potentially represent the common ancestor of muscle and nerve cells. Light information from receptor proteins can be used to change the direction of flagellar beat, as was demonstrated in *Euglena* (Lebert and Hader, 1997).

It appears plausible that the evolution of the zootype did not pass diploblasty but assembled for functional reasons from the beginning the three different germ layers. Genes, gene networks, or gene cascades that were established within the myosensory cell could have been co-opted in forging the basic Bauplan. In this view it is not astonishing that myogenesis and eye development are controlled by a similar synergistic genetic network (Heanue et al., 1999). It would also explain why Otx is used in muscle cells of medusa whereas it occupies nerve specific roles in Bilateria (Müller et al., 1999). Locomotion and feeding have certainly been of high selective value in evolution and therefore been tightly coupled to the development of muscle and nerve cells. With the evolution of the anatomy for fast locomotion sensory adaptations became necessary leading to diverse eye structures. In essence it does not matter how those sensory structures are realized, the already evolved genetic cascade from the myoepithelial-neuro-sensory ancestor was implemented and the specific members of these gene families selected for specific functions. A discussion about the homology of highly evolved eye structures is therefore redundant if it can be demonstrated that the simplest animals with eye-like organs, jellyfish, used the same genetic inventory to develop eyes.

Are all eyes homologous?

What precisely does the term homology signify when there are so many differences among the descendant structures and between them and the ancestral eye form? If in the view of classical phylogeny, a character shared between two species was present in the common ancestor (convergent) of the two species, it is termed homologous, if not it is an analogous character. Homologies are further divided into ancestral and derived homologies. If a character is homologous it must have the same fundamental structure and the same relationship to surrounding characters and development in the two species. A gene performs a homologous function in two animals if at least some of its upstream linkages or both remain the same in the two genomes, and the function it performs is descendant from their common ancestor (Davidson, 2001). Wilkins (2002) tries to answer the question of homologous eye structures as he demands some changes in the way the term homology is used. Morphological traits and genes need not to share tight, invariant relationship. There exists a continuity of genetic information and therefore the basic concept (of homology) of shared possession remains intact. In this context the eyes of insects and vertebrates are homologous even though they look different from each other, develop differently and may have arisen independently in separate lineages from ancestors lacking eyes. What they share is the inherited regulatory machinery and the ancestral function of that machinery for light sensing or some rudimentary form of vision.

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Cnidarians – the model organism of choice

The research of cnidarians has a long history. In the 1740s the Swiss scientist Abraham Trembley (1710-1784) started to work with the freshwater polyps, today known as Hydra. He discovered that hydra could regenerate heads and feet, and if cut into small pieces, all of them would regenerate to form new individuals. He was able to split the head of the polyp longitudinally and allow two heads to regenerate. By repeatedly splitting the new heads, he was able to generate a multiheaded animal that he named Hydra in reference to the mythological creature. Trembley was one of the first scientists to demonstrate that animals could reproduce asexually.

Cnidaria are well positioned to study questions related to evolution. They represent an old phylum with tissue level anatomy but already have differentiated striated and smooth muscle tissues, complex nerve rings and sense organs. Furthermore their big potential for regeneration and transdifferentiation can be additionally exploited for the evolution of development and gene interacting networks. Therefore, this phylum occupies a unique position with respect to information from basal genomic characteristics like gene structure, the ancestral function of genes and the gene complement of the common ancestor (Miller and Ball 2000).

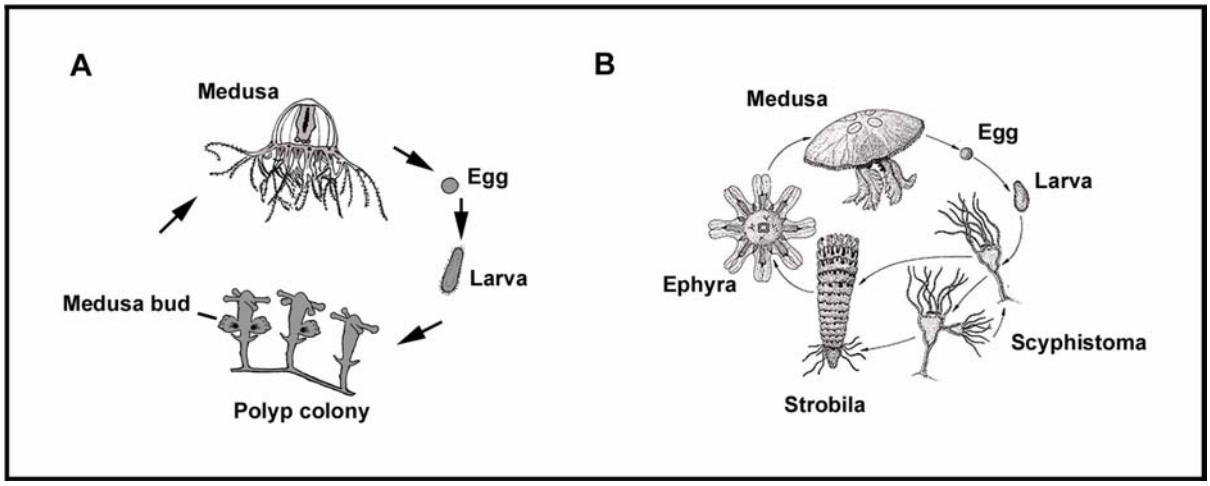

Figure1 The life cycle of one representative of the Hydrozoa, *Cladonema radiatum* (A), and a representative of the Scyphozoa, *Aurelia aurita* (B).

The phylum cnidaria comprises four different classes: Anthozoa, Hydrozoa, Scyphozoa and Cubozoa. Their relationships are still controversial. These mostly marine carnivorous animals show a primary radial symmetry and hence it is considered to place the cnidarians separately into the Radiata (Ruppert and Barnes, 1994). Fossil records are poor but those fossils found date back to the Precambrian. Most of the animal phyla that are represented in the fossil record first appear "fully formed" some 550 million years ago in the Cambrian (Ruppert and Barnes, 1994). Therefore the origin and early diversification of the various animal phyla must have occurred in the Precambrian between 600 and 1000 million years ago.

Approximately 9500 living species are combined in the cnidarian phylum, making it the seventh largest (Miller, 2000). Most cnidarians exhibit a metamorphic life cycle including a planula larva, a stationary benthic polyp stage and a free-swimming medusa, the sexually reproducing form. The variation of life cycle forms is outstanding with the highest degree of diversity within the Hydrozoa.

The polyp shape (Fig. 2) is in essence that of a tube with one end carrying the mouth and the other a basal disc that attaches the animal to the substratum. There is only one body

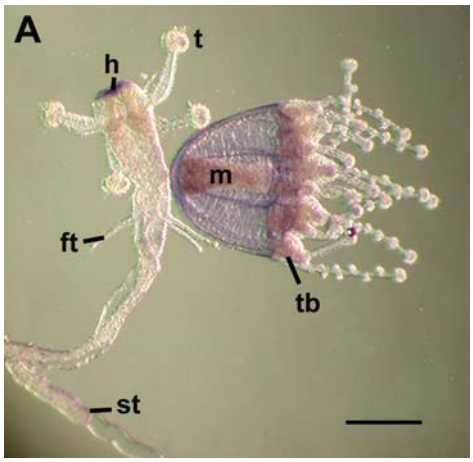

Figure 2 A polyp of *Cladonema radiatum* bearing a medusa bud, stained with an antisense RNA probe for *Six1/2-Cr*. The message localizes to exumbrella, striated muscle and hypostome (mouth region). h, hypostome; ft, filiformous tentacle; m, manubrium; st, stolon; t, tentacle; tb, tentacle bulb. Bar is 100 μm.

opening serving for food intake and ejection of indigestible material. The body wall consists of two tissue layers separated by an ECM (mesogloea). The mesogloea is composed of collagen (type IV), fibronectin, heparan sulfate proteoglycan, and laminin (reviewed in Schmid and Reber-Müller, 1995). Epitheliomuscular cells, interstitial cells, cnidocytes, gland cells and sensory nerve cells are the main cell types found interspersed in the two layers. The cnidocytes contain the stinging structures, particulary nematocysts, and are unique among the metazoans. But nematocyst-like structures are not an exclusively cnidarian feature in that similar organelles are present in a number of protists (Myxozoa, Microspora and Dinophyta). Nerve cells are associated in an irregular nerve net or plexus in the polyp. The medusa is the more elaborate life stage and its tissue architecture is completely different to that of the polyp. The mesogloea of the medusa bell is increased and striated muscle tissue and sense organs differentiate only in the medusa. In general the nervous system of the medusa is more highly specialized than that of the polyp. At the margin of the bell nerve cells are organized in a nerve ring. Some medusae contain even two nerve rings. It has been shown that these nerve rings can contain large motor neurons that connect to the swimming muscles. The

nerve ring should contain also the peacemakers, the center for rhythmic pulsation (Mackie and Meech, 1995). In *Polyorchis* it has been demonstrated that central neurons of the inner nerve ring respond to light (Anderson and Mackie, 1977). The nervous system of cnidarians is remarkable in the multifunctionality of the nerve cells: all neurons are sensory-motor-interneurons with neurosecretory granules (Koizumi, 2002). True sense organs of the medusa are the light sensitive ocelli and statocysts.

In Hydra germ cells arise from interstitial cells (Tardent, 1969). Cnidarian sperm with one exception lack an acrosome (Carré, 1984), but many contain several mitochondria. Fertilization is predominantly external in the water, but internal fertilization is known and in Cubozoa even an example of copulation is described (Brusca and Brusca, 1990). Fertilization takes place when oocyte meiosis is completed. Several different cleavage patterns are known and gastrulation is very diverse in cnidarians. By the end of gastrulation a bilayered ciliated planula larva is formed. Planulae swim for several hours to several days. They are planktonic and serve for dispersal. Planulae can be planktotrophic (many Anthozoa) or lecithotrophic (many Hydrozoa). It is believed that the planulae receive an external stimulus that signals the entry to metamorphosis. The body plan of the cnidaria is regarded to be as successful neither did it give rise to any other phlya nor any other known animal group derive from Cnidaria. It was suggested that the ancestry of the cnidarians must lie within the protista.

Anthozoa

Anthozoa are generally believed to be the most ancient cnidarians as the structure of mitochondrial DNA, mitochondrial 16S ribosomal DNA sequences as well as 18S ribosomal DNA sequence data suggest (Bridge et al., 1995). Only anthozoans have circular mitochondrial genomes. Those of the other cnidarian classes are linear and often fragmented. The medusoid stage is completely absent in this largest cnidarian class. The Anthozoa can

form large solitary or colonial polyps. The most familiar cnidarians like sea anemones or corals belong to the Anthozoa.

Hydrozoa

In contrast to the Anthozoa most people are unaware of the existence of the Hydrozoa (see Fig. 3). Hydrozoa are of small size and they often grow attached to rocks where they are usually dismissed as seaweed. A few freshwater species belong to this class with the most popular Hydrozoan *Hydra*. *Hydra* is an untypical member of this class. It not only lost the medusa stage and its polyps are solitary, it also owns an infrequent and unpredictable sexual reproduction. Without any doubt the freshwater *Hydra* can not serve as a representative of this overwhelmingly marine phylum. The uniting characters of the Hydrozoa are the lack of cells in the mesogloea, the gastrodermis contains no cnidocytes and the gonads are mostly of epidermal origin (Ruppert and Barnes, 1994). The hydromedusae are typically small ranging from half of a centimeter up to six centimeter in diameter. The medusa bears a velum. The most primitive hydrozoans are probably species in which the pelagic actinula develops directly into an adult medusa (Ruppert and Barnes, 1994). The polypoid state is missing. This life cycle is realised in the order of the trachymedusa.

Scyphozoa

Scyphozoa-medusae are similar to the hydromedusae but differ in the following: the manubrium is tentaculate, the medusa lacks a velum, the mesogloea can contain amoebocytes and gonads are gastrodermal. It is reported that nerve rings in Scyphozoan medusa are rare (Ruppert and Barnes, 1994). Pulsation control is centered on the marginal concentrations of neurons in structures called rhopalia (Hyman, 1940). Rhopalia carry also statocysts and

sometimes ocelli. The polyp forms a scyphistoma that buds by fission ephyra larvae. Ephyra larvae form juvenile medusa.

Cubozoa

The representative of the cubozoan medusa investigated is *Carybdea marsupialis* (Fig. 3). Cubomedusa are fast swimmers and known to possess a vicious sting. Cubozoa are commonly called box jellies because they have a cubical shape. There are about 20 known species found in tropical and semitropical waters. The medusa is the dominant phase in the life cycle. The very small polyp produces a single medusa by complete metamorphosis. Cubozoa have evolved the most elaborate ocelli of this phylum and the only medusa whose behaviour can be studied (Nielson, pers. communication).

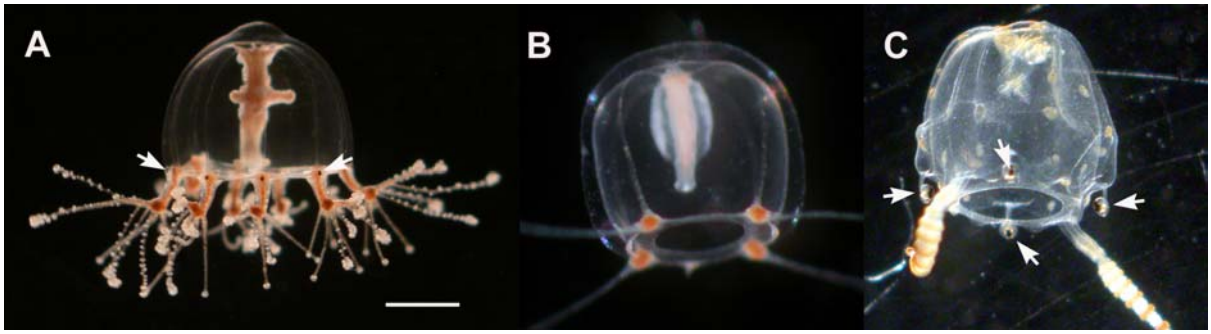

Figure 3 The species used for this thesis: (A) *Cladonema radiatum*, (B) *Podocoryne carnea*, (C) *Carybdea marsupialis*. *Cladonema* and *Podocoryne* are members of the Hydrozoa whereas *Carybdea* is a representative of the Cubozoa. The lens eyes are located at the margin of the bell (arrows in A, C). Note that the eyes in *Carybdea* are stalked and arranged together with additional ocelli. *Podocoryne* does not bear any eyes. Bar is (in μm) 350 in (A), 240 in (B), 330 in (C).

The eye – a classical model system for evolutionary studies

The vertebrate eye is one of the classical models used to demonstrate many important principles, including the concepts of inductive tissue interactions first investigated in the early 1900s. Developing imaginal discs from *Drosophila* have been described already in 1864, but the real study of the development of the compound eye started in the mid-seventieths of the 20th century (Moses, 2002). During the past 30 years there has been an explosion in the study of the fly eye. One of the most astonishing discoveries in the last years has been the molecular homology between invertebrates and vertebrates, especially the specification of the eye via the Pax6/Eyeless (Quiring et. al., 1994).

Photoreception must be phylogenetically very old. A consequence of the phylogenetic antiquity of photoreception is its near ubiquitousness in the animal kingdom. The slight variability of visual systems within a species is a sign of high selection pressure. Invertebrates have evolved a greater variety of evolutionary adaptations of their light sensitive organs than vertebrates. It is not only the huge number of species which suggests this multiformity of eye structures but also the remarkably different biotopes they occupy. Each phylum has pecularities of its own, in morphology as well as in physiology and behavior. A sedentary habit can lead to a complete absence of eyes, although some molluscs like pecten have highly developed eyes. Invertebrates have evolved adaptations that are never or only rarely found among vertebrates for example to detect the polarization of light and to orient by the pattern of polarization. As animals evolved new activity patterns, changing from the diurnal habit to a preferance for twilight and finally to life in the dark, a large number of adaptations developed. Frequently larval animals have well-developed eyes that later degenerate to a considerable extent (f.e. Ascidians). Many endoparasites completely lack eyes.

Many aspects of eye development can be investigated with the fly eye. *Drosophila* as a model system has been invaluable to elucidate eye development. A discussion of eye development without concerning results from this system seems to be impossible. However this genus lacks some developmental aspects. Arthropods precede the evolutionary appearance of neural crest, an embryonic structure from which much of the anterior of the vertebrate eye is formed. In *Drosophila* it has been estimated that more than 2500 genes may be required to construct the visual system (Gehring and Ikeo, 1999).

Note there are also a number of important eye structures that have barely been investigated, work on cornea and anterior chamber development has lagged behind lens and retina and almost nothing is known on the molecular level regarding development of the glands associated with the anterior of the vertebrate eye (see Moses, 2002).

Definition of an eye

The gradation from light-sensitive single cells through localized groups of such cells that serve as photoreceptive organs, to more complex organs with focusing devices is so continuous that it is difficult to define what an eye is. The presence of pigmentation is not a prerequisite for photoreceptors. Gehring and Ikeo (1999) propose the definition of a prototype eye: a prototype eye consists of at least two cells, a pigment cell and a photoreceptor cell carrying the visual pigment.

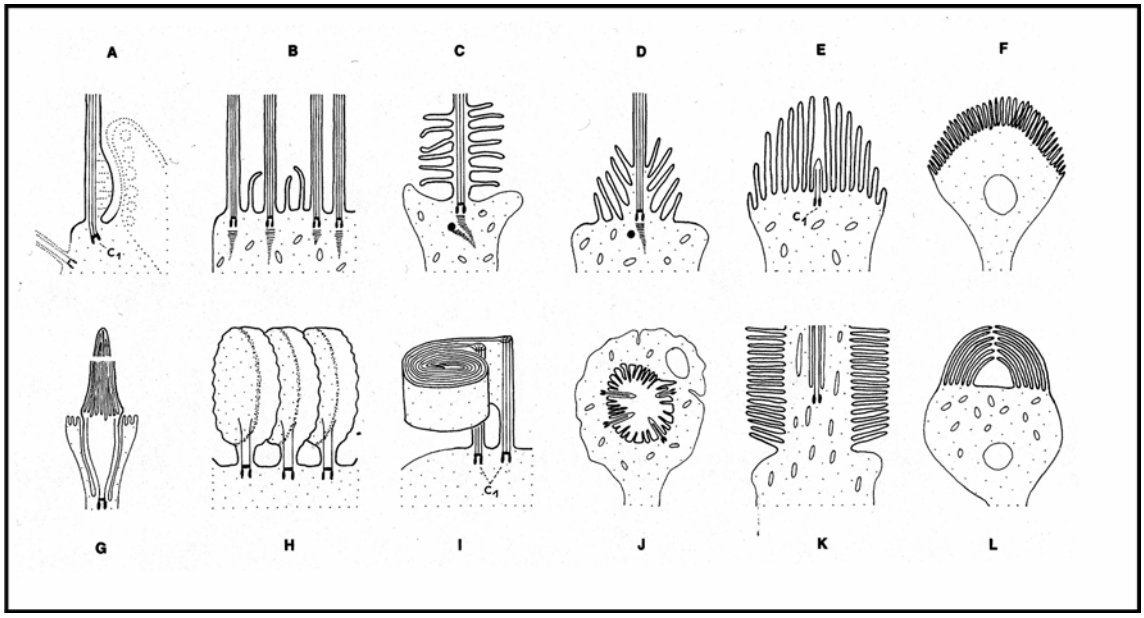

Figure 4 Different photoreceptor types from (A) Protozoa, Phytoflagellata, (B) Bryozoa, (C) Cnidaria, Hydrozoa, (D) Echinodermata, Astereoidea, (E) Mollusca, Gastropoda, (F) Cephalochordata, Branchiostoma, (G) Chaetognatha, (H) Polychaeta, (I) Placophora, (J) Annelida, Clitellata, (K) Onychophora, (L) Rotatoria. Figure modified after Salvini-Plawen and Mayr (1977).

The most basic function of a photoreceptor (Fig. 4) is to measure changes in light intensities. Two basic forms underlie photoreceptor structure: either cilia are present and the visual cell is modified in various ways, or the membrane of the visual cell is greatly enlarged by microvilli or lamellae. The visual cells of vertebrates are quite uniform. All of them contain ciliumlike structures with the typical 9+2 arrangement. In contrast, invertebrates form many different structures. These falls into two categories: (1) the ciliary photoreceptors and (2) the rhabdomeric photoreceptors. The membrane of the cilium can be enlarged in various ways: tunicates show disc-like processes of the cilium, the ocelli of sea stars are of ciliary type but from their cilia arise irregularly twisted microvilli, lamellar processes are found in the polyplacophoran mollusc *Onithochiton neglectus* while *Euglena* shows paraflagellar bodies. Micorvilli can also be arranged in a remarkable variety of ways. Ciliary and rhabdomeric photoreceptors occur side by side in *Pecten* and *Lima*.

However there exist photoreceptors that do neither contain microvilli nor cilliary structures. A great number of freshwater and marine decapod crustaceans have a paired photosensitive neuron in the sixth abdominal ganglion that lacks both optical structures. Neural photoreception is known from numerous invertebrates like the giant ganglion cells of *Aplysia* or in the metasoma of scorpions (Salvin-Plawen and Mayr, 1977). Pigment cup cells in *Branchiostoma* (Hesse's cells) are secondarily modified ganglia cells which never bear cilia. Light sensory cells of some Nematoda appear to be modified bipolar neurons.

One aspect in which the photoreceptors of vertebrates and invertebrates differ markedly is the nature and function of their receptor membrane. Vertebrate photoreceptor cells respond to light with hyperpolarization whereas invertebrate photoreceptors depolarize to light. The optical characteristics of the eye are primarily determined by whether it is used in air or water, or whether it is used under diurnal or nocturnal conditions. Lenticular structures show nearly as much diversity as receptor cells (Salvini-Plawen and Mayr, 1977).

Different eye types in the animal kingdom

Photoreceptors are present in most animal groups (see Fig. 5). Some dinoflagellates (*Erythropis*, *Liarnovia*, *Glenodinium*) are described to possess highly differentiated photosensitive structures. A number of phytoflagellates, i.e. *Euglena* (including the spermatozoids of Phaeophyceae) bear eyespots (Salvini-Plawen and Mayr, 1977). Minchin (1896) described some unicellular photoreceptors in the larvae of *Leucosolenia* (Porifera) situated in the central portion of the organism. Many endoparasitic species may have lost secondarily eye-like structures.

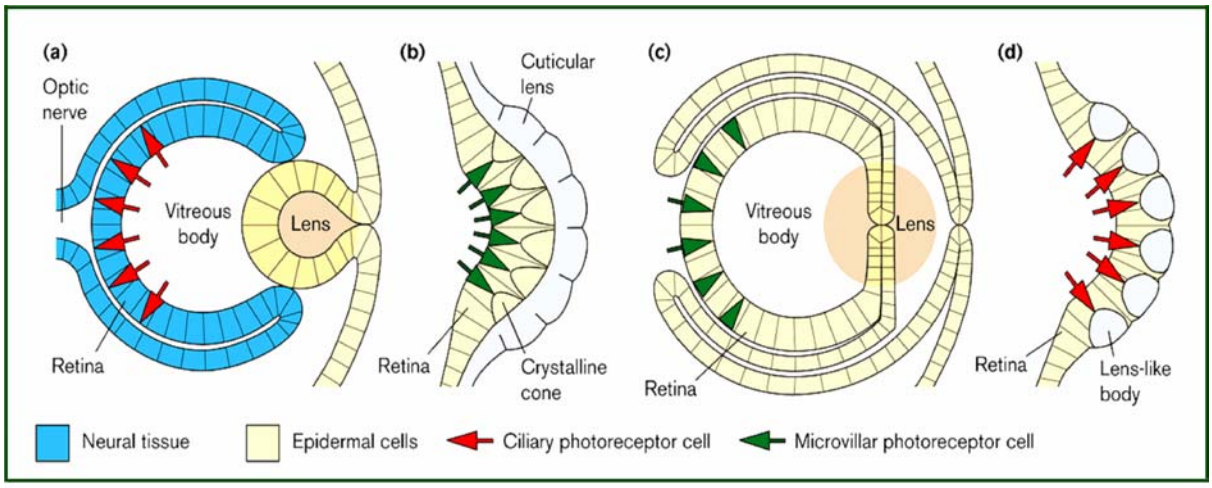

Figure 5 Building plans of four types of eye. (a) A vertebrate eye. (b) An arthropod compound eye. (c) A cephalopod lens eye. (d) A compound eye in polychaete tube-worms and arcoid clams. Note that the construction of eyes varies considerably. For example, in chordates, photoreceptor cells differentiate from the central nervous system, whereas cephalopod and arthropod eyes differentiate from the epidermis. In addition, the retina is inverse (e.g. photoreceptors are at the back of the eye) in vertebrates and everse (e.g. photoreceptors are at the front of the eye) in cephalopods. Figure taken from Fernald, 2000.

The fine structure of vertebrate eyes had been studied since the beginning of histology. Therefore the development of the vertebrate camera eye is well understood. Molluscs display the greatest diversity in the differentiation of eyes among all groups of animals. The best understood eye of invertebrates is the compound eye of *Drosophila*.

The arthropod compound eye

The compound eye of *Drosophila* contains approximately 800 individual light-sensing units called ommatidia. An ommatidium consists of 19 cells including 8 photoreceptors, 4 cone cells, 6 pigment cells and a mechanosensory bristle. The position of each cell within an ommatidium is precisely stereotyped so that each unit is an exact replica of its neighbors. Compound eyes have a single lens for each ommatidium. There are about 16000 viable cells in the adult eye. Approximately 2000 of the cells generated during eye development are

eliminated by apoptosis (Wolff and Ready, 1993) so finally there have been almost 18000 cells created during eye development. Cell death occurs in a tight band just ahead and following of the advancing morphogenetic furrow (see Fig. 6) (Wolff and Ready, 1991). The adult eye develops from the so called eye imaginal disc (monolayer epithelia), a structure which derives from about 20 cells set aside during embryonic development. This means that there is an almost 1000 fold increase in the number of cells during eye development (Neufeld and Hariharan, 2002).

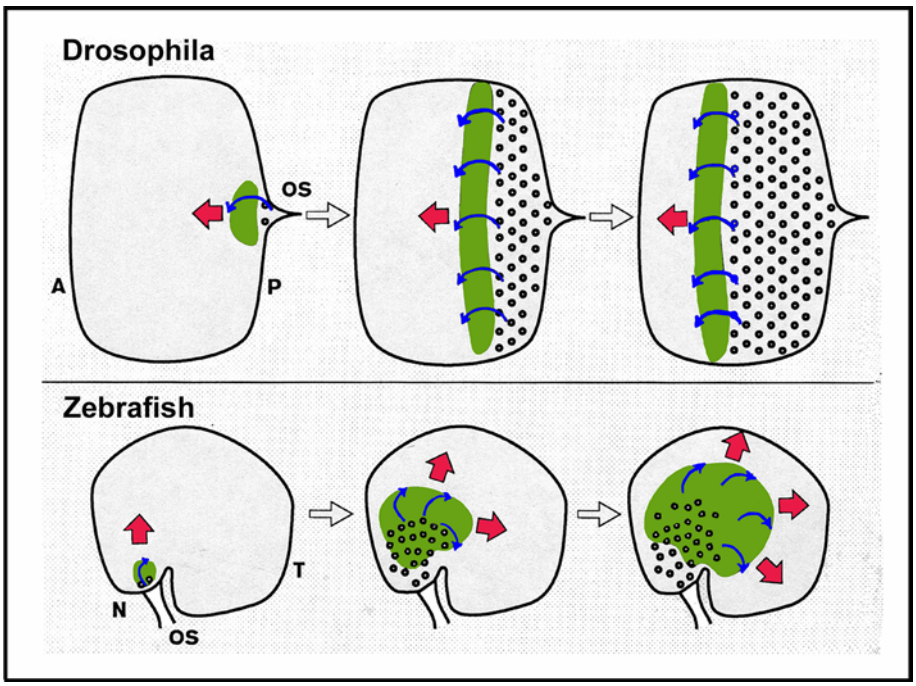

Figure 6. A wave of differentiation called the morphogenetic furrow. The way the genes are deployed in insects and vertebrates is remarkably similar. Neurogenesis in the *Drosophila* eye imaginal disc and the zebrafish inner optic cup is schematically displayed (after Jarman, 2000). Expression of atonal/ath5 (in green) precedes the appearance of initial neurons (R8 in *Drosophila*, retinal ganglion cells in zebrafish). Short-range hedgehog signaling (blue arrows), produced by newly formed neurons, appears to drive the wave of neurogenesis. Red arrows mark the direction of the wave. It remains speculative if sonic hedgehog acts via ath5 activation. A, anterior; p, posterior; N, nasal; T, temporal.

The early development of the eye imaginal disc is marked by the expression of a set of nuclear factors, the eye specification genes that will be discussed later. Eye specification does

not occur during embryonic development as previously thought, but in the second larval stage (Kumar and Moses, 2001). It is only during the second larval stage that all seven eye specification factors have overlapping expression patterns in the eye imaginal disc. The eye disc of *Drosophila* has a progressive pattern of differentiation. During embryogenesis and the first two larval instars, cells within the eye imaginal disc are unpatterened and undifferentiated. Differentiation of photoreceptors starts at the posterior margin of the eye disc and proceeds anteriorly. Prior to their differentiation cells constrict and show an apical-basal contraction that leads to an indentation which is called the morphogenetic furrow (MF; Ready et al., 1976). Anterior to this dorso-ventral groove the cells are unpatterned and divide actively. In the MF cells are arrested in the G1 phase while posterior to the MF cells undergo one round more of division, the second mitotic wave. Photoreceptor differentiation takes about 2 days. R8 cells are the first to differentiate in each ommatidium and are required for recruitment, mostly mediated via Epidermal growth factor receptor (EGFR), of other cells. R8 specification is dependent on atonal, a basic Helix-Loop-Helix (bHLH) transcription factor. Atonal null mutants lack nearly all the eye. It is expressed in a period of less than three hours within the corresponding specifying ommatidial column and controls the levels of EGFR signaling (Baker et al., 1996). EGFR is a tyrosine kinase membrane receptor and its activity leads to the RAS/MAPK signaling cascade. R8 cells require EGFR signaling only for their maintenance after the proneural gene atonal is downregulated, a function that is separable from roles in specification (Kumar et al., 1998). It has been shown that EGFR signaling is also needed to suppress programmed cell death in the eye (Bergmann et al., 1998). The receptor protein Notch plays several roles patterning the atonal expression and activated Notch expression can abolish atonal expression. On the other hand a reduced function of Notch leads to the differentiation of more than one R8 cell at the same place (Baker and Zitron 1995). EGFR and Notch signaling pathways control therefore the initiation

of the MF. It has been shown that those pathways have homeotic functions that are genetically upstream of the eye specification genes (Kumar and Moses, 2001). The complete homeotic transformation of the eye into an antenna can be induced by a hyperactivation of EGFR or a downregulation of Notch signaling (Kumar and Moses, 2001). After founding of the R8 cells development of the ommatidia seem to be self-organizing. Photoreceptor cells R2, R3, R4, R5 and R8 differentiate but those cells do not divide any more. After passing of the MF all other cells enter a synchronous round of cell division (Wolff and Ready, 1991) and differentiate into the remaining photoreceptor cells (R1, R6, R7), cone cells, pigment cells and cells of the interommatidial bristle. BarH1 is critical for the differentiation of the pigment cells (Hayashi et al., 1998). Six of the photoreceptor cells, R1-R6, extend the full depth of the retina whereas the remaining two, R7 and R8, are restricted to the upper and lower halves of each ommatidium. Each photoreceptor cells contains a specialized microvillar structure that is the site for light reception and phototransduction. The rhabdomeres are functionally equivalent to the outer segments of human rod and cones. Both R1-6 cells and rods are very sensitive to light, express a single visual pigment, and make up the majority of photoreceptor cells. By contrast, R7-8 cells and cones are less sensitive to light, express multiple visual pigments and comprise a high-acuity system. R7 and R8 cells occupy the central region of the ommatidia and are smaller in cross-sectional area. The outer photoreceptors R1-6 are mainly responsible for image formation and contain a visible light sensitive opsin, Rh1 (Papatsenko et al., 1997).

Cnidarian eye types

In general it is the medusa stage that carries eye-like structures, although polyps from all cnidarian classes are described to be light-sensitive (Tardent, 1969). One identified polyp (Stylocoronella riedli, Scyphozoa) seems to have multicellular light-detecting organs. This

interstitial living polyp has up to 24 pigment spot ocelli, located at the base of the tentacles, composed of monociliated sensory cells and pigment cells (Blumer et al., 1995). However its life cycle classifies this polyp as a member of the stauromedusae (sessile medusa). Recently a Cubozoan larva was reported to possess one-celled ocelli that even lack nerve cells (Nordström, 2003). The ocelli of the larva disappear as they settle to form polyps. Cnidarian photoreceptors range from simple eyespots and eyecups to complex eyes with a lens (Fig. 7). The number of such ocelli varies from a few to sometimes several hundreds (*Spirocodon saltatrix*). Extraocular photosensitivity is widespread throughout the cnidarians, with neurons, epithelial cells, and muscle cells mediating light detection.

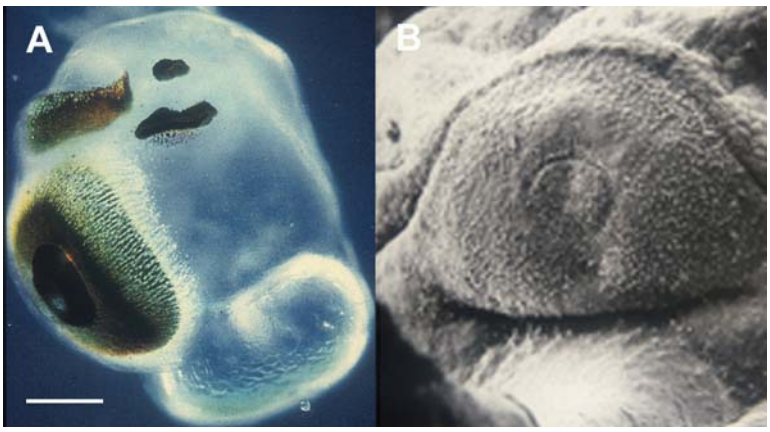

Figure 7 Cnidarian lens eyes. Stalked rhopalium of a Cubomedusa (A) and an electron microscopic picture of a hydrozoan eye. Bar is (in µm) 50 in (A), 15 in (B).

Cubomedusae have four rhopalia, each with a statocyst, two slit eyes, two pit eyes and two lens-eyes (Pearse and Pearse, 1978). The rhopalium can twist and swing back and forth.

Salvini-Plawen and Mayr (1977) argue based on morphology that cnidarian photoreceptors evolved independently in four or five different lines. All photoreceptors of jellyfish are of the ciliary type (Eakin and Westfall, 1962; Eakin, 1963) hyperpolarizing in response to light like vertebrate photoreceptors do. It has therefore been suggested that the photoreceptors of Cnidaria belong to the same evolutionary line as those of vertebrates (Eakin 1963, 1968, 1979). Photoreceptor cells of Hydrozoans are coupled to each other through gap junctions

(Singla and Weber, 1982). Such electrical coupling allows amplification of low-intensity light. Nerves and synapses operate in much the same way as those of higher animals (Mackie and Meech, 2000). Eyes seem to be directly coupled to the muscles. Photoreceptor cells have axonal contacts onto second-order neurons that group together to form an ocular nerve. Those ocular nerves enter into the main net of the animal, the nerve ring (see Fig. 9A, B). These nerve rings could be understood as the animal's central nervous system (Macki and Meech, 1995). The nerve ring neurons are large to facilitate fast transmission around the bell margin (Mackie and Meech, 1995). The precursor of the photoreceptor cells in cnidarians was probably a photosensitive ciliated ectodermal cell (Martin, 2002).

Physiological studies demonstrate that the photoreceptor cells of cnidarians respond to light intensity with graded potentials which are directly proportional to the range of changes in the light level. It has been shown that some cnidarian ocelli are most sensitive to blue-green and green light with spectral curves ranging from 363 to 675 nm (Weber, 1982). Furthermore it is believed that Cubomedusae are able to distinguish light spectra ranging from ultra-violet to deep red light (pers. communication D. Nilsson), a capacity requiring several rhodopsin types. The spectral range covered from the cubozoan eyes would be greater than from any other known animal. Behavioural experiments suggest that changes in illumination influence the movements of the animal (Hyman, 1940). From the evolutionary point of view it remains unclear why so anatomically simple structured, but certainly not primitive animals evolved sophisticated lens eyes. It remains enigmatic as the question about their quality of vision.

Cladonema radiatum is a benthic hydrozoan jellyfish that undergoes the full life cycle consisting of asexually reproducing polyp colonies liberating gonochoristic medusae. The medusa stage carries eight to twelve genuine anatomical lens eyes in the tentacle bulbs (at the margin of the bell) that derive exclusively from the ectoderm (Fig. 8).

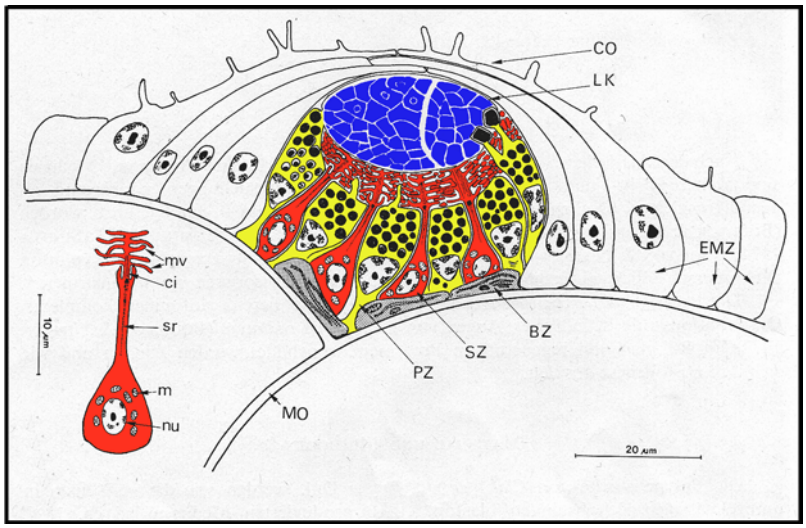

Figure 8. Schematic cross-section of a *Cladonema* lens eye. Ciliary-type photoreceptor cells are in red, melanin containing pigment cells are in yellow and the tripartite biconvex lens is in blue (modified after Weber, 1978). ci, cilium; CO, cornea; EMZ, epithelial muscle cell; LK, lens; m, mitochondria; MO, mesogloea; mv, microvilli; Nu, nucleus; PZ, pigment cell; sr, striated root; SZ, photoreceptor cell.

Structure, development and regeneration of those ocelli have been studied in detail (see Weber, 1981a, b). An ocellus has a diameter of 45-55 µm and contains a tripartite, biconvex lens. The lens body originates from the apical portion of the pigment cells whose pigmentation has been identified as melanin (Weber, 1981a, b). It is generally believed that all metazoans share the same visual pigment rhodopsin although it has never been shown to be the case for cnidarians, nor is the sequence of any cnidarian opsin available.

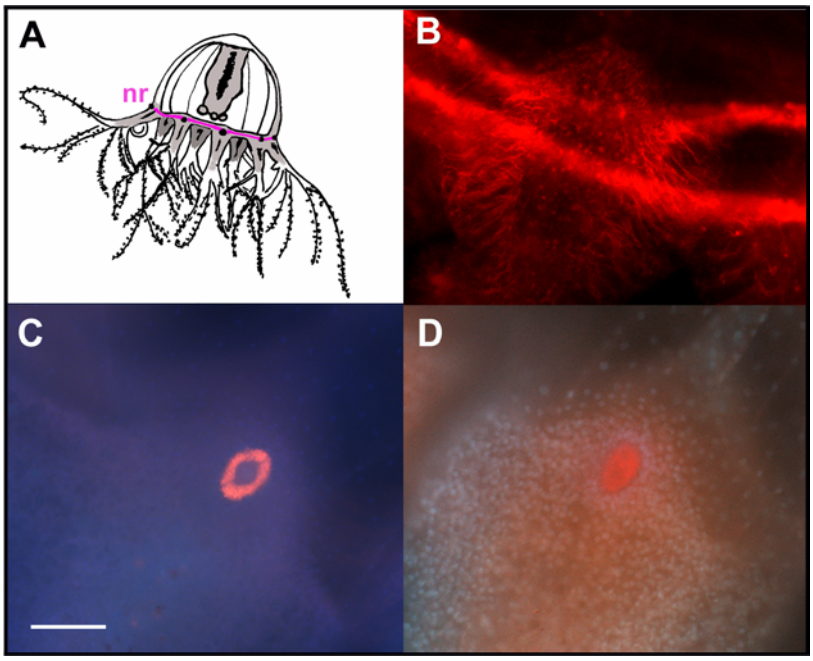

Figure 9 (A) A scheme of a *Cladonema* medusa displaying the localization of the nerve ring (in pink) along the ring canal. Tyrosine-tubulin positive nerve fibers (B) connecting the innervation of the eye to the nerve ring. (C) Cross-reaction of a monoclonal rhodopsin antibody developed against Drosophila rhodopsin 1. Note that the lens area is free of the antibody labeling (C) whereas a polyclonal squid anti-opsin antiserum stains the whole eye area (D). Nuclei are stained with DAPI. Bar is (in µm) 450 in (A), 180 in (B), 50 in (C), and 60 in (D). Immunohistology is described in chapter 1.

A *Drosophila* monoclonal antibody directed against *Drosophila* rhodopsin 1 stains specifically photoreceptor cells of *Cladonema* (Fig. 9C). Its circular staining pattern can be explained by the central position of the lens and therefore fits perfectly with the electron microscopic analysis of the eye (see Weber, 1981a). The staining pattern is specific and any artefact staining can be excluded. The specificity permits the use of this antibody as a differentiation marker. A polyclonal squid anti-opsin antiserum, originally developed against a *Loligo* eye extract, stains also specifically *Cladonema* eyes (Fig. 9D). Its crossreaction is not as strong and precise as with the insect monoclonal antibody, staining probably also pigment cells or a different opsin type. The epitope of both antibodies used is not known.

The eye specification genes

All of the master control genes are expressed anterior to the morphogenetic furrow and before the initiation of the neural differentiation in *Drosophila*. With the exception of sine oculis (so) any of those eye specification genes are sufficient to initiate the entire programme of retinal development when they are ectopically expressed. Synergistic induction of ectopic eye formation can be observed by most combinations of ectopic gene expressions. Physical interactions of the encoded proteins of these eye specification genes have been observed. Each gene of the eye specification network (Fig. 10) is absolutely required for ectopic eye induction.

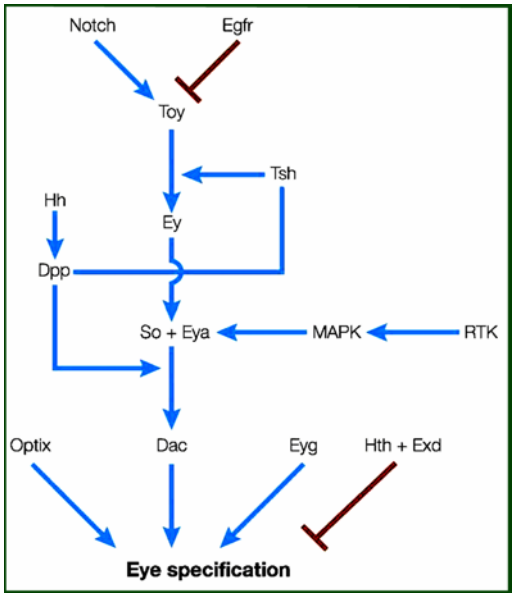

Figure 10

Genetic control of eye specification in Drosophila. A set of nuclear proteins, patterning pathways and signal-transduction cascades form a complicated regulatory network and are together required to specify the compound eye in Drosophila. The arrows indicate the direction of the genetic, molecular and biochemical relationships.

(taken from Kumar, 2001)

Removal of any of the eye specification genes results in drastic reduction or deletion of the adult compound eye and loss-of function of one gene can result in a loss of expression of another (summarized in Fig. 13). There are several feedback loops to ensure normal eye development. The genes do not function in a linear pattern but rather in a complex network of interactions that constantly cross-regulate. Transcriptional and post-translational regulation of

those eye specification genes is achieved through interactions within the network and with extracellular signaling pathways, including EGFR/RAS/MAPK, TGF-β/DPP, Wingless, Hedgehog, and Notch.

eyeless/Pax6

Pax genes are transcription factors characterized by a DNA binding motif called the paired domain. The paired domain (PD) is a stretch of 128 amino acids named after the prototypical Drosophila segment polarity gene paired in which it was first identified (Bopp et al.,1986). The PD is organized into distinct N- and C-terminal subdomains, termed PAI and RED respectively. Each subdomain consists of three alpha-helices arranged in a helix-turn-helix motif (Xu et al., 1995) and encodes a sequence-specific DNA binding activity. Both the N- and C-terminal subdomains make contact with the DNA (Xu et al., 1999). Some Pax genes contain in addition other conserved domains such as a complete or partial paired type homeodomain (HD), or an octapeptide. The octapeptide is located between the PD and the HD. The paired type homeodomain found in Pax genes is characterized by the presence of a crucial residue found at position 50, a serine (S_{50}), whereas most homeoproteins including all Hox proteins bear a glutamine at this position. In human nine different Pax genes have been identified that can be grouped into four different classes: (1) Pax1 and Pax9, (2) Pax3 and Pax7, (3) Pax4 and Pax6, (4) Pax2, Pax5 and Pax8.

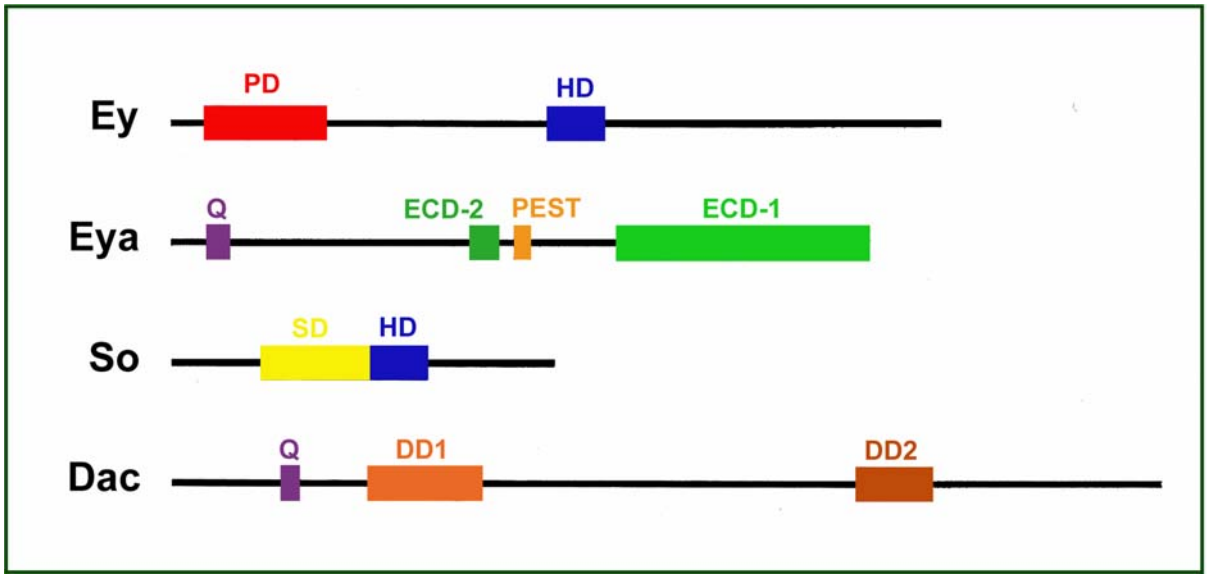

Figure 11 Predicted structures of the proteins encoded by the core eye specification genes in Drosophila. Figure modified after Kumar, 2001.

Pax6 is essential for the development of tissues including the eyes, central nervous system and endocrine glands of vertebrates and invertebrates. Pax6 takes also part in the early formation of the neural tube and the olfactory epithelium. It regulates the expression of a broad range of molecules, including transcription factors, cell adhesion and short-range cell-cell signaling molecules, hormones and structural proteins (Simpson and Price, 2002). Pax6 is involved in many biological processes like cell proliferation, migration, adhesion, signaling and oncogenesis. A number of human allelic variants of the Pax6 gene have been identified like aniridia, Peter's anomaly, keratitis, foveal hypoplasia and ectopia pupillae (Simpson and Price, 2002). But the proposed reciprocal inductive signals from presumptive lens ectoderm to presumptive retina are not dependant on Pax6 (Treisman and Lang, 2002). Initially Pax6 was identified in mouse (Walther and Gruss, 1991) but to date homologues have been isolated in a broad range of species. It is known from human, rat, chick, *xenopus*, zebrafish, ascidians, sea urchins, cephalopods, *C.elegans*, *Drosophila*, planarians. Injections of Pax6 RNA into Xenopus embryos induce optic lenses, the majority without associated neural tissue

(Chow et al., 1999). The activation of Notch signaling in Xenopus embryos causes eye duplications and proximal eye defects which are also induced by over-expression of eyeless (ey) and twin of eyeless (toy) (Onuma et al., 2002). In mice, a naturally occurring mutation in the Pax6 gene have characteristic small eye and experiments using those mutants indicate a requirement for Pax6 only in the surface ectoderm and not in the optic vesicle for lens induction (Fujiwara et al., 1994). Vertebrate Pax6 mRNA comprises 15 exons, the first four of which are noncoding (Glaser et al., 1992). Several of these exons are alternatively spliced, some of them without a PD and at least 5 different Pax6 products have been characterized in quail (Carrière et al. 1995). Pax6 may be regulated by a diverse array of factors, including retinoic acid (Hyatt et al. 1996). A series of transcriptional control elements are characterized in the Pax6 gene. Both the ectoderm enhancer (a conserved region 531 bp located 3.5 kb upstream of the first promoter) and the SIMO element (135 kb 3' to Pax6 in the last intron of the adjacent gene) mediate *Pax6* expression during the placodal phase.

The Drosophila homologues of the vertebrate Pax6 gene are toy and ey. Both are located near to each other on the fourth chromosome and share splice sites that are not found in Pax6 genes from other species (Czerny et al. 1999). It seems likely that toy and ey arose as a result of a gene duplication event during arthropod evolution (Czerny et al. 1999). Toy has to date only been described from arthropods. The early expression of toy precedes expression of ey in the embryo (Quiring et al, 1994). It has been shown that the ability of the *Drosophila* ey to induce ectopic eyes is heavily dependant upon Dpp signaling (Chen et al. 1999).

The paired domain of Ey is sufficient for its function in eye development. The role of the homeodomain is less clear. Punzo et al. (2001) showed that the eyeless homeodomain is dispensible for eye development in *Drosophila*. When ey is ectopically expressed it is the homeodomain but not the paired domain that is required to repress the antennal and leg determinant Distal-less (Dll). In human patients with aniridia or other anterior segment

defects, multiple mutations in the Pax6 paired domain have been found while only one homeodomain missense mutation is known to cause a mild eye phenotype (Hanson et al., 1999).

371 genes, mainly transcription factors involved in photoreceptor specification, signal transducers, cell adhesion molecules and proteins involved in cell division are expressed in the eye imaginal discs and up-regulated when an eye morphogenetic field is ectopically induced in the leg discs (Michaut et al., 2003). Only 40% of the genes ectopically induced by ey in the leg discs were also found to be transcribed in the eye discs.

To date no *bona fide* Pax6 homolog has been found in cnidarians (see chapter 2). A Pax6-like fragment of a paired-like HD could be isolated from *Cladonema* (see Appendix), but all attempts to elongate this fragment failed. However, several Pax genes have been characterized from Cnidaria and PaxB and/or PaxC have been suggested to play the ancestral role of a Pax6 in this phylum (Miller et al., 2000; Kozmik et al., 2003).

Sine oculis / Six genes

Six proteins are characterized by a Six domain and the Six-type homeodomain (see chapter1). Six genes have been characterized from various different phyla. *Drosphila sine oculis (so)* is required for the development of the entire visual system with its main function in the establishment of the MF (Cheyette et al., 1994). It was shown to be a direct target for both ey and toy (Punzo et al., 2002). The *Drosophila* orthologous gene for mouse Six3 is *optix*, which is involved in eye morphogenesis by an ey-independent mechanism (Seimiya, 2000). The vertebrate six gene responsible for eye development is *Six3/6*. Six genes are involved in several genetic diseases in humans like holoprosencephaly, anophthalmia and myotonic dystrophy. Recently it has been reported that Six1 has an essential role in determining the

metastatic fate of rhabdomyosarcoma, the most common soft-tissue sarcoma in children (Yu et al., 2004).

Three different Six genes could have been identified from *Cladonema* and two different six genes from *Podocoryne*. These are the only known Six genes from cnidarians.

Eyes absent

Eya family members are defined by a conserved 275 aa motif referred to as the Eya domain. The Drosophila *eya* gene, which is also termed *clift*, is required for the survival of eye progenitor cells at a critical stage in morphogenesis (Bonini, 1993). Ectopic expression of eya together with ey is more effective in eye formation and additionally can occur on genitalia, a condition which has never been observed when either gene is ectopically expressed alone (Bonini, 1997). Clift has been identified as a regulator of Drosophila gonadogenesis, it determinates somatic gonadal precursor cells. Later it turned out that clift and eya are identical. To date target genes of *eya/clift* are not discovered and therefore it remains open, if this gene unifies a role in eye and mesoderm.

Ectopic eyeless expression in the context of eyes absent or sine oculis mutations results in apoptosis (Clark et al., 2002). Mutations in EYA1 are responsible for cataracts and anterior segments defects, branchiootic syndrome and branchio-oto-renal syndrome (Abdelhak et al., 1997). There is an amino acid sequence similarity between the Eya domains and enzymes of the halocid dehalogenase (HAD) superfamily. This family includes a class of dephosphorylating enzymes (phosphatases) of which some remove phosphate groups specifically from serine amino acids in target proteins.

Eya protein is a tyrosine phosphatase (Tootle et al., 2003). Eya3 can dephosphorylate RNA polymerase II and has also an autocatalytical dephosporylation activity (Li et al., 2003). It is speculated that Eya3's phosphatase activity is required to switch Dach1 from a repressor to

an activator. Eya has probably phosphatase-dependent and –independent biological activities (Tootle et al., 2003). It is the first example of a transcription factor with intrinsic phosphatase activity and it is suggested to represent a method for fine-tuning transcriptional regulation (Tootle et al., 2003).

To date no eya homolog has been reported from Cnidaria. The hunt for eya in *Cladonema* was not successful although an antibody cross-reaction with a monoclonal *Drosophila* anti-eya antibody could have been observed (Fig. 12B). Labeling is restricted to cells in the manubrium, the feeding and sex organ. The staining protocol was performed according to the one described in chapter 1. It remains unclear if *Cladonema* does posses an Eya homolog and if the obtained labeling pattern of the cross-reaction is specific or not.

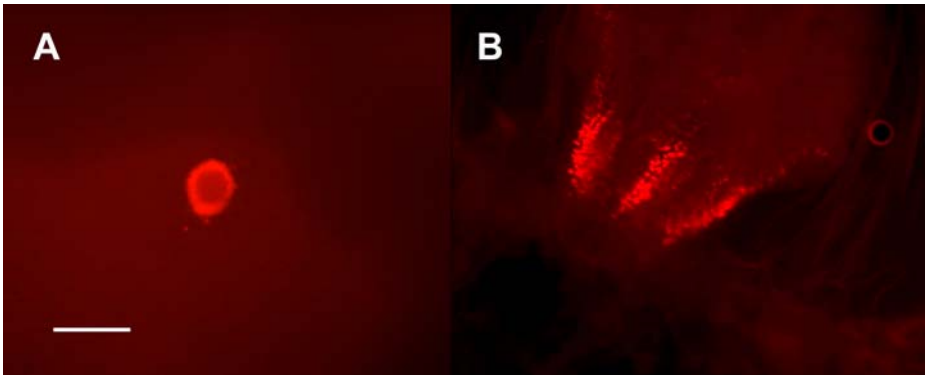

Figure 12 Immunohistology displaying the cross-reaction of a monoclonal antibody developed against *Drosophila* Dac (A) and of a monoclonal antibody developed against *Drosophila* Eya (B) with *Cladonema radiatum*. The labeling obtained with the anti-dac antibody seems to be restricted to the eye whereas anti-eya labels cells located close to the manubrium lips. Bar is (in µm) 65 in (A) and 100 in (B).

Dachshund

The fly mutant phenotype of dachshund has extremely short legs in relation to its body length and therefore inspired for the name. Dachshund was originally identified as a dominant suppressor of a mutation of the epidermal growth factor receptor *Ellipse*. *Drosophila*

dachshund is necessary and sufficient for comopound eye development and is required for normal leg and brain development (Davis et al., 2001). Dac function is required for the normal movement of the MF. In dachshund mutants cells fail to adopt a neural fate and remain in an undifferentiated state and die eventually (Mardon, 1994). Dac may be a direct target of ey. Remarkably, the external morphology of the adult ocelli in Drosophila appear normal in all dachshund mutants (Mardon, 1994). It is differentially expressed in the male and female genital discs, and plays sex-specific roles in the development of the genitalia. (Keisman and Baker, 2001). Three zebrafish dac homologues have been characterized (Hammond et al., 2002). All three are expressed in sensory organs, the central nervous system and pectoral fin buds. Its expression overlaps extensively with those of zebrafish pax, eya and six family members. A mouse homologue is expressed in the developing retina and limbs, suggesting functional conservation. Homozygous mouse mutants survive to birth but exhibit postnatal lethality associated with a failure to suckle, cyanosis and respiratory distress (Davis et al., 2001). Histological examination of the eyes reveals no abnormalities in these mice. The DACH protein exhibits two domains (DD1 and DD2) highly conserved from *Drosophila* to human, although the function of these two domains is unknown. The amino-terminal domain DD1 has approximately 35% amino acid identity to the Ski/Sno family of oncoproteins (Ayres et al., 2001). No targets of Dach1 transcriptional activity have been identified in vertebrates and the regulation of Dach1 expression by growth factors has not yet been characterized. The mouse DACH1 protein was detected in several organs in which epithelial/mesenchymal interactions are known to be important in patterning and cell fate determination, including the developing kidneys, eyes, limb buds (Ayres et al., 2001). DACH1 protein and message was detected in cells of the optic cup and in some of the mesenchymal cells surrounding the eye (Ayres et al., 2001). The expression of mouse Dach2 suggests a partially redundant role of the dach genes (Davis et al., 2001). The expression of

Dach2 in the forebrain of Pax6 mutants and in dermamyotome mutants of Pax3 is not detectably altered (Davis et al., 2001).

Ayres et al. (2001) found that DACH1 is expressed in association with other retina determination genes in the developing mammalian eyes, inner ears, limbs, and kidneys. No mutations in human DACH contributing to human disorders have been identified (Ayres et al., 2001). Human DACH maps to a chromosome region that has been associated with digital abnormalies. DACH represents an attractive candidate gene for limb malformations because it is expressed in the distal limb bud during digital patterning (Ayres et al., 2001). Dach1 in mice acts as a corepressor and as a co-activator of Six1 and Eya proteins (Li et al., 2003).

There is no cnidarian Dach homolog identified yet but preliminary data indicate, that Cnidaria seem to posses a dachshund gene (not shown). A monoclonal *Drosophila* anti-dac antibody cross-reacted specifically with the *Cladonema* eye (Fig. 12A).

Teashirt

Teashirt encodes a transcription factor with zinc finger motifs and it was originally identified for the specification of the trunk segments in *Drosophila.* The targeted expression of teashirt in imaginal discs is sufficient to induce ectopic eye formation in non-eye tissues and teashirt and ey induce the expression of each other (Pan et Rubin, 1998). It is suggested that teashirt acts upstream of eya, so and dac in ectopic eye development (Pan et Rubin, 1998). Nothing is known about a teashirt homolog from Cnidaria.

Eyegone

Eyegone, originally called Lune, is a Pax protein but contains only a partial PD, with an incomplete PAI subdomain and a complete RED subdomain. The linker region between PAI

and RED is most closely related to Pax2, Pax5, Pax8 but its helix-turn-helix region is most related to Pax6. It contains a paired-class HD with a characteristic serine at position 50. Eyg promotes eye development primarily by repressing wingless. Both eya and eyg are required for the activation of dpp in the retinal tissue. Eyg seems to be involved in growth and specification of the fly eye independently of ey (Dominguez, 2004). It regulates probably different target genes than those regulated by ey but it was speculated that eyg and ey could form heterodimers via their HD. In vertebrates no homolog of eyg has yet been identified. It has been speculated that eyg probably plays a role equivalent to the vertebrate Pax6-5 isoform (Jang et al., 2003). No eyg homolog has been identified yet from Cnidaria.

Gene	Loss of function	Gain of function
twin of eyeless (*toy*)	Not reported	Strong ectopic eye induction
eyeless (*ey*)	Reduced or no eyes	Strong ectopic eye induction
eyes absent (*eya*)	Reduced or no eyes	Weak ectopic eye induction
sine oculis (*so*)	Reduced or no eyes	No phenotype
dachshund (*dac*)	Reduced or no eyes	Weak ectopic eye induction
teashirt (*tea*)	No phenotype in the eye	Weak ectopic eye induction
optix (*optx*)	Not reported	Weak ectopic eye induction
eyegone (*eyg*)	Reduced or no eyes	Ectopic PRs in the eye disk
homothorax (*hth*)	Ectopic PRs in eye disk clones	Loss of PRs
extradenticle (*exd*)	Ectopic PRs in eye disk clones	Loss of PRs

Figure 13 *Drosophila* eye specification genes and results of their loss of function and gain of function experiments. Figure taken from Kumar and Moses, 2001.

Secreted factors required for eye development in *Drosophila*

The secreted factors encoded by hedgehog (hh), dpp and wingless (wg) are required for normal development of the Drosophila eye but these genes do not specify cell fate directly. The ectopic expression of those genes does not change the imaginal disc fate but their misexpression causes pattern duplication.

Hedgehog

Members of the Hedgehog family are key mediators of many fundamental processes in embryonic development and their activities are central to the growth, patterning and morphogenesis of many different regions within the body plans of vertebrates and insects. In *Drosophila* hh is a central patterning signal in the wing and eye discs as well as regulating several other processes like germ cell migration, development of the optic lamina and gonad, abdomen and tracheal system (reviewed in Ingham and McMahon, 2001). *Drosophila* has only one hh gene, there are several related genes in vertebrate species. One notable exception is the nematode *C. elegans*, which has no hh ortholog but does possess several genes encoding proteins homologous to the hh receptor Patched (Kuwabara et al., 2000). The vertebrate Sonic hedgehog (Shh) is involved in the separation of the eye fields and the formation of the optic stalk (Chiang et al., 1996; Perron et al., 2003). A wave of sonic hedgehog (Shh) patterns the zebrafish retina, as in the fly eye.

The intensive hunt for an hh-homolog from *Cladonema* was not successful. No cnidarian hedgehog homolog is known although previously believed differently.

Decapentaplegic

In the fly decapentaplegic (dpp) is responsible for the dorsal/ventral polarity, for the definition of boundaries between segmental compartments, between appendage compartments assuring correct anterior/posterior polarity and functions analogous in the development of the eye. In the eye it is primarily responsible for the progression of the MF. Bmp4 has been identified as a potential lens inducer (Furuta and Hogan, 1998) by regulating the expression of Sox2, a transcription factor that has been implicated in the regulation of crystallin genes. Pax6 and Sox2 form a complex that can regulate δ-crystallin gene

expression in the chick (Kamachi et al., 2001). Bmp4 regulates early differentiation in the lens lineage and Bmp7 null mice have eye defects ranging from microophthalmia to anophthalmia. Bmp and fibroblast growth factor (Fgf) signaling pathways cooperate in some way. Fgf receptor and Bmp7 signaling probably combine upstream of the placodal phase of Pax6 expression in a genetic pathway defining lens induction.

Dpp homologs and different members of the BMP/TGF-β family have been identified from Cnidaria. Although a Dpp as well as a BMP 5-8 homolog could have been identified in *Podocoryne*, the *Cladonema* representatives are still unknown.

Wingless

Like several other pathways wingless (wg) signaling has several functions in eye development. In Drosophila wg signaling establishes the border between the retina and adjacent head structures by inhibiting the expression of the eye specification genes eya, so, dac (Baonza and Freeman, 2002). Ectopic wg signaling leads to a repression of these genes and the loss of eyes (Baonza and Freeman, 2002). Wnt proteins function by binding to seven-pass transmembrane receptors belonging to the frizzled family. Wnts acitvate frizzled receptors by binding to the cysteine-rich extracellular domain of the receptor. The frizzled signaling is both necessary and sufficient to regulate eye development in Xenopus (Rasmussen et al., 2001). These findings demonstrate a requirement for wnt/frizzled signaling in regulating vertebrate eye development. Stump et al. (2003) report a role of Wnt signaling in lens epithelial differentiaion.

Wnt signaling has been reported from hydra (Hobmayer et al., 2000) and identification of Wnt from *Cladonema* has been successful (not shown).

Crystallins

Crystallins are soluble proteins in eye lenses, which play an important role in the maintenance of lens transparency, optical clarity and refractive index. They are essentially defined by their abundance, collectively 80-90% of the water-soluble proteins in the lens (Piatigorsky et al., 2001). In terrestrial vertebrates about one third of refraction is done by the lens whereas it accounts for all the refraction in aquatic vertebrates (Tomarev and Piatigorsky, 1996). Lens crystallins represent a surprisingly diverse group of multifunctional proteins and some display taxon-specificity. In general, vertebrate crystallins have been recruited from stress-protective proteins, like heat-shock proteins, and a number of metabolic enzymes (Tomarev and Piatigorsky, 1996). The crystallins (α and βγ crystallins) that show sequence similarity to small heat-shock proteins of *Drosophila*, are ubiquitously used in vertebrates, and must therefore have occurred in a common ancestor and be quite ancient (Janssens and Gehring, 1999). As different visual systems became more elaborate the more recent taxon specific crystallins must have arisen. All invertebrate crystallins examined so far are different and novel proteins. A stress protective metabolic enzyme, glutathione S-transferase (GST) seems to provide the major cephalopod crystallins. GSTs share sequence motifs with the γ subunit of the eukaryotic elongation factor 1 (EF1γ). Cephalopods have at least two taxon-specific crystallins related to aldehyde dehydrogenase and related to a superfamily of lipid-binding proteins.

In the acellular corneal lens of *Drosophila* three calcium binding taxon-specific crystallins have been found, while antigen 3G6 is a highly conserved protein present in the ommatidial crystallin cone and central nervous system of numerous arthropods (Tomarev and Piatigorsky, 1996). Drosocrystallin is a secreted protein that shows sequence similarities to some insect cuticular proteins. It is expressed in the brain as well as in the ommatidia and it is therefore likely that it serves for an additional function. There are suggestions that crystallins

of compound eyes of arthropods are expressed outside of the lens. Aldehyde dehydrogenase is an example of an enzyme-crystallin that is used by both the vertebrates and invertebrates (Piatigorsky et al., 2000). The gene sharing strategy to use multifunctional proteins for refraction may have occurred in invertebrates as it did in invertebrates (Tomarev and Piatigorsky, 1996).

A rabbit antiserum from a soluble protein of the bovine lens was able to produce immunofluorescence specifically in the lens of *Cladonema radiatum* (Weber, 1981). It is possible that the immunofluorescence was due to one or more common epitopes between jellyfish and vertebrate crystallins. A lens crystallin of the cubomedusa *Tripedalia cystophora* has been identified and shows sequence similarity to vertebrate saposins (Piatigorsky et al., 2001). Its message was detected in embryonic and larval stages as well as in the rhopalia. A GST has been characterized from *Cladonema* and its expression analysed to verify a role as a crystallin in jellyfish. However, GST from *Cladonema* is expressed in the manubrium but not in the lens (Fig. 14). The existence of a βγ-crystallin-type gene from the sponge Geodia cydonium has also been reported (Di Maro et al., 2002).

Figure 14

In situ hybridization with an anti-sense GST Dig RNA probe. The staining is restricted to the upper part of the manubrium, mainly the gonads (arrow). The presence of message in the eyes is too weak as it could play a role of a possible lens crystallin in jellyfish. Bar is 450 μm.

Phototransduction

Phototransduction is one of the fastest known G protein-coupled signaling cascades. In *Drosophila* exposure of the photoreceptor cells to light leads to activation of the light-induced cation influx channels, transient receptor potential and transient receptor potential-like within 20ms. In less than 100ms after cessation of the light stimulus the deactivation of the cascade is completed (Ranganathan et al., 1991). Recent studies have provided evidence that many of the components functioning in *Drosophila* phototransduction form a supramolecular signaling complex, consisting of a minimum of seven signaling proteins bound to scaffold protein referred to as INAD. These include rhodopsin, phospholipase C-β, protein kinase C, calmodulin, the myosin III NINAC, and two light-sensitive ion channels, TRP and TRPL. More than 40 genes function in phototransduction. *Drosophila* and vertebrate phototransduction have some notable similarities and differences. Both *Drosophila* and vertebrate visual transduction are initiated by the light-induced isomerization of the photopigment rhodopsin and subsequent interaction with the heterotrimeric G protein. The effector for the *Drosophila* G protein is a PLC, which catalyzes PIP2 to IP3 and DAG and the activation of the PLC leads to a Na and Ca influx as a result of the opening of the cation influx channels. In contrast, the effector for the G protein in vertebrates is a phosphodiesterase, which hydrolyzes cGMP to GMP and as a consequence closes the cGMP-gated ion channels and hyperpolarizes photoreceptor cells by termination of the Na and Ca influx (Montell, 1999).

Rhodopsin consists of two components: a protein containing seven transmembrane segments (opsin) and a chromophore, typically 11-cis-retinal, which is covalently attached to a lysine residue in the seventh transmembrane domain (Montell, 1999). Retinal is the molecule transducing light energy into electrical signals and opsin is the covalently bound protein carrier. Exposure to light results in conversion from the cis to the trans configuration in the

chromophore, the only step in phototransduction that is directly regulated by light. This cis-trans isomerization causes a conformational change in the opsin moiety. A major difference in the rhodopsin cycle between fly and vertebrates is the regeneration of rhodopsin.

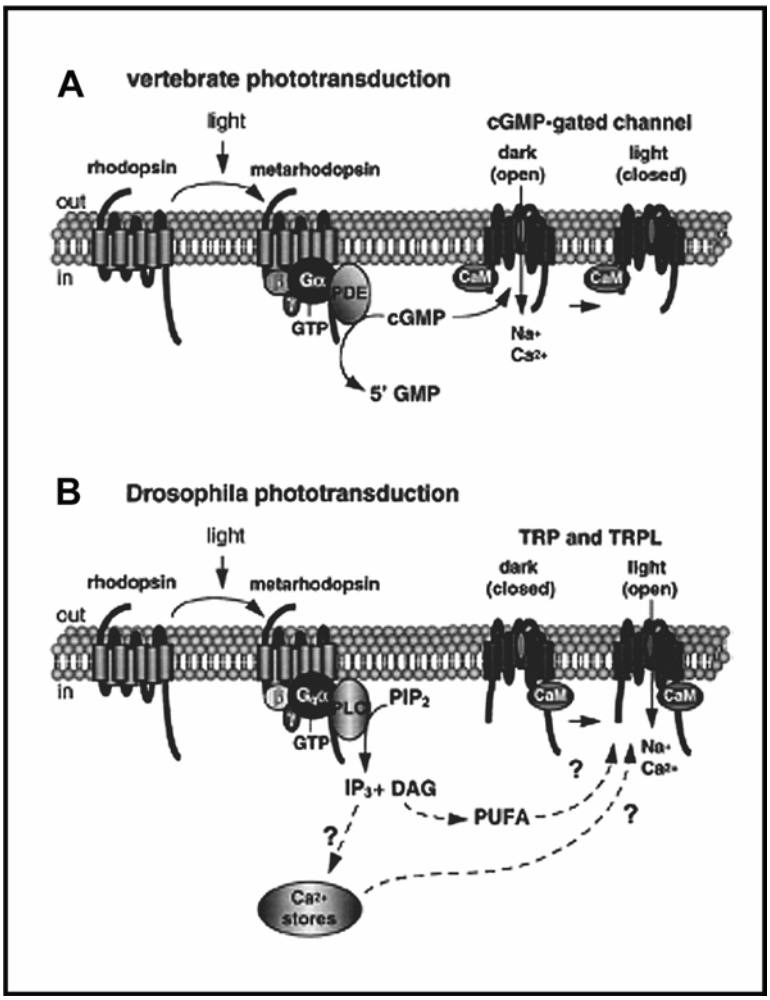

Figure 15. A scheme comparing vertebrate and *Drosophila* phototransduction (taken from Montell, 1999).

Following exposure to light, the chromophore in *Drosophila* photoreceptor cells does not dissociate from the opsin whereas vertebrate opsin and 11-cis retinal dissociate after arrestin binding. Vertebrate opsins fall into five fundamental subfamilies. It is speculated that the phototransduction proteins seem to have co-evolved as a system (Hisatomi and Tokunaga, 2002). Many vertebrates have duplicate photoreceptor type cells, rods and cones, responsible for twilight and daylight vision respectively (Hisatomi and Tokunaga, 2002). Phototransductory molecules such as opsins and arrestins are directly regulated by Otx- and Pax6 transcription factors (Kimura et al., 2000).

Drosophila and vertebrate phototransduction results in opposite effects on the ion channels, opening versus closing (Fig. 15). But both cascades share several features like sensitivity over a vast range of light intensities, high speed and temporal resolution, and enormous signal amplification.

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CHAPTER 1

The *Sine oculis/Six* class family of homeobox genes in jellyfish with and without eyes: Development and eye regeneration

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Abstract

The development of visual organs is regulated in Bilateria by a network of genes where members of the *Six* and *Pax* gene families play a central role. To investigate the molecular aspects of eye evolution we analyzed the structure and expression patterns of cognate members of the *Six* family genes in jellyfish (Cnidaria, Hydrozoa), representatives of a basal, non-bilaterian phylum where complex lens eyes with spherical lens, an epidermal cornea and a retina appear for the first time in evolution.

In the jellyfish *Cladonema radiatum*, a species with well developed lens eyes in the tentacle bulbs, *Six1/2-Cr* and *Six3/6-Cr* are expressed in the eye cup. *Six4/5-Cr* is mainly expressed in the manubrium, the feeding and sex organ. When the eye is removed, its regeneration requires expression of *Six1/2-Cr* and *Six3/6-Cr*, but not of *Six4/5-Cr*. Furthermore, expression is also found in other tissues, notably in the striated muscle. All three *Six* genes are expressed in different subsets of epidermal nerve cells, possibly of the RFamide type which are part of a net connecting the different eyes with each other and the effector organs. In *Podocoryne carnea*, a jellyfish without eyes, *Six1/2-Pc* and *Six3/6-Pc* are also expressed in the tentacle bulbs, *Six1/2-Pc* additionally in the manubrium and striated muscle, and *Six3/6-Pc* in the mechanosensory nematocytes of the tentacle.

The conserved gene structure and expression patterns of all *Cladonema Six* genes are consistent with monophyly of a basal upstream regulatory network for visual organs.

Introduction

Perception of light appears to be tightly coupled to the earliest steps in the evolution of life and is realized in all kingdoms. The next step, the evolution of photoreceptors and their specialized organs is achieved only in eukaryotes but has produced a large variety of morphological structures which stretch from the lens equipped eye spots of unicellular organisms to the many different types of eyes found in protostomes and deuterostomes (Land and Nilsson, 2002; Arendt and Wittbrodt, 2001). The molecular aspects of eye evolution have recently been the focus of much work since early parts of eye development appear to be conserved in all Bilateria (Halder et al., 1995; Gehring, 2002; Pichaud and Desplan, 2002). The same toolbox of transcription factors composed of homologs of the *Drosophila sine oculis* (*so*), *twin of eyeless* (*toy*), *eyeless* (*ey*)[*Pax6*], *eyes absent* (*eya*) and *dachshund* (*dac*) orchestrates eye development in a wide range of animals. The synergistic and regulatory manner of this network led to the idea of a monophyletic evolution of the eye (Gehring and Ikeo, 1999).

Cnidaria form the closest out-group to the Bilateria (Medina et al., 2001) and represent the most primitive metazoans with striated muscle tissue, centralized nerve rings, ganglia and different sense organs of high complexity, including lens eyes. This raises several important questions. How and when did Cnidaria acquire these structures? Do they originate from a common already eye bearing ancestor of Cnidaria and Bilateria or did eyes originate independently in Cnidaria by taking advantage of a shared common pool of genes and by assembling the same networks and gene cascades? Or is the basal network conserved for other reasons (myogenic-neurogenic pathways) and co-opted for eye formation in both lines leading to striking similar constructions but embedded in different anatomy? Only the free swimming medusa stage (Fig. 1) differentiates photoreceptor organs which range from

simple ocelli to highly evolved lens eyes (Land and Fernald, 1992). Recently even a planula larva of Cubozoa was shown to have single-celled ocelli (Nordström et al., 2003). The sessile polyps of all cnidarian classes respond to light (Tardent and Frey, 1969) but until now no photoreceptive structures or specialized cells for light detection have been identified, although immunoreactivity was reported for opsin (Musio et al., 2001).

We investigated structure and expression pattern of the *Six* family genes in *Cladonema radiatum*, a hydrozoan jellyfish with well developed and well studied lens eyes (Weber, 1981a, 1981b) and in *Podocoryne carnea*, a jellyfish without eyes. The *Six* genes, a family of transcription factors characterized by a six domain (SD) and a six-type homeodomain (HD), were originally identified by homology to the *Drosophila sine oculis* (*so*) gene, which is required for the development of the entire *Drosophila* visual system (Cheyette et al., 1994). Both domains have been shown to be involved in DNA binding. The SD is required for direct interaction and nuclear translocation of members of the eyes absent (*Eya*) gene family (Pignoni et al. 1997). The family can be classified into three major subgroups designated as Six1/2, Six3/6 and Six4/5 (Seo et al. 1999). *Six* genes have been identified from various animal phyla where, beside other functions, they are mostly engaged in eye development or derivatives of the mesoderm, including muscle (Kawakami et al., 2000). Three different *Six/so* genes are known from *Drosophila*, four from *C.elegans*, three from tunicates (Wada et al., 2003) and mammals seem to have six different members, but until now they have not been found in Sponge, Cnidaria or unicellular organisms.

We isolated and analysed 3 different *Six* genes from *Cladonema*, one of each subclass and Six1/2 and Six3/6 from *Podocoryne*, and studied their expression in the medusa and throughout eye regeneration in *Cladonema*. The conservation of sequence structure and the expression patterns of all three different *Six* genes in eye development, myogenesis and eye regeneration support the hypothesis of an archetypical Six cluster (Boucher et al., 2000)

which was already functionally assembled in the last common ancestor of Cnidaria and Bilateria. Otherwise we would accept that from a common pool of genes the assembly of similar interacting networks of regulatory genes occurred repeatedly in metazoan evolution to give rise to a similar result concerning the basal Bauplan of the phylum.

Materials and Methods

Animals

Cladonema radiatum Dujardin and *Podocoryne carnea* M. Sars (Cnidaria, Hydrozoa, Anthomedusae) colonies were reared in artificial sea water at 20°C and fed every second day with 2 day old artemia.

Molecular Cloning and Phylogenetic Analysis

Molecular biology procedures were performed according to standard protocols (Sambrook and Russell, 2001). We conducted homology PCR on medusa cDNA using the following degenerate primers for Six1/2 and Six4/5: forward (5'-TGG YTN RAR GCN CAY TAY-3'); nested forward (5'-ATH TGG GAY GGN GAR GAR AM-3'); reverse, (5'-CKN CKR TTY TTR AAC CAR TT-3'); nested reverse (5'-KTY TCY TCN CCR TCC CAD AT-3'). PCR conditions were 20x (15 seconds at 94°C, 25 seconds at 37°C, 1 minute at 72°C) then 10x (15 seconds at 94° C, 25 seconds at 40°C, 1 minute at 72°C) followed by nested PCR 40x (15 seconds at 94°C, 25 seconds at 50°C, 1 minute at 72°C). Degenerate primers used for Six3/6: forward (5'-GCN ATG TGG YTN GAR GCN CAY TA-3'), nested forward (5'-TGG GAY GGN GAR CAR AAR CAN CA-3'), reverse (5'-CAT RTT DCC WAC YTG NGT NGG-3') nested reverse, (5'-TGN GTY TTY TGY TCN CCR TCC CA-3'). PCR conditions are described above. The full coding sequences were obtained by RACE (rapid

amplification of cDNA ends) on cDNA prepared from polyadenylated RNA of medusae and medusa budding polyps with a SMART RACE cDNA amplification kit (Clontech) or by library screening as described (Müller et al., 1999). The *Cladonema* cDNA library was constructed with the Stratagene Zap cDNA Synthesis Kit and Zap cDNA Gigapack III Gold Cloning Kit and reactions were performed according to the recommendations of the manufacturer. Sequence analyses, Blast searches and phylogenetic trees were performed as described by Müller et al. (2003).

Real Time-PCR Expression Analysis and Whole Mount *in situ* Hybridzation

Real Time-PCR expression analysis was done at least three times on independent cDNA templates on the Light Cycler (Roche) as described by Müller et al. (2003). For the *Cladonema Six* genes the following primer-sets were used: Six1/2-Cr forward (5'-CAA CCG TCA GTG GCG AAT TTC ACG-3') and Six1/2-Cr reverse (5'-GTC GTC GTA ACT CGG TAA CGA CCA-3'), Six3/6-Cr forward (5'-GAA ATC AAA AGC AGC AAA GTT TAC-3'), Six3/6-Cr reverse (5'-CTG TGA TGT ATA TAC ACG CCC GAA G-3'), Six4/5-Cr forward (5'-CGT GCT AAA AGC AAG AGC TCA TGT-3') and Six4/5-Cr reverse (5'-GCT AAC AAT CTT TTA TCT TGA GGT GTT GG-3').

Podocoryne Six1/2-Pc and *Six3/6-Pc* levels of expression were surveyed with the following primer sets: Six1/2-Pc forward (5'-CAC TCC AGA ACA AGT CGC ATG TG-3'), Six1/2-Pc reverse (5'-GAT TTC GCG ACC AAG ACG GAC TCG- 3') and Six3/6-Pc forward (5'-AGA TGA CGA TAT ATC CGA CAG TG- 3'), Six3/6-Pc reverse (5'-CCT GGT GTG TAA AAA ACT ACC TAT-3'). Elongation factor 1 alpha (GenBank accession number for EF1α-Cr: AY542532) was used as a reference to compensate for variations in quality and quantity of the preparations. *EF1α* was amplified from the *Cladonema* samples with: EF-Cr forward (5'-AGC TGT TCC TGG AGA TAA TGT TGG-3'), EF-Cr reverse (5'-GGA TGA

TTT AAG ATG ATG ACC TGG-3'), and from the *Podocoryne* samples with: EF-Pc forward (5'-ACG TGG TAT GGT TGC CTC TG-3'), EF-Pc reverse (5'-TGA TAA CGC CAA CGG CTA CG-3').

In situ hybridization was performed as described previously (Müller et al., 2003) with minor differences. Fixation was performed on anaesthetized pre-cooled animals (1:1 Sea water / 7% w/v $MgCl_26H_2O$ for 5 min on ice) with freshly prepared 4% Paraformaldehyde or Lavdowsky (Gröger and Schmid, 2001). Hybridization was performed at 58°C. All probes excluded the HD, and the Six3/6-Cr probe excluded the SD. DNA templates were prepared by PCR with the following primers for Six1/2-Cr (forward, 5'-ATG GAT ATC GCA CCG TCG GCA TAT G-3'; reverse, 5'-CCA TAT CGT TCT AGG TAA CGG GTA C-3'), for Six3/6-Cr (forward, 5'-GAA ATC AAA AGC AGC AAA GTT TAC -3'; reverse, 5'-CTG TGA TGT ATA TAC ACG CCC GAA G-3') and for Six4/5-Cr (forward, 5'-ATG AGC ATC AGT CTT GAT ACG TC-3'; reverse, 5'-ATG GGC ATC ATG CCA CAT AAG CTG-3') and the DIG RNA Labelling Mix (Roche). *Podocoryne* probes excluded the HD. *Podocoryne* DNA templates for probe synthesis were prepared using the following primers: for Six1/2-Pc (forward, 5'-ATG GCA TCT TCA CAA ATC GTC CAA TC-3'; reverse, 5'-CCA TAT TGT GCG CGG TAG CGG ATG-3') and for Six3/6-Pc (forward, 5'-CCT CAC ACC AAC ACT ACG CAT TC-3'; reverse, 5'-GGA CGT CCT CGT AAT CTT TCA GCC-3').

Regenerations and dissecting experiments

Anaesthetized animals were operated with ophthalmologic scissors (Figs. 3, 4, 7) as described in Weber (1981a).

Immunohistology

Immunohistology was done as described previously (Gröger and Schmid, 2000, 2001). Specimen were incubated with RFamide antiserum (rabbit antibody diluted 1:2000 in PBS, kindly provided by Dr. C.J.P. Grimmelikhuijzen) or with a monoclonal anti-tyrosin-tubulin antibody (a mouse antibody, clone TUB-1A2, Sigma; diluted 1:2500 in PBS containing 10% fetal calf serum).

Results and Discussion

Cladonema and *Podocoryne* medusae are produced asexually by budding from polyps (Fig. 1), a process which can be viewed as a continuation of development (Spring et al., 2000; Spring et al., 2002). Only medusae differentiate striated muscle tissue and sense organs, including eyes of different complexity (Linko, 1900). The eyes of jellyfish are always located at the bell margin, usually at the base of the tentacles (Fig. 1A, B), and are occasionally stalked in the Cubozoa (Berger, 1900). Structure (Fig. 1C), development, regeneration process, neurobiology, and electrophysiology of the lens eyes were studied in detail in different jellyfish species (Mackie, 1971; Yoshida, 1973; Singla, 1974; Weber 1981a, 1981b). Only the ectoderm is involved in the genesis of the ocellus. The retina is build of melanin-containing pigment cells and of ciliary type photoreceptors. The retina and the lens body derive from a compact cup shaped primordium consisting of interstitial or dedifferentiated somatic cells. The eye is covered by a cornea that develops from intensely vacuolated, non-pigmented cells derived from the differentiating ocellus. In contrast to the depolarizing rhabdomeric type of photoreceptors found in protostomes, jellyfish have

hyperpolarizing cilia-derived photoreceptors as vertebrates do (Eakin and Westfall, 1962; Eakin, 1963). According to Eakin's theory (1963, 1968, 1979) the photoreceptor cells of Cnidaria belong to the same evolutionary line as those of vertebrates. Although *Podocoryne* medusae show positive phototaxis they differentiate no recognizable eye–like structures.

Survey of the *Six* genes from jellyfish

In search for jellyfish *Six* genes, PCR was conducted with degenerate primers corresponding to different parts of the homeobox. The obtained fragments were extended by RACE and by screening a cDNA library (as described in Spring et al., 2000). This process led to the identification of three different *Six* genes in *Cladonema* which can be classified into the three main subfamilies (Fig. 2). From *Podocoryne* we identified two different *Six* genes. The predicted protein sequences are highly conserved (see supplementary Fig.) and sequences are available in the GenBank with the accession numbers AY542527-8 for Six1/2-Pc and Six3/6-Pc, AY542529-AY542531 for Six1/2-Cr, Six3/6-Cr, and Six4/5-Cr respectively.

***Cladonema Six* genes**

The Six1/2 subclass is defined by the presence of the diagnostic amino acid sequence (ETSY) from position 3 to 6 in helix 1 of the homeodomain (HD) (Seo et al., 1999). Six1/2-Cr is a 235 amino acids protein with highly conserved six domain (SD) and with the diagnostic motif in the six-type homeodomain. The HD is 83% identical to the HD of Human Six1 and Six2, 85% to the HD of *Drosophila sine oculis* and 77% identical to that of Ceh-33 from *C.elegans*. The sequence conservation in the SD is 69% to Human and *Drosphila*.

The Six3/6 family is characterized by the tetrapeptide QKTH in the HD N-terminus (Seo et al., 1999). The presence of this tetrapeptide motif as well as the high sequence conservation of the rest of the protein clearly assigns Six3/6-Cr to the Six3/6 subfamily. Six3/6-Cr is a protein of 327 amino acids with a sequence identity within its HD of 88% to Human Six3 and 84% to *Drosophila* D-Six3/optix. Within the SD, the *Cladonema* sequence is best conserved to the Human OPTX2 or Six6 (68% identity) and to *Drosophila* optix SD (64% identity). The Six3/6-Cr shows a few short homopolymers in the C-terminus as it was described also for the *Drosophila* D-Six3 (Seo et al., 1999).

The Six4/5 family is characterized by the tetrapeptide ETVY domain. The Six4/5 subfamily candidate gene from *Cladonema* (Six4/5-Cr) has an amino acid substitution in the tetrapeptide (ETIY) relative to the described Six4 and Six5 families. It displays an isoleucine at position 5 in helix 1 instead of the typical valine. The gene encodes a protein of 214 amino acids with a shorter C-terminal region in comparison to vertebrate Six4/5 members. It is not clear whether this short C-terminal region reflects an alternative splicing or a cnidarian specificity. When compared to the databases (GenBank) the full length sequence exhibits the highest similarity to Human Six4 with 57% identity. Within the HD sequence identity is 70% to Human and *Drosophila* Six4. The SD is more divergent with sequence identities of 53-58% from insects to humans.

A phylogenetic tree was constructed on the basis of the full SD and HD (Fig. 2). The tree is congruent with the assignment of identities.

Podocoryne Six **genes**

The *Podocoryne Six1/2-Pc* encodes a protein of 296 amino acids containing the diagnostic amino acids in the HD characteristic for this group. The HD is 86% identical to that of

Human and *Drosophila* sine oculis or *Dugesia japonica* and 79% to Ceh-33 of *C.elegans*. The SD is 72% identical to that of Human Six2 and *Drosophila* sine oculis.

Six3/6-Pc is a 290 amino acids protein that shows an amino acid substitution in the characteristic tetrapeptide QKTH in the HD N-terminus. At position 5 in helix 1 the six type-homeodomain displays an alanine instead of the described threonine (supplementary Fig.; Seo et al., 1999). The HD of Six3/6-Pc is 86% identical to Human Six3 and 82% identical to the HD of *Drosophila* optix. The SD is 73% identical to Human Six3 and 65% to *Drosophila* D-Six3/optix.

Although *Cladonema* and *Podocoryne* are both hydrozoan Anthomedusae and group close to each other (Collins, 2002), the size of the *Six* genes differs considerably. The regions outside the HD and SD are not conserved. The comparison of *Cladonema* Six1/2-Cr to *Podocoryne* Six1/2-Pc shows 91% identity within the SD and 98% identity within the HD but only 65% identity when the full length jellyfish sequences are compared. The HD of Six3/6-Pc is almost 97% identical to the HD of Six3/6-Cr and the SDs are 91% identical. In both species the unique peptide sequence CFKE adjacent to the family specific tetrapeptide (Seo et al., 1999) is present (see supplementary Fig.).

Expression patterns

Expression was analyzed for both species by Real Time-PCR of excised medusa parts (Fig. 3, 4), and in *Cladonema* also during eye regeneration (Fig. 7). Expression patterns were also investigated by *in situ* hybridization of all *Six* genes for both species (Fig. 5, 6). The connexion between the eye and the nervous system was visualized by immunohistology (Fig. 5B, C and 6H, I).

***Six1/2*:** The Real Time-PCR data from *Cladonema* (Fig. 3) show that the gene is strongly expressed in the exumbrella and the subumbrellar striated muscle layer. This is also

confirmed by *in situ* stainings (not shown). Very weak expression is occasionally detected in the tentacle bulbs (Fig. 3B) where a few radially arranged cells stain in close proximity to the lens (Fig. 5D, G). The completeness of the radial pattern and the number of the individually stained cells varies within the same animal from tentacle bulb to tentacle bulb and often shows no staining cells. In cross sections staining cells are observed from the surface of the cornea to the base of the eye cup (Fig. 5G). This variation explains the weak expression observed with Real Time-PCR and overcomes the non-consistency of the two data sets (Fig. 3B, 5D, 5G). Staining is seen also in cells which are arranged along the ring canal (Fig. 6A-C) and in cells which encircle the tentacle base and enter the tentacle ganglion (Fig. 6D-F; Mackie, 1971). With the exception of the staining in the eye cup, a similar pattern can be observed for *Six3/6* and *Six4/5* too (not shown). The cell shape of the stained cells resembles that of nerve cells, and in addition immunostainings for RFamide and tyrosin-tubulin nerve cells clearly co-localise with the *in situ* hybridization pattern (Fig. 5B, C and 6H, I). We conclude that the *Six* genes stain nerve cells, possibly a subset of the RFamide or tyrosin-tubulin positive nerve cells. A nerve cord along the ring canal connecting the eyes has been described to fulfill central information processing functions (Anderson and Mackie, 1977) as jellyfish lack any brain-like structure. The described *Six* gene expression pattern has some similarity with the observation of Pineda and Saló (2002) who report the presence of GtSix3 in brain branches of planarians.

In comparison to Bilateria the *Cladonema Six1/2-Cr* gene appears structurally and functionally conserved. It is involved in both, the myogenic/mesodermal (striated muscle) and the neurogenic line (nerve, eye). In this latter role it correlates with *Drosophila sine oculis* (Cheyette et al., 1994) and planarian *Gtso* (Pineda et al., 2000). Expression in the muscle layers in both jellyfish species is also similar to the non-neural expression of *Six1*

and *Six2* of mouse (Oliver et al., 1995; Ohto et al., 1999) and Xenopus (Ghanbari et al., 2001) where the genes are expressed in head mesenchyme, somites and limb mesenchyme.

In comparison to *Six1/2-Cr* the Real Time-PCR expression data of *Podocoryne Six1/2-Pc* reveal a strong presence of this message in tentacle bulbs, the manubrium, and some message is in the striated muscle (Fig. 4B). The *in situ* stainings (Fig. 6J) do not specify a distinct cell type in this tissue. Diffuse staining is seen in the endodermal and ectodermal part of the bulb where intensive cell proliferation occurs and where all cell types for the tentacles differentiate, mostly nematocytes and nerve cells (Tardent, 1978).

***Six3/6*:** The gene is strongly expressed in the tentacle bulbs in both species (Fig. 3B, 4B). In *Cladonema* tentacle bulbs, very strong staining is restricted to the eye cup but also includes the adjacent corneal tissues (Fig. 5E, H) and in addition the striated muscle, the manubrium and the tentacles (Fig. 3B). In *Podocoryne* the tentacle bulbs' staining is similar to that of *Six1/2-Pc* (Fig. 6K). Expression is also detected in the tentacle (Fig. 4B) where nematocytes seem to stain (Fig. 6L). In vertebrates the Six3/6 homolog is responsible for eye development whereas in planarians this role is fulfilled by Six1/2 (Pineda et al., 2000; Pineda and Saló, 2002). *Drosophila sine oculis* (Six1/2 subclass) can induce ectopic compound eyes only in cooperation with *eya* dependent on *ey* activity whereas *optix/D-Six3* has been shown to induce ectopic eyes by an *ey* independent mechanism (Seimiya and Gehring, 2000). The murine ectopic expression of *Six3* promotes the formation of ectopic optic vesicle-like structures (Lagutin et al. 2001) and an increase in eye size and expansion of the retina territory could have been observed in *Xenopus* embryos after ectopic *XOptx2/Six6* expression (Zuber et. al 1999).

***Six4/5*:** *Six4/5-Cr* is mainly expressed in tentacles and the manubrium (Fig. 3B) where young oocytes stain (Fig. 6G). Staining is almost absent in the eye cup (Fig. 3B, 5F, I). Some

scattered cells stain in the tentacle bulbs and along the ring canal as seen for the other *Six* genes (Fig. 6A-F). Isolation of the corresponding *Podocoryne* gene was not successful.

From *Drosophila* it has been postulated that D-Six4 is involved in cell recognition events required for myoblast fusion and for the formation of the precursor of follicle cells (Kirby et al., 2001). Jellyfish striated muscle is mono-nucleated and therefore the expression pattern observed in *Drosophila* for myoblast fusion invalid for comparison. However, the correlation in expression in the gonads is astonishing, especially since Cnidaria appear to have no germ line and the gametes can be formed from both germ layers (Schmid, 1982; Bouillon, 1994).

Regeneration of eyes in *Cladonema*

In contrast to the short lived *Podocoryne* medusae, *Cladonema* can live and grow considerably for many months (Fig. 1). During this growth period the eyes enlarge correspondingly. It has been shown that *Cladonema* medusa can easily regenerate entire eyes (Weber, 1981a). To initiate eye regeneration, the whole ocellus has to be sucked off with a glass capillary, as partial excision would lead only to a wound healing response (Weber, 1981a). Ectodermal cells surrounding the edges of the wound start to move and close the wound. Five minutes after extirpation the hole is closed. The damaged mesogloea (ECM) to which the eye cells adhere (Fig. 1C) is repaired six hours after the operation and twenty-four hours after extirpation the cornea starts to form. By the same time, about one day after extirpation, a few presumptive sensory cells can be identified ultrastructurally (Weber, 1981a, b). Pigment and sensory cells differentiate three to six days after the operation and the lens body starts to form, and ten to fifteen days post-operation the eye is re-established. The *Cladonema* lens can regenerate from the pigment cells by transdifferentiation (Weber,

1981a) as is reported to occur also during lens regeneration in amphibia (reviewed in Okada, 1991; Kodama and Eguchi, 1995).

We used *Cladonema* to further investigate the expression pattern of *Six* genes in eye regeneration. The entire eye area was removed and then the regenerating eye area excised at different time intervals and investigated by Real Time-PCR (Fig. 7A). After 14 days the expression values are back to the level measured in the non-regenerating intact eye bulb, used as a control (Fig. 7B). *Six1/2-Cr* is strongly up-regulated during eye regeneration and reaches its maximum values one week after the eye has been removed showing that it is involved in eye regeneration but not in eye maintenance (Fig. 3B). The up-regulation of the *Six1/2-Cr* expression precedes the *Six3/6-Cr* expression for at least one day. This time shift of the *Six* gene expression during eye regeneration could indicate that *Six1/2-Cr* is needed for structural different functions than *Six3/6-Cr* and/or that *Six1/2-Cr* is hierarchically situated above *Six3/6-Cr* in a possible genetic network. It should be noted that 15 minutes after eye removal almost no *Six3/6-Cr* message could be detected. This observation suggests a restriction of *Six3/6-Cr* expression in the eye tissue. The results demonstrate convincingly that both *Six1/2-Cr* and *Six3/6-Cr* are involved in the formation of the new eye (Fig. 7B) whereas no expression of *Six4/5-Cr* was observed during the entire regeneration process (not shown).

Six genes in the evolutionary context

It has become evident that a good part of the evolution of animal diversity was not accomplished by the invention of new genes *de novo*, but largely by duplication and subsequent modification of existing genes (Meyer and Schartl, 1999; Suga et al., 1999) and remodelling and redeploying of already existing genetic networks (Peterson and Davidson, 2000). Therefore the question is how and when these basic developmental networks were

formed, only once for all phyla, or repeatedly when the evolutionary conditions were favourable? Furthermore and tightly connected to this question, we have to ask what mechanisms favoured the assembly of the genetic networks found in the genetic toolbox of the hypothetical common ancestor. Since up to now no fossils exist from this early precambrian times only analysis of molecular developmental genetics in "old" extant phyla appear promising. In this context Cnidaria as representatives of an old well suited bilaterian out-group, they exhibit diversity in life stages ranging from simple structured sessile polyp forms to the highly motile and differentiated medusa stage.

We recently demonstrated that jellyfish use the same bilaterian gene cascades and comparable developmental expression patterns to differentiate the striated muscle (Spring et al, 2000, 2002; Müller et al., 2003). Therefore we concluded that Cnidaria either derive from an already mesodermate-like ancestor or they managed to reassemble in parallel to the bilaterian trait the myogenic cascade of key regulatory genes and a comparable pattern of development. Furthermore, recent observations in anthozoan larvae demonstrated a bilaterian like *Dpp* expression pattern what further indicates that the position of Cnidaria as an outgroup to the Bilateria needs to be discussed (Hayward et al., 2002). In this context we investigated *Six* family genes in eye and muscle formation in jellyfish. The family belongs to another conserved bilaterian gene network consisting of *Pax* (paired box), *Six* (*sine oculis*), *Eya* (*eyes absent*) and *Dac* (*dachshund*) which are used in the development of sensory cells/organs. Additionally, some members of those networks are involved in the differentiation of mesodermal derivatives.

The data demonstrate that Cnidaria have at least one member of each of the three *Six* family subclasses. Therefore the family of *Six* genes arose before the Urbilateria and the Cnidaria separated, but after the first big wave of gene duplications occurred, predating the Parazoa and Eumetazoa split some 980 million years ago (Miyata and Suga, 2001). We regard it as

likely that after the first round of duplications and the separation of the Parazoa (Suga et al., 1999) sufficient genomic material was available to gradually select new developmental structures and the corresponding networks of regulatory genes. The product of this process was assumingly a non-sessile organism which had invented a muscle contraction based locomotion (Spring et al., 2002; Müller et al., 2003), invented a gut system and consequently knew predation on fellow organisms other than prokaryotes. It had evolved an anterior-posterior body axis (Yanze et al., 2001) and an anteriorized nervous system (Gröger and Schmid, 2001) which was used to control sensory input and directed locomotion. Since sexual development predated metazoan evolution, the putative non-sessile organism was likely of direct development (Wolpert, 1999). This hypothetical organism could be the source of a possible zootype (Slack et al., 1993). When the history of earth offered new niches these basic cassettes of developmental genes were available as functional networks and could be co-opted (Davidson, 2001) to further add and refine developmental patterns and anatomical structures thus providing the base for the rapid evolution of the different phyla (Miyata and Suga, 2001). We believe that the Cnidaria share with Bilateria a good part of this process. Cnidaria already have a representative of each subclass of the *Six* family genes and they use them correspondingly to Bilateria to differentiate eyes and mesodermal derivatives like muscle. It is noteworthy that the dual role of jellyfish *Six1/2* and *Six3/6* in eye formation and differentiation of mesodermal elements appears to be conserved through such long time in evolution. This is also the case for the *Six4/5* which in *Drosophila* is expressed in the gonads and in *Cladonema* in the manubrium which differentiates the gametes (Brändle, 1971; Bouillon, 1994).

Heanue and co-workers (1999) showed that the genetic network of *Pax, Dach, Eya* and *Six* genes has been used not only for eye development but also for myogenesis. The myogenic network includes gene family members that are not directly homologous to those used for

eye development, for example *Pax3* instead of *Pax6*. These functional connections between the neurogenic/sensory and myogenic pathways in the *Six* and *Pax* family indicate that muscle and nerve arose from the same genetic network which participated in the evolution of the protomyocytes (Mackie, 1990). Jellyfish appear to have conserved this ancestral situation, *Pax* genes are myogenic and neurogenic (not published) and the medusa cognate of the neurogenic bHLH gene *Atonal* has functions in both developmental lines (Seipel et al., 2004). Jellyfish *Six1/2* and *Six3/6* are involved in eye formation, as they are in *Drosophila sine oculis* and *D-Six3*/*optix* (Cheyette et al., 1994; Seimiya and Gehring, 2000) whereas in vertebrates it is only the *Six3/6* gene (Lagutin et al., 2001; Loosli et al., 1999). Remarkably *Six1/2* and *Six3/6* are expressed in *Podocoyne* in the same tissue where in *Cladonema* eyes differentiate but where expression is restricted to the eye area. Additionally, our data suggest that Six3/6-Pc could be used to differentiate nematocytes, a cell type with mechano-sensory function (Galliot et al., 2003). In the context of the above formulated hypothesis we assume that *Podocoryne* once had eyes but lost them, maybe due to the identified mutation in the homeodomain (supplementary Fig.).

Conclusions:

Although we do not know yet the full interacting regulatory network of eye determinating genes for jellyfish the high degree of *Six* gene conservation in structure and function and the observations on the molecular control of muscle formation by bilaterian-like gene cascades (Spring et al., 2002; Müller et al., 2003) suggest that these networks assembled before the ancestor of jellyfish split from the bilaterian line. Our data do not contradict the hypothesis that the upstream network of genes regulating eye formation is monophyletic. We conclude that the last common ancestor of Cnidaria and Bilateria was not a primitive diploblast planuloid type (Holland, 2000) but a motile organism of considerable complexity in body

organization. Otherwise we would opt for repeated evolution of these networks of developmental genes, a possibility which is difficult to imagine given the complexity of problems to be solved when an early metazoan gradually evolved into the zootype.

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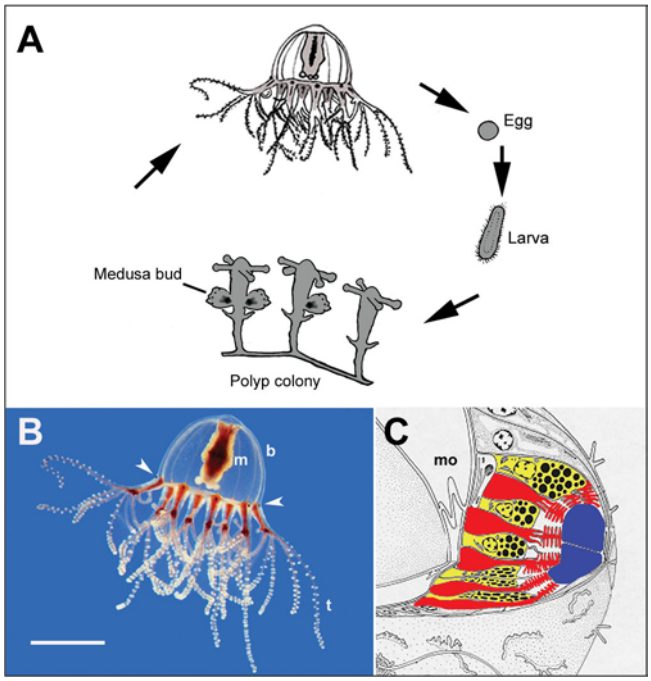

Fig. 1. (A) Schematic life cycle of *Cladonema radiatum*. The sexually mature medusa liberates gametes. The embryo develops into a free swimming ciliated planula larva, which attaches to the substrate and transforms into a polyp. Polyps bud asexually medusae. (B) shows an adult medusa with lens eyes located at the base of the tentacles at the margin of the bell (arrowheads). The structure of the lens eye is displayed in (C) (modified after Weber, 1981a). In blue is the tripartite lens, in red are the photoreceptor cells and in yellow the pigment cells. b, bell; m, manubrium (feeding and sex organ); mo, mesogloea (ECM); t, tentacle. Bar is (in µm) 700 in (B), 10 in (C).

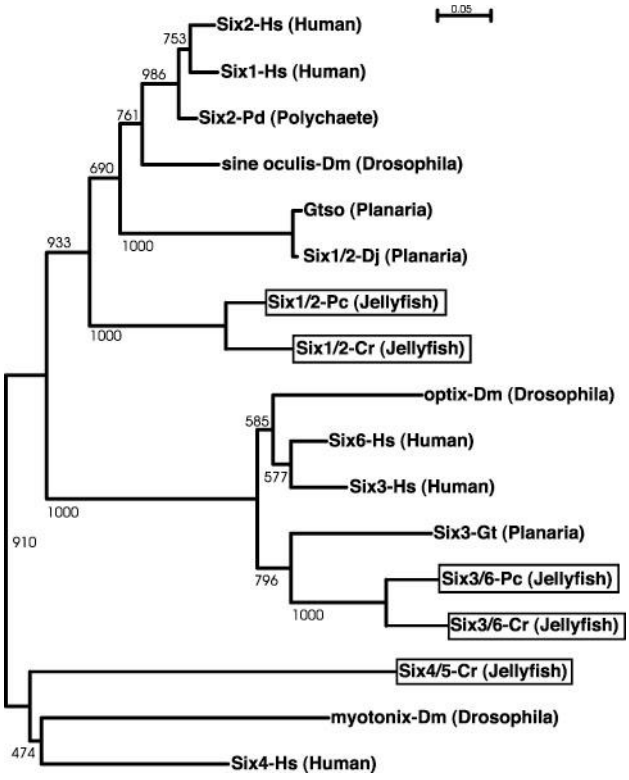

Fig. 2. Phylogenetic analysis of the jellyfish *Six* genes confirms their classification into the three main subfamilies. The full SD and HD were used as a basis for analysis. The phylogenetic neighbour joining tree was calculated with ClustalX and 1000 bootstrap replicates (Jeanmougin et al., 1998).

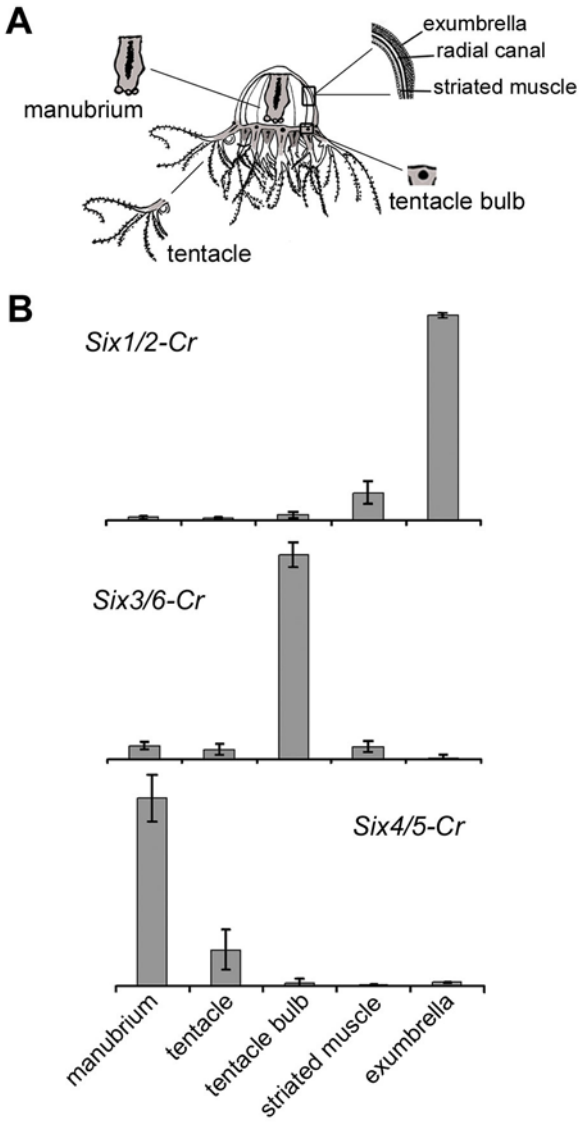

Fig. 3. *Six* gene expression analysis of *Cladonema* medusa parts. (A) Portions of the medusa are isolated by microdissection. (B) *Six* genes expression levels are measured by Real Time-PCR. Graphs display relative values normalized to elongation factor expression level.

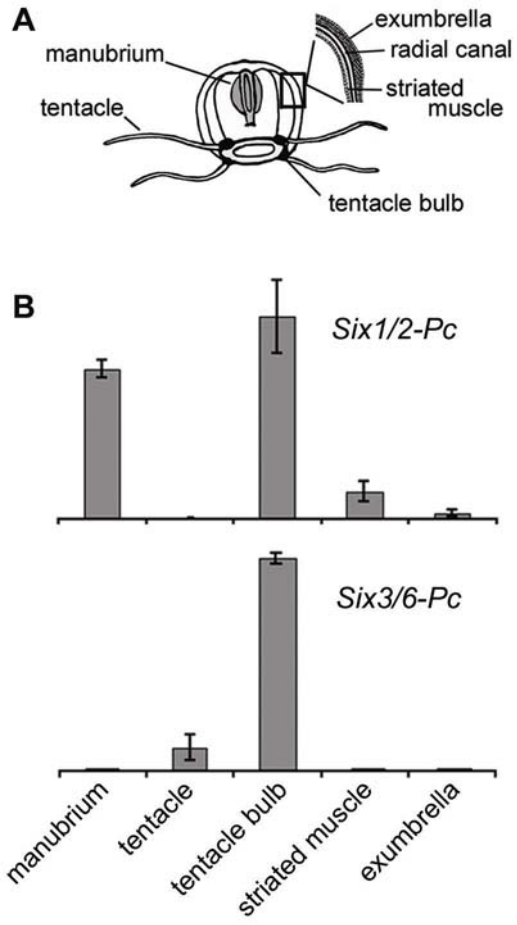

Fig. 4. *Six* gene expression analysis of *Podocoryne* medusa parts. (A) Portions of the medusa are isolated by microdissection. (B) Gene expression levels are measured by Real Time-PCR. Graphs display relative values normalized to elongation factor expression level.

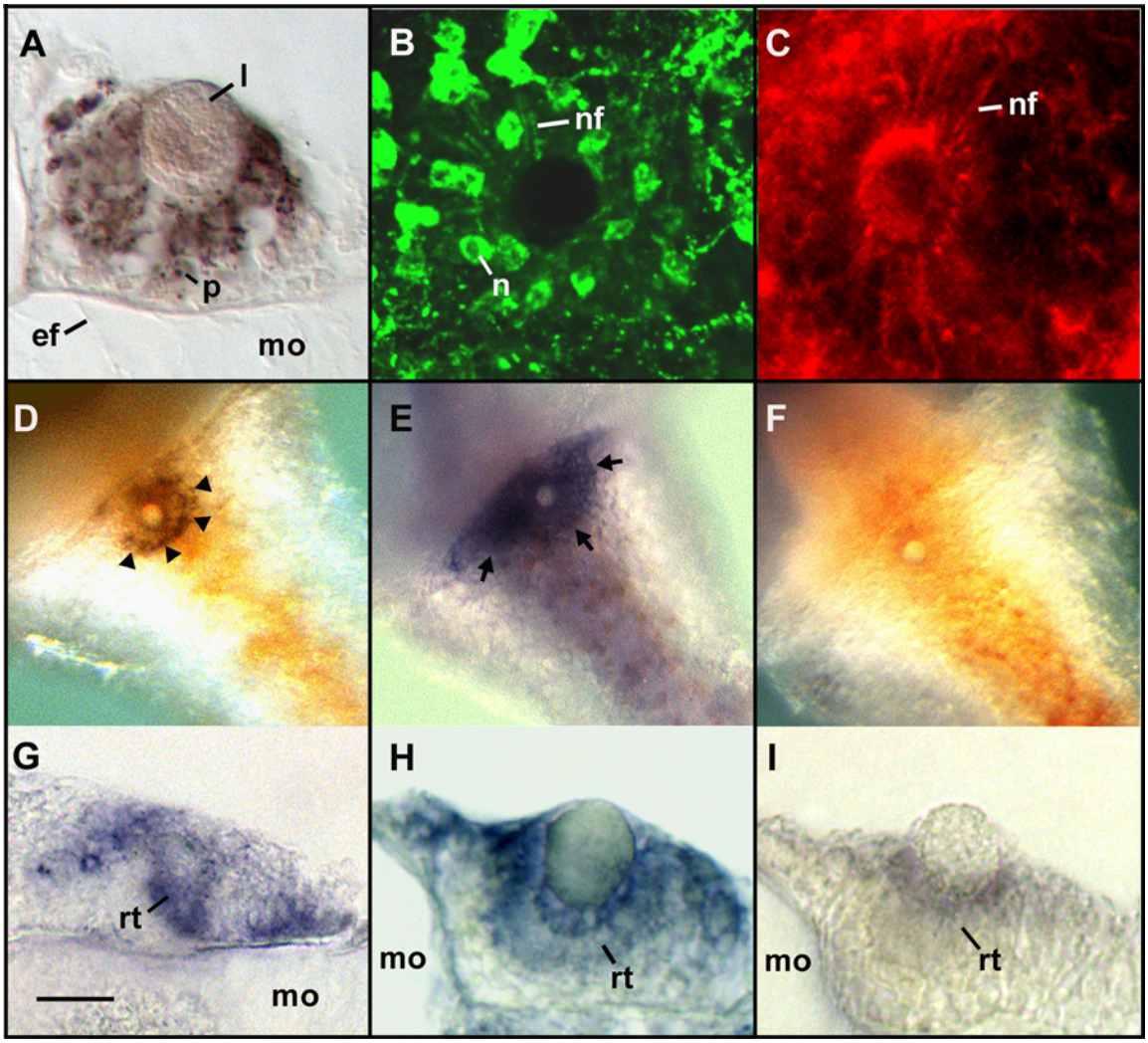

Fig. 5. Expression analysis and immunohistology of *Cladonema* eyes. (A) Cross section of an eye displays the intrinsic retina colouring (compare Fig. 1C). RFamide (B) and tyrosine-tubulin (C) positive staining cells in the eye cup. *In situ* hybridization with antisense RNA probes for *Six1/2-Cr* (D, G), *Six3/6-Cr* (E, H), and *Six4/5-Cr* (F, I). Top view on tentacle bulb displays radial arranged cells (D, arrowheads) around the lens expressing the *Six1/2-Cr* message, corresponding to the paraffin section (G, section is outside of the lens body). Six3/6-Cr stains the entire corneal part of the eye (E, cross-section in H). Arrows point to the margin of the cornea (E). Very weak residual staining of *Six4/5-Cr* is present in the eye (F, I). ef, ECM-fiber; l, lens; mo, mesogloea (ECM); nf, nerve-fibers; n, cell body of RFamide positive nerve cell; p, pigment cell; rt, retina; Bar is (in µm) 20 in (A), 30 in (B), 20 in (C), 100 in (D, E, F), and 25 in (G, H,I).

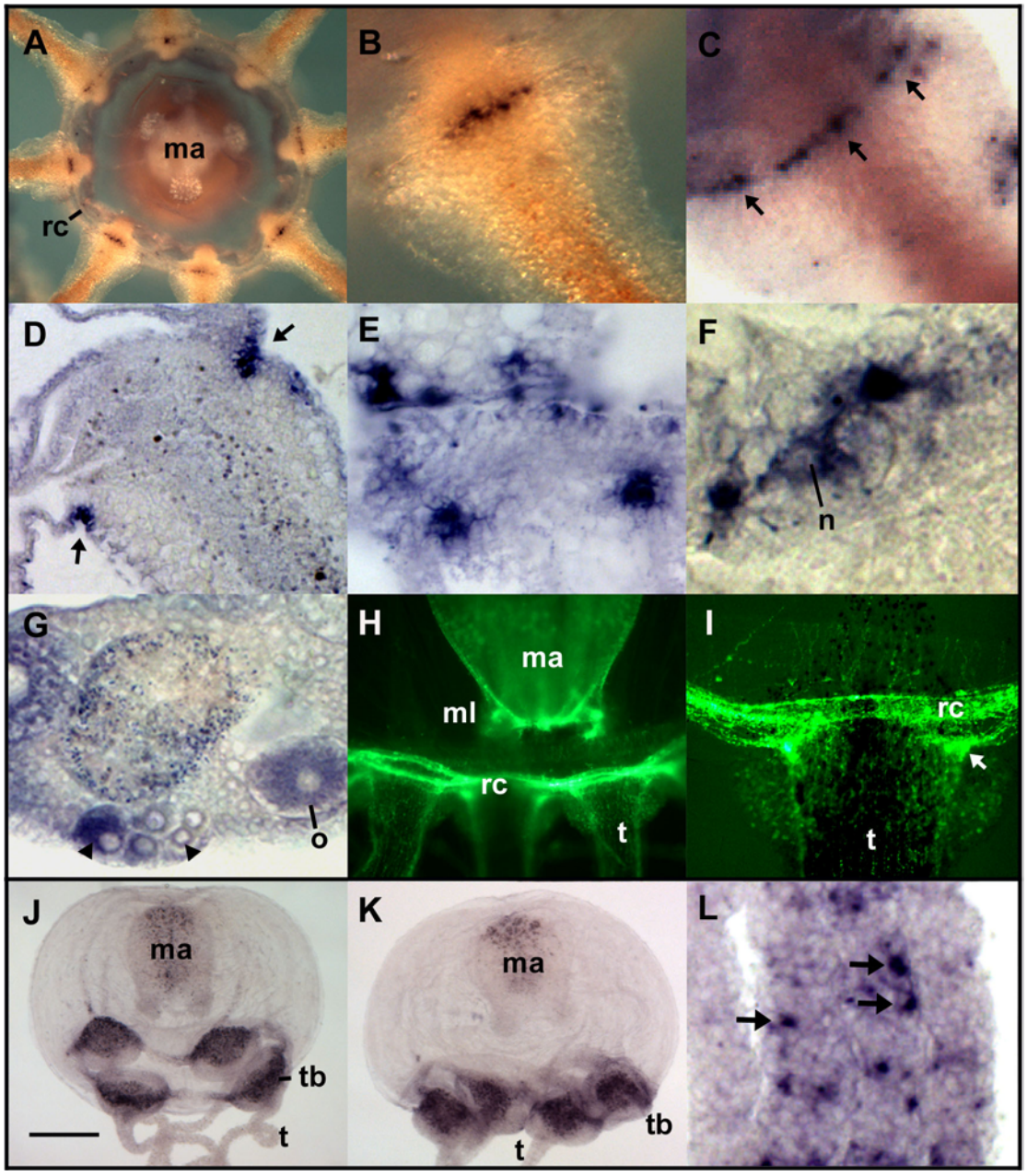
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Fig. 6. Expression analysis and immunohistology in *Cladonema* (A-I) and *Podocoryne* (J-L). *In situ* hybridization experiments reveal the correlation of *Six1/2-Cr* expression (A-F) to a subset of RFamide positive nerve cells (H, I). Bottom view of medusa (A) and tentacle bulb area (B,C) displays the staining along the ring canal. Cross section of a tentacle bulb (D). Arrows point to the expression at the rim (D). In tangential sections of the ring canal (E) and of the rim (F) cells of nerve cell appearance are stained. Young oocytes in the manubrium express *Six4/5-Cr* (arrowheads, G). RFamide positive nerve cells accumulate at the tip of the manubrium, along the ring canal (H) and encircle the tentacle base (H, arrow in I). Arrow points to the accumulation of nerves in the rim (I) at the tentacle base which corresponds to arrows in (D). *In situ* hybridization with anitsense RNA probes for *Six1/2-Pc* (J) and *Six3/6-Pc* (K,L). Diffuse staining for both genes was found in the tentacle bulbs (J, K). *Six3/6-Pc* is also expressed in potential nematoctyes of tentacles (arrows in K, four aligned tentacles). ma, manubrium; rc, ring canal; n, nerve cell; ml, manubrium lips; t, tentacle; tb, tentacle bulb. Bar is (in μm) 280 in (A), 55 in (B), 40 in (C), 50 in (D), 20 in (E, F), 100 in (G), 195 in (H), 70 in (I), 190 in (J), 26 in (K), and 160 in (L).

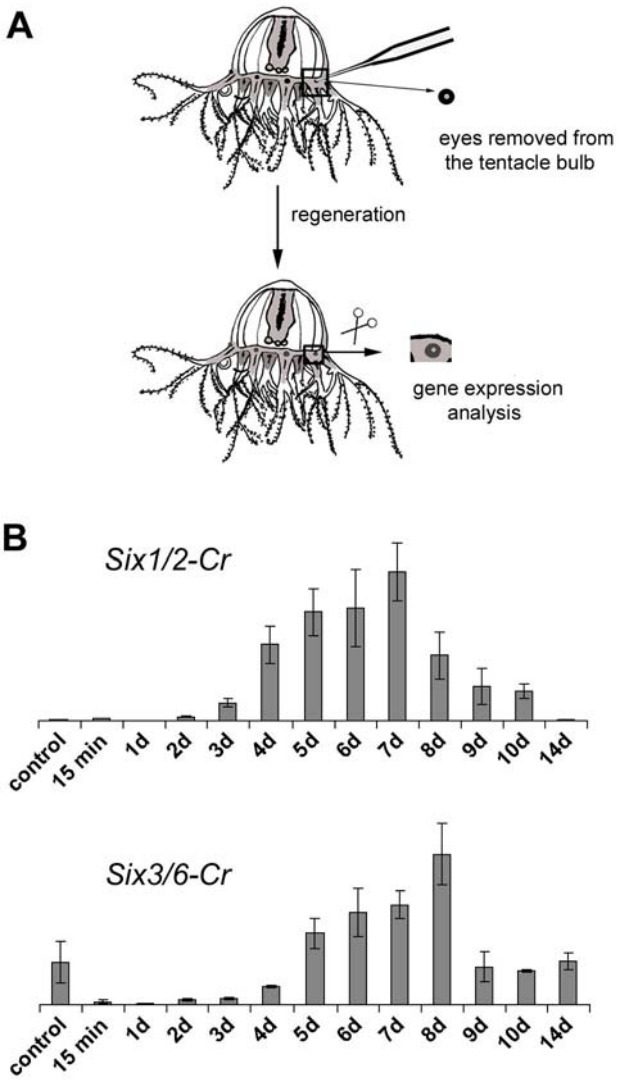
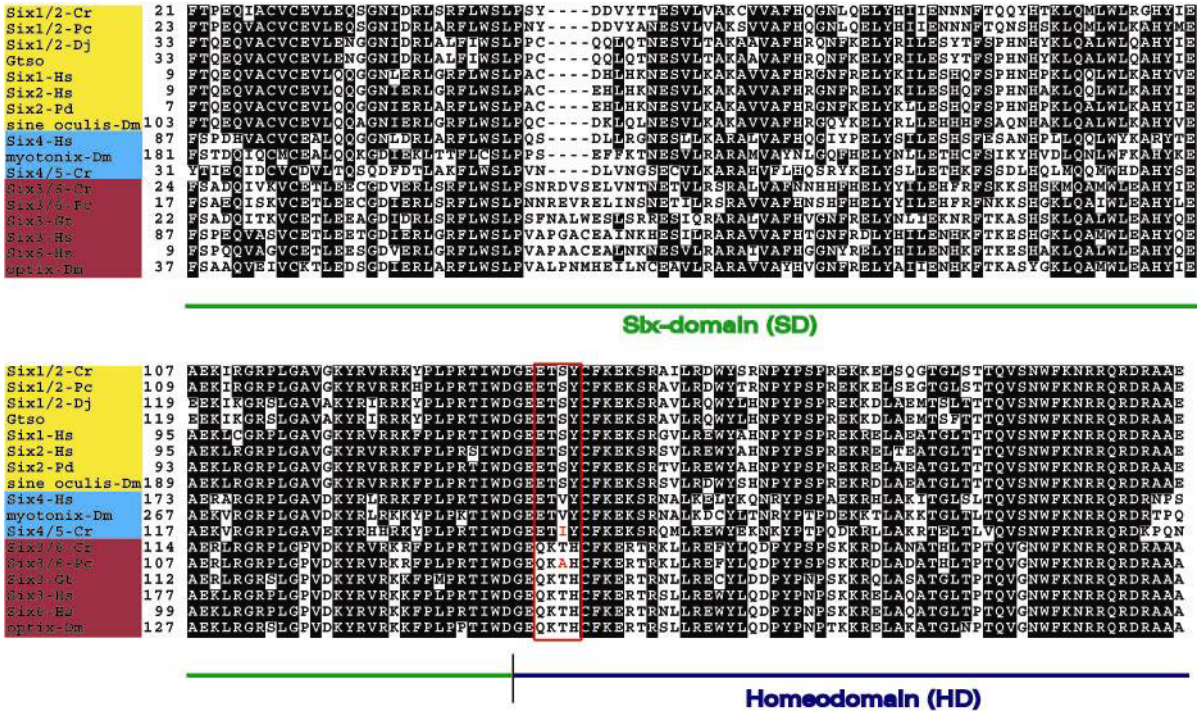


Fig. 7. Real Time-PCR expression of *Six* genes during eye regeneration. (A) Eyes are removed from the tentacle bulb using a glass capillary. At different time points, tentacle bulbs are excised from the medusa for RNA extraction. (B) *Six1/2-Cr*, *Six3/6-Cr* and *Six4/5-Cr* levels of expression are evaluated by Real Time-PCR and presented relative to the normalizing value of elongation factor. Control corresponds to the gene expression in the intact tentacle bulb. *Six4/5-Cr* expression was not detected in the eye regeneration process (data not shown). After 14 days, the eye was completely regenerated (Weber, 1981a, b).



Supplementary Fig. Sequence alignment of the entire Six and Homeodomain with a range of related proteins. *Cladonema* (Cr) and *Podocoryne* (Pc) domains are compared to *Dugesia* (Dj), *Girardia* (Gt), Human (Hs), *Platynereis* (Pd) and *Drosophila* (Dm). The Six1/2 family is boxed in yellow, the Six3/6 family in blue, and the Six4/5 in red. Characteristic tetrapeptide sequences are framed and the jellyfish amino substitutions are coloured in red. Accession numbers for published protein sequences used for sequence comparison are as follows: Six1/2-Dj, AJ557022; Gtso, AJ251660; Six1-Hs, AAH08874; Six2-Hs, AAH24033; Six2-Pd, CAC86663; sine oculis-Dm, Q27350; Six4-Hs, Q9UIU6; myotonix-Dm, AAF63760; Six3-Gt, AAN77127, Six3-Hs, AAD15753, Six6-Hs, AAD49844; optix-Dm, Q95RW8.

CHAPTER 2

Pax genes in jellyfish – Pandora's Box of eye evolution?

Abstract

We isolated two different Pax family members from the hydrozoan jellyfish *Cladonema radiatum* (Cr) and from the cubozoan jellyfish *Carybdea marsupialis* (Cm). Both species have well developed lens eyes. Gene structure and expression pattern were analyzed and in *Cladonema* additionally expression was surveyed during eye regeneration by Real Time PCR and *in situ* hybridization.

Cladonema PaxA-Cr is almost exclusively expressed in the eye. *PaxB-Cr* is not expressed in the eye but in other body parts. During eye regeneration only *PaxA-Cr* is up-regulated. In the cubomedusan rhopalia consisting of slit eyes, pit eye, lens eyes and statocyst, *PaxB-Cm* expression is restricted to the statocyst. However no *PaxC-Cm* expression was observed in the rhopalia. *PaxB-Cm* and *PaxC-Cm* are also strongly expressed in other medusa tissues.

Our data suggest that at least four different Pax genes exist in all cnidarians and that neither cnidarian PaxB nor PaxC can be an ancestor of Pax6. *PaxA-Cr* expression corresponds with a potential Pax6 ancestor candidate although its protein structure contradicts this hypothesis. Therefore the hunt for a *bona fide* Pax6 in Cnidaria is still left open.

Introduction

Pax genes, a paired domain containing family of transcription factors are involved in a plethora of control events in development, mainly nervous and myogenic systems, and oncogenesis (Simpson and Price, 2002; Chi and Epstein, 2002). Pax family members have been cloned from all metazoan phyla, including sponge (Hoshiyama et al., 1998). Their classification occurs depending on the presence and structure of a paired-type homeobox and of an octapeptide.

The ubiquitous expression and high sequence conservation of the well-characterized Pax6 throughout the Metazoa and results from its ectopic expression (Halder et al., 1995) led to the paradigm of a master control gene for eye development, and as a conclusion, monophyletic eye evolution has been proposed (Gehring and Ikeo, 1999; Gehring 2002). To date, it remains an open question whether non-bilaterian animals, such as Cnidaria possess a *bona fide* Pax6 gene, although several *Pax* genes have been reported from this phylum (Sun et al., 1997; Miller et al., 2000; Gröger et al., 2000; Sun et al., 2001, Kozmik et al., 2003). It is considered that cnidarian paired domains exhibit as a general property a broader binding specificity than other paired domains (Sun et al., 2001). Hence, they could be multifunctional controlling several pathways, uniting functions in one gene that are split in higher Metazoa into two or more genes (Kozmik et al., 2003).

Cnidarians occupy a key position representing the most primitive metazoan animals with muscle tissue, a nervous system and sense organs of great complexity like eyes. Photoreceptive organs in Cnidaria range from simple ocelli up to highly evolved lens eyes found in Hydrozoa and Cubozoa. The surprisingly elaborate eye types of cnidarian medusa are known for a long time (Schewiakoff, 1889; Pearse and Pearse, 1978). In general it is the free-swimming medusa that bears eye-like structures although a cubozoan larva with single-

celled pigment-cup ocelli has been described (Nordström et al., 2003). The sessile polyps in all cnidarian classes are photosensitive but have no eyes (Tardent and Frei, 1969).

Cnidarian Pax genes are generally termed as *PaxA*, *-B*, *-C*, and *-D*. *PaxA* lacks a homeobox and is viewed to be orthologous to *Drosophila poxneuro*, *PaxB* seems to belong to the Pax2/5/8 class, containing a full homeobox and an octapeptide. *PaxC* has been classified to the Pax4/6 class and *PaxD* resembles the Pax3/7 class.

The anthozoan *Acropora millepora* is the only cnidarian from which the full repertoire of *Pax* genes is known (Catmull et al., 1998; Miller et al., 2000). Anthozoa lack the medusa generation and therefore eyes, but PaxC is expressed in putative nerve cells during larval development. From all other cnidarians studied so far, only *PaxA* and *PaxB* homologs have been isolated. Available expression data originate from the hydrozoan *Podocoryne,* a jellyfish without eyes, in which PaxB seems to be involved in nerve cell differentiation (Gröger et al., 2000) and from the cubomedusan *Tripedalia* where PaxB is expressed in the swimming larvae, the lens, retina, and statocyst of adult rhopalia (Kozmik et al., 2003).

To further clarify the origin and role of *Pax* genes in eye evolution we searched for *Pax* genes in jellyfish with lens eyes, in the hydrozoan *Cladonema radiatum* (Cr) and the cubozoan *Carybdea marsupialis* (Cm). We isolated two Pax genes, *PaxA-Cr* and *PaxB-Cr* from *Cladonema radiatum*, a close relative to *Cladonema californicum* (Sun et al., 2001), and analyzed their expression pattern in the medusa and during eye regeneration. Furthermore we cloned and characterized the expression pattern of *Carybdea PaxB-Cm* and *PaxC-Cm*. Our data suggest that *PaxA* could be a *Pax6* ancestor and that all cnidarians have at least four different Pax genes.

Materials and Methods

Animals

Cladonema radiatum (Cnidaria, Hydrozoa) and *Carybdea marsupialis* (Cnidaria, Cubozoa) are reared in artifical seawater, *Cladonema* at 20°C in aerated aquaria and *Carybdea* between 24 and 28°C for medusa production in 200 ml culture bowls. Animals were fed every second day with two day old *Artemia* naupli.

Molecular cloning and phylogenetic analysis

Standard protocols (Sambrook and Russell, 2001) were performed for molecular biology procedures. Degenerate Pax primers (forward, 5'-GGC GGT GTT AAY CAR YTN GGN GG-3'; nested forward, 5'-TTC GTT AAT GGN MGN CCN YTN CC-3'; reverse, 5'-AAC AAA CGT TCN CKD ATY TCC CA-3'; nested reverse, 5'-GCA ACT TTT GGY TTN SWN CCN CC-3') were used for PCR conducted on cDNA obtained from entire medusa or eye tissue: 20x (30 seconds at 94°C, 45 seconds at 40°C, 1 minute at 72°C) then 10x (25 seconds at 94°C, 30 seconds at 40° C, 1 minute at 72°C) followed by nested PCR 40x (25 seconds at 94°C, 30 seconds at 50°C, 1 minute at 72°C). The full length coding sequence was acquired by RACE (rapid amplification of cDNA ends) on cDNA prepared with the SMART RACE cDNA amplification kit (Clontech). Sequence analyses, Blast searches and phylogenetic trees were performed as described (Müller et al., 2003).

Real Time-PCR Expression Analysis

Real Time-PCR expression analysis was done as described (Müller et al., 2003). For *PaxA-Cr* the primer pair (forward, 5'-GGG AGG CCG CTA CCG GAT TAC ATG CGC C-3'; reverse, 5'-CGA TCC GCC AAT AGC ACC AGG GCG TAC AG-3'), for *PaxB-Cr*

(forward, 5'-ATG AGC CAT GTG GTT CCA TTT GAA G-3'; reverse, 5'-CAT GGT CGA ACT CCT TGA CTA GAC-3'), for *PaxB-Cm* (forward, 5'-CAC AAA TGC CTG GTG GAT TTC CTG GTG-3'; reverse, 5'-CCC TGG AAC ATT GGA TTT TGT GGA GAC-3') and for *PaxC-Cm* (forward, 5'-TTG CCA GAT CAC AAG CGA CAG AGA-3'; reverse, 5'-ACC AGA GCG TAG AAG TCC AGA TTC-3') were used.

Immunohistology

Immunohistology was done as described previously (Gröger and Schmid, 2001) with minor modifications. Animals were fixed during 30 minutes at room temperature in 4% PFA, pH 7.4, washed twice in PBS-Triton for 20 minutes. Specimen were incubated with a monoclonal chicken Pax6 antibody (chicken Pax6, aa 1-223, recombinant protein made in E.coli, developed in mouse) diluted 1:150 in PBS for two hours at room temperature.

Whole Mount *in Situ* Hybridization

In situ hybridization was performed as described previously (Müller et al., 2003) with the following modifications: fixation was performed during 2-4 hours on anesthetized pre-cooled animals (1:1 Sea water/ 7% w/v $MgCl_26H_2O$ for 5 minutes on ice) with freshly prepared 4% paraformaldehyde. Hybridization was performed at 58°C in a Hybridization buffer containing 50% formamide, 5x SSC, 100 µg/ml tRNA, 100 µg/ml heparin, 0,1% Tween20, 10mM DTT, 10% (w/v) Dextran. The Pre-Hybridization solution was free of the Dextran component. DNA templates excluded paired- and homeobox, were prepared by PCR with the following primers for *PaxA-Cr* (forward, 5'-GCA AAA TTC AAA CAA GAG GAG CTT C-3'; reverse, 5'-GGG GTG ATA TAT CAG CAG CAA GAA G-3'), for *PaxB-Cr* (forward, 5'-AAT CAA GAC CAT ACC AAA AGT GAT GCC-3'; reverse, 5'-CCT GAA CCC GCT GCT CAG GGA TTT GAC-3'), for *PaxB-Cm* (forward, 5'-CAA TAG TAC TGG AAA

AGA AGA TGG TAT G-3'; reverse, 5'-CCA CCA CGT TTG CCA ACC TGT CCA TTT G-3') and for *PaxC-Cm* (forward, 5'-GCG GCG GAA AAG GCA GCA CAA CAC GC-3'; reverse, 5'-CAT TCT TTC GAA GTC ATC TTT TCT AC-3') in conjunction with the DIG RNA Labeling Mix (Roche).

Regeneration and dissecting experiments

Anesthetized animals (1:1 Sea water/ 7% w/v $MgCl_26H_2O$ for 5 minutes) were operated with ophthalmologic scissors as indicated in the Figs. and as is described in Weber (1981a).

Results and Discussion

Cnidaria can be portrayed by the presence of two main phenotypes, polyp and medusa, which differ considerably in ecology, morphology and ultrastructure. In Hydrozoa and Cubozoa most species have both life stage specific phenotypes. The medusae develop asexually from polyps, in *Cladonema* (Fig. 1A) by budding and in *Carybdea* (Fig. 1B) through metamorphosis from single polyps each giving rise to one single medusa (Fig. 1). Both species bear highly evolved lens eyes at the margin of the bell, which are stalked in case of the Cubozoa. Morphology, electrophysiology and regeneration of jellyfish eyes have been studied in detail (Mackie, 1978; Yoshida, 1973; Singla, 1974; Weber, 1981a, b). Whereas *Cladonema* has a simple lens eye, Cubomedusae have four rhopalia, each with a statocyst, two slit eyes, two pit eyes and two lens-eyes (Pearse and Pearse, 1978).

Isolation and Characterization of jellyfish Pax genes

To identify *Pax* genes from jellyfish with eyes, medusa cDNA and cDNA derived from eye tissue was surveyed by homology PCR with degenerate primers corresponding to different

parts of the paired box. Obtained fragments were extended by RACE and by screening a cDNA library (as described in Spring et al., 2000). Two different Pax genes could be isolated from *Cladonema*, *PaxA-Cr* and *PaxB-Cr*, and two different Pax genes from *Carybdea*, *PaxB-Cm* and *PaxC-Cm*. The predicted protein sequences are highly conserved. A phylogenetic tree was conducted based on the paired domain (PD) sequences (Fig. 2).

The obtained PaxA-Cr fragment has a length of 224 amino acids and resembles *Drosophila pox neuro* in lacking a homeodomain (HD) and an octapeptide. It has been speculated that cnidarian PaxA has no clear cognate in other phyla and therefore may be cnidarian-specific (Galliot et al., 1999). However Czerny et al. (1997) presented a partial PD from a sea urchin that clearly clusters to the PaxA/pox neuro group. PaxA-Cr has a sequence identity within its PD of 99% to *Hydra* PaxA and 85% to PaxA from *Acropora*. In the phylogenetic analysis the anthozoan PaxC groups closer to the cnidarian PaxA genes than to other PDs. This observation is consistent with results obtained by others (Gröger et al., 2000; Sun et al., 2001). The sequence conservation of PaxA-Cr PD is 75% to *Acropora* PaxC. Remarkably, PaxA-Cr contains C-terminal of its PD a stretch of fifteen glutamine amino acids. Besides PaxA from *Hydra*, a similar stretch of glutamine amino acids was found also in the protein of *Drosophila* eye gone (eyg). The function of such a sequence is unclear as it characterizes secreted proteins, which is unlikely for transcription factors.

PaxB genes encode all three motifs, paired domain, octapeptide and homeodomain. PaxB-Cr is a protein of 675 amino acids and shares high sequence identities to the PaxB proteins described from *Cladonema californicum* (Sun et al., 2001). Both PD and HD are 99-100% identical within the two *Cladonema* species. Due to a possible polymorphism, one single amino acid is substituted within the PD of PaxB-Cr. However, comparison with other available cnidarian PaxB proteins reveals that the cnidarian sequences are more different than expected. The PD of PaxB-Cr is 86% identical to the hydrozoan *Podocoryne* and 79%

identical to the cubozoan *Tripedalia*. The HD shows 82% identity to *Podocoryne* but only 57% to the cubozoan *Tripedalia* HD.

The *Carybdea* PaxB-Cm encodes a protein of 448 amino acids containing a full PD, an octapeptide and a full HD. When compared to the databases (GenBank) the sequence of the PD is best conserved to PaxB from *Tripedalia* (82% identity) and to PaxB from *Podocoryne* (76%). The HD is 91% identical to the HD of PaxB from *Tripedalia* but only 60% identical to the HD of PaxB from *Podocoryne*. Cubozoan PaxB genes are therefore highly diverged compared to PaxB genes from other cnidarians. The assignment of identities is congruent with the phylogenetic tree (Fig. 2).

The sequence of the *Carybdea* PaxC is only partially available (230 amino acids) but sequence identities show clearly the relation to the cnidarian PaxC genes (not shown in Fig. 2). The highest sequence identity (70%) within the PD was obtained when compared to PaxC from *Acropora*. The presence of a PaxC ortholog in Cubozoa, the most highly evolved cnidarians let assume that in all cnidarian classes at least four different pax genes could exist.

Expression patterns

Real Time-PCR of excised medusa parts and *in situ* hybridization was performed to analyze expression patterns. Additionally expression patterns during in *Cladonema* eye regeneration were investigated by Real Time PCR.

PaxA-Cr: Real Time PCR data show an exclusive expression of *PaxA-Cr* in eye bulbs (Fig. 3B). *In situ* hybridization confirms this finding (Fig. 5A). The message is restricted to the eye and most strongly expressed in cells adjacent to the lens, in pigment and photoreceptor cells, although the lens itself is free of any staining (Fig. 5A).

PaxB-Cr: *PaxB-Cr* is expressed in the manubrium, tentacles and exumbrella (Fig. 3B). Real Time PCR detects a low signal in eye bulbs but no staining was observed in the eye (arrow)

but putative nerve cells of the tentacle bulb stain (Fig. 5B, asterisks). These putative nerve cells could be the source of variation detected by the Real Time PCR.

PaxC-Cm: *PaxC-Cm* from *Carybdea* is not expressed in eyes (Fig. 5C) but strongly in the manubrium and also in tentacles (Fig. 4B).

PaxB-Cm: The cubomedusan *PaxB-Cm* is expressed in rhopalia and tentacles but also in the manubrium and muscles (Fig 4B). *In situ* hybridization of the rhopalia shows no staining in the ocelli (Fig. 5D) but in the statocyst (Fig. 5D, arrowhead).

Regeneration of eyes in *Cladonema*

Expression patterns of pax genes were surveyed during eye regeneration in *Cladonema*. The process of eye regeneration as well as the experimental procedure is described in Fig. 6 or elsewhere (Weber et al., 1981a, b; Stierwald et al., submitted). Shortly after eye removal no *PaxA-Cr* message could be detected. During eye regeneration, *PaxA-Cr* is strongly up-regulated and reaches its maximum values four days after eye removal. Weber (1981a) described the start of differentiation of pigment and sensory cells three to six days after extirpation. Sensory and pigment cells regenerate from the same group of cells. The *PaxA-Cr* up-regulation precedes the up-regulation observed for Six genes (Stierwald et al., submitted). This time shift indicates that PaxA is upstream of the Six genes in the genetic hierarchy. Since PaxA-Cr and Six1/2-Cr and Six3/6-Cr are engaged in eye regeneration we conclude that in full or in part jellyfish use the same upstream network of genes to regulate eye formation. No signal of *PaxB-Cr* was detected during eye regeneration.

Evolution of Pax genes

In recent years several conflicting hypothesis were presented concerning the primordial Pax protein and their roles in eye evolution. It was assumed that Pax genes have a monophyletic

origin. Galliot et al. (1999) proposed that Pax genes arose from a paired-like ancestor via fusion of a paired-like homeobox gene with a gene encoding only a paired domain. Therefore Cnidaria would contain genes representing the before and after fusion events. Prior the divergence of sponge a *PaxA-like* ancestor fused with a homeobox giving rise to the *PaxB/2-5-8/sparkling family* (Galliot and Miller, 2000). A second independent homeobox capturing event occurred before the cnidarian-triploblast split leading to the PaxC/1-9/3-7/4-6 family group. Breitling and Gerber (2000) proposed a different Pax evolution scenario featuring a single homeobox capturing event and an early duplication of Pax genes before the divergence of porifera. The paired box would have originally been derived from a transposase and therefore shortly after the emergence of the metazoa, the DNA binding domain of a proto-Pax transposase fused to a homeodomain. Therefore current HD-free Pax proteins including cnidarian PaxA would indicate a secondary loss of the homeobox of a PaxC-like protein and PaxA would represent a derived status. To date only one Pax gene has been identified from sponge, encoding a paired-type homeobox and an octapeptide in addition to the paired domain (Hoshiyama et al., 1998). Beside sponge evidence was found for the existence of a *PaxB-type* gene in the placozoan *Trichoplax adherens* (Gröger et al., 2000). The homeodomain and the octapeptide of the sponge Pax gene are highly diverged. Based on phylogenetic analysis, Hoshiyama et al. (1998) conclude that gene duplications gave rise to different Pax subfamilies, PaxB/2-5-8/, pox neuro, and Pax6 are very ancient, going back to dates before the divergence of parazoa and eumetazoa. It remains unclear how Pax genes evolved and which Pax resembles the most ancient, but if Pax gene duplications preceded the parazoa/eumetazoa split further Pax genes should be found from sponges. The presence of a PaxB-like gene in porifera and placozoa led to the suggestion that PaxA genes evolved specifically in cnidarians upon gene duplication and were recruited for new functions (Gröger et al., 2000). One possible newly adopted function could be correlated to the appearance of

sophisticated eye-like structures. In the Cubozoa *Tripedalia* PaxB is involved in eye development but it seems to have additional functions in the tentacle, manubrium or the larva (Kozmik et al., 2003). *Carybdea* has at least two different Pax genes and we can't exclude the presence of additional Pax genes in its genome. It is therefore unlikely that *Tripedalia* would have only PaxB. Besides morphological similarities *Cladonema californicum* and *Cladonema radiatum* share very high sequence identities. We have been successful in cloning PaxA from *Cladonema radiatum* and so it is reasonable to assume that *Cladonema californicum* should contain the same number of pax genes as *Cladonema radiatum*. Except PaxD, at least one member of each cnidarian Pax subfamily has been identified from jellyfish. Anthozoa the most basal cnidarians already have four different Pax genes. The medusa represents the more elaborate stage concerning tissue architecture, nervous system and behavior than the polyp. It seems unlikely that jellyfish lost secondarily Pax genes and we therefore conclude that all cnidarians contain at least four different Pax genes. Furthermore we suggest that several subfamilies of Pax genes were already present in the last common ancestor of cnidarians and bilaterians.

Is PaxA a possible ancestor of Pax6?

The observation that PaxB is involved in the maintenance of the eye can only be applied for cubomedusae, not for hydromedusae. Remarkably *PaxB-Cm* stains the lumen of the statocysts like *PaxB* from *Tripedalia* (Kozmik et al., 2003). In contrast to *PaxB* from *Tripedalia*, ocelli of *Carybdea* do not express *PaxB-Cm*. It seems plausible that *PaxB* in cubozoan may be involved in the development of additional eye structures that are not present in the hydrozoan eye.

Pax6 does not have an octapeptide but contains a homeodomain. In triploblasts an octapeptide mediates transcriptional repression (Smith and Jaynes, 1996) and it was also found in several non-paired class proteins, even in cnidarians (Grens et al., 1996). There is not a clear and simple relationship between cnidarian Pax proteins, the Pax6 and Pax2/5/8 classes of bilateral animals (Plaza et al., 2003). *PaxB-Am* and *PaxC-Am* from *Acropora* have both been able to induce ectopic eyes in *Drosophila* only as a chimera with Ey, and both cnidarian proteins bound to the same range of sequences *in vitro* (Plaza et al., 2003).

The expression pattern, the structure of the paired-domain which is Pax2-like, the presence of an octapeptide and the complete absence during eye regeneration in *Cladonema* indicates, that cnidarian PaxB can not be an ancestor of Pax6. Neither is it the case for PaxC as it is not expressed in the cubomedusa eye.

The C-terminal region of Pax6 is proline/serine/threonine (PST) rich and functions as a potent transactivation domain when fused to a heterologous DNA-binding domain of the yeast transcription factor, Gal4 (Tang et al., 1998). This region seems to be of great importance as experiments with cnidarian Pax/ *Drosophila* Ey chimeras displayed (Plaza et al., 2003). Although PaxA-Cr does not have a PST rich region comparable to vertebrate Pax6, the C-terminal region is rich of proline/serine/threonine amino acids. PaxA-Cr lacks a homeodomain, which is dispensible for eye development in the flye (Punzo et al., 2001), and its paired-domain is more closely related to *Drosophila pox neuro* than to another paired family class. But eye regeneration results and expression data support PaxA as a Pax6 ancestor candidate. Remarkably, a monoclonal chicken Pax6 antibody (made against the full PD of chicken Pax6) cross-reacts specifically with the eye of *Cladonema* (Fig. 7). To date it remains unclear with which cnidarian Pax protein the antibody cross-reacts, but the possibility for the existence of a so far unknown Pax gene is left open. Other data indicate (see appendix) that Cnidaria contain true Pax6-like HDs. This raises several questions: if

PaxA-Cr is not the ancestor of Pax6 does a *bona fide* Pax6 exist in cnidarians? Or did a Pax6 consisting of PD and HD evolve after the Cnidaria and the Pax6 specific functions are split in genes containing either PD or HD? We will not know for sure before the entire genome of *Cladonema* is analyzed.

Our data do not contradict the hypothesis of monophyletic eye evolution as Cnidaria do have a Pax gene that is specifically expressed in the eye during maintenance and regeneration. Furthermore cnidarians have *sine oculis/Six* class genes that are involved in eye regeneration and maintenance like in Bilateria (Stierwald et al., submitted). Our data provide elements for an eye determination network of cnidarians and that suggests that its components predated the Cnidarian/Bilaterian split.

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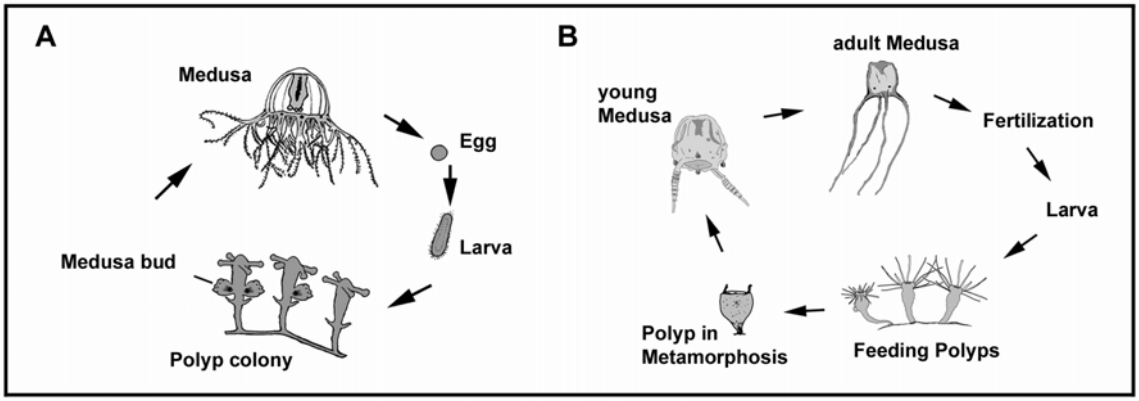

Fig. 1. Schematic life cycle of the hydrozoan Cladonema radiatum (A) and the cubozoan Carybdea marsupialis (B). In both species the sexually mature medusa liberates gametes. The embryo develops into a free swimming ciliated planula larva that attaches to the substrate and transforms into a polyp. Medusae develop asexually from polyps, in Cladonema by budding (A) and in Carybdea through metamorphosis from single polyps giving rise to one medusa.

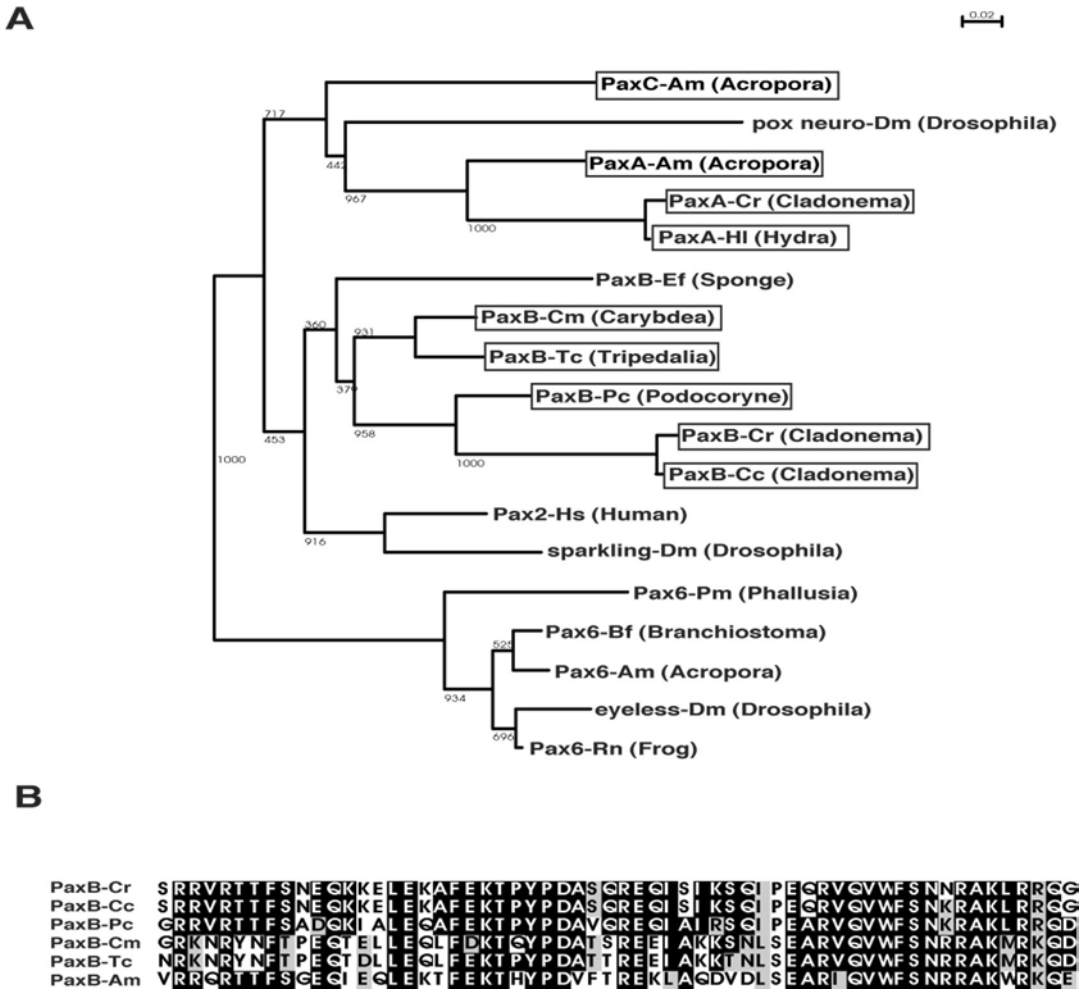

Fig. 2. Phylogenetic tree (A) and alignment (B) of cnidarian Pax genes. Phylogenetic analysis of the PD reveals the clustering of the cnidarian Pax genes (A). The anthozoan PaxC-Am groups closer to the cnidarian PaxA/pox neuro group than to the Pax4/6 subfamily. The alignment of the HD of cnidarian PaxB genes displays the heterogeneity within the cnidarian paired-like HD (B). Am, *Acropora millepora*; Cc, *Cladonema californicum*; Cr, *Cladonema radiatum*; Pc, *Podocoryne carnea*; Tc, *Tripedalia cystophora.*

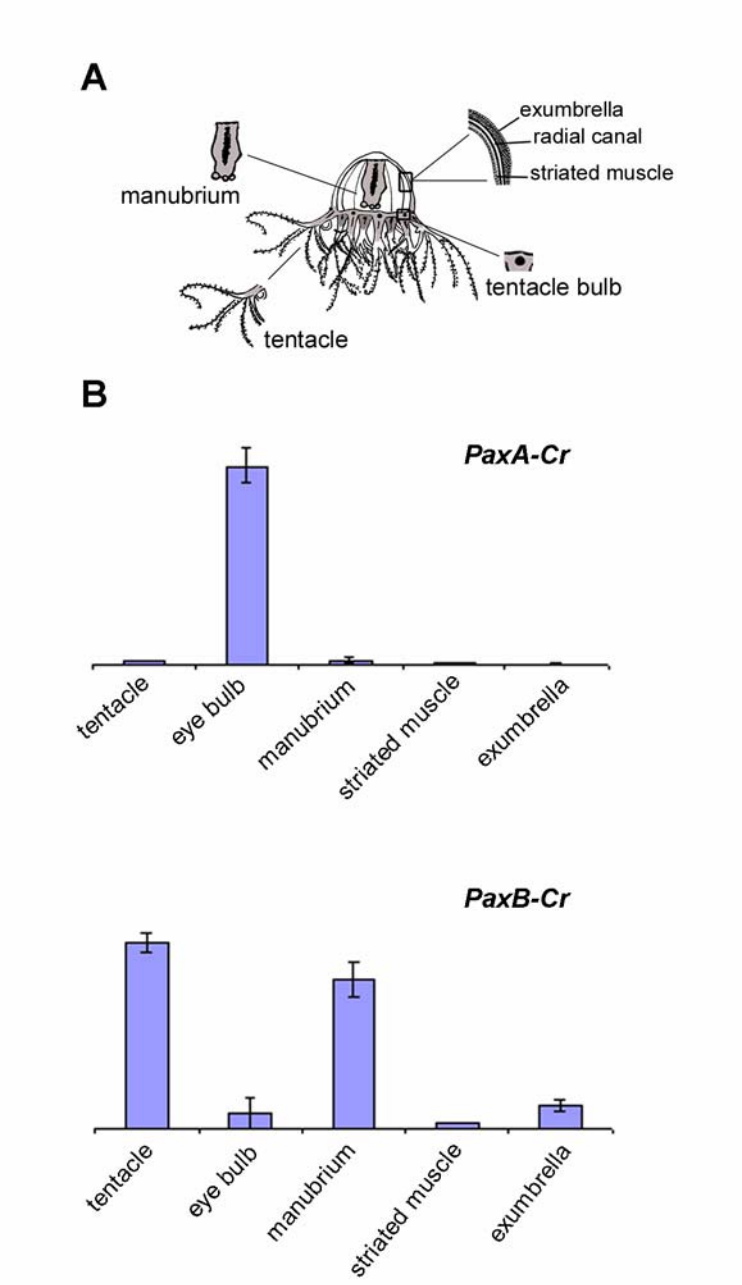

Fig. 3. Pax gene expression analysis of *Cladonema* medusa parts. (A) Portions of the medusa are isolated by microdissection. (B) Pax gene expression levels are measured by Real Time-PCR. Graphs display relative values normalized to elongation factor expression level.

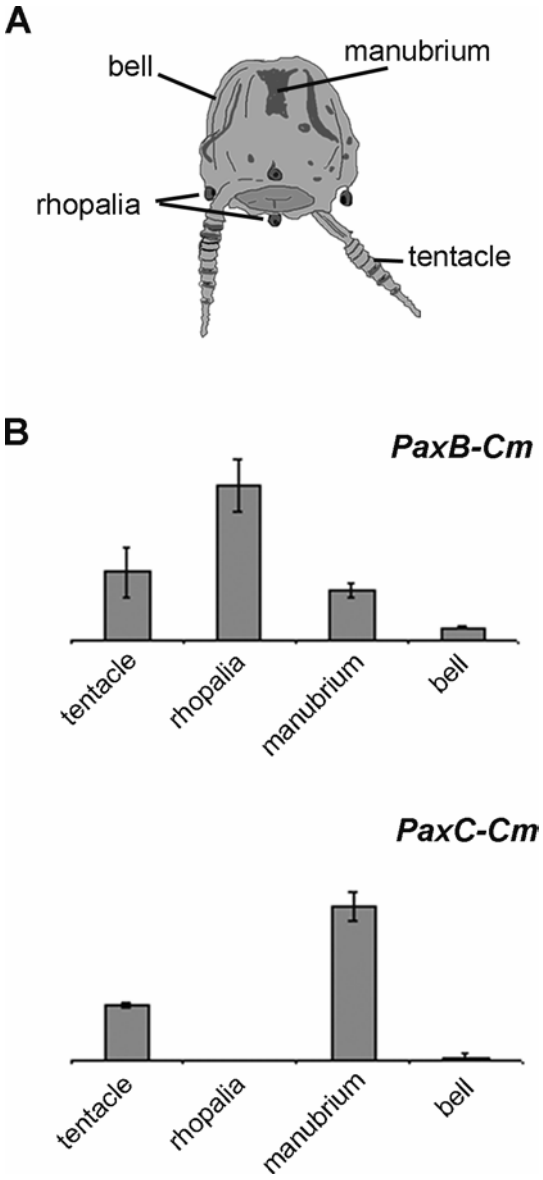

Fig. 4. Pax gene expression analysis of *Carybdea* medusa parts. (A) Portions of the medusa are isolated by microdissection. (B) Pax gene expression levels are measured by Real Time-PCR. Graphs display relative values normalized to elongation factor expression level.

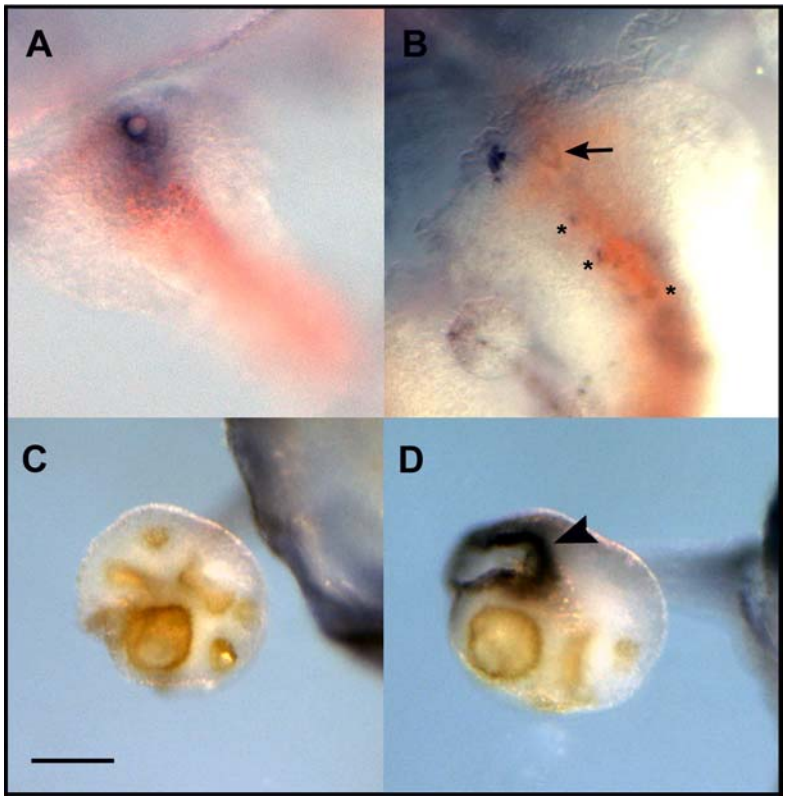

Fig. 5. Expression analysis in *Cladonema* (A,B) and *Carybdea* (C,D). *In situ* hybridization with antisense RNA probes for *PaxA-Cr* (A), *PaxB-Cr* (B), *PaxC-Cm* (C), and *PaxB-Cm* (D). Top view on tentacle bulb displays the restricted expression in the eye of *PaxA-Cr* (A). *PaxB-Cr* is not expressed in the eye (B, arrow points to the eye with intrinsic pigment cell staining) but in nerve cells of the tentacle bulb (asterisks). *PaxC-Cm* is not expressed in the rhopalia (C) whereas *PaxB-Cm* stains the statocyst (D, arrowhead). Bar is (in μm) 120 in (A, B), 100 in (C, D).

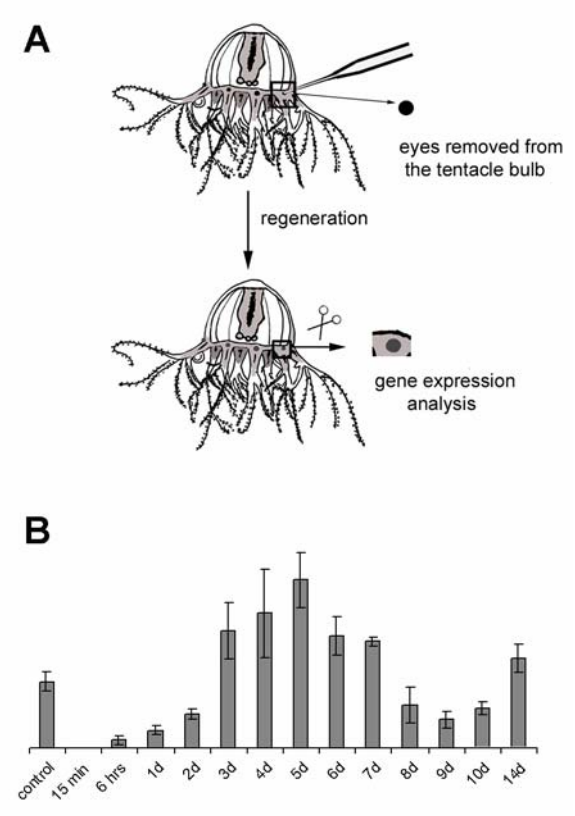

Fig. 6. Real Time-PCR expression of *Pax* genes during eye regeneration. (A) Eyes are removed from the tentacle bulb using a glass capillary. At different time points, tentacle bulbs are excised from the medusa for RNA extraction. (B) Expression level of *PaxA-Cr* is evaluated by Real Time-PCR and presented relative to the normalizing value of elongation factor. Control corresponds to the gene expression in the intact tentacle bulb. *PaxB-Cr* was not detected during eye regeneration (data not shown). After 14 days, the eye was completely regenerated (Weber, 1981a, b).

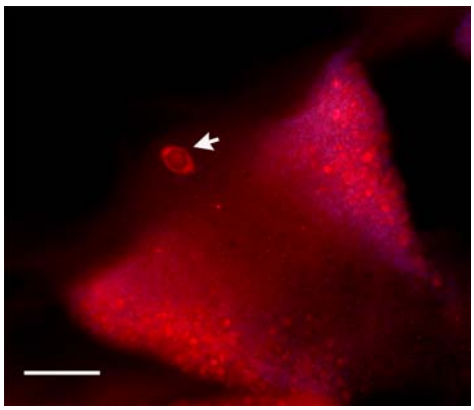

Fig. 7. Crossreaction of a monoclonal chicken Pax6 antibody with *Cladonema*. The entire eye (arrow) including the lens and additional cells in the tentacle bulb stain specifically. Bar is 100 μm.

CHAPTER 3

The Mesoderm Specification Factor Twist in the Life Cycle of Jellyfish

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ABSTRACT

The basic helix-loop-helix (bHLH) transcription factor Twist is highly conserved from Drosophila to vertebrates and plays a major role in mesoderm specification of triploblasts. The presence of a Twist homologue in diploblasts such as the cnidarian Podocoryne carnea raises questions on the evolution of mesoderm, the third cell layer characteristic for triploblasts. Podocoryne Twist is expressed in the early embryo until the myoepithelial cells of the larva differentiate and then again during medusa development. There, the gene is detected first when the myoepithelial cells of the polyp dedifferentiate to form the medusa bud and later Twist is found transiently in the entocodon, a mesoderm-like cell layer which differentiates into the smooth muscle and striated muscle of the bell. On the other hand, in later bud stages and the medusa, expression is seen where non-muscle tissues differentiate. Experimental analysis of in vitro transdifferentiation and regeneration demonstrates that Twist activity is not needed when isolated striated muscle regenerate medusa organs. Developmental roles of Twist are discussed with respect to early animal evolution from a common ancestor of cnidarians and bilaterians.

INTRODUCTION

The induction and specification of mesoderm as a unifying feature of all triploblastic animals have engaged many developmental biologists for over a century. The molecular and morphogenetic mechanisms underlying the processes of mesoderm specification were shown to be remarkably conserved from flies to humans (reviewed in Harland and Gerhard, 1997). However, little is known about the evolution of the mesoderm itself and the relation of so-called diploblasts, which should not have a mesoderm, to higher animals. The recent identification of a homologue of the mesoderm transcription factor Brachyury in the freshwater polyp Hydra (Technau and Bode, 1999) and the fact that jellyfish have a well-developed striated muscle tissue, which is comparable in its histological appearance and expression of structural genes to that of vertebrates (Gröger et al., 1999), raise the question on the phylogenetic position of this type of tissue and of Cnidaria. Besides Brachyury, the bHLH transcription factor Twist is a key regulator in specifying the mesoderm lineage. Twist was first identified as a gene playing a crucial role in mesoderm formation of the Drosophila embryo (Thisse et al., 1988). Single orthologs have been cloned in invertebrates from Caenorhabditis elegans (Harfe et al., 1998) to amphioxus (Yasui et al., 1998) while two Twist-like paralogs, called Twist (Wolf et al., 1991) and Dermol (Li et al., 1995) appear to be present in vertebrates. In Drosophila Twist is activated by Dorsal, activating itself Snail and Rhomboid and later on in the visceral mesoderm Tinman(reviewed in Baylies et al., 1998). Twist was shown to be involved in muscle differentiation by directly activating the MADS-box transcription factor Mef2 in Drosophila (Cripps et al., 1998) andin patterning of the adult muscle (Cripps and Olson, 1998). Vertebrate Twist expression and function are similar to those seen in Drosophila but there are significant differences. In vertebrates Twist was shown to inhibit myogenesis (Hebrock et al., 1994; Spicer et al., 1996). Later it was also shown in

Drosophila that Twist can negatively regulate muscle differentiation in flight muscles (Anant et al., 1998) and that other bHLH proteins are involved in mesoderm formation (Gonzalez-Crespo and Levine, 1993). Also in C. elegans the role of Twist is quite complex (Harfe et al., 1998). The initial comparison of Twist as a mesoderm regulator with the MyoD family of muscle differentiation factors must be revised. Twist functions also as a potential oncogene that inhibits apoptosis (Maestro et al., 1999) and is involved in the regulation of histone acetyltransferases (Hamamori et al., 1999). Homozygous Twist null mouse phenotypes are relatively mild and exhibit failure of neural tube closure and heterozygosity for Twist mutations show minor skull and limb anomalies (Chen and Behringer, 1995), comparable to those of the human Saethre-Chotzen syndrome caused by human TWIST mutations (El Ghouzzi et al., 1997; Howard et al.,

1997). The persistence of mesoderm in Twist-deficient mice could be due to partial redundancy with the second Twistlike gene Dermo1 or other related bHLH genes such as Scleraxis, where null embryos show no trace of mesoderm (Brown et al., 1999).

We isolated and characterized a Twist homologue from the hydrozoan jellyfish Podocoryne carnea and analyzed its expression pattern by in situ hybridization, RT-PCR, and under different experimental conditions in vitro. Twist expression appears to be correlated to muscle differentiation in larva and the formation of mesoderm-like structures in medusa formation. However, it is also expressed when nonmyoepithelial tissue is formed in medusa development. The high sequence conservation of Twist in Cnidaria and triploblasts and its expression in the formation of a third cell layer between ectoderm and endoderm during medusa bud development point toward a high potential for animal complexity already before the separation of the basal metazoan lineages.

MATERIALS AND METHODS

Animals

P. carnea M. Sars (Cnidaria, Hydrozoa, Anthomedusae) colonies are reared in the laboratory as described elsewhere (Schmid, 1979). Medusa parts and buds were obtained by microsurgery and the buds were staged according to Frey (1968). Eggs and larval stages were obtained as described in Reber-Müller et al. (1995).

Molecular Cloning and Sequence Comparison

Molecular biology procedures were carried out as described (Müller et al., 1999). Homology PCR for a Twist homologue of Podocoryne was done on genomic DNA. In the first round of PCR the primer combinations TwiF1 5'-ATG GC(AGCT) AA(CT) GT(AGCT) (AC)G(AGCT) GA-3'. and TwiR1 5'-CC (CT)TC CAT (AGCT)C(GT) CCA (AGCT)AC-3'. were used. One microliter of the first round product was used as template in the second round of PCR combining the following primers TwiF2 59-AT(CT) AT(CT) CC(AGCT) AC(AGCT) (CT)T(AGCT) CC-3. and TwiR2 5-9A (AGCT)A(AG) (AG)AA (AG)TC (AGT)AT (AG)TA-39. For each PCR round standard conditions were used, except that the annealing temperature was 40°C and the cycle number was 30 for the first and 40 cycles for the second round. A PCR product of the expected size was gel-purified with a Qiaquick column (Qiagen), subcloned into pCRII vector (Invitrogen), sequenced, and used as a probe to screen cDNA libraries as described (Müller et al., 1999). The nucleotide sequence of the 665-bp clone with the complete coding sequence has been submitted to the DNA databases with Accession Number AJ276245. Nucleotide and deduced amino acid sequences were analyzed using the GCG software package. BLAST searches (Altschul et al., 1997) were performed using the BLAST network service at the NCBI (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and phylogenetic trees based on the neighbor-joining method

were generated with Clustal X (Jeanmougin et al., 1998). Mouse bHLH names are often different from different labs and from human nomenclature and do not reflect the subfamily structure. For simplicity, subfamily members were labeled according to the most common name compatible with human and mouse gene names at the LocusLink interface of the NCBI (http:// www.ncbi.nlm.nih.gov/LocusLink) and numbered, e.g., Twist1 and Twist2 for Twist and Dermo1. Mouse sequences were used since data for bHLH proteins are more complete for the mouse, but available human or other vertebrate and invertebrate sequences confirm the phylogenetic relationship. Mouse accession numbers for Twist-related subgroups (Atchley and Fitch, 1997) were retrieved from NCBI with an advanced BLAST search with the bHLH domain of Twist as defined in Swiss-Prot. The almost complete genomes of C. elegans (The C. elegans sequencing consortium, 1998) and Drosophila (Rubin et al., 2000) were searched for additional Twist-related homologues. No C. elegans orthologs were found for the Hand, Msc, Scx, and Lyl subfamilies at the NCBI or at the C. elegans BLAST server at the Sanger Centre. Drosophila orthologs were found for all known Twist-related subfamilies and two novel subfamilies, CG5952 and CG6913. Unfortunately, the two novel proteins as well as the Hand and Scx orthologs appear to contain mistakes due to inaccurate exon-intron predictions. A human ortholog of CG5952 appears to be located just 20 kb upstream of TWIST on a BAC clone of the chromosomal location 7p15 (AC003986), indicating a complex relationship of true Twist genes with Twist-related genes containing the single amino acid insert in the bHLH domain. For clarity, other species, unfinished sequences, the NeuroD or atonal families, Mist, Tal2, and less related bHLH proteins were omitted in the final figure.

RT-PCR Expression Analysis

The mRNA was extracted using the Dynabeads mRNA direct kit (Dynal) and reverse-transcribed into single-stranded cDNA with AMV reverse transcriptase and random hexamer primers (firststrand cDNA synthesis kit for RT-PCR (AMV), Roche). The resulting first-strand cDNA was used as a template for PCR analysis, based on SYBR Green I technology with the LightCycler as described by the manufacturer (Roche). In the presence of SYBR Green I, the LightCycler produces a melting curve by monitoring the fluorescence profile during slow denaturation of the end PCR product. The calculated negative first derivative of the fluorescence versus the temperature is displayed in a graph as temperature versus 2dF/dT. Each sample is independently analyzed, the instrument calculates the Tm of the melting peak and its corresponding area. The peak area is used to measure the relative concentration of the product after 40 cycles of amplification. To minimize the side product and the primer dimer formation, several sets of primers were tested under different amplification conditions. In order to validate the value of PCR product in the plateau phase (mostly the case after 40 cycles) we monitor the amplification profile on a graph that displays the log of the fluorescence versus the number of cycles. In most cases the crossing point (threshold point) confirms the relative value in the plateau (peak area value). Representative samples were also loaded on agarose gels after quantification with the LightCycler, which confirmed that LightCycler data can be compared to gel-based data but are more sensitive.

As a control for first-strand cDNA quality and quantity, elongation factor 1 alpha was included in all experiments to normalize the samples. All primer combinations were designed to span an intron, which allows detection of putative genomic DNA contamination of the samples. Experiments were repeated three times on two different mRNA preparations with the primers TwF2 5'-GGT GGA ACA GAC TGA TCA GG-3', TwF3 5'-TTC GAC GAT GAA TCA CGT GAC GA-3', TwR1 5'-CAT AGC CGC CAA TCT GAG C-3', TwR4 5'-

ATG CAT AAC TCA GCC TCT CTT GCG-3', TwR6 5'-GCA GAA TTA ACA GCC TCA ATA CCT-3', EF1AF 5'-ACG TGG TAT GGT TGC CTC TG-3', and EF1AR 5'-TGA TAA CGC CAA CGG CTA CG-3'.

In Situ Hybridization and BrdU Labeling

Basically we have used the protocol as described by Gröger et al. (1999). Fixation was done overnight at 4°C in Lavdowsky to which 0.2% glutaraldehyde was added. Proteinase K, postfixation, and RNase treatments were omitted and hybridization was done for 18 h at 50°C. The fragile tissues require a graded and stepwise change of solutions where osmotic pressure is high. Stained samples were investigated either as whole mounts or as gelatine or paraffin sections (Gröger and Schmid, 2000). DNA replicating cells were labeled with BrdU and specimens were processed for histochemistry as outlined in Plickert and Kroiher (1988).

Experimental Analysis

Striated muscle and cells from the subumbrellar plate were isolated, activated for transdifferentiation, and cultured as presented in Fig. 6 and described in Schmid (1992). For the grafting experiments polyp extracellular matrix (ECM) pieces were isolated by PBS treatment of the animals and removal of the cell debris by washing the ECMs in distilled water. Then the ECMs were air-dried on pieces of coverslips and the isolated tissue fragments grafted onto the substrate as outlined in Schmid (1992).

RESULTS

Identification of a Highly Conserved Twist Homologue from a Diploblast

In previously known Twist-related proteins the bHLH domain and a C-terminal motif are highly conserved. Primers for homology PCR were designed within these regions. According to the expected size a 79-bp band was isolated and sequenced. The fragment was more similar to Twist family members than to other bHLH factors and was used to screen cDNA libraries. A positive clone with an insert of 665 bp contained the complete coding sequence as verified by 5. and 3. RACE experiments. Analysis of the sequence revealed a putative start codon at position 52 that is followed by an open-reading frame of 597 nucleotides that ends at position 648 with a TGA stop codon. The protein of 199 amino acid residues encoded by this clone is most similar to vertebrate Twist proteins. Podocoryne Twist contains a basic DNA binding and dimerization motif common to bHLH proteins from amino acid 51 to 103 and a C-terminal motif characteristic for the Twist subfamily (Fig. 1A). This second motif of 14 perfectly conserved residues between Podocoryne and mouse is less conserved in Drosophila and almost not recognizable in C. elegans, but absent from all less-related bHLH proteins. Since the tryptophane-arginine (WR) dipeptide is the most conserved part of the stretch it was called the WR motif. In addition, Podocoryne Twist can be aligned with mouse Twist (Twist1) and Dermo1 (Twist2) up to the N-terminus, but here sequence similarities are even low between mouse paralogs. The length and domain structure of Podocoryne Twist are more similar to vertebrate Twist proteins than to invertebrates (Fig. 1B).

The bHLH domain of Podocoryne Twist is 80% identical to vertebrate sequences but shares only 60% identity to the C. elegans sequence, which is only slightly higher than the 57% matches to mouse Ptf1 or Scleraxis. However, the closest relatives of the Twist subfamily of bHLH proteins all share a one amino acid insertion in the loop of the bHLH domain when compared to Twist. The otherwise lessrelated MyoD subfamily shares this feature with the

Twist subfamily (Fig. 1C). A phylogenetic analysis of the bHLH domain of Twist-related proteins confirms that the Podocoryne sequence is a true Twist ortholog and that it is equally similar to mammalian Twist1 and Twist2 (Fig. 1D).

Expression of Twist in the Life Cycle and in Medusa Development

Formation of the planula larva. Within 48 h after fertilization a planula larva develops. The planula is bilayered and contains the ectodermal and endodermal epithelial muscle cells and different types of nerve cells and nematocytes. The larva already has the tissue organization and the cell types of the future polyp. RT-PCR and in situ hybridization data (Figs. 2A and 3) demonstrate that the Twist message is not present in the fertilized egg, appears in the embryo and early planula larva, and disappears when the larva becomes competent for transformation to the primary polyp after 2–3 days. The gastrozoid or feeding polyp gives no signal.

Formation of the medusa. Strong expression is observed in the gonozoid, the polyp which forms the medusa buds, in the medusa and in all isolated stages of medusa bud development (Fig. 2B). In situ hybridization of whole mounts and tissue sections demonstrate that the gene is not expressed in the gonozoid polyp itself but in medusa development and the adult medusa at places where BrdU staining demonstrate high cell proliferative activity (Fig. 4). Development of the medusa was staged according to histological criteria (Kühn, 1910; Frey, 1968; Boelsterli, 1977) and we have used this staging to follow Twist expression by sections of whole mount in situ hybridization.

Stage 1. Formation of medusa occurs in a small subhypostomal zone of the gonozoid polyp (Figs. 4C and 4D) where polyp cells dedifferentiate to form a protrusion of ectodermal and endodermal tissues (Boelsterli, 1977). At this early stage or shortly later at bud stage 1 (Fig. 5), some staining is present in both the ectodermal and the endodermal layer of the bulging polyp tissues. Staining does not extend into the budding zone of the polyp ectoderm but is restricted to the bud tissues.

Stage 2. At stage 2 (Fig. 5) the distal ectoderm of the bud generates a new mesoderm-like cell layer (Fig. 5; Kühn, 1910; Boero et al., 1998). The cell layer bulges inward into the endodermal layer and completely separates from the ectoderm by the formation of a mesoglea or ECM (Boelsterli, 1977). This new layer is called entocodon. The entocodon consists of highly proliferative undifferentiated cells and after the formation of a cavity (stage 3) essentially gives rise to all smooth and striated muscle cells which line the inner side of the bell. Beside the bud ectoderm and endoderm the Twist message is also present in the entocodon (Fig. 5C).

Stages 4 and 6. While the entocodon cavity enlarges a further epithelial layer is formed. The developing entocodon partitions the endoderm into the four radial canals (rc; Fig. 5) and between the canals the subumbrellar plate (p; Fig. 5) will differentiate. It consists of a thin monotypic epithelium layer sandwiched between the outer and inner ECM and connects the developing endodermal radial canals. The plate cells strongly express Twist from the very first moment on and continue to do so until the medusa detaches from the gonozoid (Fig. 5). Expression of Twist in the entocodon-derived muscle layers gradually decreases as these tissues differentiate and from stages 6–8 on expression is absent. Additionally, expression is

observed in the most distal part of the medusa bud where the tentacle anlage and the bell opening with the velum forms.

Stage 9 and the medusa. Twist expression in the developing bell ectoderm, the exumbrella (ex; Fig. 5) concentrates in later bud stages to the distal parts of the buds where tentacle bulbs, tentacles, and velum are formed and proximally to the side where the stalk connects the bud to the bell. In adult medusa expression is found in the ectodermal margin of the bell consisting of the ring canal and the tentacle bulbs, and depending on the state of maturity, in the developing gonadal tissue which are localized in the ectoderm of the manubrium, the feeding and sex organ of this medusa (Figs. 4 and 5).

Experimental Analysis of Twist Expression in the Isolated Subumbrellar Plate

In medusa development Twist is expressed in the plate tissue, even in stage 9 when the medusa is almost ready to detach; however, no message is detectable in the liberated medusa after 1 day (Figs. 3 and 4). To investigate the stability of Twist regulation we isolated the plate tissue (Fig. 6), cultured it (Schmid et al., 1982) and monitored gene expression. When the striated muscle layer is removed and the bell fragments are excised from the animals, the isolates round up, and the plate cells aggregate in a central position. They can be isolated by removal of the outer epithelium (exumbrella) as outlined (Fig. 6). We have studied the effect of the isolation procedure and the culturing conditions on Twist expression in plate cells, first, as freshly excised interradial fragments (Fig. 7A, control); second, when the striated muscle and the inner ECM were freshly removed either mechanically by microsurgery or by enzyme treatment (Fig. 7A, a); third, after 18 h of culturing these fragments consisting of exumbrella, outer ECM, and the plate cells (Fig. 7A, b); and finally when the exumbrella was removed from such precultured explants immediately or after 7, 31, and 84 h or 7 days (Fig. 7A, c– g). Twist expression is absent in the freshly excised interradial fragments; however,

the gene is reexpressed in the rounded up isolates (Fig. 6) and in all later stages of culturing the isolated and grafted plate tissue. Expression gradually decreases and is absent after 7 days, which is shortly before the isolates disintegrate. To test the possibility that reexpression of Twist in the cultured plate tissue is due to the grossly altered tissue configuration from a monoepithelium (in vivo configuration, Fig. 6) to the aggregate state (in vitro) we grafted plate cell aggregates 24 h after isolation onto isolated and stretched ECMs of polyps. The plate cells immediately started to spread onto the grafted ECM and formed a monolayer. This caused repression of the gene whereas in the not grafted controls, expression continues (Fig. 7B).

Twist Expression in Isolated Striated Muscle

In contrast to the expression in the plate cells, Twist expression disappears in the developing striated muscle already in bud stages 6–8 (Figs. 3 and 4). Striated muscle tissue from medusa can be isolated and cultured (Fig. 6). When activated by enzyme treatment the isolated striated muscle starts DNA replication and transdifferentiates into smooth muscle and FMRF-amide-positive nerve cells; occasionally they even regenerate manubria and tentacles (Fig. 6; Schmid and Alder, 1984; reviewed in Schmid, 1992). In the latter case the transdifferentiating striated muscle cells group into two layers, an inner cell layer which will form the endoderm, and an outer layer, which will differentiate into the ectoderm of the regenerate (Fig. 6). The layers are separated by a thin ECM. The inner layer will form a flagellated endoderm where digestive cells and gland cells differentiate. Additionally, smooth muscle, nerve cells, nematocytes, and interstitial cells form by transdifferentiation from striated muscle cells in the ectoderm. Because Twist is expressed in the differentiating manubrium in the bud stages 3–5 (Figs. 3 and 4) we expected the gene to be expressed in the regeneration of manubria from striated muscle. RT-PCR studies and in situ hybridizations demonstrate that the gene is

not expressed neither in the transdifferentiation from striated to smooth muscle and nerve cells nor when tentacles and manubria regenerate.

Stability of Twist Expression in Grafts of Subumbrellar Plate and Striated Muscle

Whereas plate cells cannot transdifferentiate or regenerate autonomously they will do so when combined with activated striated muscle (Schmid et al., 1982). When the muscle is not activated prior to grafting and therefore remains stable, regeneration will not occur and the grafted plate cells gradually disappear. When the muscle is activated prior to grafting manubria and tentacles will regenerate. As was shown previously the activated muscle induces the plate aggregate to form a flagellated cavity, initiates DNA replication, and supports the regeneration process by transforming plate cells to new cell types. Since in vitro regeneration of manubria and tentacles from striated muscle occurs without Twist expression we wanted to know how Twist expression in the plate cells is affected when combined with either not activated mechanically isolated or activated isolated striated muscle tissue (Fig. 6). When the plate aggregate is grafted to stable, mechanically isolated muscle the latter quickly covers the plate tissue; however, the plate cells do not form a flagellated endodermal cavity and no regeneration occurs. In this case Twist expression is maintained but gradually decreases with time (Fig. 8A). However, when the plate aggregates are grafted to Pronase-activated striated muscle tissue, a flagellated endoderm forms after 2–3 days and manubria and tentacles regenerate within 5– 8 days. One hour after grafting Twist expression is already strongly reduced compared with the not combined plate aggregates (Fig. 8B) and after 3 h or later is no longer detectable. This confirms the observations from the regeneration experiments with activated striated muscle alone, that formation of new cell types does not

need Twist expression. On the other hand it indicates that the inactivation of Twist expression is needed for plate cells to be able to contribute to the regeneration process.

DISCUSSION

The Evolutionary Origin of Mesoderm

It has become a widely accepted notion that genes are shared throughout the animal kingdom and that animal diversity is largely based on differential use of the same components (Duboule and Wilkins, 1998). However, almost all relevant studies are about bilaterian animals. While it is still truly amazing that worms, flies, fish, mice, and humans can be so well compared, they seem to represent just variations of a common bauplan. To study the original invention of this bilaterian bauplan, investigations must be extended to animals of sister groups of the bilaterians. A large set of data from cnidarians suggests that also these animals make use of the same set of genes (Shenk and Steele, 1993), although often for quite divergent functions. Homologues of developmental regulator genes such as Hox (Schummer et al., 1992; Aerne et al., 1995; Masuda-Nakagawa et al., 2000), Pax (Gröger et al., 2000), Brachyury (Technau and Bode, 1999), or Otx (Mu¨ ller et al., 1999) were cloned from cnidarians, but it is not clear how to compare head-specific expression in cnidarians with bilaterian axis specification or brain development (Galliot and Miller, 2000).

In an alternative classification of animals bilaterians are called triploblasts and the term diploblast is often used collectively for the leftover phyla. While the bilayered freshwater polyp Hydra consists only of an ectoderm and an endoderm, and therefore can be well described as a diploblast, this is not true for the jellyfish stage which is present in three out of

four cnidarian classes, or the other nonbilaterian phyla Ctenophora, Placozoa, and Porifera. It appears

that mesoderm invention predates the split between cnidarians and bilaterians. Therefore, a reevaluation of old descriptions of a third cell layer in medusa bud development (Kühn, 1910) is needed in conjunction with studies taking into account our knowledge of known mesoderm specification factors from animals with a true mesoderm.

Twist, a Mesoderm Specification Factor in Diploblasts?

Sequence comparisons suggest that Podocoryne Twist is a true ortholog of bilaterian cognates (Fig. 1). The bHLH region is most similar to real Twist family members, even more similar to vertebrate Twist members than C. elegans Twist, and contains the same loop length, in contrast to other Twist-related subfamilies. In addition to the bHLH domain, the WR motif of 14 amino acids is perfectly conserved in Podocoryne and mammals. A WR motif was not found in any unrelated protein and is barely recognizable in the C. elegans Twist sequence. Furthermore, there is residual sequence similarity at the N-terminus of Podocoryne and vertebrate Twist proteins, where mouse Twist appears to interact with the p300 coactivator (Hamamori et al., 1999).

A phylogenetic analysis of the bHLH domain confirms that the bHLH family can be reliably subdivided into subfamilies with multiple vertebrate paralogs for each invertebrate bHLH gene, reminiscent of the Hox clusters and many other duplicated gene families (Spring, 1997). In such a natural classification of bHLH proteins (Atchley and Fitch, 1997) Twist belongs to a different subgroup than the MyoD, NeuroD, or the achaete-scute families, from which the only other cnidarian bHLH protein CnASH is known (Grens et al., 1995). Still, within its subgroup with the Ptf, Hand, Msc, Scx, Nhlh, and Lyl subfamilies, Twist is the

only member with the same loop length as the MyoD family. Whether this has any influence on the direct interaction and competition of Twist and MyoD family members with E proteins (Kophengnavong et al., 2000) is not known. The robustness of the Twist branch of the phylogenetic tree and the high conservation of Podocoryne Twist suggest that gene duplication within the bHLH family had occurred before the split of cnidarians and bilaterians. Therefore, orthologs for all subfamilies can be expected in both lineages. This is the case in the Drosophila genome while C. elegans appears to have lost four of the subfamilies. Apparently, genome sequences are needed to detect all the members of gene families as decades of Drosophila studies have only revealed two (Varterasian et al., 1993) of the eight Twist-like subfamilies found in the genome (Rubin et al., 2000), possibly due to partial redundancy of duplicated genes.

Medusa Development as Completion of Gastrulation?

Podocoryne Twist is present from early cleavage stages until myoepithelial cells form, but expression decreases in larva competent to transform into the primary polyp. Feeding polyps are basically made up of the same two myoepithelial cell layers as larvae and also lack Twist expression, even during colonial growth. However, development is only completed with the formation of the medusa, the sexually mature animal, and here again Twist is expressed strongly, but transiently in the proliferating undifferentiated cell mass that will also give rise to the medusa-typical muscles.

The formation of the medusa starts with highly proliferative, undifferentiated cells generated by dedifferentiation of epithelial muscle cells of the mother polyp (Boelsterli, 1977). In a process comparable to gastrulation, migration of tissues and cells, formation of body cavities, and consecutive morphogenesis and differentiation processes finally lead to the formation of

the adult stage, the sexual medusa (Kühn, 1910; Frey, 1968; Brändle, 1971; Boelsterli, 1977). The expression of Twist is first noticed in the bulging ectodermal and endodermal epithelial muscle cells of the polyp (Figs. 3 and 4). It is active at the same time when Otx (Müller et al., 1999) or Cnox1-Pc (Aerne et al., 1995) start to be expressed. When muscle differentiation progresses beyond stages 4–5 Twist expression gradually disappears in both muscle tissue layers whereas expression of Otx (Müller et al., 1999) and Cnox1-Pc (Aerne et al., 1995) is maintained even in the striated muscle of the adult medusa. The disappearance of Twist expression correlates well with the declining rate of DNA replication (Schmid, 1972).

All the Twist-expressing tissues lack organized contractile myofilament systems and staining is strong where cell proliferation is high. It appears that Twist is expressed either transiently in development, including myogenic tissues, or permanently in the adult medusa at places where cell proliferation continues and nonmyogenic tissues differentiate, like the growth zone at the margin of the bell (Fig. 4). Although Twist function cannot be surmised from temporal and spatial expression patterns, the results demonstrate on one hand that there exists a correlation between formation of myoepithelial cells in early muscle development in the larva and in the entocodon derived muscle systems, and, on the other hand, its expression clearly correlates with the formation of nonmuscle tissues such as the subumbrellar plate in later bud stages and the medusa. In this context it is interesting to note that epithelial muscle cells of the gonozoid polyp start to express Twist when they dedifferentiate to form the highly proliferative nonmuscle cells of the early medusa buds.

Experimental Manipulation of Twist Expression

The experiments with the isolated subumbrellar plate cells demonstrate that Twist expression can be regulated by cell-ECM interactions (Fig. 7). This alteration in Twist expression correlates with changes in tissue connectivity. Previous investigations have demonstrated that

the gap junctions which connect the plate cells in vivo disappear in the collapsed aggregate state and are reexpressed when the plate cells stretch on the host ECM (Weber and Schmid, 1984). Whereas isolated striated muscle when tested in similar grafting experiments showed no activation of Twist expression, regulatory genes such as Otx or Cnox1-Pc and structural genes such as the tropomyosin Tpm2 or the myosin heavy chain Myo1 change as long as the striated muscle cells are migrating (Yanze et al., 1999). Similar observations were done in other systems (reviewed in Boudreau and Bissell, 1998; Chicurel et al., 1998). In the cultured plate cell aggregates Twist remains active until the isolates degenerate after a few days.

It is known that cultured plate cell aggregates do not undergo DNA replication or transdifferentiation (reviewed in Schmid, 1992). However, when combined with activated striated muscle, plate cells too are able to transdifferentiate (Fig. 6). How the grafted muscle itself activates the plate cells is not yet investigated, but it is known that transdifferentiating striated muscle tissue is proteolytically active and can degrade grafted ECM (Schmid et al., 1993). Twist is not expressed when the combined tissuestransdifferentiate and regenerate manubria and tentacles. The regenerates are bilayered, formed by epithelial muscle cells like the larva and the polyp, and also contain essentially the same cell types. In general, regeneration processes are widely regarded as a repetition of the corresponding ontogenetic steps. In the case of Twist this seems not to be the case as the developing manubrium expresses Twist in the ectodermal epithelial layer. This indicates that regeneration, at least in part, can occur by different regulatory pathways than the corresponding processes in ontogeny or that Twist regulates very basic determination steps upstream of the regulatory cascades which specify organogenesis and cell differentiation of manubria and tentacles. Similar observations were made in regenerating newt limbs, where HoxD gene expression is different in development and regeneration (Simon, 1999).

Twist in Evolution

In Drosophila Twist was seen as a factor specifying early mesoderm and required for somatic myogenesis (Baylies and Bate, 1996) while in vertebrates Twist was shown to inhibit myogenesis (Spicer et al., 1996). Later it was also shown in Drosophila that Twist can negatively regulate muscle differentiation in flight muscles (Anant et al., 1998). This discrepancy is reflected in the more complex role of Twist in C. elegans where this factor is not required for embryonic mesoderm but appears to be restricted to the mesodermal blast cell M. During larval development undifferentiated descendants of M express Twist until they differentiate into body wall or sex muscles (Harfe et al., 1998). Developmental roles for Twist have evidently varied rather dramatically in evolution, but it appears that an activating function of potential muscle precursors and an inhibitory function of muscle differentiation are shared from C. elegans and Drosophila to vertebrates. The high sequence conservation and the expression pattern of Twist in Podocoryne are at least consistent with related roles in cnidarians and bilaterians. Since gene functions cannot be tested directly in Podocoryne yet, further investigations are needed to elucidate the role of Twist-like bHLH factors in animal evolution.

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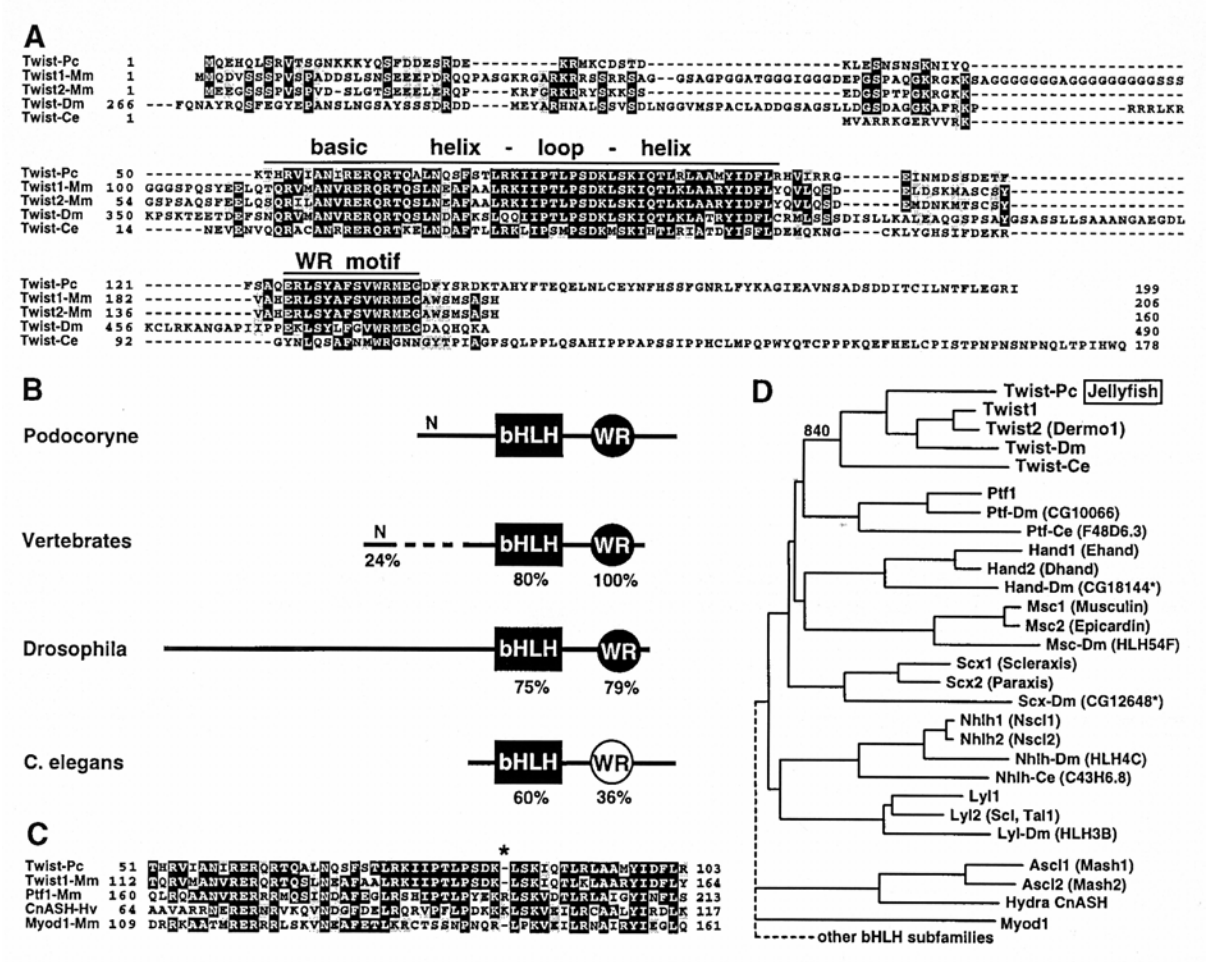
A
basic helix - loop - helix
WR motif
B
Podocoryne
Vertebrates
Drosophila
C. elegans
N
bHLH
WR
24%
80%
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79%
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36%
C
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Twist-Pc
Jellyfish
Twist1
Twist2 (Dermo1)
Twist-Dm
Twist-Ce
840
Ptf1
Ptf-Dm (CG10066)
Ptf-Ce (F48D6.3)
Hand1 (Ehand)
Hand2 (Dhand)
Hand-Dm (CG18144*)
Msc1 (Musculin)
Msc2 (Epicardin)
Msc-Dm (HLH54F)
Scx1 (Scleraxis)
Scx2 (Paraxis)
Scx-Dm (CG12648*)
Nhlh1 (Nscl1)
Nhlh2 (Nscl2)
Nhlh-Dm (HLH4C)
Nhlh-Ce (C43H6.8)
Lyl1
Lyl2 (Scl, Tal1)
Lyl-Dm (HLH3B)
Ascl1 (Mash1)
Ascl2 (Mash2)
Hydra CnASH
Myod1
other bHLH subfamilies

FIG. 1. High conservation of Podocoryne Twist. (A) The complete sequence of Podocoryne Twist was compared to representative family members. Only the bHLH domain and the Twist-specific WR motif are conserved in all species. The Podocoryne and the mouse proteins Twist1-Mm (Twist) and Twist2-Mm (Dermo1) could also be aligned at the N-terminus. (B) The domain structure of Podocoryne Twist is more similar to vertebrate proteins than to Drosophila (Twist-Dm) which is more than twice as big as C. elegans (Twist-Ce) where the WR motif appears to deteriorate. Even the bHLH region of C. elegans shares only 60% identical residues with Podocoryne as compared to 80% in vertebrates. (C) The length of the loop in the bHLH domain of Twist is conserved, and is one amino acid shorter than in the most related bHLH proteins such as Ptf1 or the only other known cnidarian relative CnASH from Hydra. The Twist proteins share this gap (asterisk) with the MyoD family, which is otherwise not closely related. (D) A phylogenetic analysis of the bHLH domain confirms that Podocoryne Twist corresponds to single orthologs from Drosophila or C. elegans and two paralogs in vertebrates. The neighbor-joining tree was calculated with Clustal X, but alternative methods confirm the placement of the Podocoryne sequence within the bilaterian Twist branch. The bootstrap value 840 of 1000 replicates for the Twist subfamily increases to 994 if C. elegans is omitted. Mouse sequences of the relevant subfamilies, Ptf, Hand, Msc, Scx, Nhlh, and Lyl were compared to Drosophila and C. elegans sequences as described under Materials and Methods. The only other known cnidarian bHLH protein, the achaete-scute-like factor Hydra CnASH, was included with the mouse homologues Asc11/2, and mouse Myod1 as outgroups. Representatives of all Twist-related subfamilies can be found in the Drosophila genome (asterisks indicate inadequate protein prediction), while C. elegans homologues are usually more derived, as in the case of Twist, or even absent from the complete genome as in the Hand, Msc, Scx, and Lyl subfamilies.

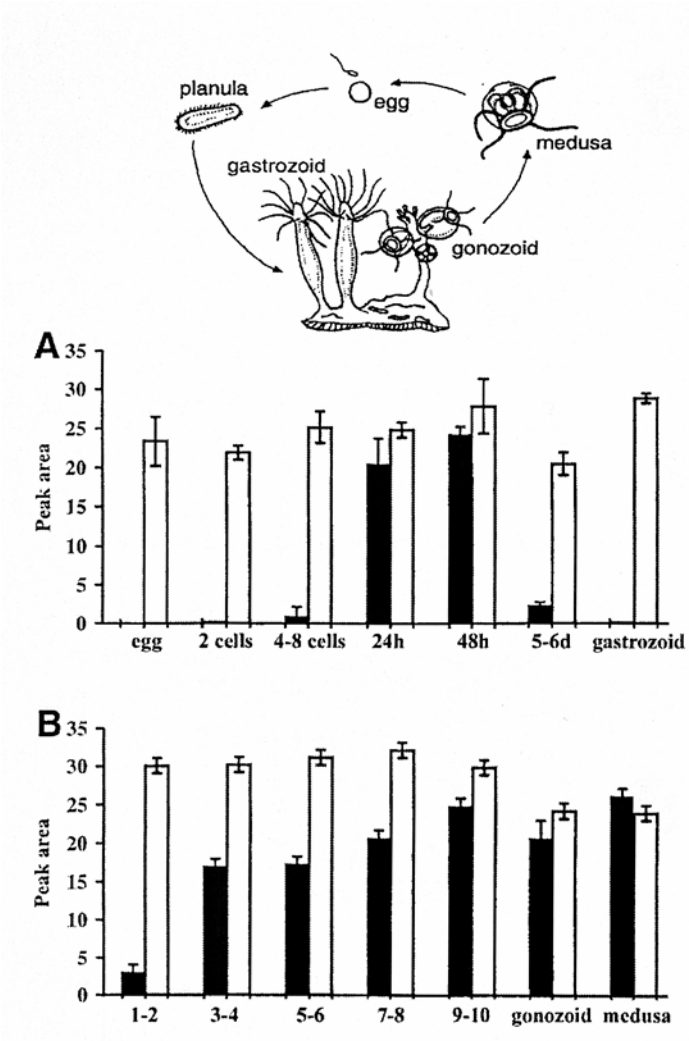


FIG. 2. RT-PCR expression analysis of Twist in the life cycle. (A) Twist expression (shaded bars) is compared to elongation factor 1 alpha (empty bars) in eggs, embryonic and larval stages, and the gastrozoid. (B) Medusa bud stages 1–10 according to Frey (1968) are compared to gonozoids and medusae. Values in the ordinate are from the LightCycler as described under Materials and Methods.

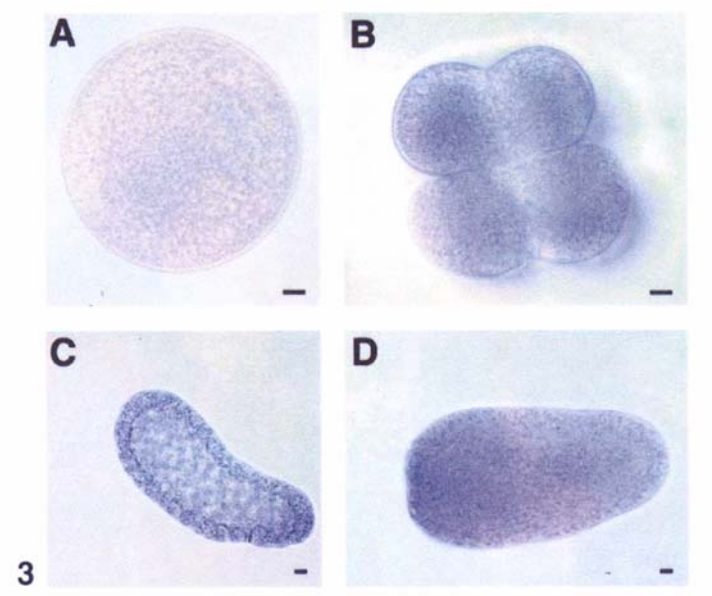


FIG. 3. In situ hybridization of Twist in embryogenesis. No expression is seen in fertilized eggs (A), while Twist is present in 8-cellcleavage stages (B) and planula larvae at 24 (C) and 48 h (D). The bars correspond to 0.01 mm.

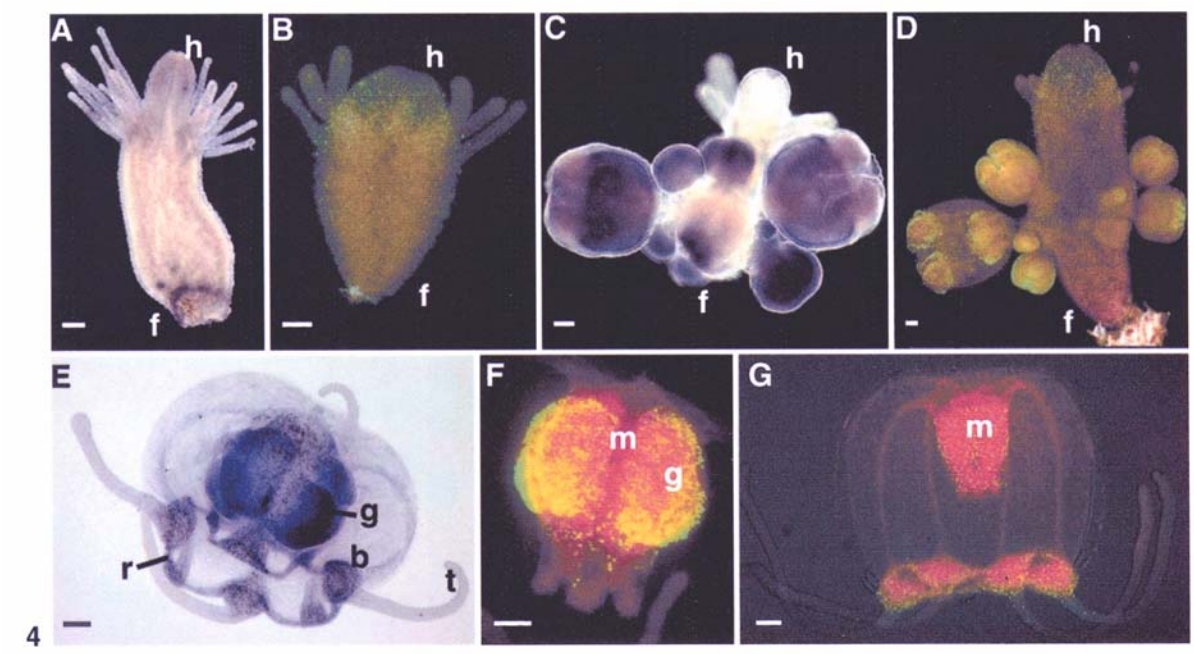
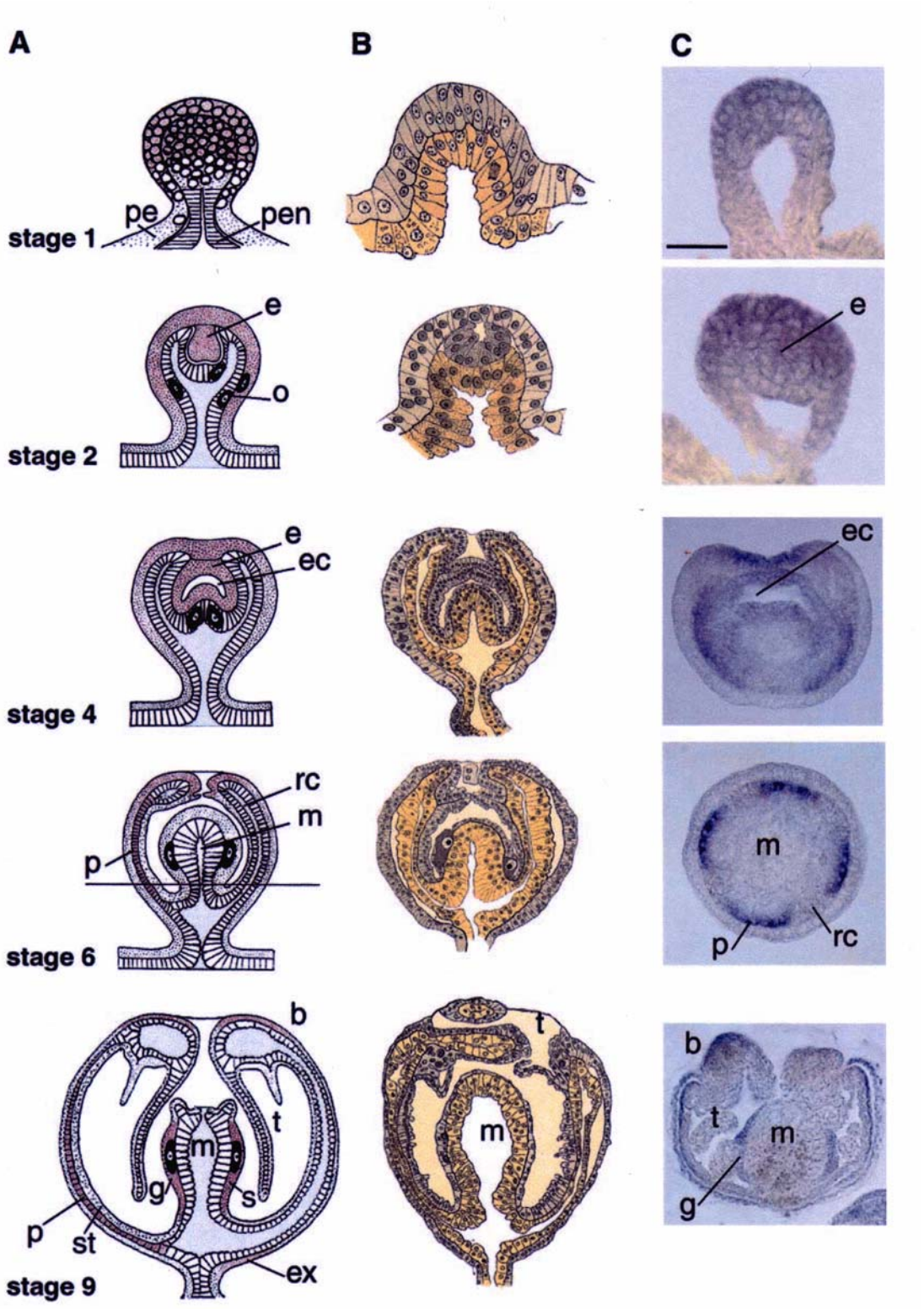


FIG. 4. Correlation of Twist expression and cell proliferation. Twist expression is detected by in situ hybridization in the gastrozoid (A), gonozoid with medusa buds (C), and sexually mature medusa (E). BrdU histochemistry reveals zones of DNA replication in the hypostomal part of the gastrozoid (B), in the gonozoid with intensive cell proliferation in the different bud stages (D), in the manubrium of the sexually mature medusa (F) and in the immature, freshly hatched medusa where cell proliferation is mainly located at the margin of the bell (G). b, tentacle bulbs; f, foot part of the polyp with adhering perisarc from the stolons; g, gonads (testes); h, hypostome (mouth) of the polyps; m, manubrium (mouth) of the medusa; t, tentacles. The bars correspond to 0.1 mm.


A
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C
stage 1
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stage 2
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stage 4
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FIG. 5. Expression of Twist in the development of the medusa as revealed by in situ hybridization of tissue sections. Column A is modified after Frey (1968) and represents schematized drawings of the corresponding developmental stages in which Twist expression as documented in column C is marked in red. Column B is an accurate histological drawing established from Podocoryne medusa buds by Ku¨ hn (1910). Circles in stage 1 in column A present dedifferentiated epithelial muscle cells from polyp ectoderm and endoderm (after Boelsterli, 1977). The plane of the cross section in stage 6 that shows intensive staining of the subumbrellar plate cells is indicated in column A. b, tentacle bulbs; e, entocodon; ec, entocodon cavity; g, gonads; m, manubrium; o, oocytes migrating from the polyp into the manubrium anlage; p, plate cells; pe, polyp ectoderm; pen, polyp endoderm; rc, radial canal; s, smooth muscle of the manubrium: st, striated muscle; t, tentacles. Bar corresponds to 0.05, 0.1, 0.2, 0.2, and 0.3 mm in stages 1, 2, 4, 6, and 9, respectively.

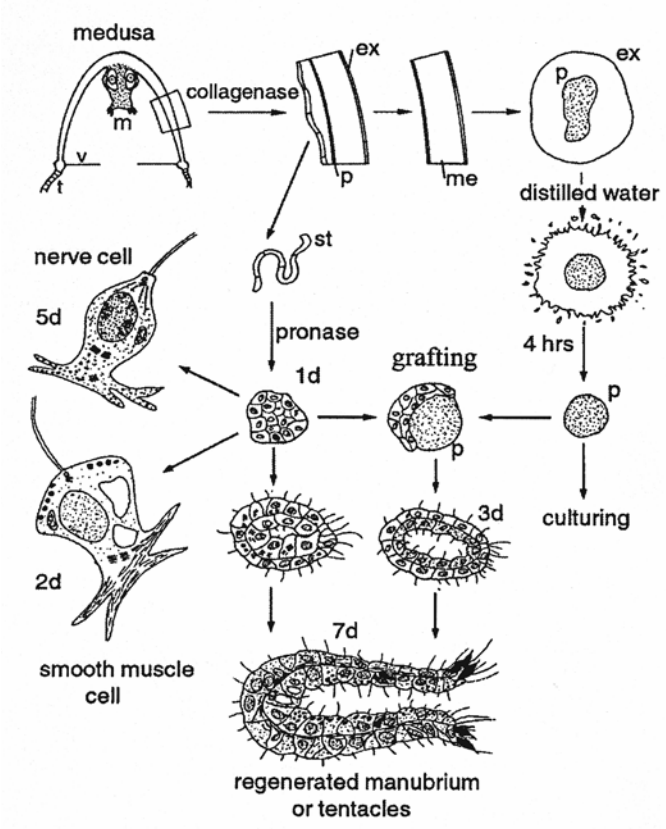

FIG. 6. Schematized drawing of the in vitro transdifferentiation and regeneration system. d, days; p, plate cells of the subumbrella; ex, exumbrella; m, manubrium; me, mesoglea; st, striated muscle; t, tentacles; v, velum (Schmid, 1992).

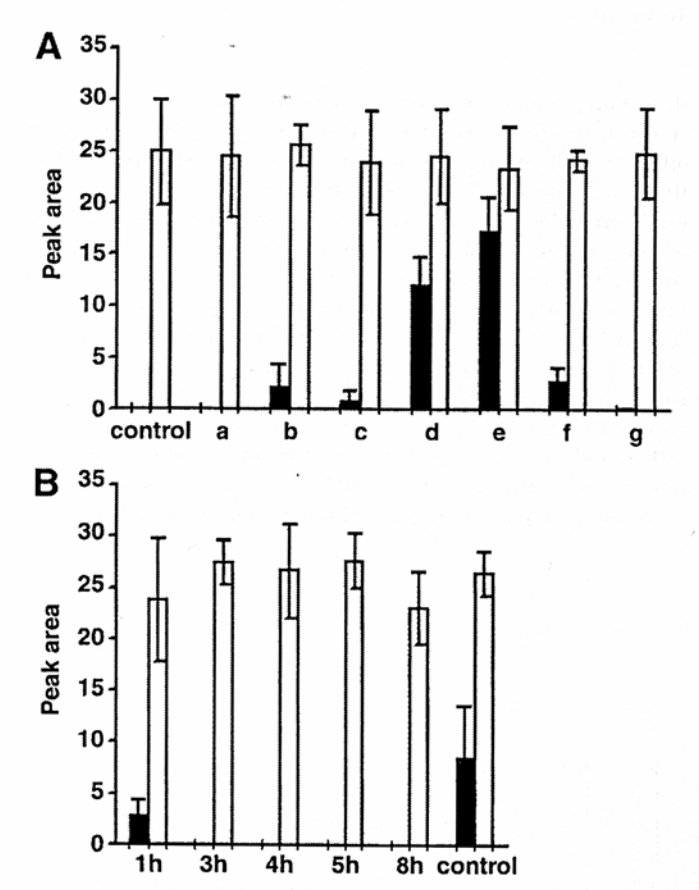

FIG. 7. Twist expression in isolated plate cells. (A) Medusa fragments and isolated plate cells cultured as tissue aggregates. Mechanically isolated interradial medusa fragments (control) were compared to interradial medusa fragments after the striated muscle was isolated with enzyme treatment (a), and after the isolated fragment was cultured for 18 h (b), or immediately (c), 7 h (d), 31 h (e), 84 h (f), and 7 days (g) after the exumbrella cells were removed (Fig. 6). (B) Cultured plate cells corresponding to sample (e) in (A) but grafted on polyp ECM and processed at various times indicated in hours for expression analysis; control is not grafted plate cells after 8 h.

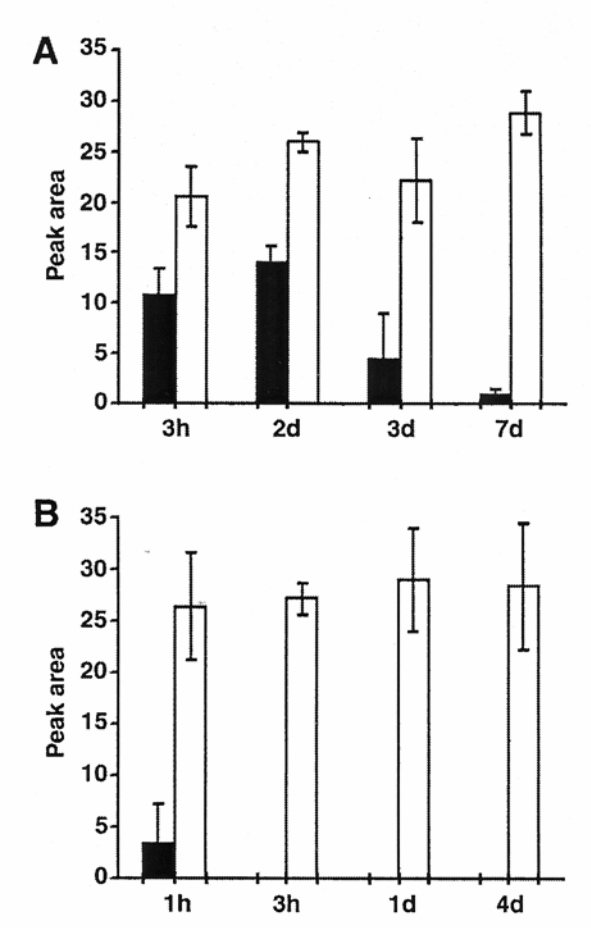

FIG. 8. Twist expression in combinations of isolated striated muscle and plate cells (see Fig. 6). Twist (shaded bars) and elongation factor 1 alpha (empty bars) expression were measured as in Fig. 2. Abscissa gives time in hours (h) or days (d) after tissues were combined. (A) Plate cells were combined with not activated striated muscle. Combinations do not form regenerates and transdifferentiation does not occur. (B) Plate cells were combined with activated striated muscle, transdifferentiation into new cell types and formation of regenerates occur.

CHAPTER 4

Evolutionary Aspects of Developmentally Regulated Helix-Loop-Helix Transcription Factors in Striated Muscle of Jellyfish

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ABSTRACT

The function of basic helix-loop-helix (bHLH) proteins in cell differentiation was shown to be conserved from *Drosophila* to vertebrates, exemplified by the function of MyoD in striated muscle differentiation. In phylogeny striated muscle tissue appears first in jellyfish and the question of its evolutionary position is controversially discussed. For this reason we have studied the developmental role of myogenic bHLH genes in medusa development. Based on their dimerization ability, four genes of the bHLH family of transcription factors were isolated from the hydrozoan jellyfish *Podocoryne carnea*. While the proteins Id and Ash group with cognate family members from bilaterians, Net-like and JellyD1 could not be unequivocally classified. *Id* is expressed during the medusa budding process and in the adult medusa, *Ash* and *Net-like* in all life cycle stages from egg to adult medusa and *JellyD1* in the blastula and gastrula stages, the planula larva and in late medusa bud stages. The dimerization specificity, the expression pattern and the conservation of two residues specific for a MyoD bHLH domain suggest that *JellyD1* is related to an ancestral *MyoD* gene. *Id, Net-like* and *JellyD1* are either expressed in the entocodon or its derived tissues, the striated and smooth muscle of the bell. These findings strengthen the hypothesis that the entocodon is a mesoderm-like structure and that the common ancestor of Cnidaria and Bilateria was more complex in cell type architecture and body organization than commonly thought.

INTRODUCTION

Striated and smooth muscle of the hydrozoan medusa bell are derived from the entocodon, a third cell layer, which forms from the early bud ectoderm during medusa development (Kühn, 1910; Hyman, 1940; Bouillon, 1994). This observation challenges the established diploblastic status of the phylum Cnidaria (Boero et al., 1998). Molecular markers for striated muscle such as myosin heavy chain (Schuchert et al., 1993) and tropomyosin (Gröger et al., 1999) and regulatory proteins such as the homeobox transcription factors *Cnox1-Pc* (Aerne et al., 1995) or *Otx* (Müller et al., 1999) show high sequence conservation with homologues from *Drosophila* and vertebrates. Recently it has been demonstrated that the *Podocoryne* cognates of bilaterian genes specific for mesoderm formation and myogenesis are highly conserved and show comparable expression patterns. The bHLH factor Twist (Spring et al., 2000) and the T-box, MADS-box and zinc finger genes *Brachyury*, *Mef2* and *Snail* (Spring et al., 2002) are expressed in the entocodon and during differentiation of striated and smooth muscle of the medusa bell. A MyoD homologue, however, the major myogenic activator (Molkentin and Olson, 1996; Baylies and Michelson, 2001), could not be isolated from *Podocoryne* by sequence based approaches.

Due to its function as a heterodimer the HLH domain is an ideal target for an interaction trap screen such as the yeast two-hybrid system (Staudinger et al., 1993; Lee et al., 1995; Hollenberg et al., 1995; Yoon et al., 2000; Massari and Murre, 2000). The *Drosophila* E-protein homologue daughterless has been used successfully in cross-phyla screens (Lee et al., 1995) and interacts with a cnidarian bHLH factor, the *Hydra* achaete-scute homologue CnASH (Grens et al., 1995). Here we used the bHLH domain of *Drosophila* daughterless to screen for a *Podocoryne* MyoD homologue.

The interaction trap screen of a *Podocoryne* cDNA library lead to the identification of the HLH transcription factor Id, and the bHLH factors Ash, Net-like, and JellyD1. Except Net-like all genes were found to interact with *Drosophila* daughterless and all four genes with vertebrate MyoD. Gene expression was analyzed through the life cycle stages: including larva formation, polyp and medusa budding. The expression and hybridization data suggest that cnidarians use HLH proteins to regulate myogenesis. These observations support the hypothesis (Boero et al., 1998; Spring et al., 2000, 2002) that the striated muscle of jellyfish is related to the striated muscle of bilaterians. We propose that the last common ancestor of Cnidaria and Bilateria was a metazoan of mesodermate body structure.

MATERIALS AND METHODS

Animals

Colonies of *Podocoryne carnea* (Cnidaria, Hydrozoa, Anthomedusae) were reared in the laboratory using artificial seawater as described (Schmid, 1979). Larval stages were obtained by the methods of Aerne et al. (1995). Medusa buds were isolated by microsurgery and classified according to Frey (1968).

Molecular Cloning and Sequence Comparison

Molecular biology procedures were performed according to standard protocols (Sambrook and Russell, 2001). A directed *Podocoryne* cDNA fusion library was constructed in the HybriZAP II phage vector (Stratagene) and library screening was performed as described (Müller et al., 1999). Clones surviving on selective media plates were amplified by PCR with the primers AD25 (5`-TAT TCG ATG ATG AAG ATA CCC CAC C-3´) and T725 (5`-GCA GTA ATA CGA CTC ACT ATA GGG C-3´) spanning the cloning site of the pAD-GAL4 vector (Stratagene). PCR products were gel purified and sequenced on an ABI PRISM 310 (Applied Biosystems). Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen), retransformed together with the bait plasmids and retested on selective media.

The full-length coding sequence of the *Net-like* gene was obtained by 5' RACE (rapid amplification of cDNA ends) on cDNA prepared from mRNA of medusae and budding polyps with a SMART RACE cDNA amplification kit (Clontech). Nucleotide and deduced amino acid sequences were analyzed using the GCG software package. Pairwise sequence comparisons were performed using the GAP program. BLAST searches (Altschul *et al*., 1997) were performed at the NCBI (National Center for Biotechnology Information, USA) using the BLAST network service (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and phylogenetic trees based on the neighbor-joining method were generated with Clustal X (Jeanmougin *et al*., 1998). Sequences have been submitted to the DNA databases with the accession numbers x-y for Id, Ash, Net-like and JellyD1, respectively.

Plasmid Construction

The plasmid Gal(AD)-MyoD (Staudinger et al., 1993) was obtained from Eric Olson. The bait plasmid pBD-Da was constructed by PCR amplification of *Drosophila* genomic DNA with the oligonucleotide primers DaF (5'-GCG AAT TCG TGA AGG CCA TTC GCG AAA AGG-3') and DaR (5'-GCT CTA GAT GCG CTC GCG CAC CTG CTG C-3'). The resulting fragment containing nucleotides 1788 to 1992 of *Drosophila daughterless* (accession number J03148) coding for aa 547-614 was digested with EcoRI and XbaI and cloned into the EcoRI and XbaI sites of the yeast vector pBD-GAL4Cam (Stratagene). Further bait plasmids were constructed by cloning PCR amplified cDNA fragments coding for selected bHLH domains into pBD-GAL4Cam. Instead of an XbaI site, a SalI site was used in the gene specific reverse primers. Primer pairs used for the construction of the bait plasmids were for *JellyD1* JeF7 (5'-CGA ATT CAA AAA ACC GCG GCA TAC TGT AC-3`) and JeR7 (5'-CGT CGA CTT ATC GAA ATC CTA ATT TAT CTT C-3`), for *Twist* TwistF1 (5'-CGA ATT CTC GAA AAA TAT TTA CCA AAA AAC-3') and TwistR1 (5'-CGT CGA CTT ATC CAC GTC GTA TAA CGT GAC G-3'), for *Net-like* MycF5 (5'-CGA ATT CGA GCA AAC AAA TTT CAA CCG-3') and MycR3 (5'-CGT CGA CTT ACA ACT CGG CAT GTA TTT TCT T-3'), for *Id* IdF1 (5'-CGA ATT CCC CGC CGC ACC CGA GAC CGC-3') and IdR1 (5'-CGT CGA CTT ACT GTC GAA GAG TTG GTC TTG C-3') and for *Ash* AshF2 (5'-CGA ATT CAG TTT GGT CGC TAC AAT GGA TCC T-3') and AshR2 (5'-CGT CGA CTT AGT ATT CTT CAA CGA CTC CTT T-3').

For β-galactosidase assays, cDNA fragments of the bHLH transcription factors were recloned into the plasmids pEG202 and pJG4-5 (Gyuris et al., 1993). The plasmids pEG202-LAR.D1D2(1275-1881) and pJG45-Trio.SIK(2485-3038) were used as positive controls in interaction assays (Debant et al., 1996). The bHLH cDNAs were cloned into the EcoRI and SalI restricted pEG202. For pLexA-Da the cDNA covered amino acids 547-614 of *Drosophila* daughterless, for pLexA-XMyoD residues 2-288 of the *Xenopus laevis* MyoD, for pLexA-MyoD-C residues 102-167 of mouse MyoD1, for pLexA-JellyD1 residues 1-109 of *Podocoryne* JellyD1, for pLexA-Ash residues 1-159 of *Podocoryne* Ash, for pLexA-Ash-C residues 59-126 of the *Podocoryne* Ash, for pLexA-Id the cloned part of *Podocoryne* Id and for pLexA-Net residues 1-149 of *Podocoryne* Net-like. The plasmids pJG-Da, pJG-XMyoD, pJG-MyoD-C, pJG-Ash, pJG-Ash-C, pJG-Id, pJG-Twist and pJG-Net were

constructed by cloning the EcoRI and SalI digested cDNA fragments into the EcoRI and SalI restricted pJG4-5 vector.

β-galactosidase Assays

The interactions of the bHLH transcription factors were quanitified by β-galactosidase assay as described by Schneider et al., 1996. The yeast strain EGY48 (*MATα, ura3, his3, trp1, LexA$_{op(x6)}$-LEU2*) was used with the plasmids pEG202, the expression vector for LexA-fusion proteins, and pJG4-5, the expression vector for Gal4 activator fusion proteins, together with the plasmid pSH18-34 carrying the lacZ reporter (Gyuris et al, 1993). The plasmid pSH17-4 encoding the LexA-GAL4 fusion was used for maximal reporter gene activation. As positive control for protein-protein interactions the plasmids pEG-LAR.D1D2 and pJG-TrioSIK were used (Debant et al., 1996).

Whole Mount in Situ Hybridization

Probes were prepared by *in vitro* run-off transcription from the full coding sequence of the novel bHLH genes and the two controls RFamide precursor (Gajewski *et al*., 1998), and *Twist* (Spring *et al*., 2000) using the DIG RNA Labeling Mix (Roche). *In situ* hybridization experiments were carried out as following: specimen were fixed over night at 4°C in freshly prepared Lavdowsky fixative (ethanol : 37% formaldehyde : glacial acetic acid : water = 50 : 10 : 4 : 36), supplemented with 0.2% glutaraldehyde. Fixative was removed by two washes in PBST (PBS ; 0.1% Tween 20). Then specimens were transferred into hybridization buffer (5 X SSC, 50 % formamide, 100 µg/ml tRNA from E. coli, 50 µg/ml heparin, 0.1 % Tween 20) in a stepwise manner going from 15 % hybridization buffer to 30, 45 , 60, 75, 90 and 100 %. Pre-hybridization, hybridization and the first washing step (5 X SSC ; 50 % formamide and 0.1 % Tween 20) were carried out at 58°C. The following washing steps were done at RT in the Wash 2 solution (2 X SSC ; 50 % formamide ; 0.1 % Tween 20), Wash 3 (2 X SSC ; 0.1 % Tween 20), and finally Wash 4 (0.2 X SSC ; 0.1 % Tween 20). Specimens were then incubated for 1 hour in a blocking buffer (Roche), then transferred in the anti-DIG antibody conjugated with the alkaline phosphatase (1:2000) for 2hours at RT. After three washes in PBST, specimens were placed in TMNT buffer (100 mM Tris-HCl pH 8.0; 50 mM $MgCl_2$; 100mM NaCl; 0.1 % Tween 20) for 30 minutes and then transferred in color detection solution (100 mM Tris-HCl pH 8.0; 50 mM

$MgCl_2$; 100mM NaCl; 225 µg/ml NBT ; 175 µg/ml BCIP). After staining specimens were extensively washed in PBST and investigated as whole mounts or as 10 µm sections of paraplast (Sigma) embedded specimens (Spring *et al*., 2002).

RT-PCR Expression Analysis

mRNA from the different life-stages and medusa buds was extracted using the Dynabeads mRNA Direct Kit (Dynal) as recommended by the manufacturer. 1st strand cDNA synthesis and real time PCR were carried out as described and the ubiquitously expressed elongation factor 1α (*EF1a*) was included in each set of experiments as a reference to compensate for variations in the quantity and quality of preparations (Spring et al., 2000). PCR analysis was done in duplicates and in independent experiments. For *JellyD1*, the primer pair JellyF (5'-CTG TTG TTA TGG AGA CTA GCA GA-3') and JellyR (5'-TAA AGA TTC GGC ATC GAG AAC CT-3') was used. *Ash* expression was evaluated using the primers AshF1 (5'-AAT GCA GCT CCT CTA TCC AAA GCC-3') and AshR1 (5'-GGT AGA AAT GGT ACC TTC TTT CGC-3'). *Podocoryne Id* was analyzed by using the primer pair PIdF1 (5'-CAT CAA CAA GGG CAA GGA GAC CG-3') and PIdR1 (5'-ATC GAA GAG TTG GTC TTG CAG CG-3'). The *Net-like* gene was amplified using the primer pair MycF2 (5'-CAT TAC AAA CTG GCC AAG AGG AGC-3') and MycR2 (5'-AGC TTG AGT TGG TTT CTT CGT TGC-3'). Primers for *EF1a* are EF1AF (5´-ACG TGG TAT GGT TGC CTC TG-3‘) and EF1AR (5‘-TGA TAA CGC CAA CGG CTA CG-3‘).

RESULTS AND DISCUSSION

Identification of Four Podocoryne HLH Transcription Factors

A cDNA fusion library with the yeast GAL4 activator was prepared from budding polyps of *Podocoryne* and screened using the *Drosophila* daughterless bHLH domain as a bait. Da represents the *Drosophila* orthologue of the ubiquitously expressed vertebrate E-proteins E-12/E-47 which form homo- or heterodimers (Murre et al., 1989) and have successfully been used as baits for bHLH proteins (Staudinger et al., 1993). A total of 3.4 x 10^7 yeast clones were screened and 518 clones grew on selective media. Eleven cDNA clones were isolated encoding four different *Podocoryne* HLH transcription factors. Id was found eight times whereas the other three transcription factors were isolated only once in the screen.

JellyD1, Ash, and Id were isolated with the *Drosophila* daughterless bait while the Net-like transcription factor was isolated using JellyD1 as bait. Id was also isolated independently using mouse MyoD1 as bait.

Sequence Analysis of the Podocoryne HLH Transcription Factors

To classify the four HLH proteins, database searches were performed and representative sequences from *Drosophila,* mouse and *Hydra*, were used for a phylogenetic analysis. The highly conserved *Podocoryne* Twist (Spring et al., 2000) was also included in the analysis. Due to the low degree of sequence conservation outside the domain the multiple sequence alignments were restricted to the basic helix-loop-helix regions. The HLH domains from the different protein subfamilies show a high degree of sequence conservation within each subfamily but only moderate conservation between subfamilies (Fig. 1).

Podocoryne Id, a HLH transcriptional repressor. Despite a low degree of sequence conservation of only about 35% in the HLH domain, the *Podocoryne* Id homologue can be classified as a member of the Id family of transcriptional repressors due to the lack of a basic region characteristic for this protein family (Norton, 2000). The 107 amino acid fragment of *Podocoryne* Id is encoded by a partial cDNA clone and is lacking the basic region N-terminal to the HLH domain from amino acids 24 to 64 (Fig. 1). Sequence similarities are relatively low with only 32-43% identity to Drosophila emc and the four mouse Id family members Id1-4 but significantly higher than to all bHLH proteins with a basic domain.

The Id protein family members heterodimerize with many different bHLH proteins and thus antagonize their functions resulting in a form that is incapable of binding DNA (Norton, 2000). Analysis of the *C. elegans* genome has revealed that the Id class of HLH transcription factors is missing (Ruvkun and Hobert, 1998). It was therefore suggested that the strategy of inhibiting the function of bHLH transcription factors by the formation of non-DNA-binding complexes evolved later during evolution (Massari and Murre, 2000). However, since *Podocoryne* contains an Id homologue, its absence in the *C. elegans* genome is better explained as a secondary gene loss in the *C. elegans* lineage.

Podocoryne Ash, a highly conserved member of the family of Achaete-scute homologues. *Podocoryne Ash* was isolated as a full-length clone and encodes a protein of

158 amino acids with the bHLH domain spanning amino acids 67 to 120 (Fig. 1). The sequence can be aligned over the entire length with the *Hydra* CnASH protein (Grens et al., 1995) with 60% of sequence identity. Within their bHLH regions the two proteins are 87% identical. When compared to other phyla only the bHLH domain can be reliably aligned showing 65% sequence identity with mouse Ash1, 59% with *Drosophila* achaete or scute and 52% with the *C. elegans* homologue T24B8.6. Interestingly, the founding members achaete and scute as well as all other insect family members have an insert in the loop region that is not present in *Podocoryne* or other phyla.

The CnASH protein from *Hydra* is able to dimerize with the *Drosophila* protein Da and binds *in vitro* to the E-box consensus sequence specific for the *Drosophila* achaete-scute complex proteins (Grens et al., 1995). Furthermore, the differentiation of *Drosophila* achaete and scute double mutants could be partially rescued by expression of the CnASH gene product (Grens et al., 1995). These results suggest that although cnidarians and arthropods diverged prior to the Cambrian explosion (Erwin and Davidson, 2002), the biochemical functions of the CnASH protein have been conserved (Grens et al., 1995).

Podocoryne Net-like, a bHLH transcription factor related to Drosophila Net or the Myc family. A partial cDNA clone coding for a Net-like transcription factor was isolated with *Podocoryne* JellyD1 as bait. The sequence was completed by 5' RACE. The gene encodes a 148 amino acid protein with the bHLH domain spanning amino acids 49 to 99 (Fig. 1). When compared to the databases, the Net-like protein is most similar to the *Drosophila* bHLH transcriptional repressor Net (35% identity, Brentrup et al., 2000) and almost equally similar to the members of the Myc and Max families (Grandori et al., 2000) The similarity to Net and Myc is lower than expected for a true orthologue. Furthermore *Podocoryne* Net-like does not contain a proline rich region thought to be important for transcriptional repression in *Drosophila* Net (Brentrup et al., 2000) or a leucine zipper domain characteristic for Myc and Max family members.

Podocoryne JellyD1, a derived MyoD homologue or a novel bHLH transcription factor? JellyD1 is a protein of 109 amino acids with the bHLH domain spanning amino acids 24 to 76. Outside this motif no further sequence conservation to other bHLH transcription factors is observed. The highest sequence similarity is seen with MyoD family members from vertebrates, *C. elegans* and *Drosophila* with 36-40% identity. However, sequence similarities

are also seen with the bHLH domains of members of the Hand and Hen families and phylogenetic analysis cannot assign JellyD1 to a specific subfamily (Fig. 1). The gene was called JellyD1 to reflect similarities as well as differences to MyoD1. JellyD1 contains two of the crucial amino acids needed for myogenic activity (Davis et al., 1990). Alanine 32 and threonine 33 correspond to alanine 114 and threonine 115 in mouse MyoD1 while lysine 124 is not conserved (Fig. 1).

The low degree of sequence similarity raises the question whether JellyD1 is a genuine MyoD homologue or belongs to the Hen, Hand or another family in *Podocoryne*. All members of the bHLH family involved in muscle determination and differentiation contain within their basic region conserved amino acid residues that are not present in other bHLH proteins. Mutation analysis has shown that the amino acids A114, T115 and K124 of mouse MyoD1 are crucial for myogenic activity. Remarkably, placing the corresponding amino acid residues from MyoD1 into its dimerization partner E-12 produces a protein that functions as myogenic activator (Davis et al., 1990). A more detailed mutational analysis has indicated that the alanine and the threonine are sufficient to mediate the myogenic specificity, while the lysine is not necessary in this context (Davis and Weintraub, 1992). JellyD1 contains these two crucial amino acid residues in the correct position in its basic region, while the lysine is replaced by a glutamic acid residue. No other bHLH transcription factor except the myogenic activators have an alanine together with a threonine residue at those crucial positions, while alanine alone is found in many bHLH transcription factors. Because Hen and Hand family members lack these crucial amino acid residues, JellyD1 is more MyoD-like than any other bHLH protein.

Interactions with Other bHLH Transcription Factors

To assess the possible functions and the structural conservation of the *Podocoryne* HLH proteins, their mutual interactions were investigated using quantitative yeast β-galactosidase assays. The strength of reporter gene activation, as measured by *lacZ* activity, is assumed to reflect the strength of the protein-protein interactions. The results of β-galactosidase assays are shown in Fig. 2A and summarized in Fig. 2B. In the absence of a coexpressed bHLH fusion protein the LexA fusion proteins were unable to activate the β-galactosidase gene. As expected, the *Drosophila* daughterless (Da) protein was able to form heterodimers with Ash, Id and JellyD1 indicating a conserved structure and dimerization

function of the *Podocoryne* bHLH proteins. The *Podocoryne* Twist protein, despite its high level of sequence conservation did not bind to Da, while *Drosophila* Twist and Da interacted (Castanon et al., 2001). Moreover, *Podocoryne* Twist interacted with JellyD1 and Id but not with the mouse or *Xenopus* MyoD fusion proteins (Figs. 2A, 2B). Mouse MyoD1 has been reported to interact with Twist, however the interaction is not a result of a conventional HLH-HLH interaction but also requires the basic domains of the two proteins (Hamamori et al., 1997). The interaction data support the idea that JellyD1 is a myogenic regulatory factor. As expected for a MyoD-like factor JellyD1 interacted with Da and with Id. Surprisingly, interactions between JellyD1 and *Podocoryne* Twist were detected while both MyoD constructs from vertebrates did not interact with *Podocoryne* Twist. Homodimerization of JellyD1 and interactions with the mouse as well as the *Xenopus* MyoD homologues were unexpected since members of the MyoD family were reported to form heterodimers (Lassar et al., 1991; Hu et al., 1992). However, the *C. elegans* MyoD homologue CeMyoD was recently found to be functional as homo-and heterodimer with the *C. elegans* daughterless homologue Ce/DA (Zhang et al., 1999). It has also been shown that vertebrate MyoD-MyoD homodimerization occurs *in vitro* and that these complexes are equally stable as MyoD-E-12 heterodimers. Homodimerization of the E-12 proteins does not occur (Maleki et al., 1997) which is consistent with our results with the *Drosophila* E-protein homologue daughterless (Fig. 2A).

Podocoryne Id was able to form heterodimers with all tested proteins, but no homodimers (Figs. 2A, 2B). This indicates that the role of Id as a transcriptional repressor has been conserved during evolution. The strength of the protein-protein interaction was not fully reciprocal. For example, *Podocoryne* Ash interacted strongly with Id when fused to LexA whereas the same proteins expressed from the opposite vectors interacted only weakly (Fig. 2A). This may be due to sterical inhibition in one of the fusion proteins. As expected for an Achaete-scute homologue, the *Podocoryne* Ash protein was able to interact with *Drosophila* daughterless. Furthermore, it formed heterodimers with the *Podocoryne* transcriptional repressor Id, consistent with the interaction of members of the achaete-scute and Id families in *Drosophila* (Cabrera et al., 1994). Formation of homodimers or interactions with tissue specific transcription factors such as JellyD1 and Twist could not be detected, indicating that *Podocoryne* Ash is able to discriminate between the different bHLH proteins. Net-like not only interacts with JellyD1 but also with Id and vertebrate MyoD constructs, no interaction with the *Drosophila* daughterless protein could be detected.

Expression Patterns

Analysis of the life cycle stages. Within 30 hours the fertilized egg of *Podocoryne* develops into a ciliated planula larva. The epidermal and endodermal smooth-muscled myoepithelia are the first cell types to differentiate in larval development, followed by nerve cells (epidermis) and nematocytes (endodermis) (Gröger and Schmid, 2001). Within 30-35 hrs all cell types have differentiated and the larva is ready to attach to the substrate at its anterior pole and transforms into the primary polyp (Fig. 3A). By stolonization from the polyp base a colony develops which is composed of feeding polyps (gastrozoids), and medusa budding polyps (gonozoids, Fig. 3A). Larva and polyp are bilayered and although different in morphology, they share many cell types. In the mid-body region of the gonozoid, medusa buds of different developmental stages form a whorl (Fig. 3A). The buds are classified according to their stage of differentiation from 1 to 10 (Frey, 1968).

Expression analysis by RT-PCR demonstrates the presence of the *Ash* and *Net-like* transcripts in the unfertilized egg, in all stages of the life cycle and in all medusa bud stages (Figs. 3B, 3C). As was already observed for the *Hox/ParaHox* genes (Yanze et al., 2001), *Twist* (Spring et al., 2000), *Brachyury*, *Mef2* and *Snail* (Spring et al., 2002) *Ash* and *Net-like* are maternal messages. *Id* is the only gene with medusa-specific expression (Fig. 3C). *JellyD1* expression is very weak in gastrulation, stronger in the planula larva and restarts again in the late medusa bud stages (Figs. 3B, 3C). In correlation with the RT-PCR data Id in general showed no staining in larval development. Occasionally, in few old larvae some cells at the most anterior pole showed some staining (Fig. 4). At the moment we can not explain the difference to the RT-PCR data. However, since no signal resulted with 6 different primer combinations we regard this Id staining as an artifact, probably due to the local most anterior gland cells. *In situ* hybridization with *Ash* results in patchy endodermal staining in 24-30 hrs old planula (Fig. 4 Ash, c), the time period when nematocytes differentiate in the larval endoderm (Gröger and Schmid, 2001). As *Ash* is involved in cnidogenesis in polyps (not shown) and medusae (Fig. 4) and has a similar expression pattern in Hydra polyps (Grens at al., 1995) we assume that it is involved in nematocyte differentiation in larvae. No specific staining in the larva was observed for Net-like. *JellyD1* stained some unidentified endodermal cells in the solid planula (Fig. 4).

The development of medusa buds. The histology of medusa bud development has been described by a number of investigators (Kühn, 1910; Tardent, 1978; Bouillon, 1994). Dedifferentiating and immigrating polyp cells from the early stages of the budding process (Brändle, 1971; Boelsterli, 1977). First, an ectodermal thickening appears at the budding site, then endoderm and ectoderm bulge to form stage 1 (Fig. 5). The undifferentiated cells of stage 1 are highly proliferative (Boelsterli, 1977). At stage 2, cells from the distal ectoderm separate from both layers. This additional layer is called the entocodon (Hyman, 1940; Fig. 5, colored in blue). Because the entocodon cells are clearly separated from the ectoderm and endoderm by the formation of an extracellular matrix (Boelsterli, 1977, m in Fig. 5) they constitute a third germ layer (Boero et al., 1998). At stage 3-4 (Fig. 5), the entocodon enlarges and the endoderm pushes distally around the entocodon forming four tubes destined to become the four radial canals. At the same time the endoderm projects centrally carrying the central entocodon before it to build the primordium of the manubrium (feeding and sex organ, Hyman, 1940). During this process the entocodon forms a cavity, the future subumbrellar space (Fig. 5). At stage 4 the four radial canal tubes which at stage 3 were connected become separated by the growing bud, however, they remain connected via the outgrowth of a thin monoepithelial cell layer, the subumbrellar plate or gastrodermal lamella (p in Fig 5, cross sections of stage 6-7). Unlike the cells of the larval and polyp epithelia, the cells of the subumbrellar plate and those of the bell ectoderm will never differentiate myofilaments. The first striated myofibers differentiate in very few cells at stage 3-4 in the outer entocodal layer (Bölsterli, 1977, red colored in Fig. 5). Myofiber differentiation strongly increases after stage 5 and ends after stage 8 when cell proliferation in the striated muscle layer has stopped (Schmid, 1972). The central layer of the entocodon, which covers the endoderm of the manubrium Anlage, will differentiate the smooth muscle of the manubrium (Fig. 5, yellow colored). How the thin threads of smooth muscle which run on top and perpendicular to the striated muscle over the radial canals differentiate in detail (Fig. 5, light green colored) is not known, however, they apparently also derive from the entocodon. The primordium of the velum (stage 5-6, v in Fig. 5) develops from the most distal bud tissues. The outside velar smooth muscle derives from the epidermis, the inner, striated muscle from the entocodon. Also distally the tentacle bulbs and tentacles will differentiate, however, without any cellular contribution from the entocodon. Up to stage 5 the entocodon cavity is completely sealed off from the outside but from stage 6-7 on the velar opening forms through which the growing tentacles are pushed into the entocodon cavity. This

process brings the striated and smooth muscle into an ectodermal position, a situation that has helped to disguise the true character of the bell muscle (Boero et al., 1998). The transformation of the late bud stages into a functional medusa occurs at stage 9. Within few minutes the mesoglea of the bell swells (Schmid, 1972). The increase in volume unfolds the bell tissues causing the tentacles to attain their external position and activates rhythmic contractions in the striated muscle, a process that liberates the medusa from the mother polyp. From stage 9 on and in the adult medusa no DNA replication occurs in the tissues which form the bell, however, cell proliferation is observed in the manubrium and in the tentacle bulbs (Schmid, 1972; Spring et al., 2000).

Expression of Id. *In situ* analysis (Fig. 5) demonstrates that *Id* staining is present throughout bud development, starting in the distal endoderm of the very first bud stage and from then on in the manubrium Anlage (feeding and sex organ) and in the endoderm of the radial canals. Between stage 4-6 the plate cells stain transiently. The entocodon of stage 2 shows no or very weak staining. When the entocodon forms a cavity at stage 3-4, entocodal staining increases and remains strong throughout the differentiation process of the striated and smooth muscle and even persists into the late bud stage 8-10 when the muscle is fully differentiated. Throughout development strong staining is seen in the gonads.

In *Drosophila* (Cubas et al., 1994) and vertebrates (Jen et al., 1997) Id associates with other transcription factors to inhibit muscle differentiation. A possible inhibitory function of Id in the formation of the bell muscle systems in *Podocoryne* is not supported by the staining pattern. Since Id is transiently expressed in the plate cells and since the plate cells lack myofilaments Id may act as an inhibitor for myogenesis in this cell type. *Twist*, another negative regulator of myogenesis in Bilateria is only weakly expressed in the differentiating muscle but strongly in the plate cells (control in Fig. 5, Spring et al., 2002). The dimerization data suggest that Id may associate with bHLH transcription factors like JellyD1 and Net-like to promote myogenesis from entocodal tissues, however, it may also associate with Twist in the plate cells (Fig. 2B) to inhibit muscle differentiation.

Expression of Ash. *Ash* staining (Fig. 5) is observed from the very early bud stages on in both epithelial cell layers and the early entocodon. At stage 5-6 cells in the interradial ectoderm and in the endoderm of the manubrium Anlage stain. In later bud stages (stage 7-10) and the adult medusa *Ash* staining is restricted to cell clusters in the manubrium

endoderm, nematocytes in the developing tentacles (Fig. 5) and nematoblasts in the ectoderm of the tentacle bulbs (Fig. 4 Ash, c), the site where nematocytes for the tentacles differentiate. No staining was observed in the plate cells and the developing muscle tissue. In *Drosophila*, members of the achaete-scute complex are proneural proteins and involved in external sensory organ formation (Jan and Jan, 1993). Vertebrate Achaete-scute homologues have a proneural function and might also be involved in the transition of precursors to differentiated cells (Lee, 1997). Although *Podocoryne* has no interstitial cells comparable to Hydra (Braverman, 1974) the expression pattern in undifferentiated cells of the early bud stages and later in differentiating nematoblasts correlates well with the expression of the *Hydra CnASH* gene which is active in a subset of the interstitial cells and in cells of the nematocyte differentiation pathway (Grens et al., 1995).

Expression of Net-like. *Net-like* has almost the same staining pattern as *Id* (Fig. 5). This correlates with the dimerization data (Fig. 2). The endoderm of the bud primordia, the manubrium Anlage, the radial canals and the plate cells stain distinctly in the young bud stages. In difference to *Id*, *Net-like* still stains the plate cells at stage 6-7. Endoderm expression ceases completely in stage 8-10. The very young entocodon of stage 2 shows no staining but in all later stages the entocodon and the differentiating and fully developed muscle of the bell stain. Staining is also found in the gonads. From the dimerization data one would expect the gene to be co-expressed with *Id* and *JellyD1* in the developing striated and smooth muscle and this is the case. Furthermore, the expression pattern is similar to that of the MADS-box gene *Mef2* (Spring et al., 2002).

Expression of JellyD1. No staining was found in young bud stages (Fig. 5). The first staining is detected in the distal endoderm of the manubrium, the developing gonads and in the differentiating striated and smooth muscle of the bell. This staining pattern persists throughout development. The expression of *JellyD1* during medusa development correlates well with that of bilaterian muscle determination factors. The *JellyD1* message is present in both of the developing muscle types similar to MyoD homologues from other bilaterian invertebrates where the gene has other functions in addition to the specification of striated muscle (Harfe et al., 1998). Furthermore, JellyD1, like MyoD, seems to represent a differentiation and not a determination factor for muscle (Weintraub, 1993). In accordance with the hypothesis that *JellyD1* represents a MyoD homologue the gene is not expressed in

the early stages of entocodon development (stages 1-4, Figs. 3B and 5) but later when the cells start to differentiate the striated myofibrils. *JellyD1* appears to have a regulatory function in determination and differentiation of striated muscle cells and like *Id* and *Net-like* it is expressed in fully differentiated muscle suggesting that its expression is needed for the maintenance of the differentiated state. Sequence analysis, dimerization assays and the expression data indicate that *JellyD1* represents a derived cnidarian *MyoD* homologue.

In *Drosophila* and vertebrates, *Twist* (Cripps et al., 1998) and *Snail* (Hemavathy et al., 2000; Manazanares et al., 2001) family members are required for mesoderm specification and act together with the Mef2 and MyoD (Baylies et al., 1998) family members in myogenesis. All these myogenic regulatory genes were shown to be structurally and developmentally conserved in *Podocoryne* (Spring et al., 2001; 2002). We propose *JellyD1* to be a member of this cascade and a downstream target of Twist and Snail in the muscle differentiation process.

Conclusions

A growing set of molecular data from cnidarians suggests that these "primitive" animals use basically the same genes for axis formation, development and for the maintenance of body functions as bilaterians (Shenk and Steele, 1993; Galliot, 2000; Hobmayer et al., 2000; Masuda-Nakagawa et al., 2000; Yanze et al., 2001; Hayward et al., 2002). Because of its functional and histological features the striated muscle of the medusa is of special evolutionary interest. In Bilateria this cell type in general is a derivative of the mesoderm, a third germ layer that is supposedly missing in Cnidaria. However, these and published data demonstrate the presence of a bilaterian-like cascade of developmentally regulated myogenic control genes in jellyfish (Spring et al., 2000,2002) which stress the evolutionary importance of the entocodon as an ancestral, mesoderm-like structure. Although sequence data do not allow us to group *JellyD1* unequivocally with the *MyoD* family members, the two conserved crucial amino acids, the conserved interaction network and the expression pattern during myogenesis indicate that *Podocoryne* JellyD1 corresponds to the MyoD of higher animals. Based on morphological and 18S ribosomal RNA data the Anthozoa appear to be the most ancestral class within the phylum (Bridge et al., 1995; Collins, 2002). Because extant Anthozoa such as corals or sea anemones all lack a medusa stage, it was assumed that the medusa evolved later. However, the unique role of *Id* in medusa formation and the sequence and expression data of the bHLH and other mesodermal

genes (Spring et al., 2000, 2002) rather support the idea that a free living, planktonic ancestor invented the striated muscle lineage and not a sessile polyp which, at least in all extant species, never differentiates this cell type. Hence, it appears that the medusa stage was reduced as a secondary event in the common ancestor of all extant Anthozoa, as is seen also in some Hydrozoa such as the freshwater polyp *Hydra* (Schuchert, 1993; reviewed in Bouillon, 1994).

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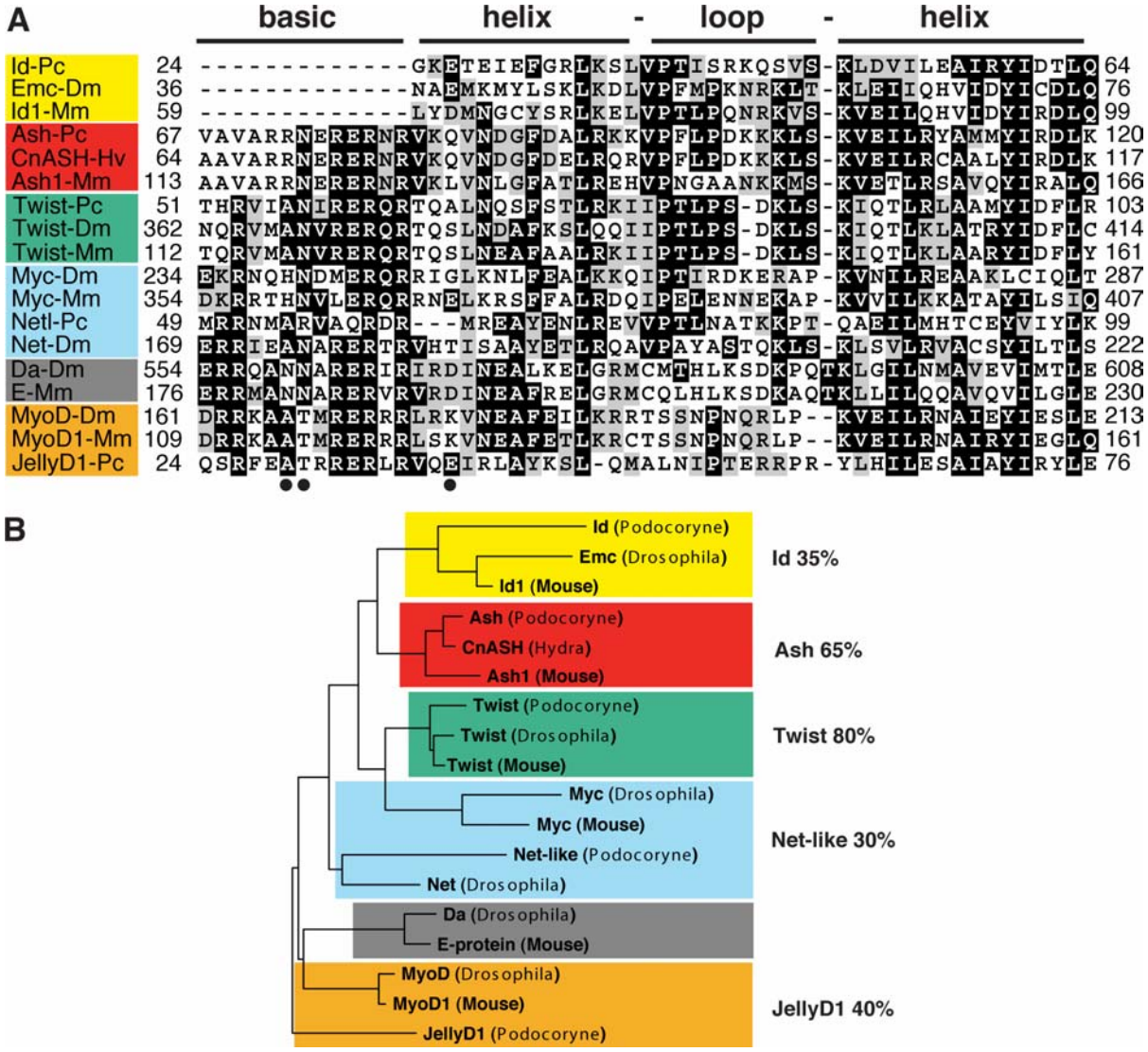
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basic
helix
loop
helix
Id-Pc 24 ----------GKETEIEFGRLKSLVPTISRKQSVS-KLDVILEAIRYIDTLQ 64
Emc-Dm 36 ----------NAEMKMYLSKLKDLVPFMPKNRKLT-KLEIIQHVIDYICDLQ 76
Id1-Mm 59 ----------LYDMNGCYSRLKELVPTLPQNRKVS-KVEILQHVIDYIRDLQ 99
Ash-Pc 67 VAVARRNERERNRVKQVNDGFDALRKKVPFLPDKKKLS-KVEILRYAMMYIRDLK 120
CnASH-Hv 64 AAVARRNERERNRVKQVNDGFDELRQRVPFLPDKKKLS-KVEILRCAALYIRDLK 117
Ash1-Mm 113 AAVARRNERERNRVKLVNLGFATLREHVPNGAANKKMS-KVETLRSAVQYIRALQ 166
Twist-Pc 51 THRVIANIRERQRTQALNQSFSTLRKIIPTLPS-DKLS-KIQTLRLAAMYIDFLR 103
Twist-Dm 362 NQRVMANVRERQRTQSLNDAFKSLQQIIPTLPS-DKLS-KIQTLKLATRYIDFLC 414
Twist-Mm 112 TQRVMANVRERQRTQSLNEAFAALRKIIPTLPS-DKLS-KIQTLKLAARYIDFLY 161
Myc-Dm 234 EKRNQHNDMERQRRIGLKNLFEALKKQIPTIRDKERAP-KVNILREAAKLCIQLT 287
Myc-Mm 354 DKRRTHNVLERQRRNELKRSFFALRDQIPELENNEKAP-KVVILKKATAYILSIQ 407
Netl-Pc 49 MRRNMARVAQRDR---MREAYENLREVVPTLNATKKPT-QAEILMHTCEYVIYLK 99
Net-Dm 169 ERRIEANARERTRVHTISAAYETLRQAVPAYASTQKLS-KLSVLRVACSYILTLS 222
Da-Dm 554 ERRQANNARERIRIRDINEALKELGRMCMTHLKSDKPQTKLGILNMAVEVIMTLE 608
E-Mm 176 ERRMANNARERVRVRDINEAFRELGRMCQLHLKSDKAQTKLLILQQAVQVILGLE 230
MyoD-Dm 161 DRRKAATMRERRRLRKVNEAFEILKRRTSSNPNQRLP--KVEILRNAIEYIESLE 213
MyoD1-Mm 109 DRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLP--KVEILRNAIRYIEGLQ 161
JellyD1-Pc 24 QSRFEATRRERLRVQEIRLAYKSL-QMALNIPTERRPR-YLHILESAIAYIRYLE 76
B
Id (Podocoryne)
Emc (Drosophila)
Id1 (Mouse)
Id 35%
Ash (Podocoryne)
CnASH (Hydra)
Ash1 (Mouse)
Ash 65%
Twist (Podocoryne)
Twist (Drosophila)
Twist (Mouse)
Twist 80%
Myc (Drosophila)
Myc (Mouse)
Net-like (Podocoryne)
Net (Drosophila)
Net-like 30%
Da (Drosophila)
E-protein (Mouse)
MyoD (Drosophila)
MyoD1 (Mouse)
JellyD1 (Podocoryne)
JellyD1 40%

FIG. 1. Classification of *Podocoryne* HLH and bHLH domains. (A) Sequence alignment of HLH and bHLH domains. *Podocoryne* (Pc) domains were compared to representative domains from *Drosophila* (Dm), mouse (Mm) and *Hydra* (Hv) bHLH transcription factors. Identical amino acids are boxed in black and similar amino acids boxed in gray when present in more than half of the sequences. Protein subfamilies are boxed in shades of gray. The Id family of HLH proteins is characterized by the lack of a basic region. Black dots indicate amino acid residues specific for myogenic bHLH domains. (B) Phylogenetic analysis of bHLH domains. Related proteins are boxed in shades of gray. The analysis confirms that *Podocoryne* Ash, Id and Twist are true orthologues of proteins from *Drosophila*, mouse and *Hydra*, while JellyD1 and Net-like are more derived and can not be grouped unequivocally. Percentages indicate average values of amino acid identity between *Podocoryne* proteins and related bHLH domains.

A

bait \ prey	Ash	Ash-C	Da	Id	JellyD1	MyoD	MyoD-C	Net-like	Twist	pJG
Ash	3.9 ± 1.2	5.4 ± 2.2	**142.9 ± 16.9**	**508.7 ± 54.6**	6.8 ± 0.4	9.8 ± 0.4	1.8 ± 0.1	10.0 ± 1.4	1.6 ± 0.5	1.3 ± 0.5
Ash-C	1.8 ± 0.2	5.3 ± 1.5	**70.4 ± 30.7**	3.4 ± 0.5	2.5 ± 0.3	2.8 ± 0.8	3.7 ± 0.2	2.9 ± 0.2	2.9 ± 0.9	4.3 ± 1.4
Da	**238.8 ± 28.7**	**305.0 ± 66.5**	1.8 ± 0.7	**668.0 ± 278.7**	**443.5 ± 210.0**	**403.0 ± 236.6**	**385.2 ± 82.1**	1.3 ± 0.2	2.5 ± 1.0	1.6 ± 0.3
Id	**36.6 ± 4.9**	**453.1 ± 162.3**	**149.1 ± 60.9**	3.2 ± 0.2	**132.0 ± 7.8**	**712.3 ± 189.8**	**197.5 ± 73.4**	**301.7 ± 88.5**	**292.6 ± 93.3**	2.2 ± 0.6
JellyD1	5.0 ± 0.4	9.9 ± 1.4	**437.6 ± 157.7**	**42 ± 10.2**	**188.1 ± 18.1**	**173.9 ± 17.8**	**43.4 ± 7.8**	**132.7 ± 43.4**	**240.5 ± 26.7**	4.6 ± 0.5
MyoD	9.3 ± 1.6	5.3 ± 0.7	**114.9 ± 34.4**	**120.1 ± 37.7**	**35.7 ± 3.3**	**235.1 ± 36.4**	**25.6 ± 3.5**	**28.2 ± 2.5**	4.1 ± 0.8	5.6 ± 1.6
MyoD-C	2.9 ± 0.2	4.3 ± 1.1	**52.4 ± 5.5**	**16.4 ± 1.3**	**66.5 ± 29.9**	**553.0 ± 334.3**	**70.6 ± 25.8**	**18.6 ± 1.7**	5.6 ± 1.6	5.2 ± 0.7
Net-like	7.3 ± 0.8	6.1 ± 1.6	12.6 ± 2.3	**30.0 ± 4.4**	**119.8 ± 29.1**	**102.6 ± 35.6**	**31.8 ± 8.7**	7.4 ± 1.3	4.4 ± 0.5	1.1 ± 0.54
Twist	3.6 ± 0.6	5.8 ± 2.0	8.2 ± 4.6	**32.8 ± 12.9**	**87.4 ± 26.4**	3.6 ± 1.3	4.5 ± 1.7	5.4 ± 1.7	5.1 ± 1.6	3.4 ± 1.11

B

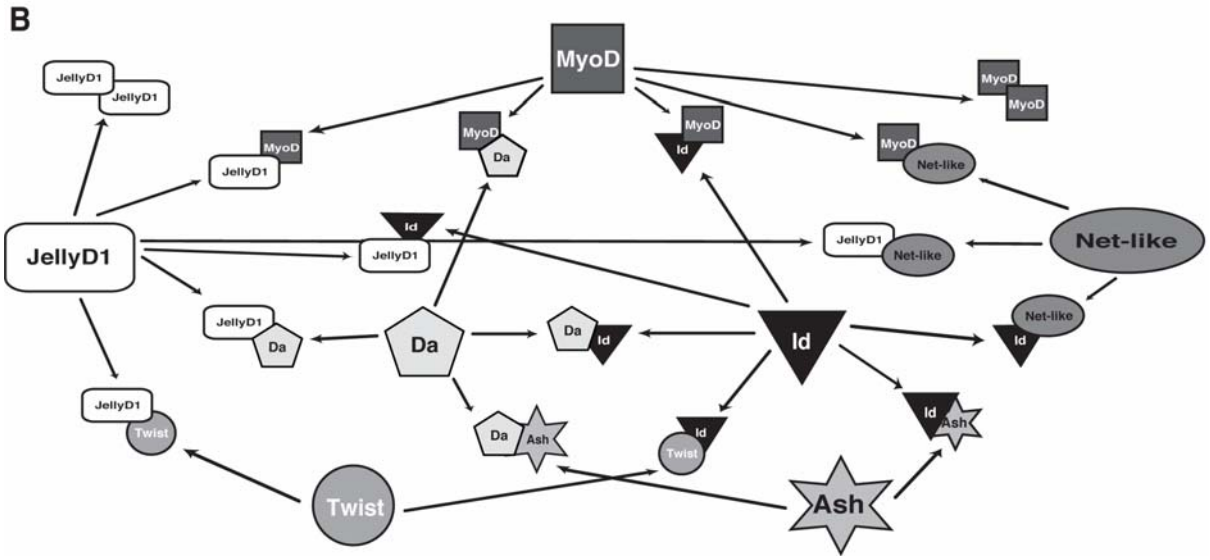


FIG. 2. Interactions between different bHLH transcription factors. (A) Relative levels of β-galactosidase activity. The yeast strain EGY48 was transformed with plasmids pEG202 (bait) and pJG4-5 (prey) expressing *Drosophila*, *Podocoryne* and vertebrate bHLH fusion proteins. Values above a threshold of 15 Miller units are set in bold. Yeast cells expressing a LexA-GAL4 fusion protein (pSH17-4) had maximal activity levels (2102 ± 884). As a positive control for protein-protein interactions yeast cells expressing pEG-LAR.D1D2 and pJG-Trio.SIK (Debant et al., 1996) had activity levels of 275 ± 139. Yeast cells containing the empty pEG202 and pJG4-5 had background activity levels of 2.18 ± 0.44. Yeast cells containing pEG-bHLH bait plasmids and pJG4-5 vector had background β-galactosidase activity. (B) Summary of bHLH protein interactions investigated with the yeast two-hybrid system. Different symbols were chosen for the seven proteins tested and arrows indicate protein interactions, e. g. JellyD1 interacts with itself and with Da, Id, MyoD, Net-like and Twist while Ash interacts only with Da and Id.

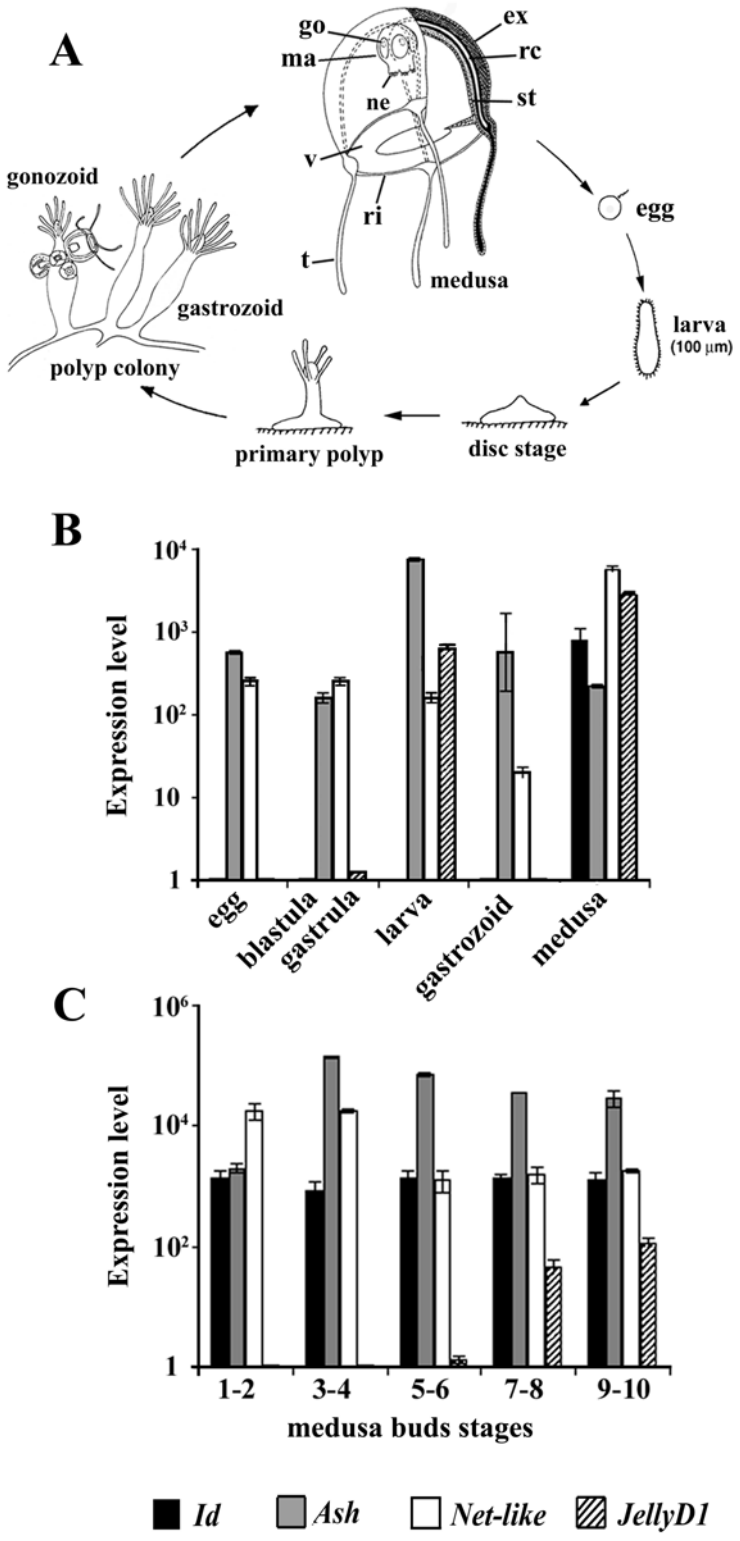

A
go
ma
ne
ex
rc
st
v
ri
t
medusa
egg
larva
(100 µm)
gonozoid
gastrozoid
polyp colony
primary polyp
disc stage
B
Expression level
10⁴
10³
10²
10
1
egg
blastula
gastrula
larva
gastrozoid
medusa
C
Expression level
10⁶
10⁴
10²
1
1-2
3-4
5-6
7-8
9-10
medusa buds stages
Id
Ash
Net-like
JellyD1

FIG. 3. (A) Life cycle of the hydrozoan *Podocoryne carnea*. Benthic polyp colonies consist of feeding polyps (gastrozoid) and polyps which asexually produce medusae by budding (gonozoid). The pelagic sexually mature medusa liberates gametes. The embryo develops into a free swimming planula larva which settles on the substrate (disc stage) to metamorphose into a primary polyp that is the founder of a new colony. Cross section of the medusa shows: ma, manubrium; go, gonads; v, velum; t, tentacle; ri, ring canal; rc, radial canal; ex, exumbrella; st, striated muscle. (B and C) Quantitative RT-PCR expression analysis of *Id* (black bar), *Ash* (grey bar), *Net-like* (white bar) and *JellyD1* (hatched bar) in the life cycle (B) and in medusa buds (C) of *Podocoryne* . The values are normalized to the expression level of *EF1a* and expressed in arbitrary units. Medusa buds were staged according to Frey (1968).

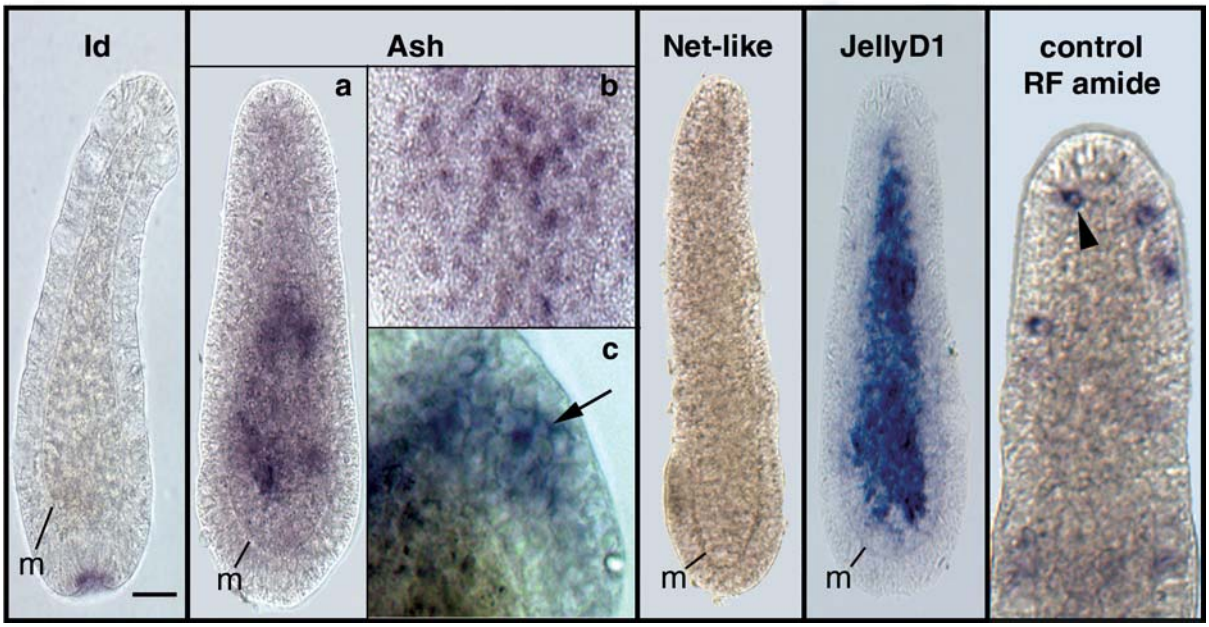
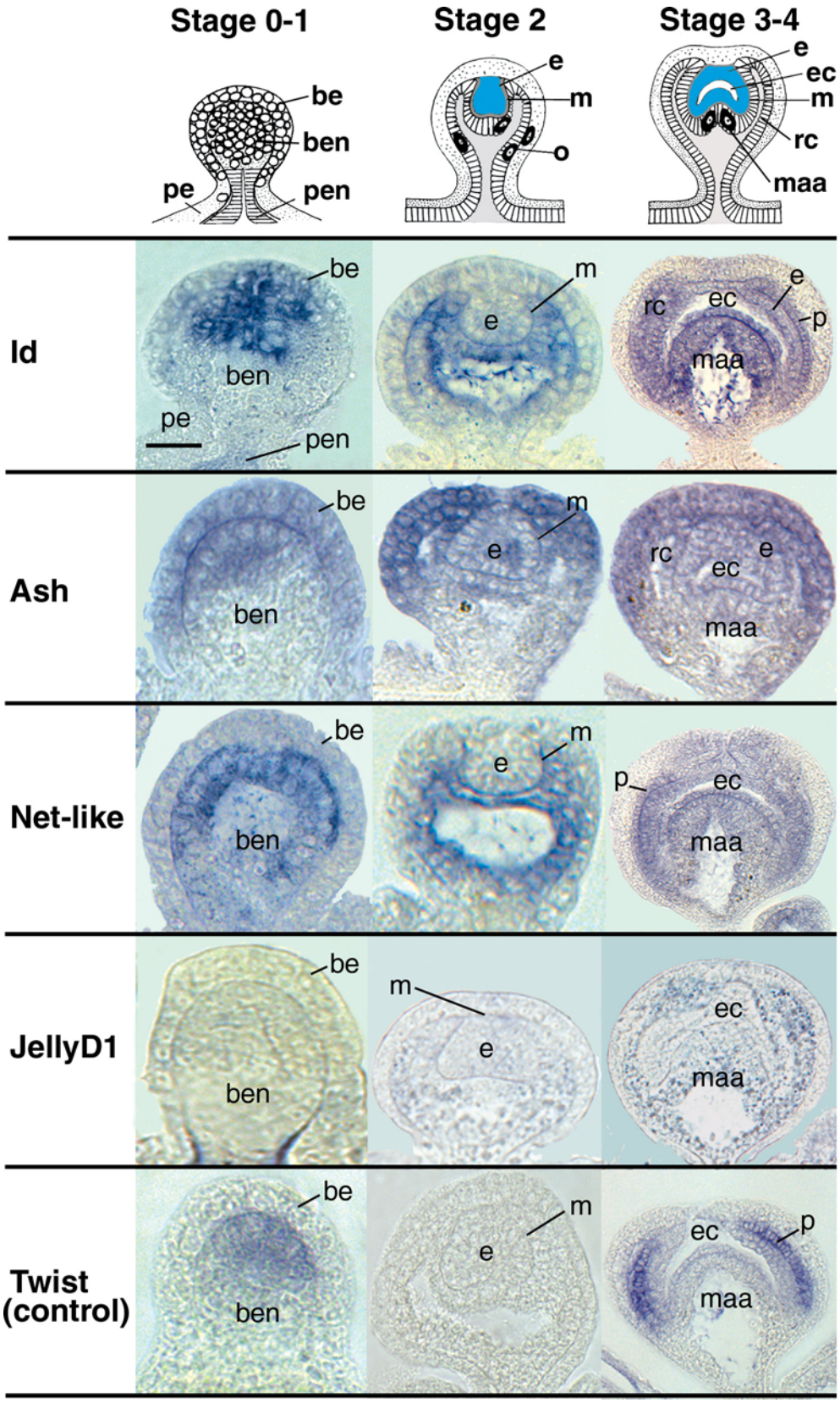


FIG. 4. Expression analysis in the planula larva of *Podocoryne.* In situ hybridization with anitsense RNA probes for *Id*, *Ash*, *Net-like*, *JellyD1* and *RFamide* (control). All larvae are oriented with the anterior pole to the bottom. *Id*, planula larva, 3 days; *Ash*, a. larva, 27 hrs, b. same after squeezing the specimen, c. cnidogenesis in tentacle bulb of medusa, arrow points to differentiating nematoblasts; *Net-like*, planula, 35 hrs; *JellyD1,* larva, 2 days. Control, planula, 35 hrs, stained with probe for *RFamide*-positive nerve cells (arrowhead). m, mesoglea separating ectodermis from endodermis. Bar in μm is 8 for *Id*; for *Ash* it is 8 in a, 20 in b, 20 in c; 8 for *Net-like*, 8 for *JellyD1* and 1.5 for *RFamide*.


Stage 0-1
Stage 2
Stage 3-4
be
ben
pe
pen
e
m
o
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rc
maa
Id
Ash
Net-like
JellyD1
Twist
(control)
p

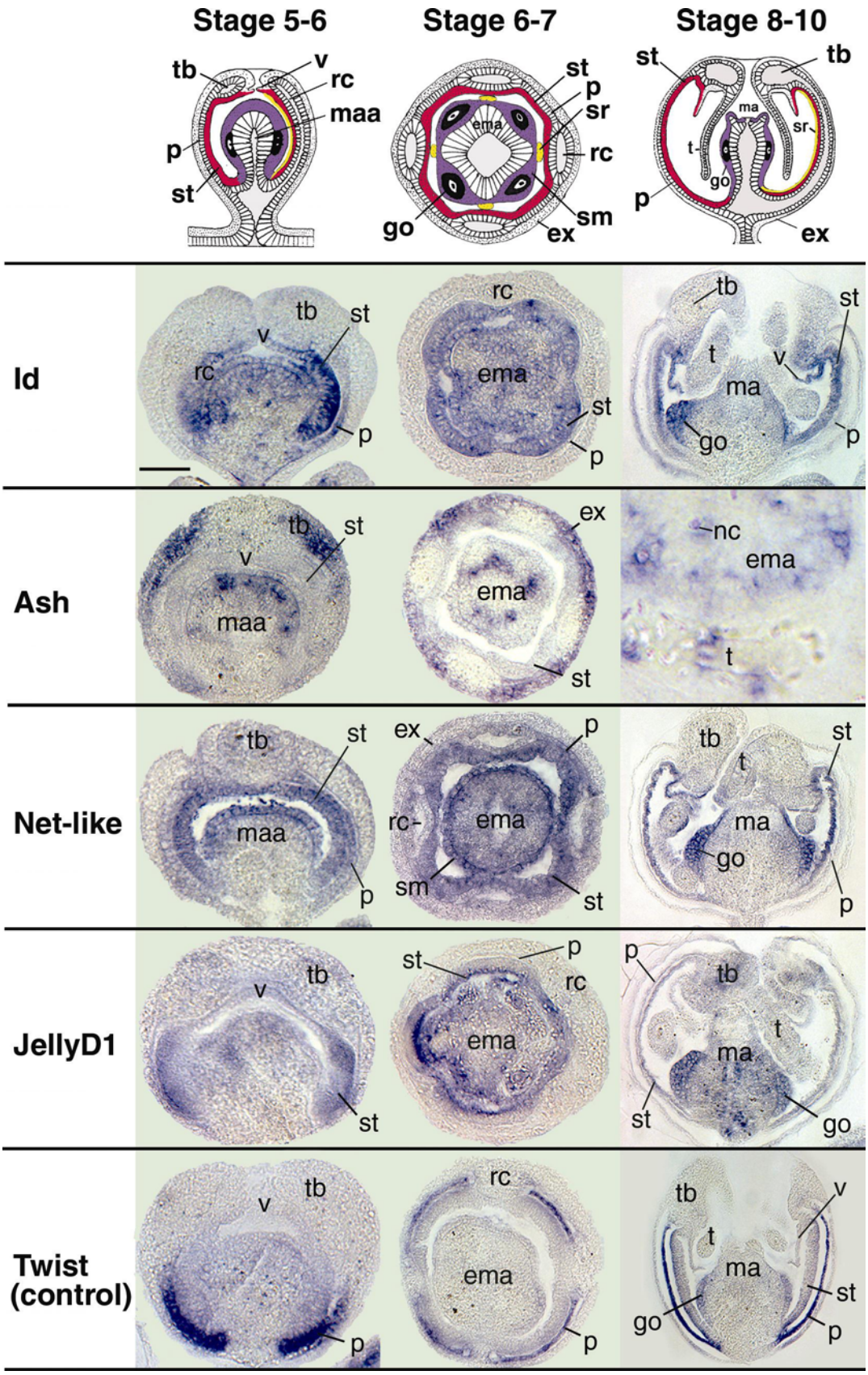
Stage 5-6
Stage 6-7
Stage 8-10
tb
v
rc
maa
p
st
ema
sr
go
ex
sm
ma
t
Id
Ash
Net-like
JellyD1
Twist
(control)
nc

FIG. 5. Expression analysis during medusa development in *Podocoryne. In situ* hybridization with antisense RNA probes for *Id*, *Ash*, *Net-like*, *JellyD1* and *Twist* (control). Medusa buds were staged after Frey (1968). Schematic drawings: Blue, entocodon; red, derivative of the entocodon which differentiates striated muscle; violet, derivative of the entocodon which differentiates smooth muscle of the manubrium; yellow, derivative of the entocodon which forms the smooth muscle over the radial canals; be, medusa bud ectoderm; ben, medusa bud endoderm; e, entocodon; ec, entocodon cavity; ex, exumbrella; go, gonads; m, designates mesoglea (ECM) separating entocodon tissues from ectoderm and endoderm; ma, manubrium; maa, manubrium Anlage; nc, nematoblasts/nematocytes in manubrium and tentacles of stage 8-9; o, oocytes; p, plate cells of the subumbrella; pe, polyp ectoderm; pen, polyp endoderm; rc, radial canal; st, striated muscle; t, tentacle; tb, tentacle bulbs; v, developing velum. Bars in μm and from top to bottom is for stage 0-1: 27 (Id), 25 (Ash), 28 (Net-like), 21 (JellyD1), 23 (Twist, control), correspondingly for stage 2 it is: 47, 43, 42, 36, 41, for stage 3-4 it is: 85, 69, 89, 72, 90; for stage 5-6 it is: 102, 112, 105, 103, 100; for stage 6-7: 125, 140, 122, 120, 133, and for stage 8-10 it is: 178, 32, 183, 174 and 210.

APPENDIX

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EXPRESSION NOTE

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Identification of the *Drosophila melanogaster* homolog of the human *spastin* gene

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Abstract The human SPG4 locus encodes the *spastin* gene, which is responsible for the most prevalent form of autosomal dominant hereditary spastic paraplegia (AD-HSP), a neurodegenerative disorder. Here we identify the predicted gene product CG5977 as the *Drosophila* homolog of the human *spastin* gene, with much higher sequence similarities than any other related AAA domain protein in the fly. Furthermore we report a new potential transmembrane domain in the N-terminus of the two homologous proteins. During embryogenesis, the expression pattern of *Drosophila spastin* becomes restricted primarily to the central nervous system, in contrast to the ubiquitous expression of the vertebrate *spastin* genes. Given this nervous system-specific expression, it will be important to determine if *Drosophila spastin* loss-of-function mutations also lead to neurodegeneration.

Keywords *Drosophila melanogaster* · Spastin · AAA domain · Embryonic CNS

Hereditary spastic paraplegia (HSP) comprises a heterogeneous group of neurological disorders. So far several loci, known as SPG loci, have been identified (reviewed in Casari and Rugarli 2001). The autosomal dominant form of hereditary spastic paraplegia (AD-HSP) is a neurodegenerative disorder characterized by progressive spasticity, mainly of the lower limbs. The disease in 40% of families with the pure form of AD-HSP is linked to SPG4 on chromosome 2p21-p22 due to mutations of a gene encoding *spastin*. This gene is expressed ubiquitously in fetal and adult human tissues (Hazan et al. 1999). The highest expression levels are found in the brain, with selective expression in the cortex and striatum. In the spinal cord, *spastin* is expressed exclusively in nuclei of motor neurons, suggesting that the strong neurodegenerative defects observed in patients are caused by a primary defect of *spastin* in neurons (Charvin et al. 2003). The human *spastin* gene encodes a predicted 616-amino-acid long protein and is a member of the large family of proteins with an AAA domain (ATPases Associated with diverse cellular Activities). When human *spastin* was identified, no clear invertebrate homologs were known, not even in the complete genome sequence of *Caenorhabditis elegans*; indeed there are a confusing number of similar sequences in humans, *C. elegans*, yeast and even prokaryotes.

With the availability of the first draft of the genome sequence of *Drosophila* (Adams et al. 2000) a predicted gene product, CG5977, with much higher sequence similarity to the human *spastin* than any other AAA domain protein, became evident. Our analysis revealed that the initial prediction, based on the genomic sequence contained errors, that a first version based on a partial cDNA clone (LD23843) was lacking the N-terminus, and that EST clones existed which appeared to extend the similarity with human *spastin* from the N- to the C-terminus. We sequenced one such clone, AT01057 (Fig. 1A). We also used this clone in order to prepare a probe for in situ hybridization. Subsequently AT01057 was also sequenced by the Berkeley *Drosophila* Genome Project. The two sequences are identical and code for a predicted protein of 758 amino acids with extended sequence similarity to human *spastin*, which is therefore called *Drosophila spastin* (*spas*).

Closer inspection of the *Drosophila* sequence and of the alignment of *Drosophila* and human *spastin* reveals, besides the two known highly conserved domains [namely the AAA domain (70% identity) of 300 residues, and the MIT (microtubule interacting and trafficking molecules, Ciccarelli et al. 2003) domain (55% identity)

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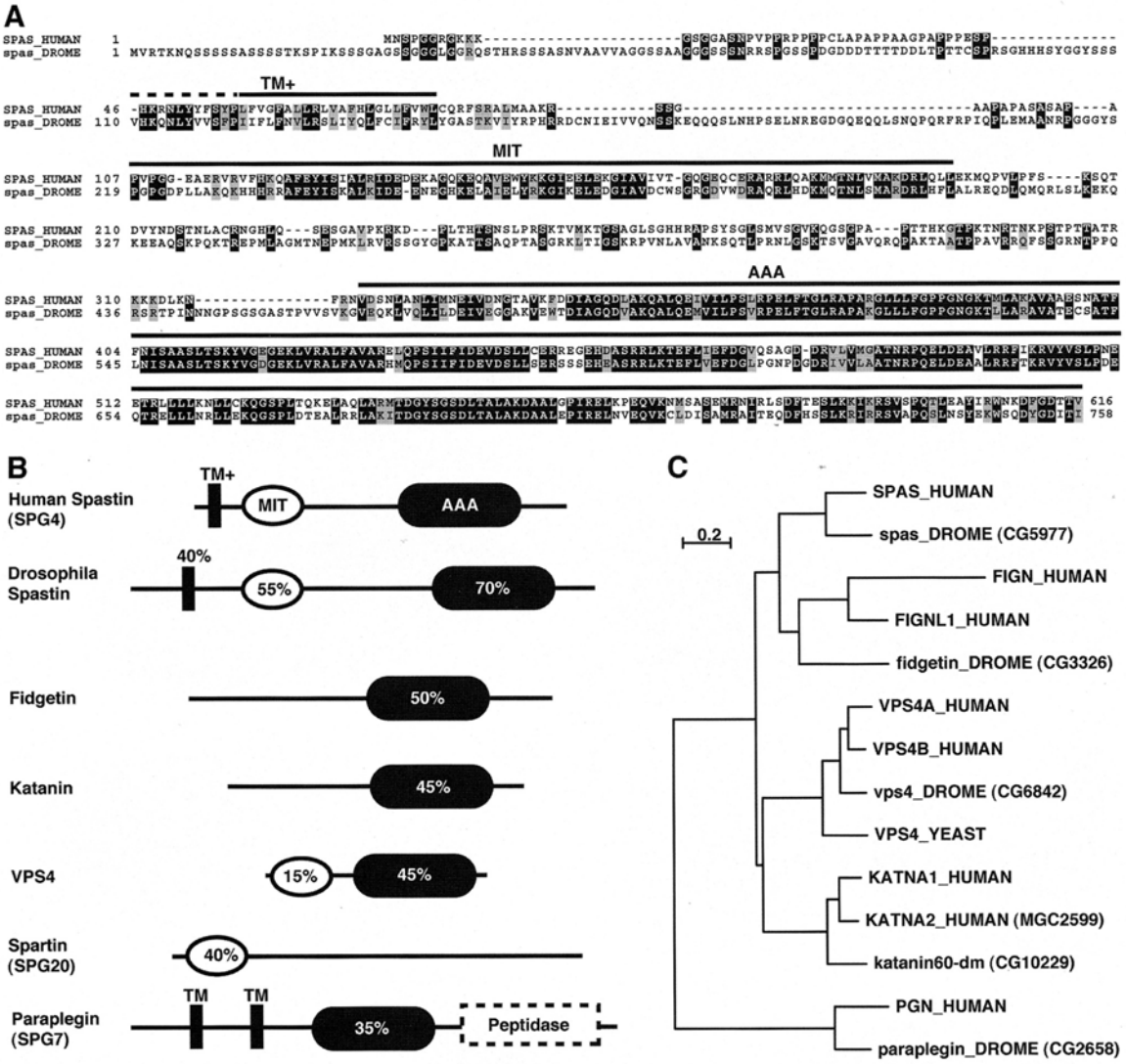

Fig. 1A–C Sequence analysis of *Drosophila* spastin. **A** Comparison of human and *Drosophila* spastin homologs reveals three regions of increased sequence conservation, the extended transmembrane domain *TM+*, the *MIT* (microtubule interacting and trafficking) and the *AAA* (ATPases associated with diverse cellular activities) domains, respectively. **B** Human, mouse and *Drosophila* spastin are the only proteins in current databases with precisely this domain structure. The fidgetin and katanin subfamilies belong to the AAA family, but lack transmembrane or MIT domains. The VPS4 subfamily lacks transmembrane domains. Spartin (*SPG20*) contains a MIT domain with 40% of sequence identity to spastin, but does not belong to the AAA family. Paraplegin (*SPG7*) belongs to a very different subgroup of the numerous AAA subfamilies with only 35% of sequence identity in the AAA domain with spastin and contains unrelated transmembrane domains and a peptidase domain. **C** A phylogenetic analysis based on the AAA domain of representative proteins reveals that spastins and fidgetins are most similar in the AAA domain, but not in the overall structure, while VPS4 proteins are less similar in the AAA domain although they contain a MIT domain and are highly conserved from yeast to humans. *Spastin* and *paraplegin* are single genes in their subfamily in *Drosophila* and humans, while *fidgetin*, *katanin* and *VPS4* are represented by two paralogs in humans

of about 100 residues], an additional conserved region of 33 residues near the N-terminus (Fig. 1A). All three domains are also recognized with SMART domain analysis (Letunic et al. 2002) as AAA, MIT and as a putative transmembrane domain, respectively. Since the similarity in the N-terminal domain extends beyond the hydrophobic core that could be a transmembrane domain, we call this a TM+ domain. This domain appears to be

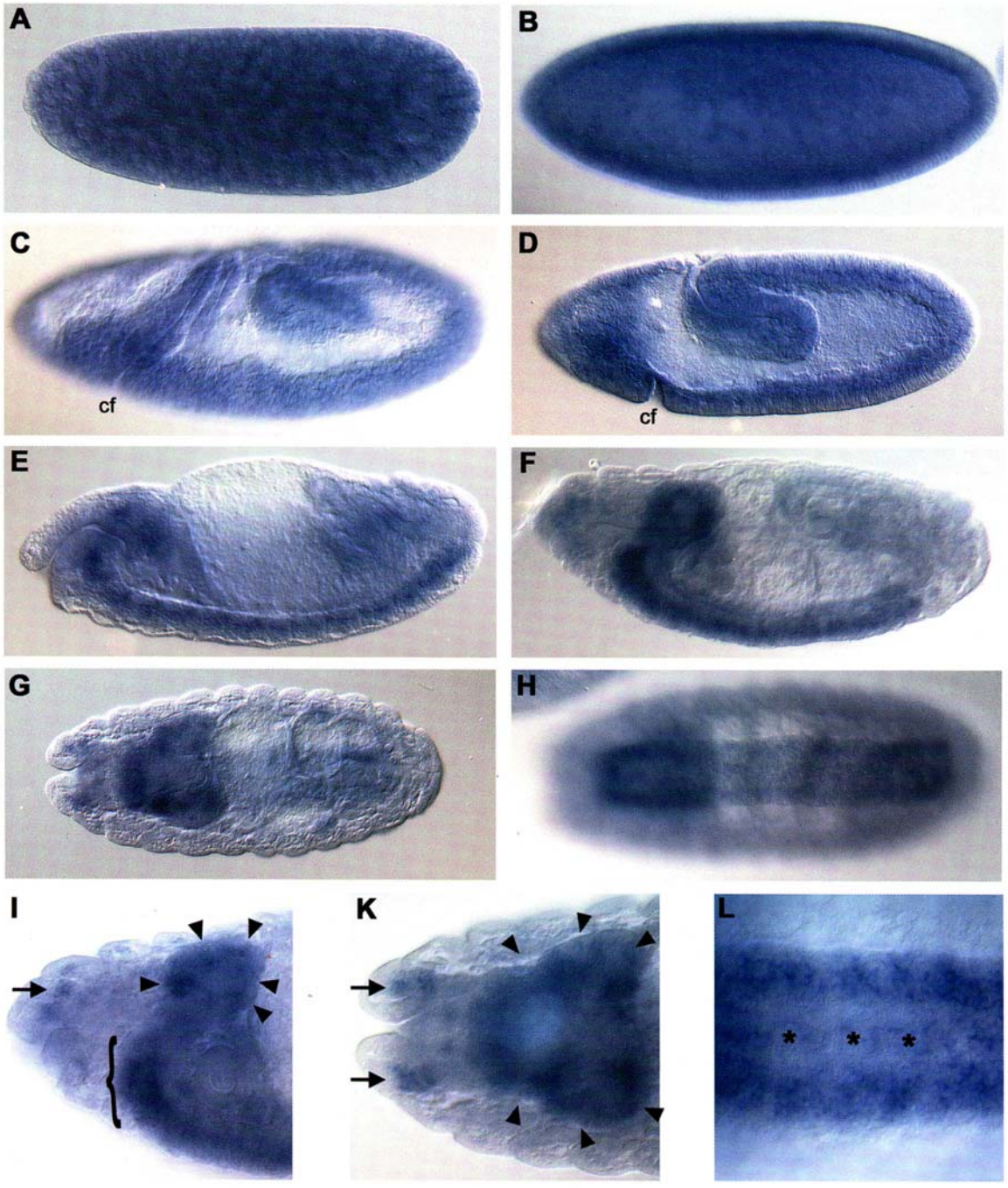

Fig. 2A–L Embryonic expression of *Drosophila spastin*. Whole-mount in situ hybridization of *spastin* transcripts in wild-type embryos. **A-F**, **I** Lateral views; **G**, **K** dorsal views; **H**, **L** ventral views, anterior is to the *left*. **A** Preblastoderm stage, **B** blastoderm stage, **C**, **D** stage 9, different focal planes, **E** stage 13, **F-L** stage 16–17 embryos; stages according to Campos-Ortega and Hartenstein (1997). **I**, **K**, **L** Enlarged views. *Arrowheads* indicate the expression in cell clusters of the supraesophageal ganglion; *bracket* expression in the subesophageal ganglion; *asterisks* expression in some midline cells of the VNC; *arrows* expression in some sensory head organs of the PNS

specific for spastin proteins. MIT domains, and to a comparable degree AAA domains, also appear in unrelated proteins; one was discovered in *spartin,* the gene responsible for SPG20 (Patel et al. 2002). The association of MIT and AAA domains, however, appears to be evolutionarily old since even the highly conserved vacuolar protein sorting 4 (VPS4) subfamily shows this domain structure. The members of the AAA family most closely related to spastin, however, are not VPS4 but members of the fidgetin subfamily (Fig. 1C), which may be of neurological interest because of the shaking phenotype in the fidget mouse (Cox et al. 2000). *Paraplegin*, the gene responsible for SPG7, however, is only distantly related to *spastin*, and the paraplegin protein is localized to the mitochondria and contains a peptidase domain (Casari et al. 1998; Fig. 1B, C). Since *spastin* was already shown not to be targeted to the mitochondria, but appears to be localized to the nucleus (Charvin et al. 2003), it will be important to test whether the potential transmembrane domain with the highly conserved neighbouring residues could be involved in localization of spastin to the nuclear membrane system. Other studies using expression systems of wild-type and mutated spastin with reporter sequences in different cell lines have suggested an involvement of spastin in microtubule dynamics (Errico et al. 2002).

The spatial and temporal expression patterns of *Drosophila spastin* were analysed by in situ hybridization. For this, the EST clone AT01057 that covers *spastin* was used to generate digoxigenin-labeled sense and antisense RNA probes (Tautz and Pfeifle 1989). These were hybridized to wild-type whole-mount embryos. Maternally contributed *spastin* transcripts characterized early embryogenesis with high expression levels seen until blastoderm stage (Fig. 2A). At the cell formation stage, expression was strongest near the basal part of the cell layer underlying the surface (Fig. 2B). During germband extension and stomodeal plate formation, expression was seen in the ventral head and trunk ectoderm, as well as in cells near the cephalic furrow and in the invaginating hindgut and midgut primordia (Fig. 2C, D). After germband retraction and delamination of neuroblasts at stage 13, transcripts were observed in subsets of cells in all neuromeres of the CNS including those of the supraesophageal and subesophageal ganglia (Fig. 2E). In later embryonic stages, marked expression of *spastin* was observed in cell clusters throughout the supraesophageal ganglion (Fig. 2F, G, I, K, arrowheads), with pronounced expression also seen in the subesophageal ganglion (Fig. 2I, bracket). In the ventral nerve cord (VNC), transcripts were seen in two broad longitudinal stripes located laterally, and weaker expression was observed in some midline cells (Fig. 2H, L, asterisks). In addition to expression in the CNS, *spastin* transcripts were also observed in some sensory head organs of the peripheral nervous system (PNS), most probably the Bolwig's organs and/or the dorsal organs (Fig. 2F, G, I, K, arrows).

Thus, during embryogenesis, expression of *Drosophila spastin* is mainly restricted to the central nervous system, in contrast to the ubiquitous expression of the vertebrate *spastin* gene (Hazan et al. 1999).

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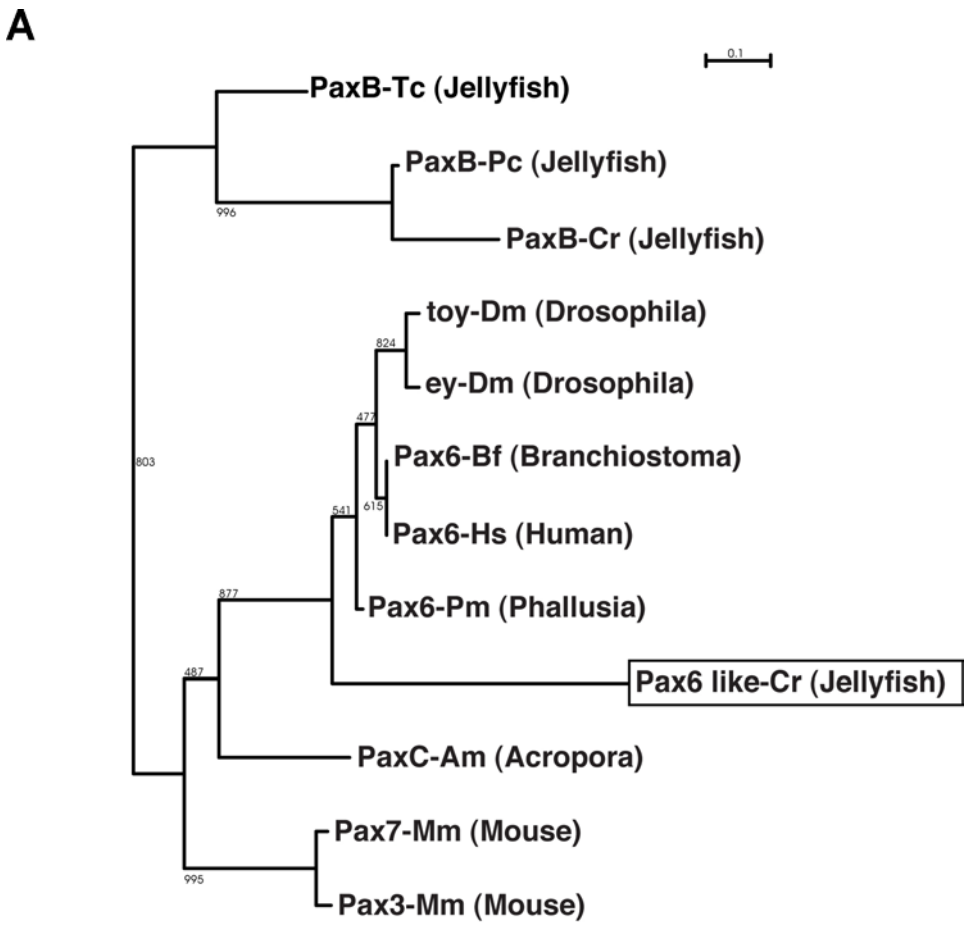
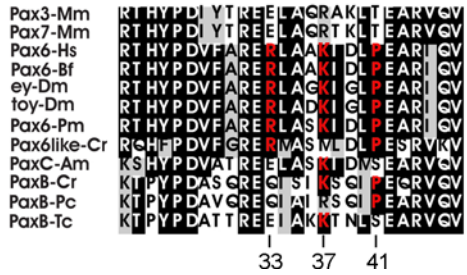
Pax6-like fragment of the HD of *Cladonema radiatum*

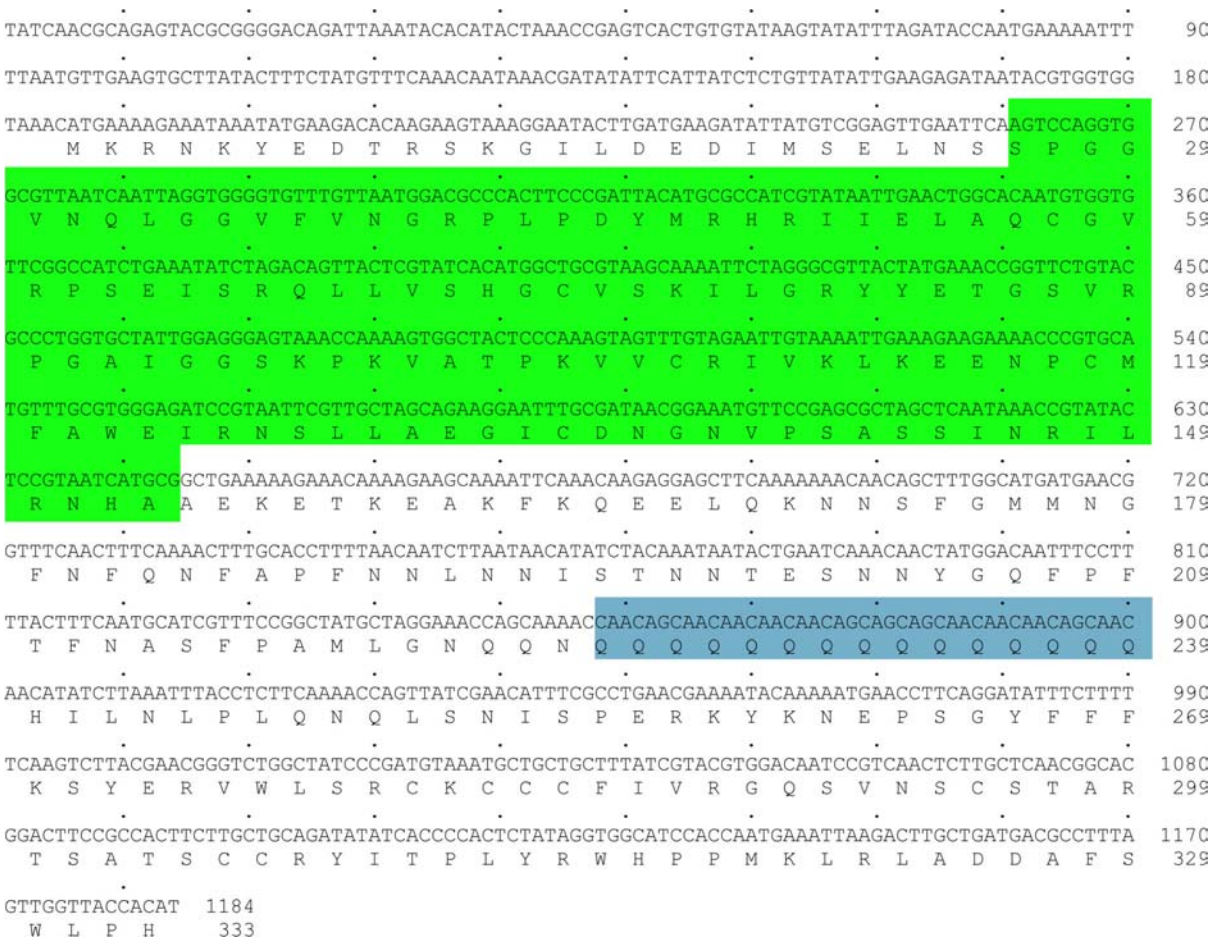


B



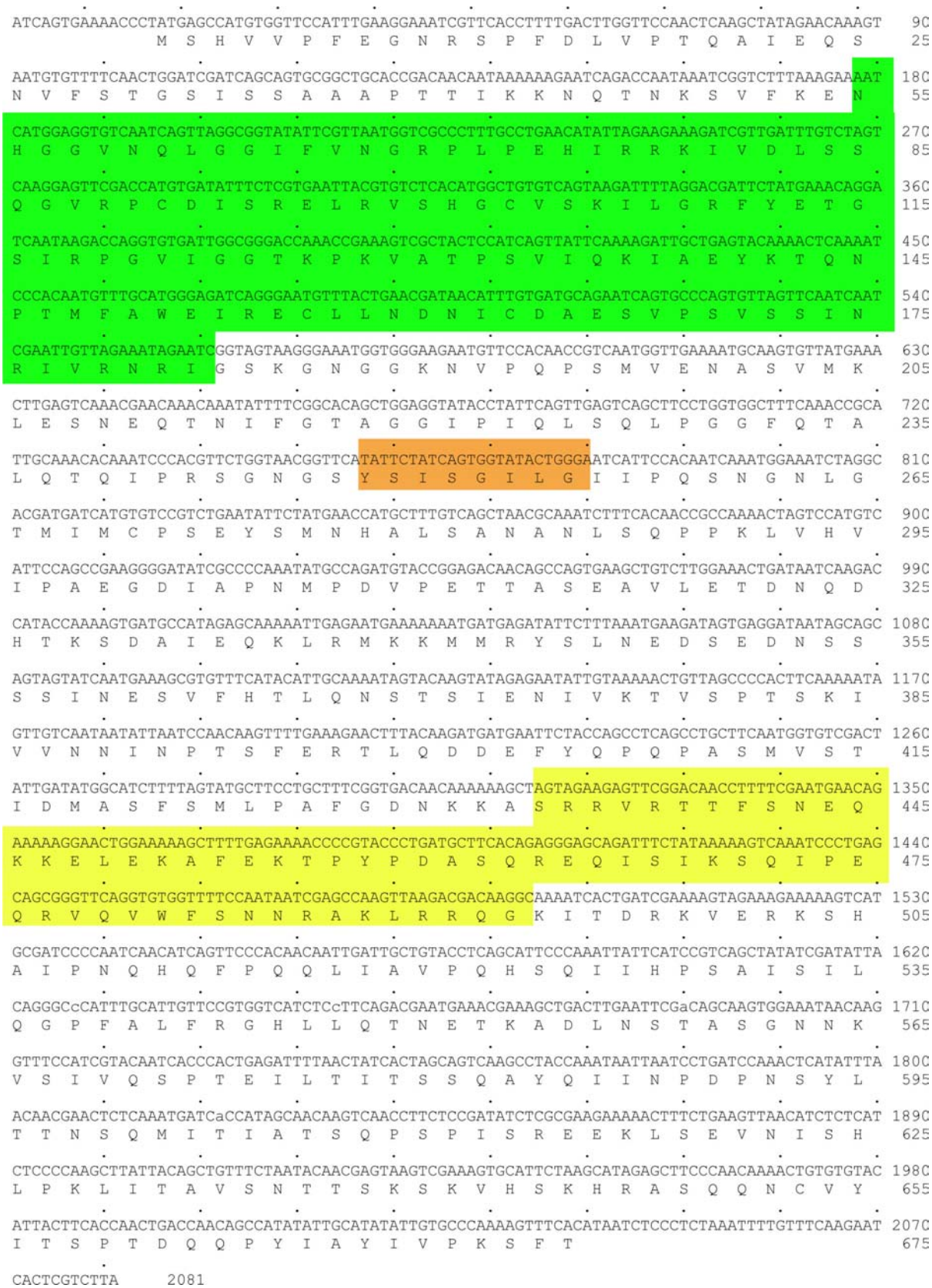
Phylogenetic analysis (A) and corresponding alignment (B) of a Pax6-like fragment of the HD of *Cladonema radiatum*. The *Cladonema* fragment groups clearly to the Pax4/6 subfamily (A). The Pax6 specific amino acids (in red) arginine at position 33 of the HD and proline at position 41 of the HD are present in the fragment. At position 37 of the HD the Pax6-like fragment of *Cladonema* has a methionine instead of the typical Lysine. Attempts to elongate the Pax6-like fragment failed.

PaxA-Cr* fragment of *Cladonema radiatum



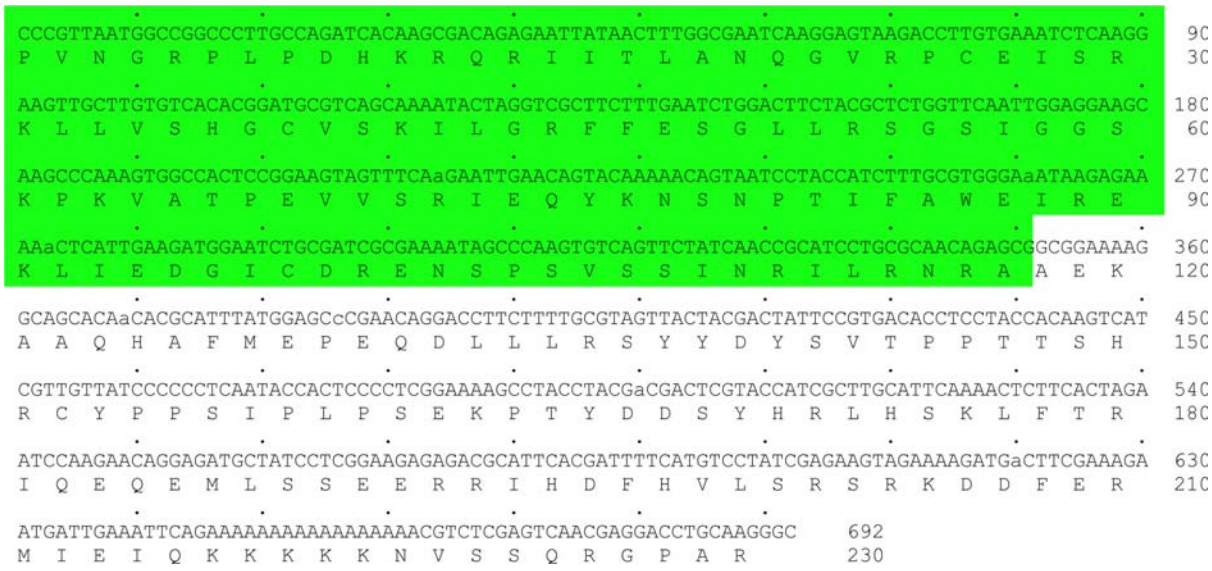
Sequence of the *PaxA-Cr* fragment from *Cladonema*. The PD is boxed in green and the glutamine amino acid stretch is boxed in blue (see chapter two for details).

PaxB-Cr* of *Cladonema radiatum



PaxB-Cr of *Cladonema* contains a full PD (boxed in green), an octapeptide (boxed in orange) and a HD (boxed in yellow) (for details see chapter 2).

PaxC-Cm* fragment of *Carybdea marsupialis



Sequence of the *PaxC-Cm* fragment of *Carybdea marsupialis*. The PD is boxed in green.

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Kammermeier, L., Spring, J., **Stierwald, M.**, Burgunder, J.M., Reichert, H., 2003. Identification of the Drosophila melanogaster homolog of the human spastin gene. Dev Genes Evol. 213, 412-415.

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