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Development of the larval peripheral nervous system in the ascidian *Ciona intestinalis*: role of the Retinoic Acid and FGF/WNT signaling pathways and of the POU transcription factors.

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Summary

ABSTRACT

The research presented in this document focuses on the development of the larval peripheral nervous system of the Tunicate *Ciona intestinalis* with two main aims: a) to understand how the interactions between Retinoic Acid (RA) and the FGF/WNT pathways control its development; b) to study the role played by transcription factors of the POU family in its differentiation. Within chordates, tunicates represent the sister group of vertebrates and their larvae have a typical chordate body plan. Notably, larval nervous system is formed by few cells whose organization mirrors that of vertebrates. For these reasons the species *C. intestinalis*, whose genome is completely sequenced, is a good animal model often used to understand the basic mechanisms of Chordate development. POU genes are an important family of transcription factors with several members that regulate the neural patterning and differentiation in both vertebrate and invertebrate embryos. *C. intestinalis* has only three genes coding for POU transcription factors: *Ci-POU-2, Ci-POU-IV* and *Ci-POU-like.* The gene *Ci-POU-IV* is specifically expressed in all peripheral nervous system (PNS) territories and in some cells of the central nervous system (CNS) during development. Since the expression of the two other genes was not previously studied in detail, a part of this research consisted in their characterization. Since the research team of Prof. De Bernardi previously described the existence of two alternative transcripts for *Ci-POU-IV*, the expression of the two isoforms was studied by *in situ* hybridization with isoform-specific probes. These showed that the short isoform is absent from the sensory epidermal neurones of the trunk. In order to study the role that the two isoforms play in neural differentiation, morpholino oligos were designed to perform isoform-specific knock-down experiments. The results from these experiments revealed that the expression of the serotonin rate-limiting synthesis enzyme, tryptophane hydroxylase (*Ci-Tph*), and of glutamate vesicular transporter (*vGluT*) in the PNS neurons could be regulated by the product of the long transcript. Moreover experiments were performed to understand the relationship between *Ci-POU-IV* and the Delta/Notch pathway. The latter was previously been shown to control the neural or the epidermal fate of the PNS precursors. The Delta/Notch pathway was inhibited both using DAPT, a pharmacological inhibitor of the γ–secretase enzyme, responsible of the correct functioning of the pathway and by electroporating the *pFOG::VeSu(H)DBM* construct that blocks the pathway activity. Embryos treated showed an abnormal development of epidermal sensory neurons and the following *in situ* hybridizations for *Ci-POU-IV* revealed an ectopic expression of the gene. I also used an *in silico* approach to search for *Ci-POU-IV* targets in order to identify the genes regulated by *Ci-POU-IV* during the PNS differentiation. The likely *Ci-POU-IV* binding site consensus sequences were inferred by bibliographic research of those known for the POU IV factors family in both invertebrates and vertebrates. These sequences have been used to build a matrix that was

employed to perform a bioinformatic research in the whole *C. intestinalis* genome with a software elaborated by the Lemaire team at the IBDML in Marseille. The search identified 19 possible targets of *Ci-POU-IV*; 8 regions, corresponding to 6 genes including *Ci-Tph*, have been preliminary selected. The activity of the selected regions is being evaluated.

The second part of the thesis has been developed during a period of research with Dr. A. Pasini at the Institut de Biologie du Développement de Marseille Luminy. The aim was to understand whether RA and the FGF/WNT pathways control antero-posterior patterning of PNS during *C. intestinalis* embryonic development. In vertebrates it was already known that during the antero-posterior extension of the body axis these pathways antagonize each other to coordinate mesoderm segmentation and nervous system patterning and differentiation. For this reason the hypothesis that an analogous mechanism could occur in other Chordates, including tunicates was tested. Thus in *C. intestinalis* I performed *in situ* hybridizations for different genes involved in these pathways. In particular results showed that the expression of the gene *Ci-Raldh* that codes for Retinaldehyde dehydrogenase, the enzyme responsible of RA synthesis, is confined to the anterior part of the tail in tailbud stage embryos. On the contrary, at the posterior extremity of the tail it is predictable the existence of a source of FGF and WNT signals as shown by the expression of *Ci-FGF-8* and *Ci-Wnt-5*. Moreover, embryos treated with RA showed up-regulation of the anterior marker *Ci-Hox-1* throughout tail epidermis and the inhibition of the posterior *Ci-Hox-12* expression. On the contrary, embryos treated with FGF showed an opposite situation with *Ci-Hox-12* activation and *Ci-Hox-1* inhibition. Moreover, quantifications of differentiated caudal epidermal neurons and meticulous analysis of their position along the tail have been performed in late stage embryos treated with RA, FGF, their respective inhibitors and an inhibitor of the enzyme *Ci-Cyp26*, responsible for RA catabolism. This showed significant alterations in both the number of neurons and their position. In particular RA treatment increased the overall number of caudal epidermal neurones but caused the loss of the most posterior ones; on the contrary FGF treatment induced a decrease in the number of neurons but maintained the posterior ones. Treatment with FGF and a *Ci-Cyp26* inhibitor mimicks the effects of RA while treatment with RA synthesis inhibitor mimicks the effect of FGF.

1. Introduction

1.1 Ascidians: an attractive model organism to study Chordate development.

Ascidians or sea-squirts are marine organisms that, when adults, are sessile filter feeders (Figure 1.1 A, B, C, D). They are a member group of the Urochordates, or Tunicates, and as such they are characterized by a protective cellulose-based structure that represents a feature unique in the animal kingdom. This structure is called tunic, is composed principally by a polysaccharide named tunicine and shows the capability to grow together with the animal. In spite of their highly derived adult morphology, ascidians have a larval development that is akin to vertebrate one, showing a prototypical chordate body plan (Figure 1.1 D). Moreover, current molecular analyses positioned tunicates as the sister group of vertebrates (Delsuc et al. 2006, Bourlat et al. 2006), replacing in this position the cephalochordates. The exact phylogenetic relationship between ascidians and other members of the chordate lineage is since years a topic of debate (Lacalli, 2004) being from time to time influenced by new biological discoveries and theories. For example a long-held view (Garstang, 1928), borne after the first descriptions of neoteny in amphibians, was that chordates, and so vertebrates, arose by neoteny along with cephalochordates from sessile, ascidian-like ancestors; there are also more parsimonious view, that the ancestral chordate was a motile animal, similar to the larvae of present-day ascidians (Wada and Satoh, 1994). Although previously a rival view held that cephalochordates were closer to the ancestral chordate condition (Holland and Holland, 2001; Lacalli, 2004), a recent analysis of nuclear gene sequences indicates that tunicates are closer to vertebrates than other basal chordates ancestral forms (Delsuc et al., 2006). Also, tunicates possess neural crest cells (Jeffery et al., 2004) and placodes (Manni et al., 2004; Mazet et al., 2005) that are lacking in cephalochordates.

Ascidian ancestors date from at least the early Cambrian (Shu et al., 2001), and, among ascidians, the family of Cionidae belongs to the most ancient group (Berrill, 1936; 198; Swalla et al., 2000), making the cosmopolitan species *Ciona intestinalis* a particularly valuable model organism (Dehal et al., 2002) for studies of chordate evolution (Zeng and Swalla, 2005), development and neurobiology (Meinertzhagen and Okamura, 2001; Meinertzhagen et al., 2004). Notably, *Ciona* embryos are made of few cells, develop quickly and according to a fixed lineage.

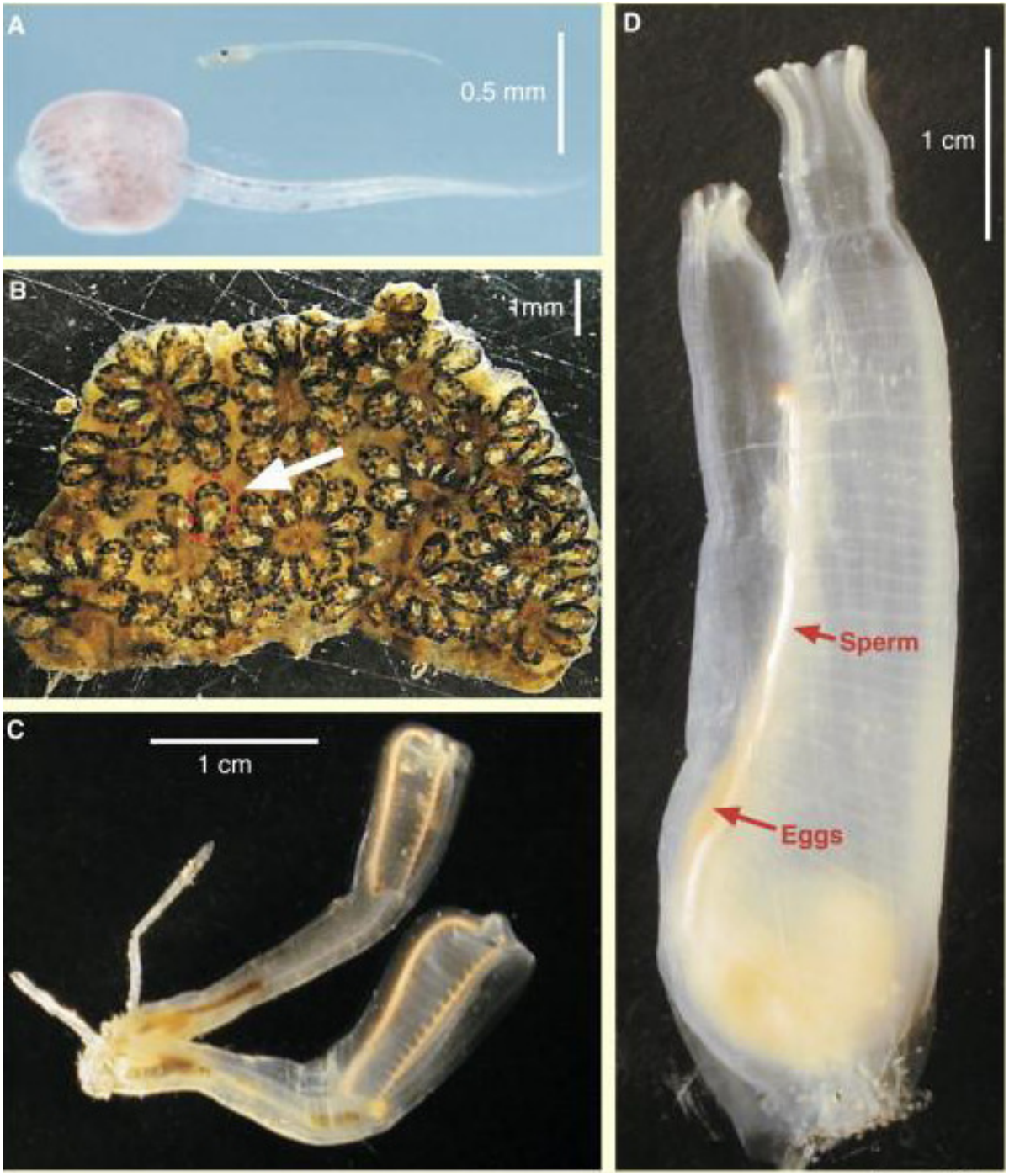

Figure 1.1: Examples showing the diversity of ascidian morphology during development. A: larval morphology of two ascidian species, including *Ciona intestinalis* (up). B: an example of a colonial ascidian species, *Botryllus schlosseri*. C and D: examples of the adult ascidian morphology. Notice the difference between larval and adult form of *Ciona intestinalis* (A (up) and D) (From Lemaire et al. 2009).

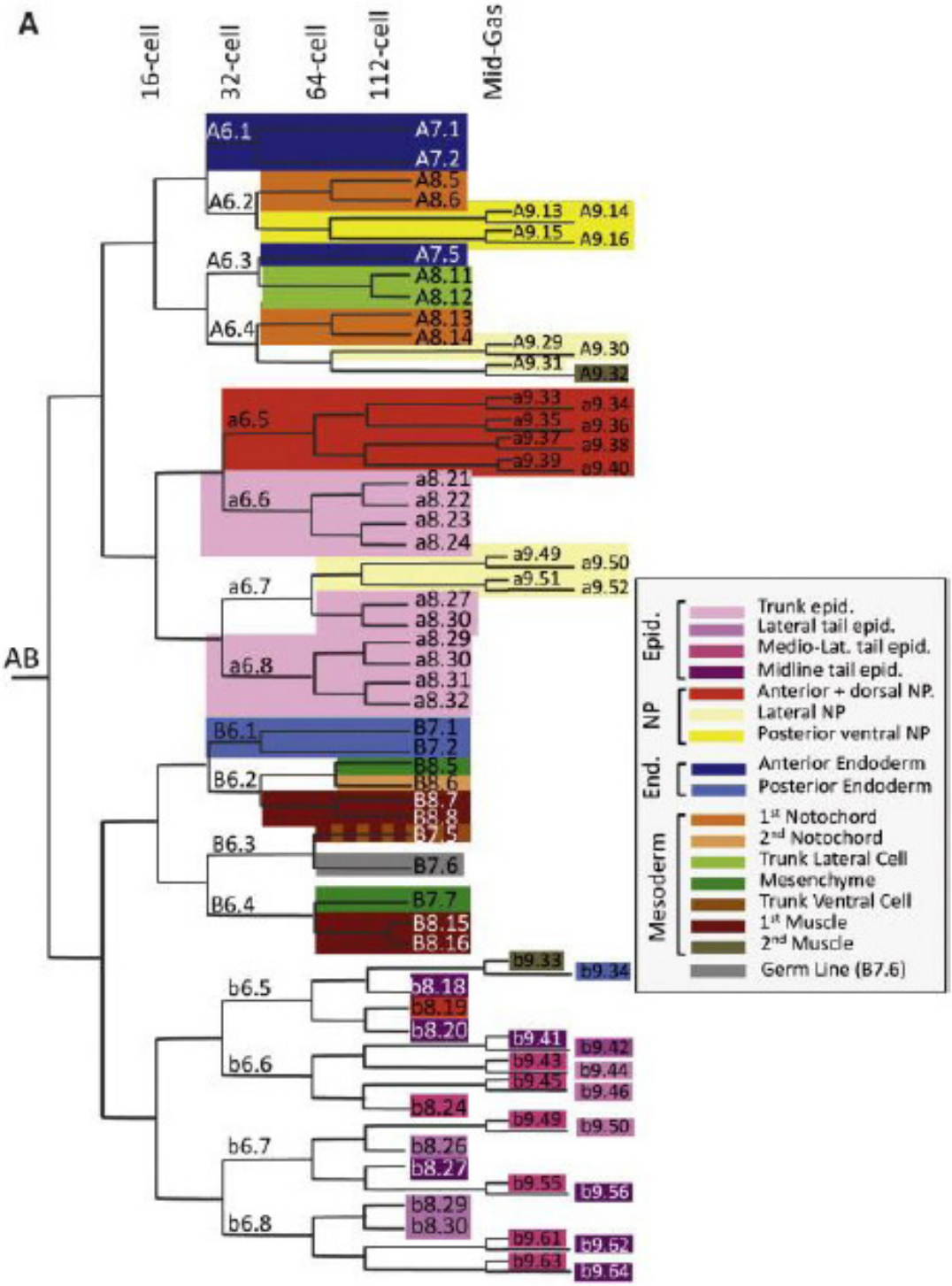

Figure 1.2: Ascidian invariant lineage until early gastrula. Colors represent the fates that each blastomere will adopt. Note that already at this stage most cells have determined their future fate. (from Lemaire, 2009).

The fact that Ascidians have a large number of eggs (which, once fertilized, develop rapidly), together with the easy access to animals (which are hermaphrodites) turned ascidians into an attractive model to study developmental processes right from late 19th century (Chabry 1887). The small number of cells and invariant cleavage pattern characteristic of ascidian embryos enabled a very precise lineage characterization (Figure 1.2).

In spite of such simplicity, ascidians maintain similarities to vertebrates, both morphologically and molecularly. This is particularly relevant when we consider a complex tissue such as the nervous system, which in *C. intestinalis* can be seen as a miniaturized version of the vertebrate nervous system (Bertrand et al. 2003) (Figure 1.3). Initial experiments with ascidian embryos led the first researchers to formulate concept of the mosaic theory of development, of which ascidians were, until recently, considered a perfect example. According to this theory, all tissues develop independently of each other, as a result of the precise inheritance of localized maternal determinants. Recent studies disputed this notion and revealed a primordial role, even in the "simple" ascidians, for cell-cell signaling in embryonic patterning (see Lemaire 2009 for a review). The fixed lineage is now being interpreted as a mechanism that allows the precise positioning of competent cells with respect to signaling sources, in addition to precisely controlling the inheritance of maternal determinants.

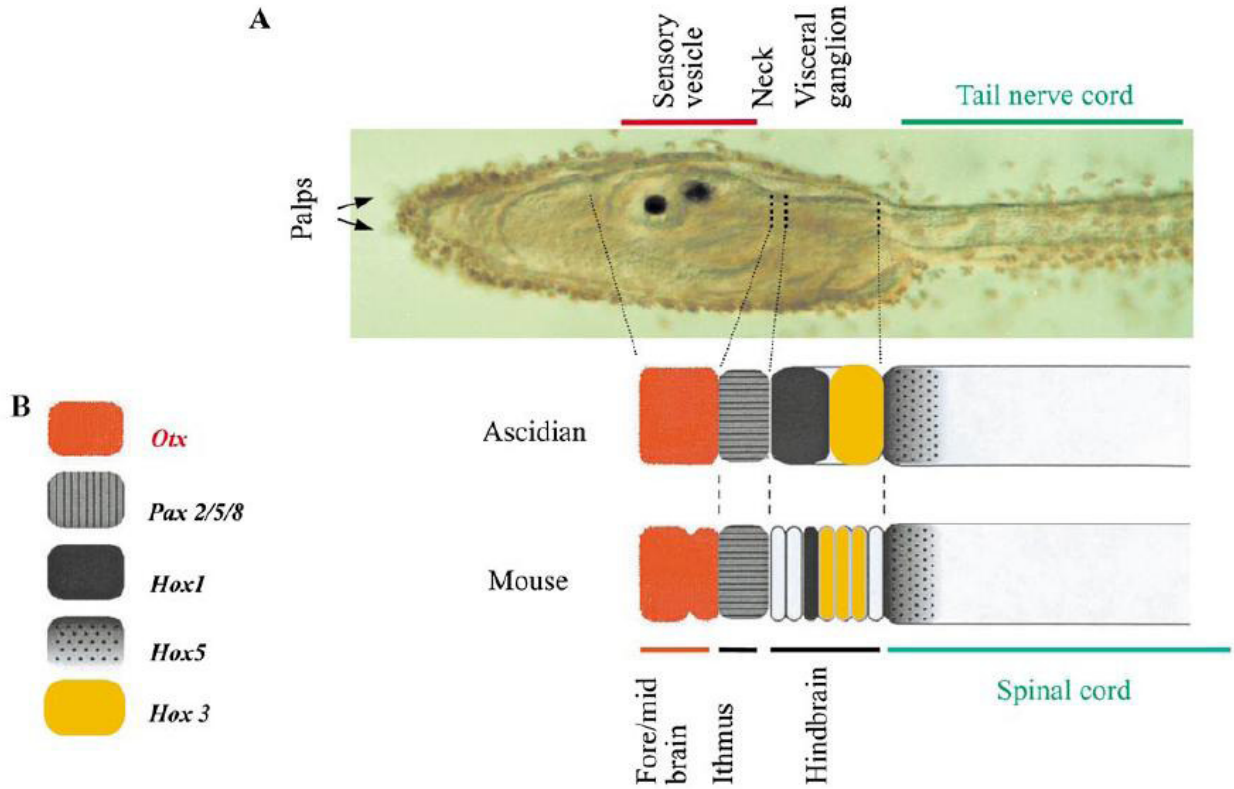

Figure 1.3. Homologous territories in the *Ciona intestinalis* and vertebrate CNS (from Lemaire et al., 2008).

Thus, ascidians have long appealed to the researchers as a simplified chordate model for developmental studies. Although the initial fervour surrounding ascidians was mostly lost in the middle of the XXth century, recent data brought again ascidians into the limelight of research in developmental biology. One important step for the revitalization of ascidian research was the sequencing of the *Ciona intestinalis* genome (Dehal et al. 2002) followed by the genome of its relative, *Ciona savignyi* (Small et al. 2007). The availability of the relatively small and compact *Ciona* genomes, combined with the emergence of powerful molecular tools (such as DNA electroporation to create transient transgenics, and morpholinos for loss-of-function studies), more than justify the recent return of ascidians as an attractive model system (Corbo et al. 2001).

Although ascidians and chordates have a very similar larval body plan, this may be achieved using very different strategies, as many differences exist, even during larval development. For example, ascidians are characterized by a limited number of cells with an invariant lineage, which contrasts with the highly variable lineages in vertebrates. Furthermore, there are no traces of an organizing center in ascidians (Kourakis & Smith 2005), which is a fundamental structure required for vertebrate development, especially for the establishment and patterning of the chordate body plan (Lemaire & Kodjabachian 1996). Finally, important differences are also observed at the genomic level. In spite of conserving most gene families, the compact ascidian genome has lost many individual genes important for vertebrate development (Holland & Gibson-Brown 2003). Furthermore, *Ciona* has also lost (or maybe never acquired) the highly conserved regions that are shared throughout the vertebrates (Vavouri & Lehner 2009), as well as most gene synteny (e.g. the Hox cluster (Duboule 2007)). Thus, an important question remains: do these differences predominate, suggesting globally distinct genetic programs of ascidians and vertebrates with only a limited set (probably inconspicuous) of commonalities that are able to generate a similar body plan, or is there a clear common chordate program?

Ciona intestinalis is a very appealing biological model for the study of different aspects of developmental biology of early Chordates; the great availability of a large number of embryos that develop synchronously and rapidly permits the planning of medium/large-scale experiments that can consider different aspects of gene regulation and expression during development. Furthermore, *C, intestinalis* has a relatively compact genome that has not experienced the multiple large scale genome duplications found in vertebrates (Dehal and Boore 2005). Since the major players in gene regulatory networks are transcription factors and signalling transducers, a detailed cataloguing of these genes was carried out, along with the careful analysis of their precise spatial and temporal expression profiles throughout embryonic development (Imai et al. 2004, Miwata et al. 2006). In subsequent work, the group of Y. Satou (University of Kyoto) has thoroughly analysed the effects of the knock-down of a set of relevant genes (regulatory genes that had restricted expression profiles during early organogenesis) on a broader set of regulatory genes. This mid-scale study led to the first initial blueprint of the global regulatory network underlying early development of a complete metazoan (Imai et al. 2006) (Figure 4).

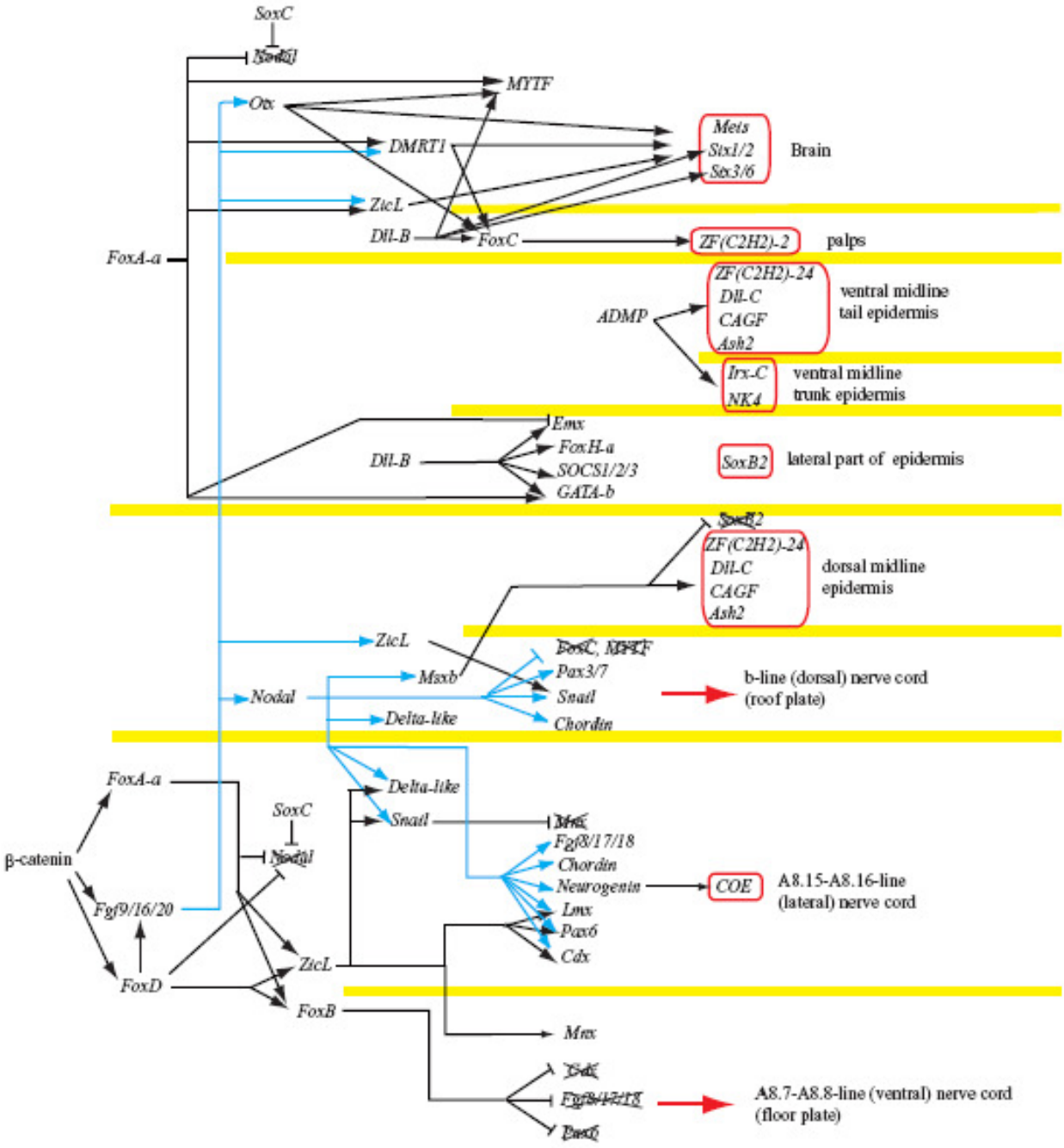

Figure 1.4. Regulatory blueprint of a chordate embryo (Imai et al. 2006).

1.2 An overview of the larval central and peripheral nervous systems in Ciona.

1.2.1 Morphological features

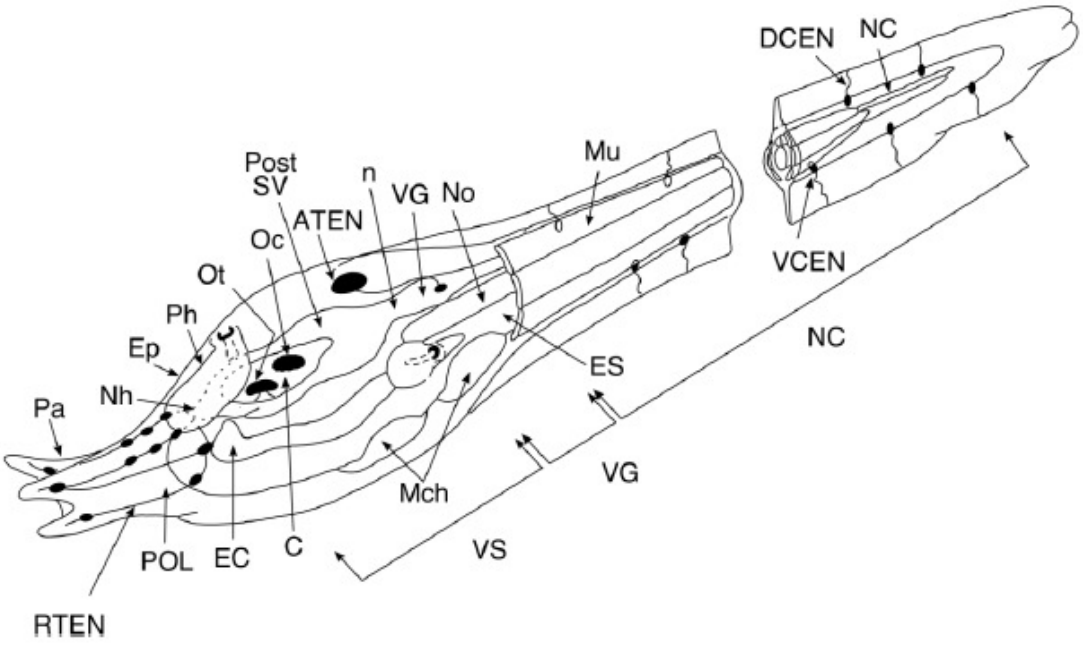

Fig. 1.5 Central and peripheral nervous system of a *C. intestinalis* larva (from Imai & Meinertzhagen 2007).

The larval CNS in *Ciona* arises from a neural plate that rolls up to form a dorsal neural tube and undergoes neurulation (Nicol and Meinertzhagen, 1988; Cole and Meinertzhagen, 2004). The entire complement of the larval CNS is approximately 330 cells, and this appears fixed, at least among sibling larvae (Nicol and Meinertzhagen, 1991).There are an additional 40 or so cells in the neurohypophysial duct (Willey, 1893; Nicol and Meinertzhagen, 1991), the first rudiment of the adult neural complex (Mackie and Burighel, 2005). Among the cell complement, about 100 cells have been predicted to be neurons (Nicol and Meinertzhagen, 1991). The CNS is essentially an epithelial tube with three subdivisions (Fig. 1). The sensory vesicle has two conspicuous pigment granules, one associated with the otolith, a presumed gravity-sensing organ (Torrence, 1986) in the left anteroventral wall of the vesicle, the other with the organ of light detection (Tsuda *et al*.,

2003b), the ocellus in the right wall. The ocellus comprises three lens cells, a pigment cell and photoreceptors (Barnes, 1971), about 18 in number (Nicol and Meinertzhagen, 1991). The visceral ganglion may function as the integrative center of the nervous system, receiving inputs from various sensory structures and initiating swimming behavior (Meinertzhagen and Okamura, 2001). Somata of the motor neurons are located in this ganglion, five pairs in *Ciona*, with axons forming the ventrolateral nerve bundles that project into the tail (Stanley MacIsaac, 1999). Finally, the caudal nerve cord comprises four longitudinal rows of ependymal cells—dorsal, ventral, and two lateral rows—enclosing a neural canal (Nicol and Meinertzhagen, 1991). The ventrolateral nerve bundles between each lateral row and the overlying caudal musculature contains progressively fewer axons as distance increases along the tail, such that no axons exist at its most distal tip (Crowther and Whittaker, 1992). With so few neurons, and the possibility that these are fixed in number and composition (Meinertzhagen, 2005), it should be possible to characterize individual cell types uniquely by a combination of morphological and other criteria and to map their contributions to neural circuits, as in other model species, such as *C. elegans* (White et al., 1986) or the circuits of the *Drosophila* visual system (Meinertzhagen and Sorra, 2001).

The neurons of the PNS are embedded within the larval epidermis (Takamura, 1998; Pasini et al., 2006) distributed as an epithelium over the trunk and tail, most with cilia that project into the tunic. In Ciona and another widely studied species, *Halocynthia roretzi*, these neurons arise from the epidermal cell lineage (Ohtsuka et al., 2001; Pasini et al., 2006), thus differing from the neurons of the CNS, which arise from the neural plate (Cole and Meinertzhagen, 2004). Secreted by the epidermis, the transparent tunic, a filamentous matrix of tunicin, ensheathes the entire larval body, flaring out as dorsal, ventral, and caudal tail fins (Burighel and Cloney, 1997). It is populated by test cells of obscure function, which remain active during the life of the swimming larva. Rostrally, the epidermis covers three bulbous adhesive papillae, which serve to attach the larva to the substrate (Cloney, 1978; Svane and Young, 1989). Various categories of PNS neurons are reported to be immunoreactive to the antibody UA301 that recognizes an uncharacterized epitope.

PNS neurons include the neurons of the adhesive papillae and three groups of ciliated neurons, which probably have a mechanosensory function. This neurons are indicated as RTEN (rostral trunk epidermal neurons) with at least three pairs of neurons situated in the dorsal epidermis between the papillae and the sensory vesicle of the CNS; ATEN (apical trunk epidermal neurons), positioned dorsally to the posterior region of the sensory vesicle and finally the CEN (dorsal and ventral caudal epidermal neurons), along the dorsal and ventral midlines of the tail (DCEN and VCEN, respectively) (Takamura, 1998; Pasini et al., 2006; Imai and Meinertzhagen 2007; Horie *et al*., 2008).

Crowther and Whittaker (1994) described at least 10 corresponding pairs of DCEN and VCEN. Pasini et al., (2006) showed that specification from the midline embryonic epidermal cells takes place in two successive steps. These involve different molecular mechanisms and will be recalled in the following chapter.

The classic study of Torrence and Cloney (1982) showed that the axons of the CENs contribute to the afferent nerve bundles below the tail epidermis, which project rostral to the visceral ganglion of the CNS. The ventral nerve makes a spiky upward turn at the level of the trunk–tail junction and enters the visceral ganglion adjacent to the dorsal nerve. Some of the CESNs anyway send relatively long axons toward the sensory vescicle.

Identifying the caudal epidermal sensory neurons (CESNs) constituting the PNS in the tail is quite easy thanks to their long projections that enter dorsally and ventrally the fin tunic; these projections are positive to β-tubulin and in the distal portion extend along the fin outline and are exposed to the seawater (Pasini et al., 2006). The cell body of every CESN is embedded in the epidermis and has a thin apical surface where the projections start. Pasini et al., (2006) found that the CESNs are always positioned as pairs with the exception of the most posterior one in the ventral tip of the tail. Also the identification of the nuclei of every pair is easy since these nuclei are smaller that those of the surrounding epidermal cells and lie always along a line that in every case derive from the embryo anteroposterior axis.

In general Pasini et al. (2006) in the larvae of *C. intestinalis* found a minimum of 8 to a maximum of 20 pairs of caudal epidermal sensory neurons with an average of 14 pairs. These are positioned along the tail at more or less regular intervals and intercalated by three to five epidermal cells with large nuclei.

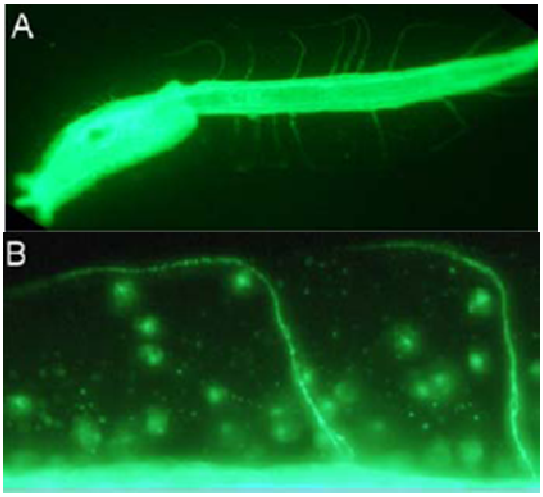

Figure 1.6. The two images (A and B) show the projections of the CESNs extending outside the tunic fin (from Pasini et al., 2006).

1.2.2. Gene network involved in PNS determination

A great number of intensive studies have looked in these decades for the developmental origins of the peripheral nervous system of both vertebrates and invertebrates. In vertebrates the origin of most sensory neurons of the PNS from the neural crest and the placodes is well known. They derive from the ectoderm situated at the borders of the neural plate. Their development is controlled by complex gene regulatory networks that control different developmental steps. These signals include bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Wnt, and Notch (Litsiou et al., 2005). Placodes and neural crests are usually thought to be innovations typical of vertebrates with a very important role in the evolution of this clade. As a matter of fact, the acquisition of the new structures derived from neural crests and placodes is a major point for the development of the typical active predatory lifestyle of vertebrates. The generally accepted theory is that these two structures take their origin from some cell populations that were already present in the protochordate ancestor. In protochordates the migratory ability or pluripotency of these cells is debated (Jeffery et al., 2008) .

The ascidian dorsal midline tail epidermis expresses homologues of neural plate border markers but not homologues of either neural crest or placodes specific genes; furthermore it gives rise to sensory neurons. Thus it has been proposed to be an evolutionary precursor of the neural crest (Meulemans & Bronner-Fraser, 2005).

Pasini and coworkers (2006) showed that dorsal and ventral midline regions are specified by Fgf 9/16/20-Nodal and Admp signals, respectively, and that the fate of the CESN precursors is

controlled by the Delta/Notch pathway. In particular, the role of Nodal has been further investigated by Mita & Fujiwara (2007) who showed its role in regulating the formation of the neural tube. About the Delta/Notch pathway Pasini *et al*. (2006) showed that it negatively controls the number of midline cells that differentiate as CESN. Moreover, the inverse relationship between the number of CESNs and epidermal cells in the midline domains strongly suggests the existence of a Notch-mediated lateral-inhibition phenomenon responsible for preventing excess midline cells from adopting a CESN fate.

Satou *et al*. (2001) performed a comprehensive analysis of gene expression profiles in *Ciona* tailbud embryos and revealed 34 genes that are expressed exclusively in the nervous system.

Currently there are at least 54 genes. expressed in the PNS of the ascidian *Ciona intestinalis* (Data from ANISEED http://aniseed-ibdm.univ-mrs.fr/). In their large scale determination of the genes specifically expressed in the larval nervous system of *C. intestinalis* Mochizuki et al., 2003, identified 56 genes, 25 of which are expressed at the larval stage. In particular the gene *Ci-Etr* has been identified by different large-scale investigation of genes involved in the nervous system patterning (Bertrand et al., 2003; Mochizuki et al., 2003; Nagatomo et al., 2003; Hamada et al., 2005; Wada et al., 2003) and has been successfully used as a marker of the central nervous system and of the caudal epidermal sensory neurons by Pasini *et al*., (2006). Its expression is first detected at the 110-cell stage embryos. At this stage the staining is confined to the a-line and A-line neural plate cells. At the larval stage expression is detected throughout most of the central and peripheral nervous systems including CENs, Palps, RTEN, ATEN. This makes it a good marker for CESNs as described in chapter 3. Horie et al (2008) studied the gene *Ci-VGLUT*, an ortholog of the vertebrate VGLUTs in *C. intestinalis*. The whole genome analysis they conducted showed that the *Ciona* genome contains only one VGLUT family gene. This gene codifys for a vesicular glutamate transporter similar to the orthologs known for both vertebrates and invertebrates even if phylogenetic analyses indicate that *Ci-VGLUT* is more closely related to vertebrate VGLUTs than *Caenorhabditis elegans* and *Drosophila* VGLUTs. They showed that papillar neurons of the adhesive organs, almost all epidermal neurons, the otolith cell, and ocellus photoreceptor cells are glutamatergic. Furthermore they suggested that glutamatergic neurotransmission plays an important role in sensory systems and in the integration of the sensory inputs of the ascidian larvae.

Another interesting gene whose expression has been detected at the level of the peripheral nervous system is the recently described gene *Ci-Pans* (Alfano et al., 2007). It is an 885-bp cDNA, that encodes a protein with apparently no sequence similarities to known proteins and shows a spatial and temporal specific expression pattern. In fact after a short and early localization in the muscle precursors, it is expressed in a dynamic fashion in the nervous system, reaching very high levels of

expression both in the CNS and PNS as the development proceeds. A further interest is that Alfano et al. (2007) also isolated the predicted promoter region of *C. intestinalis* for *Ci-Pans* that is 1kb long.

Finally Candiani et al. (2005) analyzed the interesting expression of *Ci-POU-IV* a gene prevalently expressed at the level of the PNS. The only expression detected in the central nervous system is restricted to the posterior sensory vesicle and to some motoneurons of the visceral ganglion. To the POU gene family in *C. intestinalis* is dedicated the next chapter.

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2. Interacting Retinoic Acid, FGF and WNT pathways pattern the *Ciona* embryonic posterior epidermis and peripheral nervous system.

Abstract

Vertebrate embryos rely on antagonizing gradients of Retinoic Acid (RA) and FGF/WNT to coordinate the patterning and differentiation of the posterior paraxial mesoderm and neuroepithelium with the elongation of the antero-posterior body axis. A posterior source of FGF/WNT keeps the caudal cells in an immature proliferative state, while RA produced in the somites promotes mesoderm maturation and neuroepithelium patterning and differentiation. FGF/WNT and RA signaling pathways inhibit each other, resulting in the formation of an interface between the two gradients that travels posteriorly as the embryo elongates. This allows patterning and differentiation to proceed coordinately with the extension of the embryo axis. Such a strategy appears to be common to all vertebrates, but has never been described in non vertebrate chordates.We show here that an anterior RA and a posterior FGF/WNT signalling centres exist in the tail of *Ciona intestinalis* larvae. The two signals are mutually antagonistic and control the AP patterning of the tail epidermis and the tail peripheral nervous system.

Introduction

In vertebrate embryos, the generation, patterning and differentiation of trunk and tail paraxial mesoderm and neuroepithelium occur in a rostrocaudal sequence. Paraxial mesoderm cells, generated by gastrulation or produced in the tailbud, continuously enter the posterior end of the presegmented mesoderm (PSM), are progressively displaced anteriorly within the receding PSM, mature when they cross a determination front, and finally become incorporated into the segmented somites that bud off periodically from the PSM anterior margin (Dubrulle and Pourquié, 2004). The segmented somites are then internally patterned and progressively differentiate to give rise to skeletal muscle, bone, cartilage, tendons and dermis. After exiting the posterior growth zone, the epiblast-derived spinal cord progenitors are incorporated into the closing neural tube, then undergo dorsoventral (DV) patterning and neuronal differentiation when their position along the anteroposterior (AP) axis of the embryo roughly coincides with the somite determination/segmentation front (Briscoe et al., 2000; Diez del Corral et al., 2002; Diez del Corral et al., 2003). As a consequence, different points along the embryo AP axis represent different stages in the development of the paraxial mesoderm and neuroepithelium.

It has been proposed that, to coordinate the maturation of paraxial mesoderm and spinal cord with each other and with the concomitantly ongoing processes of body axis extension and AP patterning, vertebrates adopt a strategy relying on two opposing and antagonizing gradients of FGF and retinoic acid (RA) (Diez del Corral et al., 2003; Diez del Corral and Storey, 2004). The tailbud provides a

posterior source of FGF8 that keeps the caudal PSM cells in an immature state and maintains the proliferative status of the spinal cord precursors, while at the same time preventing the onset of DV patterning and neuronal differentiation (Dubrulle et al., 2001; Diez del Corral et al., 2002; Diez del Corral et al., 2003; Sawada et al., 2001; Bertrand et al., 2000; Mathis et al., 2001). On the other hand, RA produced in the segmented somites and the anteriormost PSM by the enzyme Retinaldehyde dehydrogenase2 (Raldh2, also called Aldh1a) promotes PSM segmentation, maintains the bilateral symmetry of somite formation and triggers ventral spinal cord patterning and differentiation (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Kawakami et al., 2005; Vermot and Pourquié, 2005; Sirbu and Duester, 2006). As the embryo elongates and the FGF8-producing region moves posteriorly, the mutual inhibition between FGF and RA pathways results in the formation of a caudalward-travelling wave of RA signaling activation and consequent FGF signaling silencing. This would act as a determination front allowing paraxial mesoderm segmentation and spinal cord differentiation to proceed coordinately with the extension of the embryo AP axis (Diez del Corral et al., 2003; Diez del Corral and Storey, 2004; Moreno and Kintner, 2004; Dubrulle and Pourquié, 2004). The opposing RA and FGF signals are also involved in controlling the colinear activation of *Hox* gene transcription in the posterior central nervous system and possibly in the paraxial mesoderm: FGF appears to preferentially activate genes located at the 5' end of the Hox cluster (posterior Hox genes), while RA induces expression of the 3' end Hox cluster members (anterior Hox genes) and intermediate concentrations of FGF and RA control the expression of the intermediate cluster genes (Liu et al., 2001; Bel-Vialar et al., 2002; Diez del Corral and Storey, 2004). A posterior-to-anterior WNT canonical activity gradient has also been described, with an anterior limit corresponding to that of the FGF gradient and with a role in coordinating the PSM maturation and segmentation (Aulehla et al., 2007; Dunty et al., 2008). Moreover, Wnt8c-triggered WNT canonical signalling stimulates the expression of Raldh2 in the low FGF signalling environment of the anterior PSM, thus mediating the transition between FGF- and RA-sensitivity in the process of neuronal differentiation in the chicken spinal cord (Olivera-Martinez and Storey, 2007).

The strategy relying on opposing FGF/WNT and RA gradients to coordinate AP axis elongation with mesoderm segmentation and the DV patterning/neuronal differentiation of the posterior neuroepithelium has currently been identified only in vertebrates, and it is unclear whether it was already exploited by the last common chordate ancestor. In the cephalochordate Amphioxus, currently seen as the most basal extant chordate (Delsuc et al., 2006), embryonic AP elongation takes place from a posterior tailbud/growth zone (Holland, 2002), although somitogenesis differs

substantially from the vertebrate paradigm (Schubert et al., 2001; Holland, 2002). Spinal cord DV patterning is likely similar, but its temporal aspects have not been addressed in detail (Shimeld, 1999; Shimeld et al., 2007). Some support for the existence of a two opposing gradient system in amphioxus may come from gene expression data. The amphioxus tailbud expresses several Wnt genes and at least one Fgf8-related factor, Fgf8/17/18 (Schubert et al., 2001; Meulemans and Bronner-Fraser, 2007). In amphioxus, RA signaling has been shown to control Hox gene expression, as well as AP patterning of the spinal cord, epidermis and sensory epidermal neurones (Escrivà et al., 2002; Schubert et al., 2004; Schubert et al., 2005; Schubert et al., 2006; Campo-Paysaa et al., 2008).

In ascidians, AP elongation of the embryo takes place by spatial rearrangement of postmitotic cells without posterior growth (hence a tailbud *sensu stricto* is missing) (Katsuyama et al., 1999; Passamaneck et al., 2007); mesoderm segmentation is absent (Passamaneck et al., 2007); the Hox gene cluster is dispersed across four chromosomes and colinearity of expression is only partially retained (Ikuta et al., 2004); RA has been shown to control the expression of Hox1 (Kanda et al., 2009), but the developmental abnormalities described in RA-treated embryos are not indicative of homeotic transformation (Cañestro and Postlethwait, 2007 and references therein).

If the last common chordate ancestor already exploited opposing FGF/WNT and RA signals to coordinate AP elongation with mesoderm and neuroepithelium maturation and patterning, then such a mechanism is likely to have undergone profound modifications in the most derived tunicates: it may have been altogether lost for lack of selective constraints (as it is most likely the case for the appendicularian *Oikopleura dioica*, in which the entire RA signalling machinery is lost (Campo-Paysaa et al., 2008; Cañestro and Postlethwaith, 2007), reduced to a nonfunctional atavism, simplified, adapted to different constraints such as a determinative mode of development or recycled to fulfill novel functions. In any case, a better understanding of the molecular and functional interactions between FGF, WNT and RA pathways in the development of the posterior structures of ascidian larvae will contribute to clarify the existence of a "common minimum blueprint" for generating the chordate body plan and may provide insights into how the evolution of gene regulatory networks and other complex patterning systems accompany morphological and morphogenetic simplification in an extremely derived chordate (Holland and Gibson-Brown, 2003; Davidson and Erwin, 2006; Lemaire et al., 2008).

We show here that an anterior RA and a posterior FGF/WNT signaling centres exist in the tail of *Ciona intestinalis* larvae. The two signals are mutually antagonistic and control the AP patterning of the tail epidermis and the tail peripheral nervous system (PNS).

Material and methods

Animals

The specimens of *Ciona intestinalis* were collected by the Roscoff biologic station. Naturally spawned eggs were fertilized in vitro using a suspension of dry sperm in artificial sea water and 0.1 mM TRIS 9.5 pH. The fertilized eggs were raised in filtered artificial sea water at 18 °C. Samples at appropriate stages (in general late tailbud) were collected by low speed centrifugation and were fixed in MOPS Fix (4% PFA, 0.5M NaCl, 0.1M MOPS pH7.5) for whole-mount *in situ* hybridization and in PFA 4% for immunostaining.

Treatments

Pharmacological treatments were performed at early gastrula, late gastrula-early neurula and late neurula stages (Hotta et al., 2007). The used pharmacological agents were: RA 1.5 µM, DEAB (4-(Diethylamino)benzaldehyde) (SIGMA - Italy) 150 µM, bFGF 100ng/ml, UO126 (Roche) 10 µM, R11 10 µM, LiCl 150 mM, LiCl, 300 mM. Control embryos were treated with DMSO 10 µM.

Immunostaining

Acetylated α-tubulin and β-tubulin staining were performed with the 6–11B-1 and 2-28-33 monoclonal antibodies (Sigma-Aldrich, St. Louis, Missouri, United States). Confocal images were acquired using a Zeiss LSM510 inverted microscope. 3-D stacks were processed using the Zeiss embedded software and Imaris (Bitplane, Zurich, Switzerland).

Electroporation

We generated and electroporated some expression vectors in order to verify the RA activity in tail epidermis. The plasmids with vectors were generated using the Gateway system (Invitrogen, Carlsbad, California, United States). Entry clones were generated by PCR using Pfx polymerase (Promega, Madison, Wisconsin, United States) and designed to contain the entire ORF of interest. The plasmid pAmphiRARElacZ, containing 400bp of Amphioxus genomic DNA immediately upstream the RA receptor with a putative RARE (GGTTCAGTCAGAGTTCA) was used to generate the fusion construct p4xARARElacZ and was a gift from M. Schubert. Overexpression was performed using transient transgenesis by electroporation as described in Bertrand et al (2003), but all volumes were scaled down to a half. 30 ng to 100 ng of plasmid DNA were used.

Whole mount in situ hybridization and immunohistochemestry

Whole mount in situ hybridization and antibody staining were performed according to Hudson & Lemaire (2001).

Neurons numbering and distribution

In order to quantify the neurons we performed *in situ* hybridization with a probe for the gene *Ci-Etr* that is expressed in both central and peripheral nervous system and particularly easy to detect in the ventral caudal sensory neurons. Neurons were quantified by manual count under a Leica stereoscope. Analysis of neurons distribution was done by taking pictures of every embryo and using the program "Image J" to take standard measures. The parameters analised were: total tail length, distance of the last ventral neuron from the tail tip, distance of the first ventral neuron from the end of the trunk.

Results

To address the hypothesis that a mechanism analogous to opposing gradients of RA and FGF/WNT signals plays a role in the patterning of *Ciona* posterior larval structures, we first defined the embryonic territories where these pathways are active during the process of posterior elongation and identified the genes likely responsible for their activation.

At tailbud stage, X-gal histochemical staining of embryos electroporated with the reporter construct pCi-Hox1(intron2)/lacZ, in which expression of β-galactosidase is controlled by RA-responsive sequences of the *Ci-Hox1* gene (Kanda *et al*., 2009), reveals RA pathway activity in the trunk mesenchyme, the muscle and the anterior tail epidermal cells (Fig 2.1). These structures coincide with, or are adjacent to, the territories expressing *Ci-aldh1a1/2/3a*, the *Ciona* homologue of the RA-synthesizing enzyme Raldh2 (Cañestro et al., 2006), which is first detected in mesodermal cells at late gastrula stage, then confined to the anteriormost 3-5 muscle cell pairs throughout the process of tail extension (Fig 2.1 and Nagatomo and Fujiwara, 2003).

Immunoreactivity against diphosphorylated Erk, a hallmark of activation of the RTK-dependent MAPK pathway, has been described in epidermal cells surrounding the *Ciona* tailtip at neurula and late tailbud stages (Shi et al., 2008). At these stages, only two *Ciona* FGF gene are expressed at or around the tailtip: *Ci-fgf8/17/18*, restricted to a very small population (2 to 4 cells) of tailtip epidermal cells (Fig 2.1 and Imai et al., 2002), and *Ci-fgf9/16/20*, expressed by some tailtip muscle cells (C. Hudson and H. Yasuo, personal communication) .

Activation of the canonical WNT pathway was addressed by X-gal histochemical staining of embryos electroporated with a TOPFLASH reporter construct in which expression of β-

galactosidase is under the control of a dodecamer of consensus binding sites for the canonical WNT pathway effector, TCF. In the tail of tailbud stage *Ciona* embryos, WNT canonical activity is restricted to two territories, the endodermal strand and the posterior ventral midline epidermis, with only occasional embryos showing activity in posterior dorsal midline and/or posterior lateral epidermis. While the endodermal strand activity is the outcome of the earlier β-catenin requirement for endoderm specification (Imai et al., 2000), the posterior epidermis activity may reflect the activation of the WNT canonical pathway by either *Ci-Wnt5*, expressed in tailtip epidermal cells (Fig 2.1), or by *Ci-Wnt11-1/Ci-orphan* WNT, expressed by a few tailtip muscle cells (ANISEED ref).

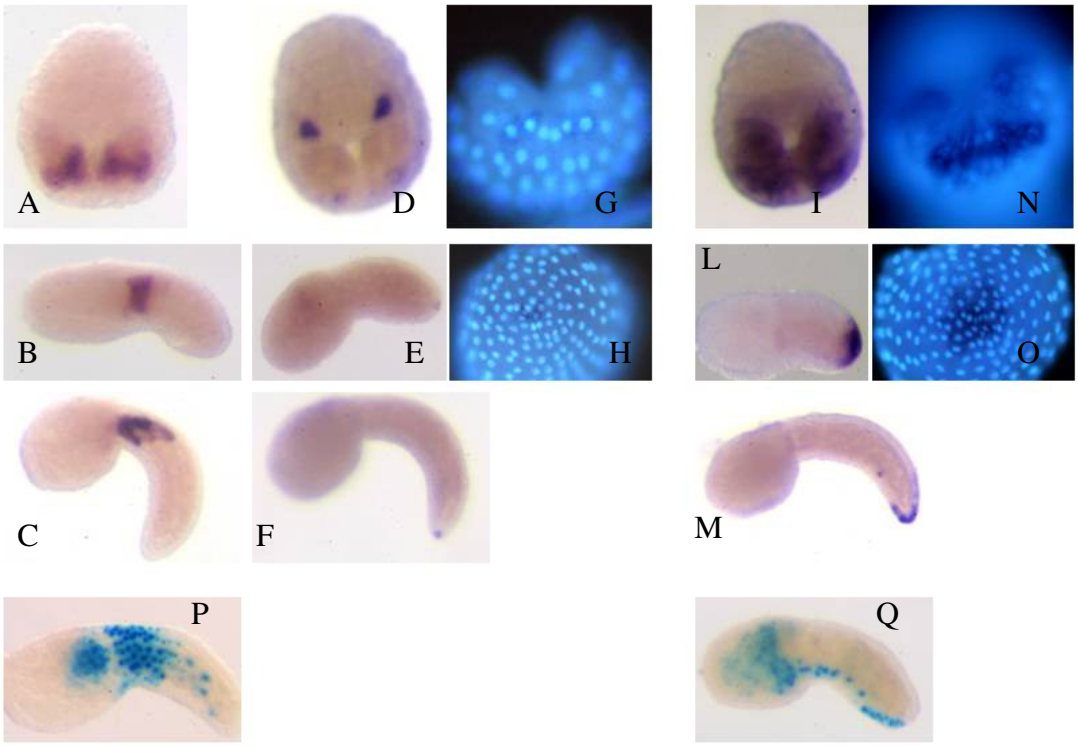

Fig. 2.1.A-C: Expression of the RA-synthesising enzyme, *Ci-Raldh*, in embryos at late gastrula (A), early tailbud (B) and mid tailbud (C) stage. D-F: expression of *Ci-fgf8* at late gastrula (D), early tailbud (E) and mid tailbud (F) stage. G, H: higher magnification of embryos at late gastrula and early tailbud stage showing expression of *Ci-fgf8* in the tailtip cells. DAPI staining to highlight the

nuclei. I-M: Expression of *Ci-Wnt5* in embryos at late gastrula (I), early tailbud (L) and midtailbud (M). N, O: higher magnification of embryos at late gastrula and early tailbud stage showing expression of *Ci-Wnt5* in the tailtip cells. DAPI staining to highlight the nuclei. P: X-Gal staining of an embryo electroporated with the construct pCi-Hox1-(intron2)::LacZ, showing activity of the RA pathway in the anterior tail epidermis. Q: X-Gal staining of an embryo electroporated with the construct pTOPFLASH, showing activity of the canonical WNT pathway in the posterior tail epidermis.

FGF/MAPK and RA pathways control the AP patterning of *Ciona* tail epidermis.

We next explored whether the RA and the FGF/MAPK pathways control the AP patterning of *Ciona* tail epidermis. In contrast to the relative complexity and striking regularity of its mediolateral regionalization (Pasini *et al*., 2006), the tail epidermis shows only a limited degree of AP patterning: in addition to the above described expression of *Ci-fgf8/17/18* and *Ci-wnt5* by cells at or around the tailtip, *Ci-Hox12* is expressed by epidermal cells in the posteriormost third of the tail, and *Ci-Hox1* by those of the anteriormost third. Moreover, these expression domains do not appear to correspond to the boundaries of tail epidermal cells clones (Pasini et al., 2006). As shown in Fig 2.2, treatment of embryos with 1.5µM RA leads to an expansion of *Ci-Hox1* expression throughout the entire tail epidermis and to an almost complete loss of both *Ci-Hox12* and *Ci-wnt5* expression, with only a residual signal in cells at very tip of the tail, while the expression of *Ci-fgf8/17/18* is not affected. Conversely, treatment with recombinant bFGF results in an anterior expansion of the *Ci-Hox12* expression domain up to the middle third of the tail epidermis and in a weaker anterior expansion of the *Ci-Wnt5* expression, mostly limited to the ventral midline epidermis (Fig. 2.2). Neither the expression of *Ci-fgf8/17/18* nor of *Ci-Hox1* appear to be affected by treatment with bFGF (Fig. 2.2).

To further analyze the role of the RA and FGF/MAPK pathways in tail epidermal patterning, embryos were treated with either DEAB (Diethylaminobenzaldehyde), an inhibitor of the RA-synthesizing enzyme Raldh2, or the MAPK inhibitor, UO126 at different stages of embryonic development. Treatment with 150µM DEAB leaded to the complete loss of *Ci-Hox1* expression, without significantly affecting the expression patterns *of Ci-Hox12, Ci-wnt5* or *Ci-fgf8/17/18*. Conversely, treatment with 10µM UO126 severely affected the expression of all the four markers, Ci-*Hox1, Ci-Hox12, Ci-Wnt5 and Ci-fgf8/17/18*: while *Ci-fgf8/17/18* and *Ci-Hox1* are lost from the tail epidermis regardless of the time at which UO126 treatment was initiated, the loss of *Ci-Hox12*

expression was complete following UO126 treatment at stages 11 to 15 (Hotta *et al.*, 2007) but only partial after stage 15. Expression of *Ci-Wnt5* was strongly downregulated and reduced to only a few cells at the tip of the tail (Fig. 2.2).

Distinct anterior and posterior subpopulations of ventral Caudal Epidermal Neurons

The caudal epidermal neurons (CENs) which constitute the posterior portion of the larval PNS are one of the most conspicuous features of the *Ciona* tail epidermis. Although their precise function is yet undetermined, these cells, which extend long cilia within the tunic and project axons towards the sensory vesicle, express neuronal markers and are generally supposed to be primary sensory neurons. Their numbers vary among individual larvae, with an average 14 pairs of CENs distributed along the dorsal and ventral midlines of the tail, their cell bodies embedded within the epidermis. Although some early reports described their distribution as regular and considered them as an instance of segmentation in ascidians, subsequent observations revealed that they are only irregularly spaced, each pair of CENs being separated from its neighbours by three to five epidermal cells. No significant difference has yet been described among the various CENs, but we could show that the dorsal and ventral CENs derive from two distinct populations of early gastrula-stage blastomeres, respectively induced by *Ci-fgf9/19/20* and *Ci-ADMP*, thus hinting to the possible existence of different morphological and/or functional neuronal subclasses located in different regions of the tail epidermis. To further explore this possibility, we searched for differences in the CENs birthdate and morphology. First, examination of embryos hybridized with the neuronal-specific marker *Ci-Etr* at stages 19-21 revealed that the posteriormost CENs are consistently born earlier than the anterior ones. Then we electroporated fertilized eggs with the reporter construct pVGluT::EGFP (Horie et al., 2008) and analysed the morphology of the labeled CENs at the hatching larva stage. We found that while all the dorsal CENs as well as the one or two posteriormost ventral CENs emit long axonal projections (or are contacted by the long axonal projections of the subepidermal dorsal bipolar cells, not very clear yet), the anteriormost ventral CENs have only short, stumpy axons or no axon at all, and are never contacted by axonal projections from the bipolar cells.

Thus, our data revealed unexpected differences among *Ciona* CENs and supported the existence of distinct anterior and posterior subpopulations of ventral CENs.

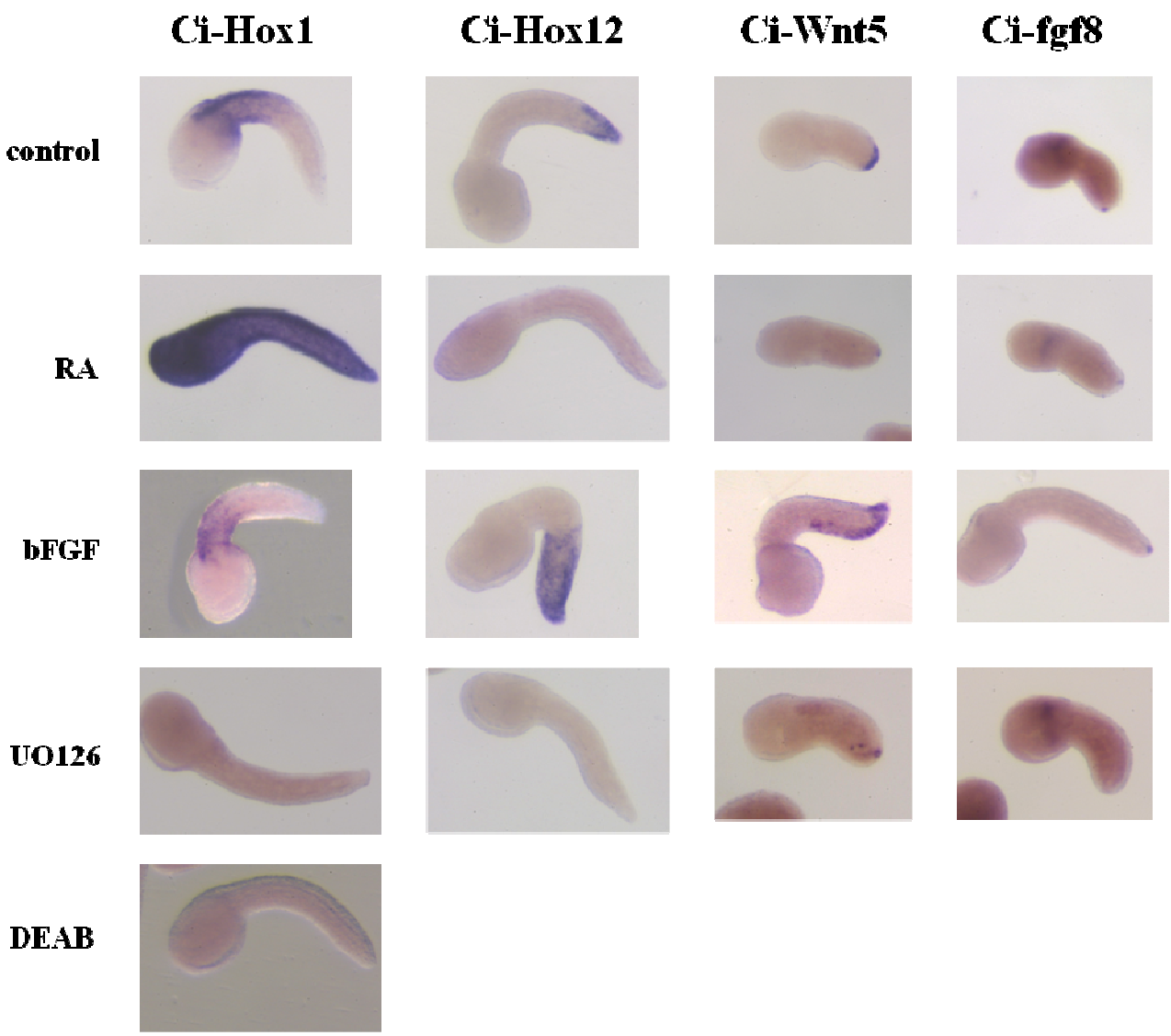

Fig. 2.2. In situ hybridizations with probes for he genes *Ci-Hox1, Ci-Hox12, Ci-Wnt5* and *Ci-fgf8*. Embryos are at tailbud and mid-tailbud stages.

FGF/MAPK, RA and canonical WNT pathways control the number of ventral CENs.

In order to understand if and how the caudal epidermal neurons (CENs) are under the control of the FGF/MAPK, RA and canonical WNT pathways, extensively numberings of the ventral CENs in specimens belonging to different treatments were performed. In particular treatments with RA increased the number of ventral neurons (Fig. 2.3). On the contrary the treatments with bFGF decreased the number of neurons (Fig. 2.3).

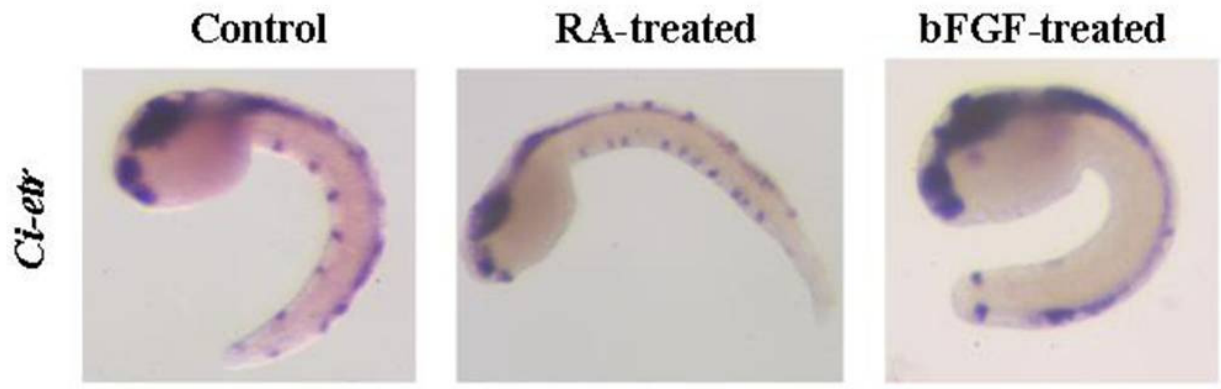

Fig. 2.3. *In situ* hybridizations for *Ci-Etr* that marks the neurons of the PNS and underlines the differences between controls, RA treated and bFGF treated embryos.

The analysis of ANOVA and the subsequent Tukey test confirmed the relevance of the differences between the number of neurons of the treated specimens and the number of neurons in the controls. These differences were in particular significant in embryos treated at the stage 11 (early gastrula), 13 (late gastrula) and 15 (neurula) described by Hotta et al., (2007). On the contrary in embryos treated at the stage 17 (initial tailbud) the differences were trivial (Fig. 2.4).

Also treatments with the respective antagonists of RA and FGF contributed to clarify the mechanism. Treatments with DEAB, the inhibitor of the RA synthesis, dramatically decreased the number of anterior ventral CENs, without affecting the posterior ones. On the other hand, treatment with UO126, a MAPkinase inhibitor, caused a limited increment of the neuron number with respect to the controls. In embryos at the stage 13 were also performed combined treatments: DEAB with FGF, DEAB with UO126, RA with FGF and UO126 with RA. These double treatments led to significant differences with respect to controls and embryos subjected to single treatments. The analysis of coupled treatments effects confirmed that FGF and RA have opposite action. In fact they showed that treatments with DEAB and FGF induced a stronger loss of neurons than FGF alone. Coupled treatments with DEAB and UO126 and with FGF and RA balanced the number of neurons while the treatments of RA and UO126 strongly augmented them, more than treatments with RA alone (Fig. 2.5).

From stage 11 to stage 17 were performed also treatments with R11, the inhibitor of Cyp26, an enzyme which degrades RA, also coupled with treatments with RA. These treatments showed a great increment in neurons number, particularly significant in the case of the combined treatments (Fig. 2.8-9). These data can lead to draw the conclusion that RA can also regulate its own activity and that inhibiting the autoregulation leads to a stronger antagonism of RA by respect to FGF/WNT.

Finally we also performed treatments with 150 and 300 mM LiCl. The effect of lithium is caused by the inhibition of glycogen synthase kinase-3β (GSK3β: Klein & Melton, 1996; Stambolic et al., 1996) which acts as an inhibitor of WNT signaling cascade, although other endogenous targets of lithium have been reported (Busa & Gimlich, 1989). The final result is a constitutive activation of WNT signaling (Klein and Melton, 1996). Only embryos treated at stage 15 and 17 were viable at late tailbud stage (stage 24). In these specimens the number of ventral CENs was very low especially in those treated with the 300 mM solution (Fig. 2.6-7).

All these results showed that altering both the FGF/MAPK, RA pathway and the canonical WNT affect the number of neurons in the PNS.

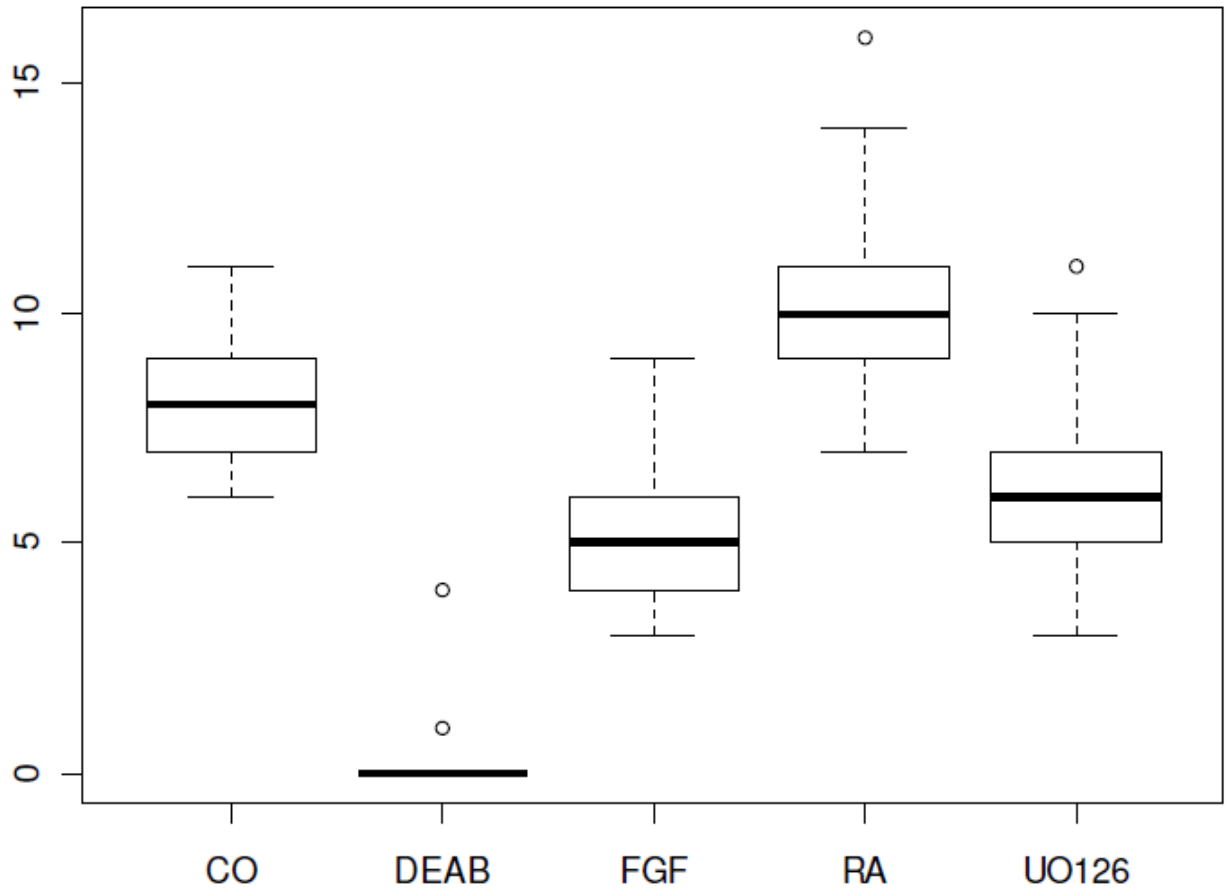

Fig. 2.4. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatments performed at the 11 stage of Hotta et al., (2007). For each treatment N > 50; ANOVA P = < 2.2e-16 ** F = 588.64

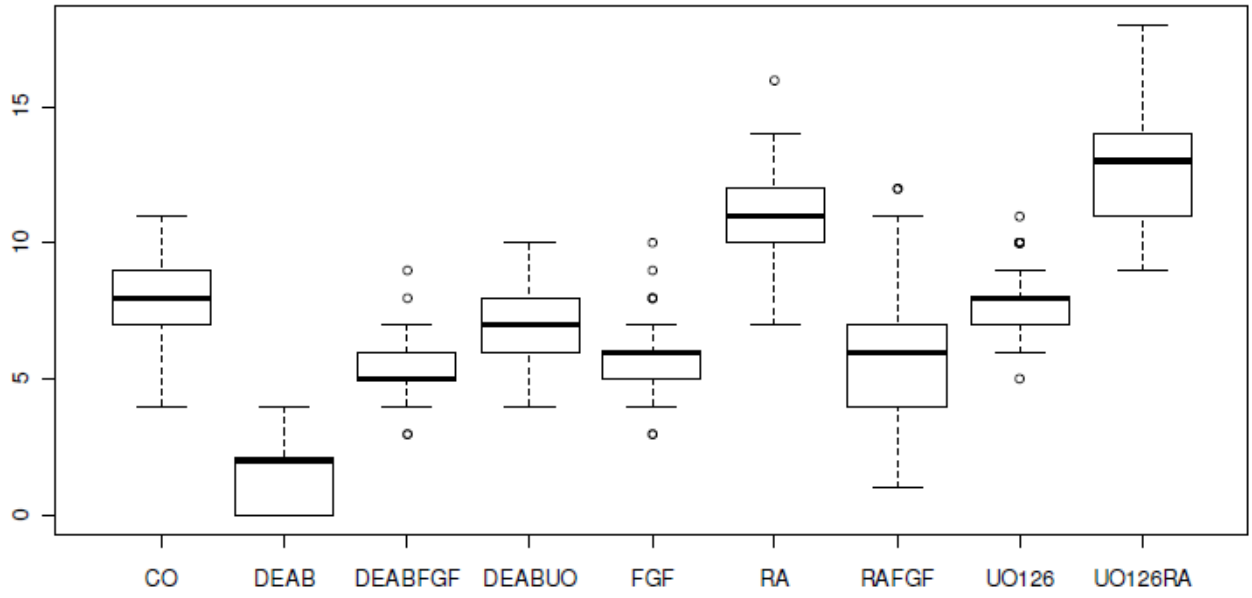

Fig. 2.5. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatments performed at the 13 stage of Hotta et al., (2007). For evrey treatment N > 50; ANOVA Pr= < 2.2e-16 ***, F = 670.67.

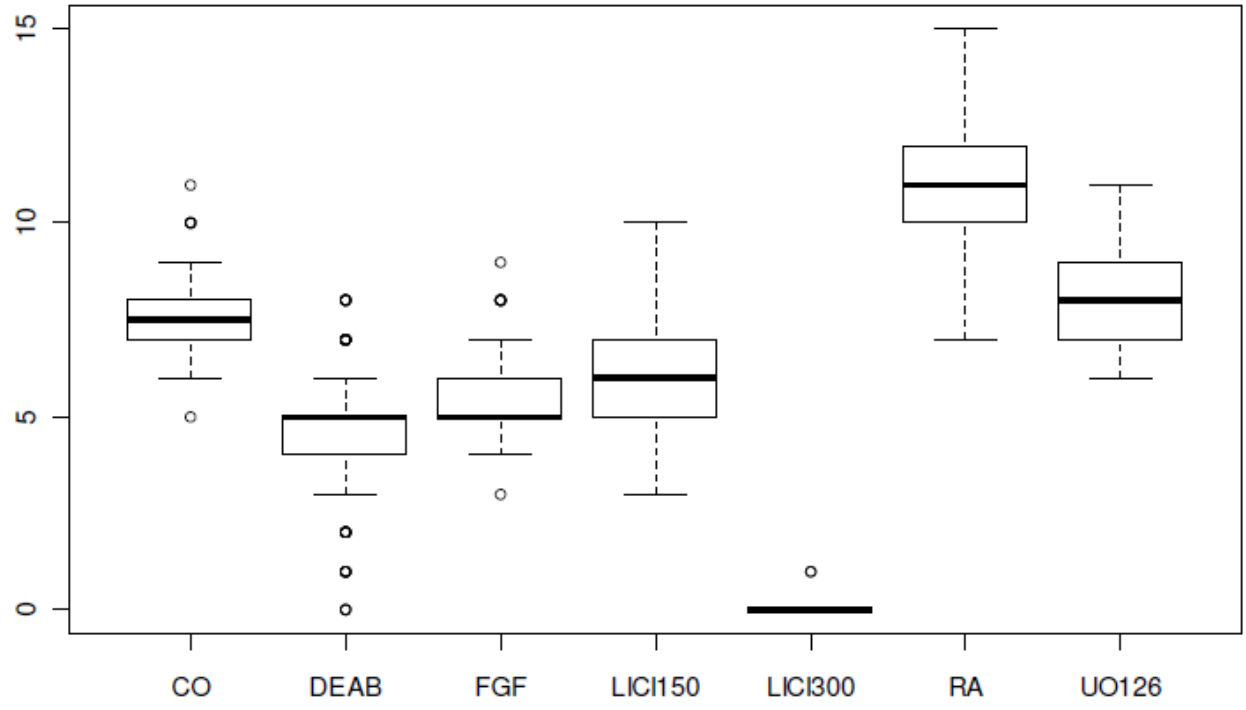

Fig. 2.6. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatment effectuated at the 15 stage of Hotta et al., (2007). For every treatment N > 50; ANOVA P = 330< 2.2e-16 ***, F = 638.7.

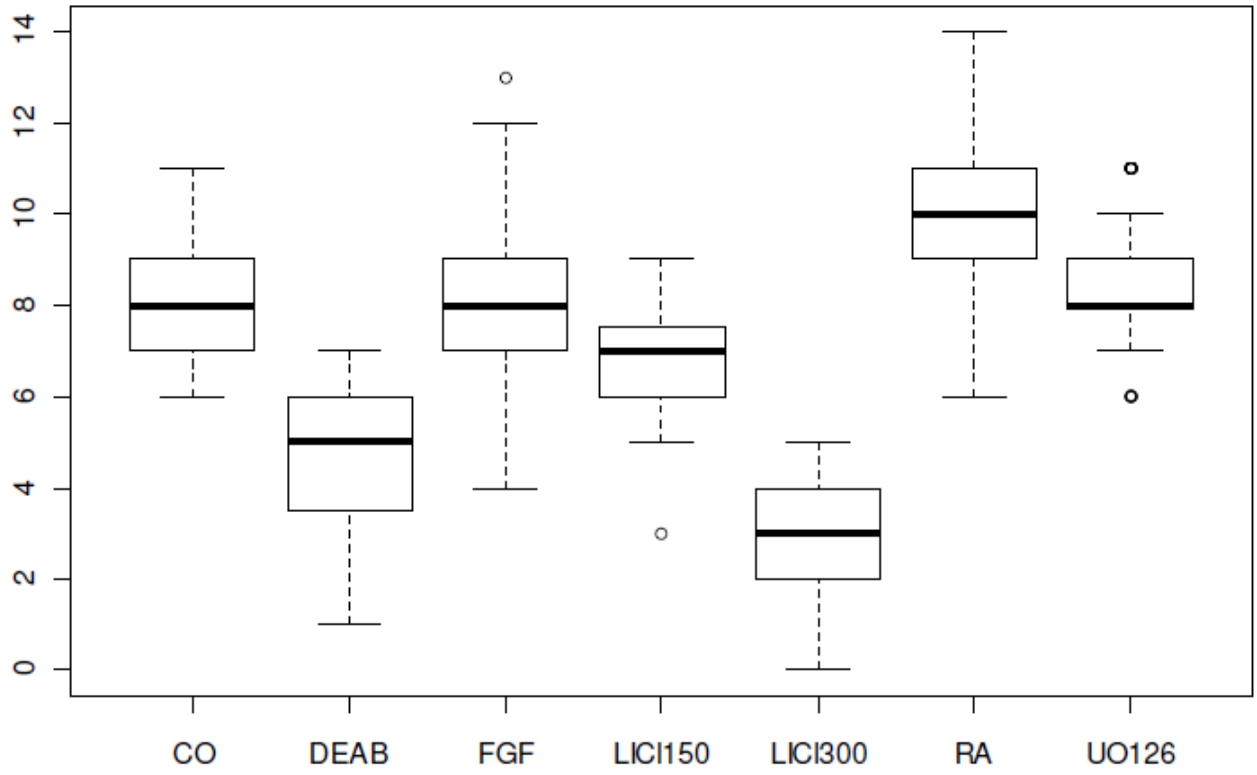

Fig. 2.7. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatment effectuated at the 17 stage of Hotta et al., (2007). For every treatment N > 50; ANOVA P = 188 < 2.2e-16 ***, F = 326.81.

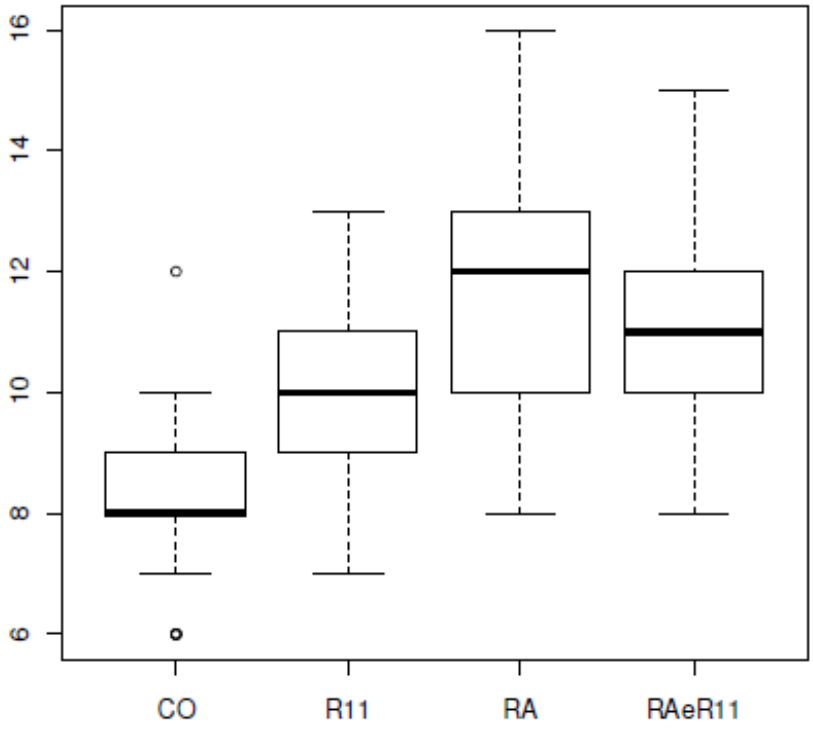

Fig. 2.8. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatment effectuated at the 13 stage of Hotta et al., (2007). For every treatment N > 50; ANOVA P = < 2.2e-16 *** F = 47.01.

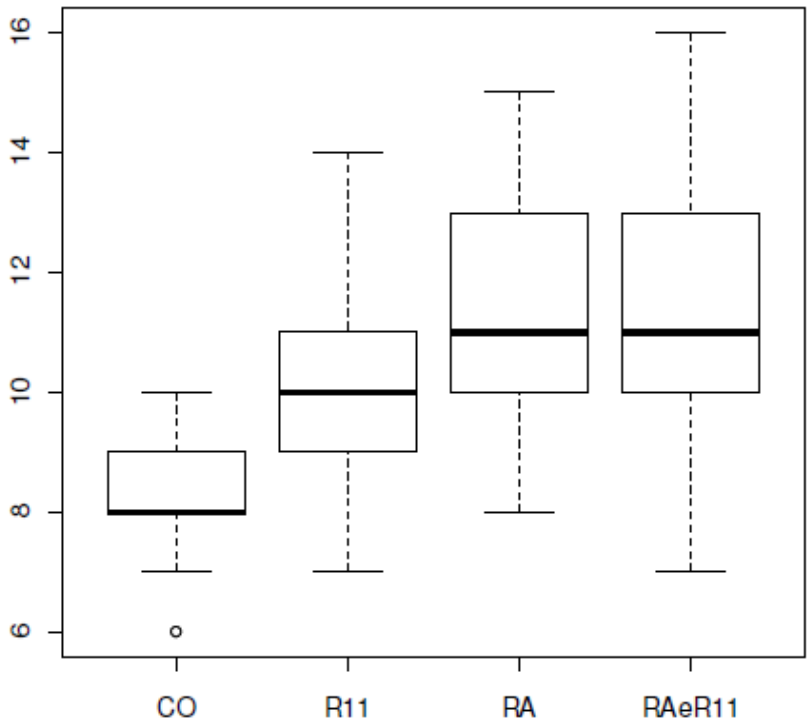

Fig. 2.9. Mean difference of ventral CESNs in late tailbud of *C. intestinalis*. Treatment effectuated at the 17 stage of Hotta et al., (2007). For every treatment N > 50; ANOVA P= < 2.2e-16 ***, F = 50.11.

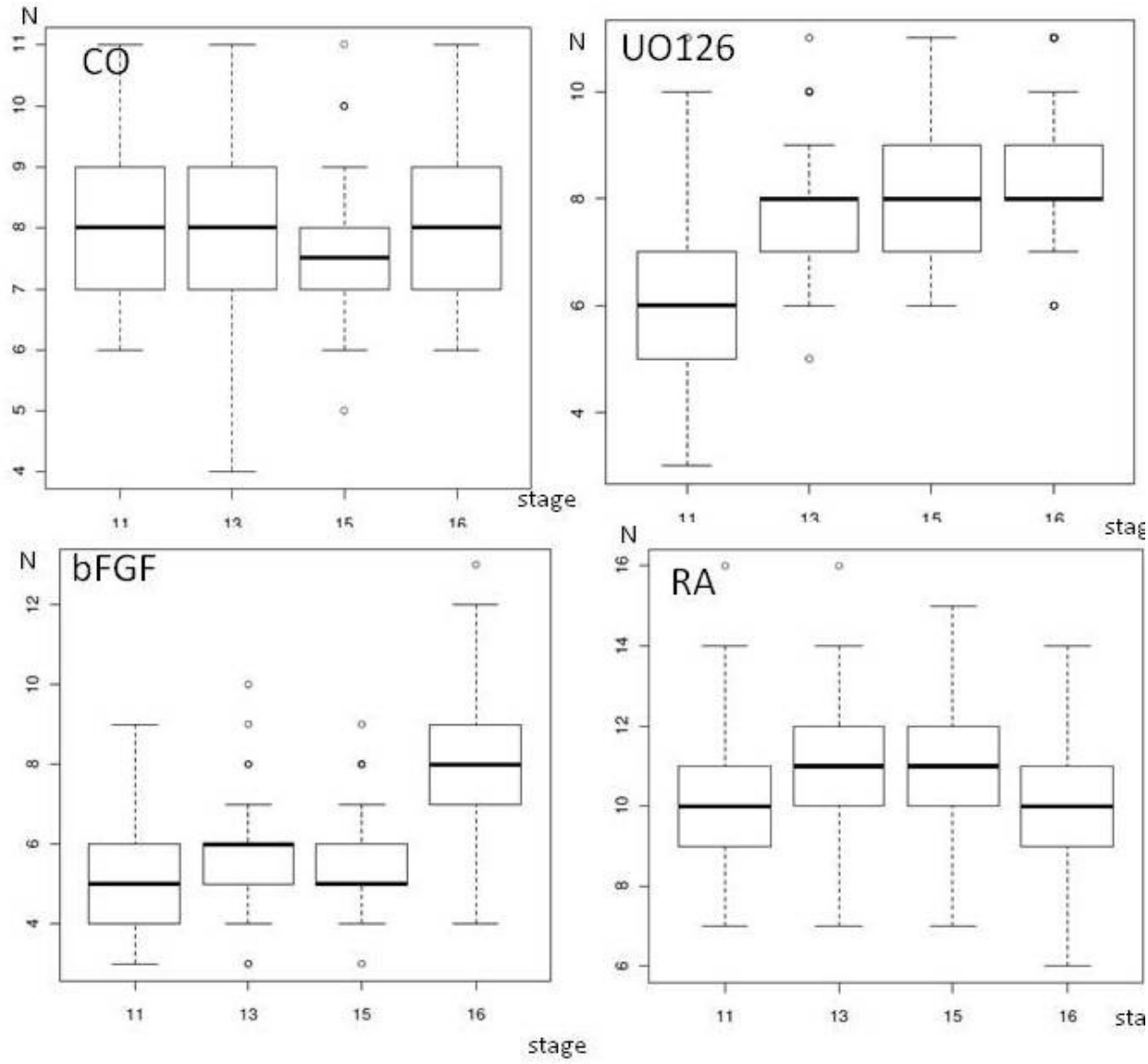

Fig. 2.10. Boxplot of the mean difference of ventral CESNs number in late tailbud of *C. intestinalis* for different treatments effectuated at 4 different stages. For every treatment N > 50.

Moreover the position and distribution of the neurons in embryos treated with RA and bFGF. In particular as shown in Fig. 3.12, there was a clear difference in neuron position according to the treatment. RA treatments increased the neuron number but determine the loss of the most posterior ones while FGF treatments induce a decrease in the neurons number but without the loss of the most posterior ones that was conserved in every embryo.

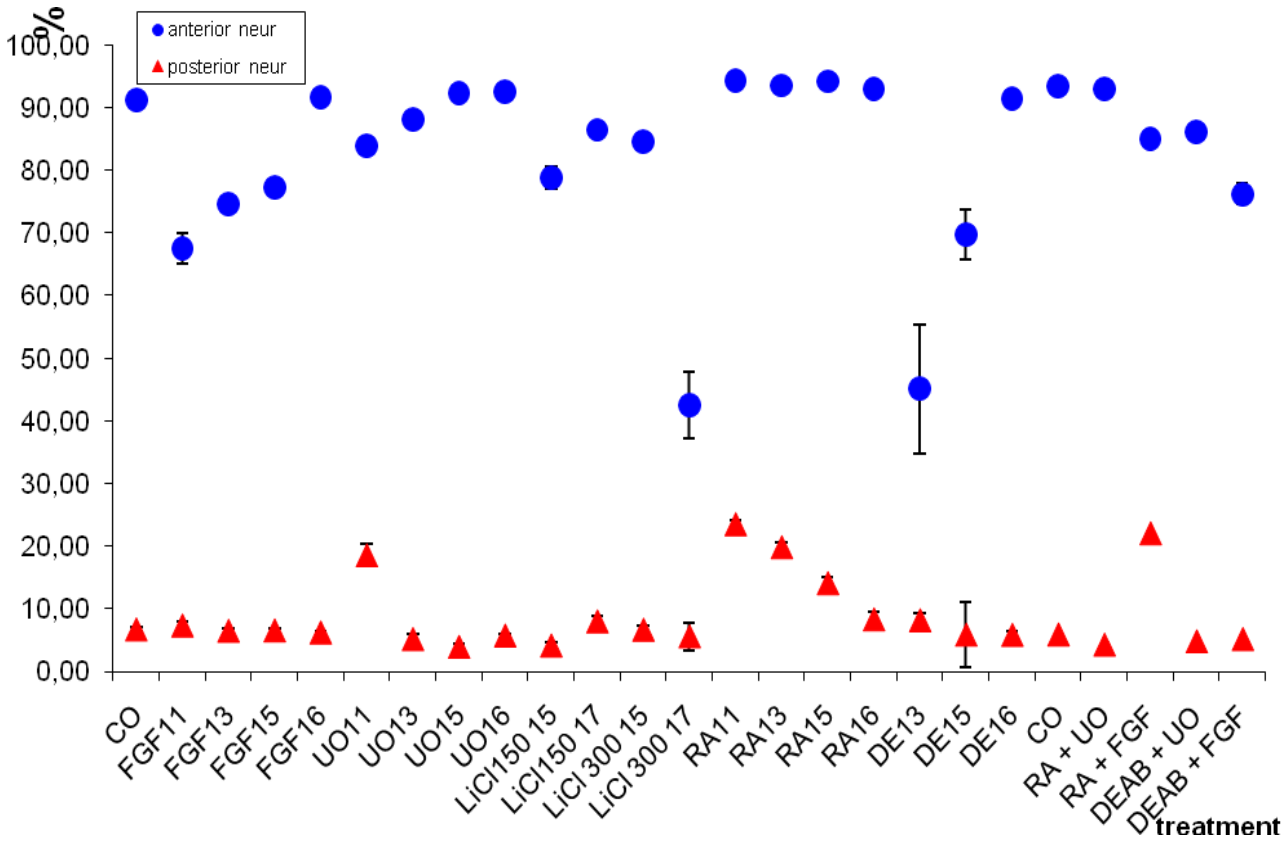

Fig. 2.11. Position of ventral CENs based on the different treatments.

Mutual antagonism

A further evidence that there is a mutual antagonism between RA from a side, and FGF/WNT pathways from the other is the fact that RA-treatment decreased *Wnt5* expression. However, the fact that RA-treatment did not decreased the expression of *Fgf8* also suggests an indirect mechanism of antagonism. Moreover the gene *Ci-Cyp26*, involved in the degradation of RA during AP axis differentiation was over expressed in embryos treated with RA(Fig. 3.13.) indicating an autroregulation exerted by RA itself.

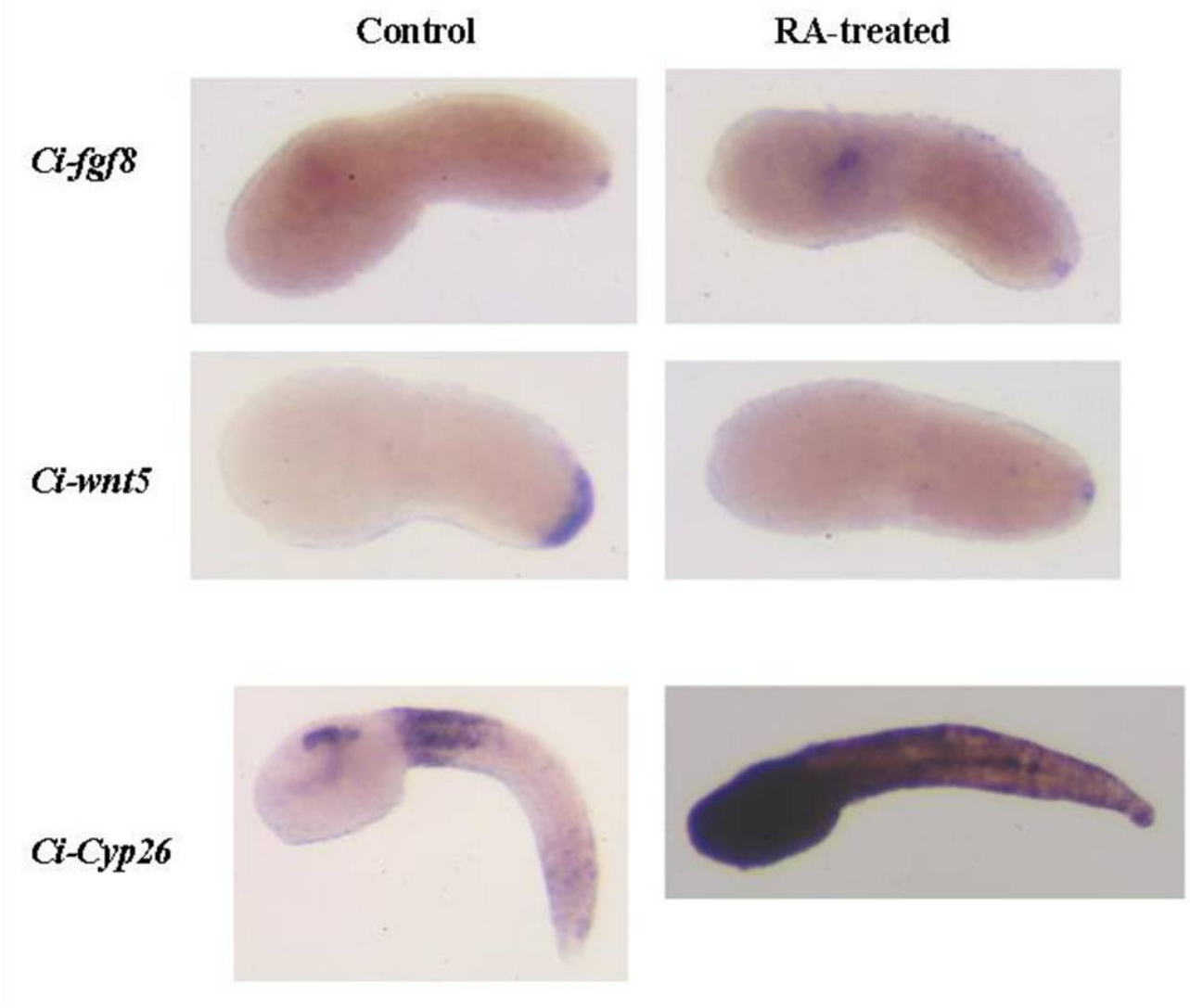

Fig. 2.12. In situ showedexpression of *Ci-Fgf8*, *Ci-Wnt5* and *Ci-Cyp26*.

These results suggested that RA treatment may affect MAPK phosphorylation. **Also,** the bFGF treatments are indirect, as don't affect directly *Ci-Raldh2* expression. A strong effects determined by the antagonist UO126 who completely inhibits the expression and the bFGF itself and affect the expression of *Ci-Cyp26*. The latter is as a matter of fact more expressed than in the controls. UO126 on the other hand doesn't totally block Ci-Cyp26 expression but seems to determine a loss in the anterior part of the tail.

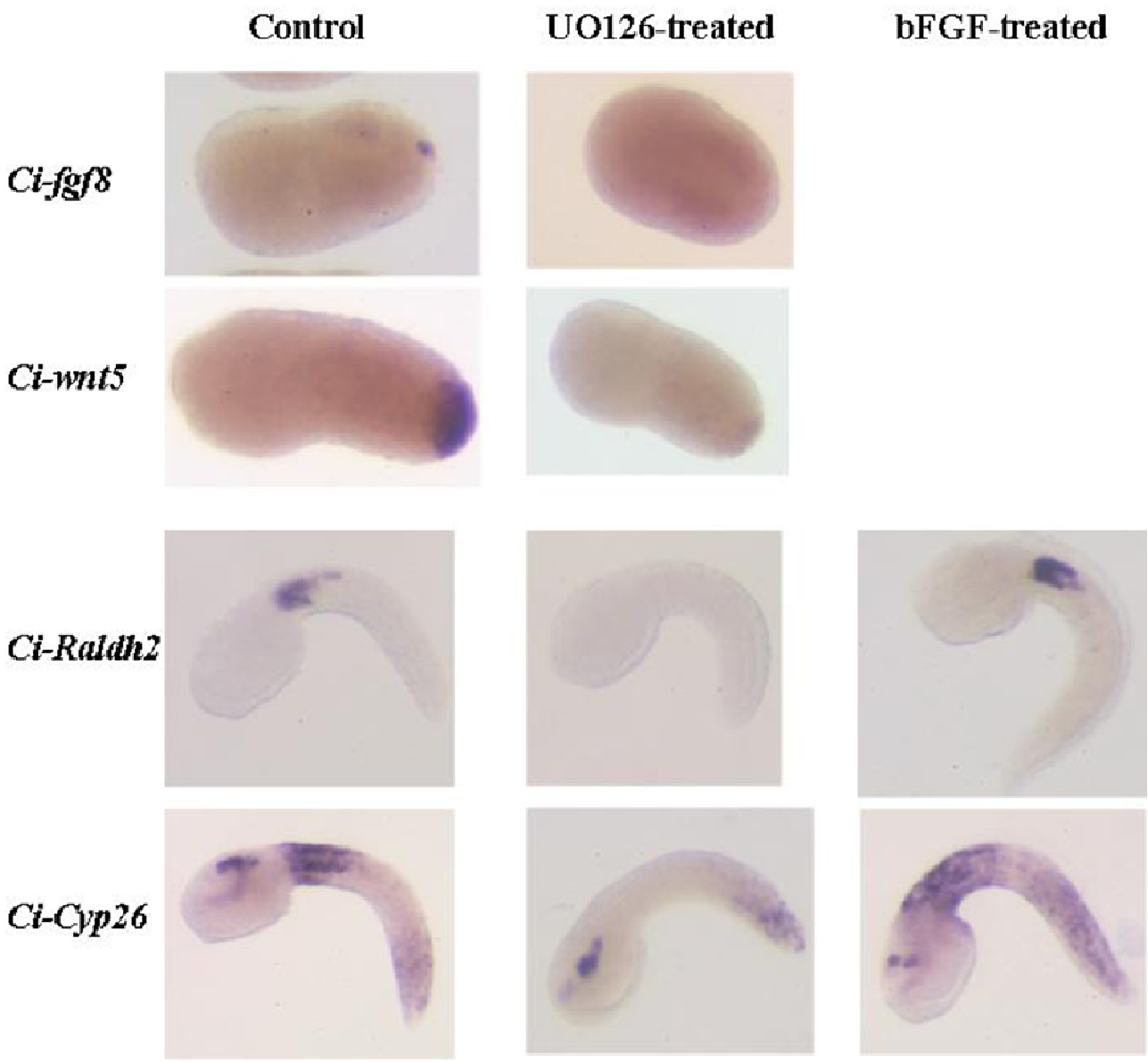

Fig. 2.13. In situ results. In the upper line there are embryos at the stage of initial tailbud. In the following two lines embryos at the stage of advanced tailbud.

Discussion

In vertebrates the existence of opposing FGF/WNT and RA gradients that coordinate mesoderm segmentation, and the DV patterning and differentiation of the posterior neuroepithelium with the process of AP axis extension has been described (Olivera-Martinez & Storey, 2007). However it is still unclear whether this mechanism was already exploited by the last common chordate ancestor. In Amphioxus there are no detailed studies on this subject, but the existence of a two opposing gradient system could be hypothesized comparing the expression data from independent experiments, even if more developmental work is required (Diez del Corral et al., 2003). This is the

first study that tries to investigate the role and the possible existence of opposing FGF/WNT and RA signals in a member of the Tunicates which are the sister group to vertebrates (Delsuc et al., 2006).

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration. Mammals express at least four distinctly regulated groups of MAPKs: extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38alpha/beta/gamma/delta) and ERK5. The level of FGF/MAPK activation (highest in the posterior PSM) serves as a positional cue within the PSM that regulates progression of the cyclic wave and thereby governs the positioning of somite boundary formation (Sawada et al., 2001; Shinya et al., 2001). In zebrafish FGF inhibitors reveal that ERK activation is totally dependent on FGF signaling (Shinya et al., 2001). The signal starts when FGF binds to the receptor on the cell surface and ends when the DNA in the nucleus expresses a protein and produces some change in the cell, such as cell division. The pathway includes many proteins, including MAPK (originally called ERK), which works by adding phosphate groups to a neighboring protein, which acts as an "on" or "off" switch.

Opposition between RA and FGF/WNT signals controls the A-P patterning of the posterior epidermis and peripheral nervous system in the urochordate *Ciona intestinalis*. This control is mediate trough a complex picture of direct an direct antagonistic interactions (fig 3.15).

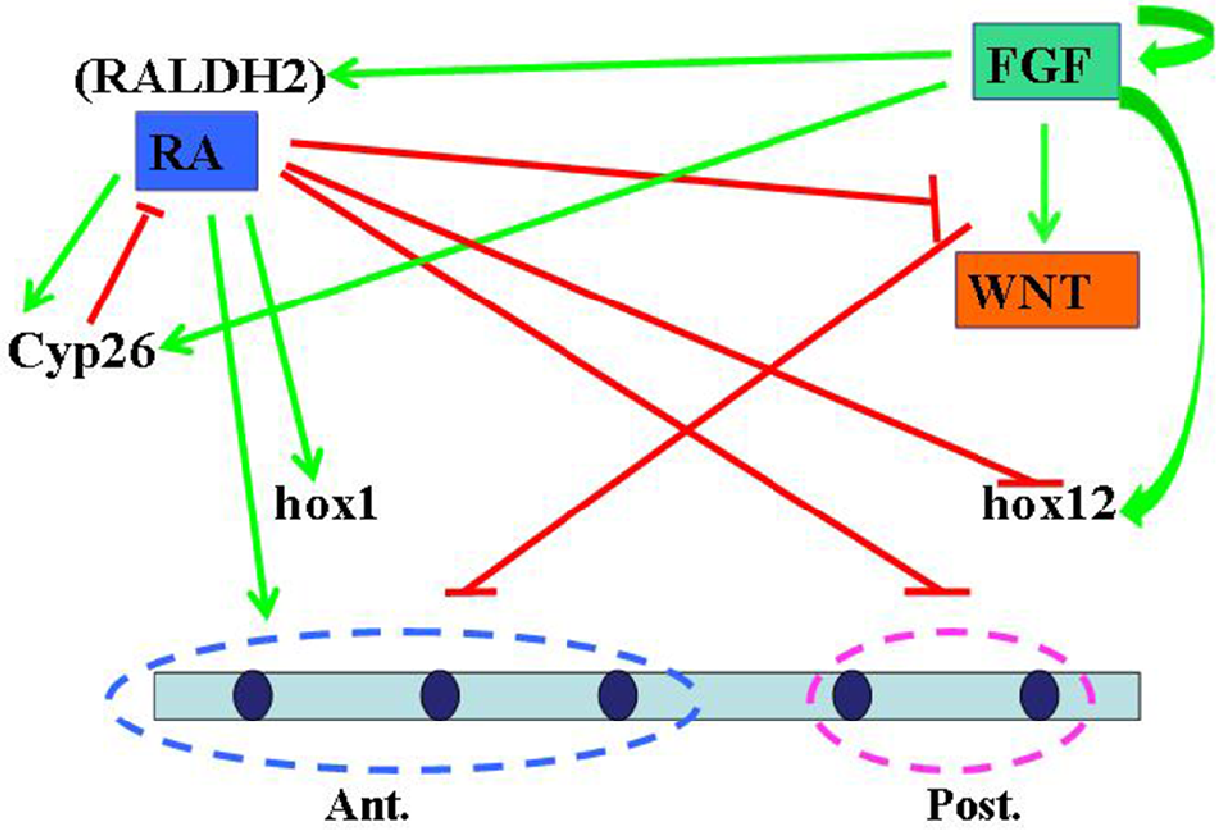

Fig. 2.14. Complex model of mutual antagonistic direct and indirect interactions during AP patterning of the tail.

In particular RA and FGF control differentiation in tail epidermis, RA promoting *Ci-Hox1* that is expressed in the anterior part of the tail and inhibiting *Ci-Hox12,* that is expressed posteriorly; FGF on the contrary activates *Ci-Hox12*. RA and FGF directly control also the number and the position of the neurons in the tail, RA promoting the differentiation of the anterior and inhibiting the posterior and FGF simply blocking the differentiation of the anterior ones. Moreover RA inhibits WNT pathway and via Ci-Cyp26 modulates its own activity. In summary there is a gradient of RA that decreases with AP axis elongation and neural differentiation; the initial high level of RA promotes anterior neurons specification, maintains the activity of *Ci-cyp26* regulating its own level and prevents the formation of the posterior structures. Posterior structures differentiate when RA level decreases and FGF, WNT gradient increases.

Definitively we can state that antagonism between RA and FGF/WNT signals controls the A-P patterning of the posterior epidermis and peripheral nervous system in the urochordate *Ciona intestinalis*. This fact lets open an important evolutionary question about the possibility that this can be a case of convergence or the proof that antero-posterior axial patterning by opposing RA and FGF/WNT gradients precedes the emergence of vertebrates. This aspect can open the perspectives of investigations in both Cephalochordates and also among other bilaterians in order to understand how widely this morphogenetic mechanism is conserved among them.

An interesting fact is the possibility that the anterior and posterior ventral CENs are two functionally distinct populations of neurons. In this study we found that the one or two posteriormost ventral CENs as well as all the dorsal CENs emit long axonal projections while the anteriormost ventral CENs have only short, stumpy axons or no axon at all, and are never contacted by axonal projections from the bipolar cells. These data support the existence of distinct anterior and posterior subpopulations of ventral CENs, fact never signalled before in the studies focusing on *C. intestinalis* PNS (Imai & Meinertzhagen, 2007).

Furthermore, the simple, well characterized *Ciona* embryos can be very useful to decipher the mechanics of the RA and FGF/WNT 'opposing gradients' patterning system also in vertebrates because it represent a suitable model to study how the evolution of a complex patterning system underlies morphological and morphogenetic simplification in a derived chordate.

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3. POU genes are important in neuronal development. Do they have a role in *Ciona* SNP formation?

Transcription factors play critical roles at every step of the embryonic development: establishment of the general body plan, progressive patterning of the embryonic tissues, organogenesis, specification and differentiation of individual cell types. Particularly interesting are transcription factors which regulate the same developmental events in phylogenetically distant organisms. In this view, the study of transcription factors has high potential both for understanding critical processes during embryogenesis and from an evolutionary point of view.

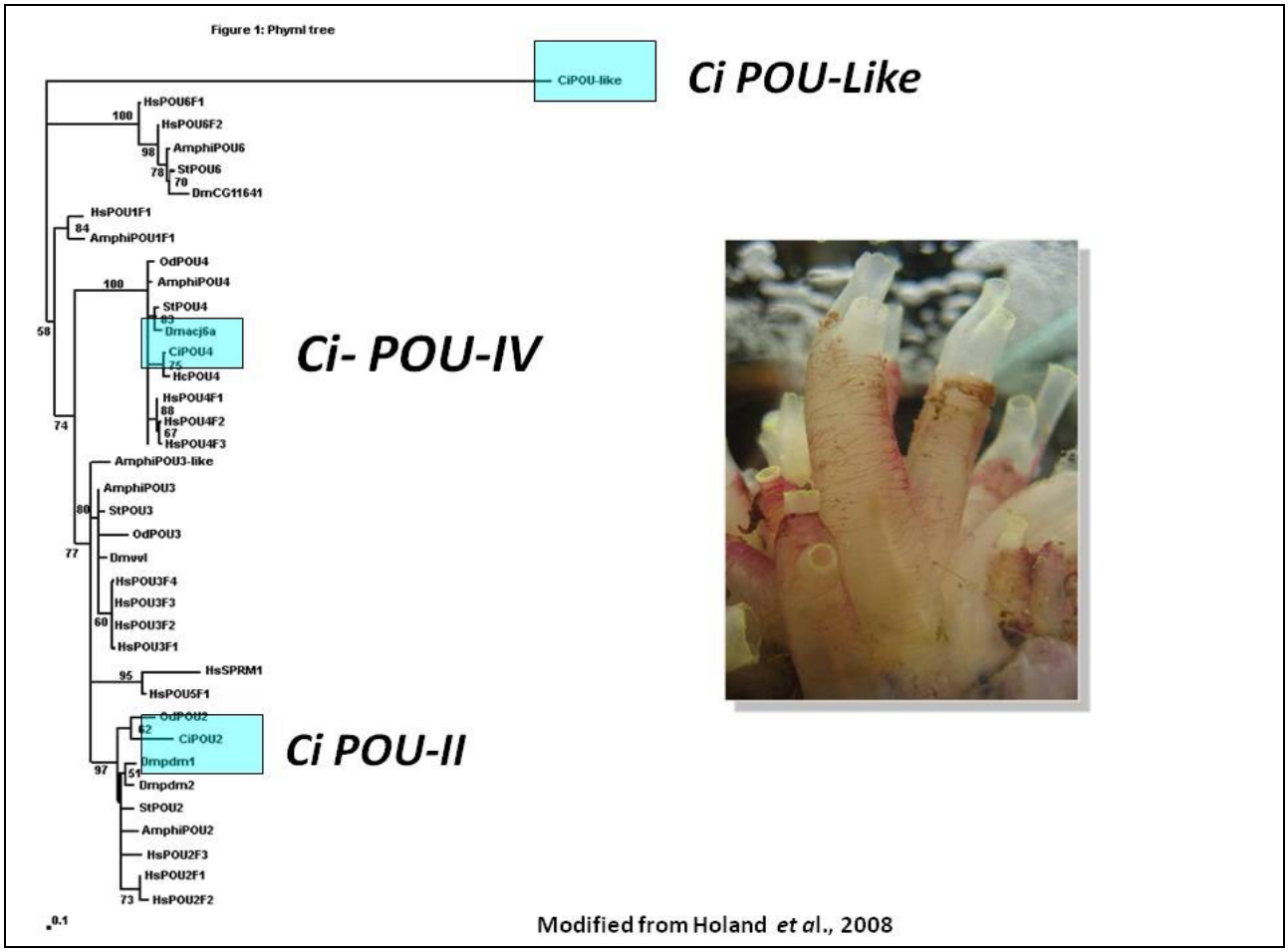
In 1997 Ryan and Rosenfeld proposed a critical and massive review of the developmental functions of members of the transcription factor family characterized by the POU DNA-binding motif.

The first description of the family of POU domain transcription factors was performed based on the observation that the products of three mammalian genes, *Pit-l, Oct-l,* and *Oct-2* and the protein encoded by the nematode *Caenorhabditis elegans* gene *unc-86* shared a region of homology (Ryan & Rosenfeld 1997). Thus this region was named the POU domain by the acronym of these genes.

The POU domain is a bipartite DNA-binding domain (Verrijzer et al., 1992), consisting of two highly conserved regions tethered by a variable linker. The amino terminal region is 75 bp long. This region was considered the POU specific domain. On the other hand the carboxy terminal region, that is 6o bp long, was called the POU homeodomain. Between these two regions there is a linker region of variable length. Both the linker region and the POU specific region of POU domains have been used to classify the POU genes that have been discovered since the first description. Currently they are grouped in six classes. These are the classes I, II, III , IV V and VI .

The *in vivo* functions of many POU proteins have been examined through targeted gene disruption in mouse and the characterization of loss-of-function and gain-of-function mutations in *Drosophila, C. elegans,* and *Xenopus.* These in vivo studies demonstrated that POU domain proteins regulate key developmental processes (Ryan & Rosenfeld, 1997). About the conformation of the POU domain on DNA, there is a high site-specific affinity of the DNA binding by POU domain transcription factors that requires both the POU-specific domain and the POU homeodomain (Verrijzer et al.1992). The two subdomains can bind DNA at the same time even when they are not joined by the linker region (Klemm and Pabo 1996). Resolution of the crystal structures of Oct-1 and Pit-1 POU domains bound to DNA as a monomer and homodimer, respectively, confirmed several *in vitro* findings regarding interactions of this bipartite DNA-binding domain with DNA and has provided important information regarding the flexibility and versatility of POU domain proteins (Ryan & Rosenfeld, 1997).

In *Ciona intestinalis*, only three POU class genes have been found during a genomewide survey of genes coding for homeobox transcription factors (Wada et al. 2003; Imai et al. 2004): *Ci-POU-IV*, belonging to the Class IV; *Ci-POU-2*, belonging to the class II and *Ci-POU-Like*, that does not belong to any known class from other organisms. A phylogenetic analysis of the position of these gene and of their relationships with the POU genes occurring in Cephalochordates are described by Holland et al. (2008). The phylogenetic tree reported in the figure 1.7 shows how different *Ci-POU-Like* is from the others.



Fi.g 3.1. Phylogenetic tree of the POU genes in both vertebrates and invertebrates. In the figure are underlined those of the ascidian *Ciona intestinalis.*

Wada et al (2003) consider *Ci-POU-like* to be a divergent member of the POU class of proteins. On the contrary *Ci-POU-2* and *Ci-POU-IV* cluster with the other POU genes of the respective classes. The classes missing in Tunicates, subclass III and VI, appeared to have been lost or were too divergent in the ascidian lineage to be identified, since both vertebrates and flies have genes of

these subclasses. On the other hand, it is possible that genes of subclass V are vertebrate innovations that appeared after the split of the vertebrate and ascidian lineages. The absence of subclass I genes in *Ciona* supports a view that this subclass also arose during vertebrate evolution, although this possibility requires more studies to be effectively tested (Wada et al., 2003).

The expression profile of *Ci-POU-IV* has been studied in detail by Candiani et al (2005). In general the class IV POU domain genes identified in mammals were immediately after their discovery related to the *unc-86* gene product in *C. elegans* (Ryan & Rosenfeld 1997). As shown in figure 1.7, this similarity is true not only for *Ci-POU-IV* but also for similar genes that are expressed in *Drosophila* and zebrafish and even in molluscs (Candiani et al., 2005; Pennati et al., 2009). Also the expression profiles of these genes share a high level of similarity in these organisms that are very distant from each other from a phylogenetic point of view. In particular, their expression is confined to the peripheral nervous system. This allows several inferences on the ancestral role for class IV POU genes in the development of the sensory cells.

In fact, in the nematode *C. elegans* the gene Unc- 86 is expressed in sensory and motor neurons (Finney and Ruvkun 1990); in the mollusk *Haliotis asinina* , transcripts of the gene *HasPOU-IV* are localized in chemo- and mechanosensory cells (O'Brien and Degnan 2002); in *Drosophila*, *POU-IV* genes are expressed in the visual and chemosensory systems (Certel et al. 2000). Furthermore, in zebrafish, the expression of *Brn3b* in the mechanosensory lateral line suggests a maintained role of a class IV POU domain protein in sensory system development (DeCarvalho et al. 2004). In mammals, each member of the Brn-3 family is described in a small subset of neurons in the brainstem and in the auditory/vestibular, somatosensory and visual system (Xiang 1998).

During *C. intestinalis* development, *Ci-POU-IV* is expressed in neural precursor cells (Candiani et al., 2005). Its expression is first detected at the neurula stage in two pairs of epidermal cells in the posterior dorsal part of the embryo. In early tail-bud embryos, the expression extends to some epidermal cells positioned ventrally along the growing tail. In the dorsal part of the embryo the expression is detected at the level of the borders of the rolling neural plate (the region, as described above, that corresponds to the neural crests of vertebrates). At the larval stage *Ci-POU-IV* is prevalent in the PNS. The only expression detected in the central nervous system is restricted to the posterior sensory vesicle and to some motoneurons of the visceral ganglion. On the contrary in the PNS, *Ci-POU-IV* occurs in all the three cell types of PNS described by Takamura (1998).

Thus it is present in two pear-shaped cells in each palps and in RTEN connecting primary neurons of the palps to the CNS. *Ci-POU-IV* mRNA is also detectable in ATEN, dorsal to the posterior part of the sensory vesicle. In the tail, pairs of *Ci-POU-IV* positive cells are localized at the midline of the dorsal and ventral epidermis and correspond to the CEN (Candiani et al., 2005).

There are no detailed study of *Ci-POU-II* and *Ci-POU-like*, with the exception of some annotations that are registered in ANISEED and that belong to the study of Imai et al, (2004).

3.1. Characterization of the expression profiles of two POU genes in the ascidian *Ciona intestinalis*: *Ci-POU-2* and *Ci-POU-like*.

Abstract

The aim of the research explained in this chapter was to define and characterize the expression profiles of both *Ci-POU-2* and *Ci-POU-like* and to infer some hypothesis on their roles during *C. intestinalis* development. Transcripts of the gene *Ci-POU-2* occur early during cleavage. In particular the expression is strong at the 4 cells stage in all the blastomers. The expression is maintained during the subsequent cell divisions even if after the 8 cell stage the intensity of the hybridization signal seems to decrease in all tested embryos. The *Ci-POU-like* expression is confined to the cells of the larval lateral mesenchyme and to their precursors during the embryonic development. the results of this research complete our knowledge about the expression of all the POU genes present in the ascidian *C. intestinalis* genome, providing the first complete description of the expression profiles of *Ci-POU-2* and *Ci-POU-Like* and demostrating that they are expressed in different territories comparing to the well studied *Ci-POU-IV* gene.

Introduction

As stated in the first chapter, a genome-wide survey of genes encoding for homeobox transcription factors carried out by Wada et al. (2003) and by Imai et al. (2004) revealed only three POU genes in *C. intestinalis*. The better characterized among these is *Ci-POU-IV* whose expression profile has been described by Candiani et al. (2005) and extensively studied during this research (see chapters 4 and 6). The other two genes are *Ci-POU-2* and *Ci-POU-like* that are not studied in detail yet. There are only some general annotations in the ANISEED database referring to the work of Imai et al, (2004). In a study of the POU genes of the tunicate *Oikopleura dioica* (class Larvacea), Cupit et al. (2006) found another POU gene (*OctB*) that they placed within the class III on the basis of their phylogenetic analysis.

Several genes of both invertebrates and vertebrates with broad expression and basic importance especially during the early stages of development belong to the class II of POU genes.

The protein Oct1 (POU2F1) is probably the most well known member of the class II. It is widely expressed in almost all vertebrate cells and is implicated in the regulation of a number of ubiquitously expressed genes such as those for histones H2B and H3B as well as small nuclear RNA (Ryan and Rosenfeld, 1997; Cupit et al., 2006). Oct1 plays also a role in a tissue specific way and, with its transcriptional co-activators, is implicated in the regulation of immunoglobulin genes and some cytokines (Shah et al., 1997). In addition to regulating transcription, Oct1 is also involved in chromatin modelling and apoptosis (Jin et al., 2001). Another important gene of the class II is Oct2 (POU2F2). It is highly expressed only in the central nervous system and in B lymphocytes,

where it is also implicated in the regulation of immunoglobulin gene transcription (Kemler and Schaffner, 1990). Another gene of the same class, Oct11 (POU2F3), contributes to epidermal stratification and homeostasis by promoting keratinocyte proliferation and differentiation (Hildesheim et al., 2001). Members of the Class II POU genes and their transcripts (or partial transcripts) have been identified and studied also in invertebrates. For example, in the genus *Drosophila* Billin et al (1991) found homologues of Oct1 (*pdm-1*, *dPOU-19*) and Oct2 (*pdm-2*, *dPOU-28*). These authors showed that these genes are highly expressed during early embryogenesis but their expression falls to lower levels during the other stages of development (Billin et al., 1991). Also in echinoderms, and specifically in the sea urchin (*Strongylocentrotus purpuratus*) there is a gene, named *spOCT*, that is closely related to the *Oct1* and *Oct2* vertebrate genes (Char et al., 1994). It is essential during the earliest stages of embryo development because it is required for early cleavage and protein accumulation (Char et al., 1994). In Urochordates other than *C. intestinalis,* the POU genes have been surveyed in *Oikopleura dioica* which posses three genes *OCtA1, OctA2* and *OctBm (Cupit et al., 2006)*. In their study Cupit and coworkers showed the inclusion of *O. dioica* OctA1, 2 within the class II branch, supported with a strong bootstrap value of 97%. The topology of their phylogenetic tree places the *O. dioica* OctA proteins, together with the gene *Ci-POU-II* of *C. intestinalis* on a branch that is ancestral to both the vertebrate *Oct1/Oct2* lineage and the vertebrate *Oct11/Skn1* lineage. Anyway, there are no data on their expression profiles. Very little is known about *Ci-POU-Like* that was identified during the already cited genomewide surveys of Wada et al. (2003) and Imai et al. (2004). Its phylogenetic position has been analyzed in the study of Holland et al. (2008) that showed that it does not belong to any known class of other organisms.

The aim of the research explained in this chapter was to define and characterize the expression profiles of both *Ci-POU-2* and *Ci-POU-like* and to infer some hypothesis on their roles during *C. intestinalis* development.

Material and methods

Adults of *C. intestinalis* were collected in the Gulf of Naples, Italy, and reared in aquaria. Gametes were used for in vitro fertilization. Fertilized eggs were allowed to develop in 9-cm Petri dishes in Millipore-filtered seawater at 18°C.

Sequence and phylogenetic analysis: A POU-2 cDNA clone named cieg42p24, present in the gene collection of Satoh (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and in an expressed sequence tags

database (ESTs) of *C. intestinalis* (Satou et al. 2001; Kusakabe et al. 2002; Ogasawara et al. 2002) was chosen from those exported in the published database at http://aniseed-ibdm.univ-mrs.fr. In order to synthesize the probe for the clone cieg42p24, a PCR was carried out using 1 µl Ciona genomic DNA in a total volume of 50 µl, containing 1.5 mM $MgCl_2$, 0.5 µM each primer and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystem, Calif., USA). The gene-specific primers used for *Ci-POU-2* were: ACCGAATCTGCCACAAAAGA, for predicted start codon and TGGGAGCATCTGATGTGGAA. Primers for the *Ci-POU-like* probe were obtained from GenBan (GenBank Accession: AK112159.1) and refer to the clone cieg024l04. they are: GCTTCTGCGACCTCTGAGG and GCCTTGTTGAATCCGCAG. PCR conditions used for *Ci-POU-2* amplification (fragment length: 962 bp) are: 94°C 2 minutes, then 35 cycles at 94 °C for 30sec, 56 C for 30 sec and 72 C for 1 min 10 sec. Extension occurred at 72 °C for 7 minutes. PCR conditions used for *Ci-POU-like* (fragment length: 1161 bp) were: 94°C 2 minutes, then 35 cycles at 94 C for 30sec, 57 C for 30 sec and 72 C for 1 minute. Extension occurred at 72 °C for 7 minutes.

Amplified PCR products were cloned using the TOPO VECTOR cloning kit (Invitrogen, San Diego, Calif., USA). The plasmid was then used to transfect competent *E. coli* cells: 2 µl of the TOPO Cloning reaction was added in a vial of One Shot Chemically competent *E. coli* (Invitrogen, San Diego, Calif., USA). Transformation was carried out by heat-shock for 30 second at 42 °C. After an hour in SOC medium cells were plated in ampicilline selective agar plates and incubated overnight at 37 °C. Successively cells were cultured in 200 ml of LB broth overnight at 37 °C. Plasmids were then purified with the Invitrogen Maxi Prep Kit (Invitrogen, San Diego, Calif., USA). Linearization was performed with EcoRV restriction enzyme for the sense and with the BamHI enzyme for the antisense sequence. 20 µl of DNA from Maxi prep were used and digested with 4 µl of the restriction enzyme in a total volume of 60 µl. The reaction was performed for two hours at 37 °C. After having verified that linearization worked by electrophoresis analysis, the linearized DNA was extracted with phenol-chloroform and precipitated in a solution containing 3M NaCl, absolute EtOH and glycogen overnight at -20 °C. After centrifugation the resulting pellet was resuspended, quantified by spectophotometry and used for in vitro transcription. The sense sequence was transcribed with 2 µl of SP6 polymerase, the antisense one with T7 polymerase. The probe was labeled using DIG-NTPs (Roche, New York). The reaction was performed at 37 °C for two hours. The DNA was then digested with DNAse for 15 minutes at the same temperature. The probes were then precipitated with 0.2 mM EDTA, 4 mM LiCl, Glycogen and EtOH overnight at -20 °C. After 45 minutes of centrifugation the pellet was resuspended in 30 µl of molecular biology grade H_2O and used for the in situ experiments.

Whole-mount in situ hybridization was carried out according to Holland et al. (1992). Larvae and embryos were fixed with 4% paraformaldehyde in 0.5 M sodium chloride, 0.1 M MOPS (morpholino propanesulfonic acid) (pH 7.5) at room temperature for 90 min, dehydrated in 30, 50 and 70% ethanol (10 min each) and stored in 70% ethanol at –20°C. All further steps were performed at room temperature unless otherwise specified. Embryos were manually deprived of their envelopes after rehydration by successive incubation in 50 and 30% ethanol and in PBST (phosphate-buffered saline containing 0.1% Tween 20). The specimens were then treated with 20 µg/ml proteinase K in PBST (30 min, 37°C) and digestion was stopped by washing with PBS three times (5 min each). The specimens were postfixed with 4% paraformaldehyde in PBS for 1 h, followed by washing with PBST three times (5 min each). Acetylation was carried out in 0.1 M triethanolamine, pH 8.0, supplemented with 0.25% acetic anhydride for 10 min and then the specimens were washed in PBST three times (5 min each). The specimens were incubated in prehybridization buffer (50% formamide, 5 × SSC, 50 µg/ml heparin, 50 µg/ml yeast tRNA, 5 × Denhardt's solution, 0.1% Tween 20) for 1 h at 42°C. Hybridization was carried out at 60°C overnight. After the hybridization, specimens were washed in 50% formamide, 4 × SSC, 0.1% Tween 20 (2 × 15 min, 60°C), then 50% formamide, 2 × SSC, 0.1% Tween 20 (2 × 15 min, 60°C) and solution A (0.5 M NaCl, 10 mM TRIS-HCl pH 8.0, 5 mM EDTA, 0.1% Tween 20; 3 × 10 min, 37°C); treated with 20 mg/ml RNase A in solution. A (30 min, 37°C) and washed with 50% formamide, 2 × SSC, 0.1% Tween 20 (20 min, 60°C), 50% formamide, 1 × SSC, 0.1% Tween 20 (2 ×15 min, 45°C), 1 × SSC/PBST 1:1 (15 min) and PBST (4 × 5 min). RNA hybrids were detected immunohistochemically. After blocking in 5% normal sheep serum (NSS) in PBST (30 min), the specimens were incubated with 1:2000 alkaline phosphatase-conjugated anti DIG-antibody (Boehringer Mannheim) in the above buffer (overnight, 4°C). The specimens were washed with PBST (4 × 20 min) and alkaline phosphatase buffer (APB; 100 mM NaCl, 50 mM MgCl2, 100 mM TRIS-HCl pH 9.0; 3 × 10 min). Signal detection was performed in APB containing 4.5 µl NBT/ml (nitroblue tetrazolium salt) and 3.5 µl BCIP/ml (5-bromo-4-choro-3-indolylphosphate) according to the supplier's instruction (Boehringer Mannheim DIG RNA Detection Kit). When satisfactory signals over the background were obtained, the solution was replaced with PBST. Larvae were thus fixed 4% PFA, used for taking pictures at the microscope and then conserved in 80% glycerol solution at –20°C.

Results

Transcripts of the gene *Ci-POU-2* occur early during cleavage. In particular the expression is strong at the 4 cells stage in all the blastomers. The expression is maintained during the subsequent cell divisions even if after the 8 cell stage the intensity of the hybridization signal seem to decrease in all tested embryos. At the 16-cells stage, when all animal and vegetal cells have undergone a fourth cleavage and there is a first sign of a clear antero-posterior polarity in the shape of embryo, the expression is detected especially in a6 and b6 lineages. At the 32-cell stage, when all blastomeres have undergone a fifth cleavage, which occurs earlier in the vegetal than animal lineages, *Ci-POU-2* is still expressed in the a6 and b6 cells. During the further cleavages the expression becomes more and more feeble and at the gastrula stage it is not more detectable (Fig 3.1.1).

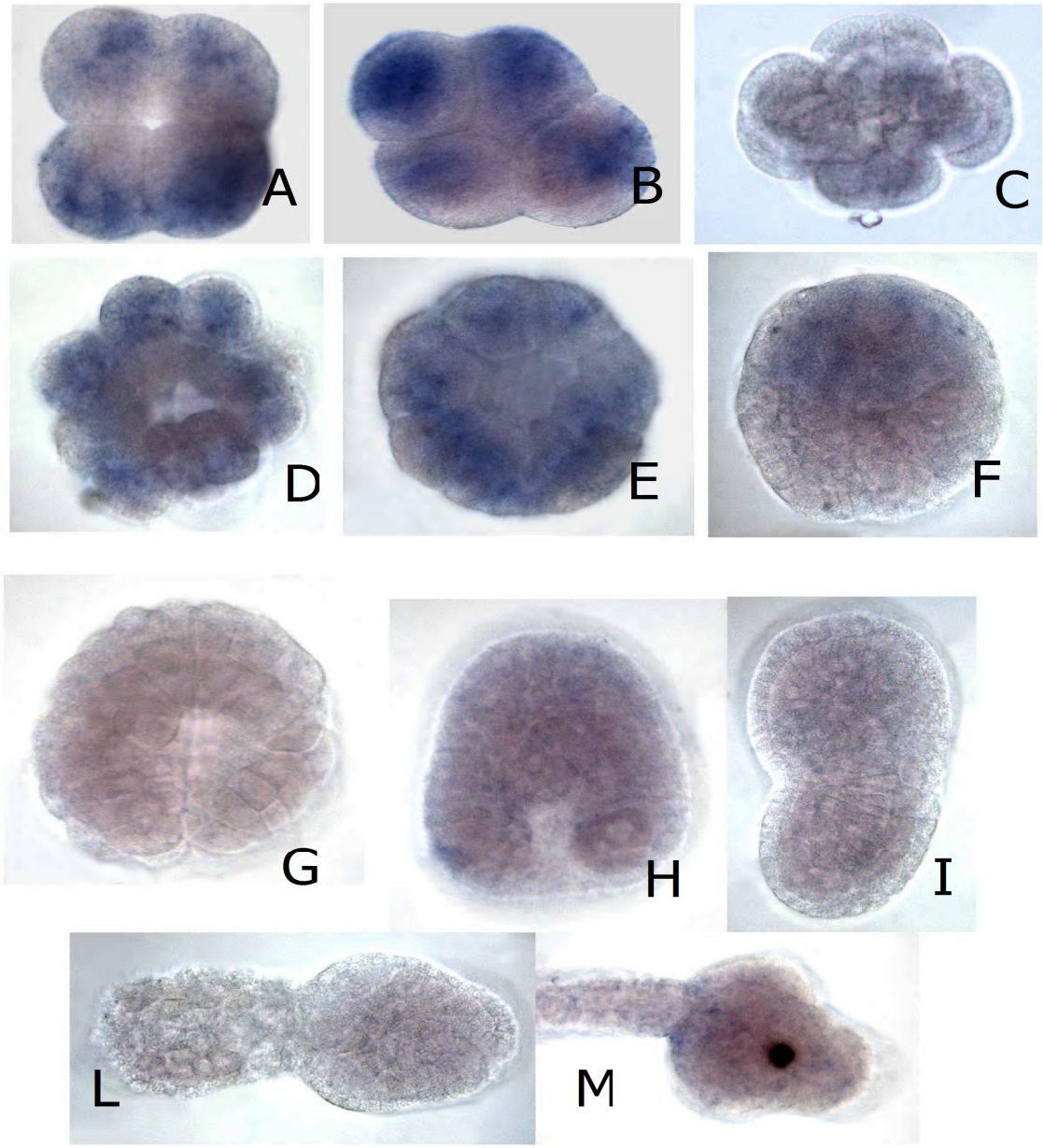


Fig 3.1.1. Expression profile of *Ci-POU-2* in *C. intestinalis* from 4 cell stage to Larva. A, B = 4 cells stage, C = 8 cell stage, D = 16 cell stage, E, F = early gastrula stage, G = mid gastrula stage, H = neurula stage, I , L = initial tailbud stage, M = larval stage.

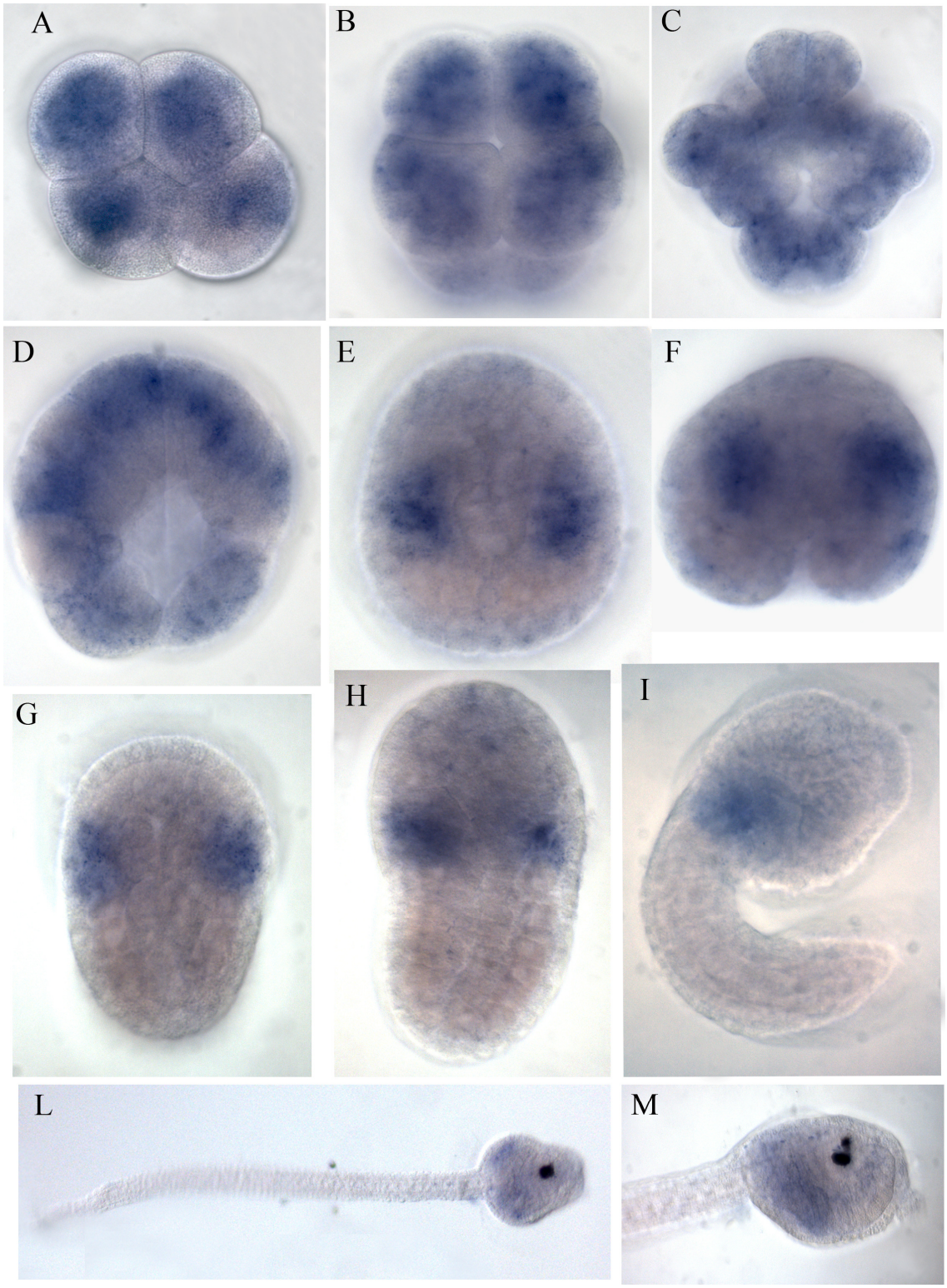


Fig. 3.1.2. Expression profile of *Ci-POU-Like.* A: four cell stage, B: eight cell stage, C: sixteen cell stage; D, E: gastrula stage, F: late gastrula stage, G: late neurula stage, H: early tailbud stage, I = mid tailbud stage, L, M: pre-hatching larva.

The *Ci-POU-like* expression is confined to the cells of the larval lateral mesenchyme and to their precursors during the embryonic development. In particular it is first detected in all the blastomers at the 4-cell stage. The expression level is strong till 110-cell stage where Ci-POU-Like occurs with an strong hybridization signal in A8.16, A8.15, A8.8 and A8.7. At this stage there are also some transcripts in the B8 lineage. At an advanced gastrula stage the expression is confined to the mesenchyme precursors and there it is detected from the tailbud stages till the larva. In this latter developmental stage the expression is still evident and easy to be detected (Fig. 3.1.2).

Discussion

In the present work, we have characterized the expression profiles of two POU genes of the ascidian *C. intestinalis* : *Ci-POU-2* and *Ci-POU-like*. In ascidians, POU genes have been initially identified in genomewide surveys of genes encoding for homeobox transcription factors that were carried out by Wada et al. (2003) and by Imai et al. (2004). These works indicated that *C. intestinalis* has only three POU genes. A successive study found in the Larvacean *Oikopleura dioica* (Cupit et al. 2006) another POU gene (OctB) that was classified within the class III. Incomplete expression profiles of *Ci-POU-2* and *Ci-POU-like* were previously reported in the ANISEED database where it was indicated that these genes are maternally expressed in the oocytes.

The early expression of *Ci-POU*-2 during cleavage up to the gastrula stage fits to what is already known about POU2 genes both in vertebrates and invertebrates. The mammalian homolog *Oct1* (or *Pou2f1*) is expressed in a variety of tissues and cell types including placental JEG-3 cells and neuronal GT1–7 cells. It is hypothesized that Oct-1 may participate in tissue-specific gene expression by interaction with either other transcription factors or tissue-specific coactivators (Dong et al., 2001). In mammals it is expressed very early, on embryonic day 8 in the egg cylinder. Also during the interaction with Pit-1 (a protein encoded by the mammalian POU gene of class I) Oct-1 protein appears to be present much earlier during pituitary development than Pit-l (Voss et al., 1991). Donner et al. (2007) identified Oct-1 as a co-factor for *Sox2* in the context of mouse lens and nasal placode induction. *Sox2* encodes an SRY-like HMG box transcription factor and is critical for vertebrate development. Anyway Oct1 is known to interact with regulatory sites in interleukin, immunoglobulin, and histone genes (Murayama et al. 2006). Oct1 also moderately stimulates gene expression reporter constructs linked to target sequences in transient transfection assays (Kang et al., 2008). This information cannot be extended to *Ci-POU-2*, given the lack of functional studies on the role of this gene in protochordates. As already stated in sea urchin, the gene *spOct* is fundamental during the earliest stages of the embryo development being required for early cleavage

and protein accumulation (Char et al., 1994). Also in Drosophila the two class II POU genes *Pdm-I* (nubbin, dpou-19) and *Pdm-2* (mitimere, dpou-28) are co-expressed in the early embryo with a gap-like pattern; they have been also detected in the peripheral sensory organs, neuroectoderm, neuroblasts and ectoderm (Lee et al., 2010).

The expression of *Ci-POU-like* is also detected early during development but it persists longer being detectable till the larval stage, where it is limited to cells of lateral mesenchyme.

The POU genes typically expressed in mesenchyme are members of class III. For example targeted mutagenesis in mice demonstrates that the POU III gene *Brn4/Pou3f4* plays a crucial role in the patterning of the mesenchymal compartment of the inner ear (Phippard et al., 1999). *Brn4* is expressed extensively throughout the mesenchyme of the developing inner ear. Phippard et al. (1999) introduced mutations for *Brn4* suppressing its functional activity. In their study the mutant animals displayed behavioral anomalies that resulted from functional deficits in both the auditory and vestibular systems, including vertical head bobbing, changes in gait, and hearing loss. Furthermore, they performed different anatomical analyses of the temporal bone, which is derived in part from the otic mesenchyme, and they demonstrated that several dysplastic features occurred in the mutant animals, including enlargement of the internal auditory meatus. Finally they suggested that *Brn4* plays a role in the epithelial-mesenchymal interaction necessary for the cochlea to develop correctly. Yet in vertebrates,such as *Xenopus laevis*, the *XlPOU 2* gene, member of the class III POU-domain family that is expressed initially at mid-blastula transition (MET) and during gastrulation in the entire marginal zone mesoderm, when it is inactivated suppresses the expression of a number of dorsal mesoderm-specific genes, including *gsc*, *Xlim-1*, *Xotx2*, *noggin* and *chordin*, but not *Xno* (Witta & Sato, 1997). As a consequence of the suppression of dorsal mesoderm gene expression, bone morphogenetic factor-4 (Bmp-4), a potent inducer of ventral mesoderm, is activated in the Spemanns Organizer structure (Witta & Sato, 1997).

These fact could suggest that in *Ciona intestinalis Ci-POU-like* could play the role that in vertebrates is usually performed by the POU genes of class III even if further studies are required. Moreover these aspects of the function of vertebrate POU genes can help to understand how conserved is the expression territory among them and the gene *Ci-POU-2* suggesting also a conserved role.

In conclusion the results of this research complete our knowledge about the expression of all the POU genes present in the ascidian *C. intestinalis* genome, providing the first complete description of the expression profiles of *Ci-POU-2* and *Ci-POU-like* and demostrating that they are expressed in different territories comparing to the well studied *Ci-POU-IV* gene.

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3.2. Role of the alternative transcripts of the *Ci-POU-IV* gene in the development of the Peripheral Nervous System of the tunicate *Ciona intestinalis*.

Abstract

Some members of the POU gene family regulate the neural patterning and differentiation in both vertebrate and invertebrate embryos. The tunicate *Ciona intestinalis* has only three genes encoding for POU transcription factors and the gene *Ci-POU-IV* is specifically expressed in all the peripheral nervous system (PNS) territories and in some cells of the central nervous system during development. The aim of this work was to study the role played in neural differentiation by the two alternative transcripts of *Ci-POU-IV*, that we discovered. We designed Morpholino oligos to perform gene knock-down experiments for the different isoforms, the short and the long one. Results from these experiments revealed that the expression of the serotonin rate-limiting synthesis enzyme, tryptophane hydroxylase (*Ci-Tph*), and glutamate transporter (*vGlut*) could be regulated by the long transcript. To verify if the alternative transcripts are expressed in different neuron populations, we synthesized a probe selective for the short isoform. The in situ hybridizations, compared to the whole expression profile of *Ci-POU-IV*, showed a lack of expression of the "short" form in the sensory epidermal neurons of the trunk.

Introduction

Ascidians are marine sessile organisms, and together with Thaliacea and Larvacea, specialized for a free-swimming planktonic existence, they form the subphylum Urochordata or Tunicata. With Cephalochordata and Vertebrata, Tunicata constitutes the phylum Chordata. The larva displays a typical chordate body plan with a dorsal tubular central nervous system (CNS) above a central notochord flanked by muscle blocks, but it is composed of a remarkably small number of cells. The tadpole larva has a trunk and tail, is completely covered by a single-layer epidermis which produces the larval tunic. The PNS of the ascidian tadpole larva comprises a population of isolated sensory neurons distributed along the trunk and the tail. They are embedded within the larval epidermis (Takamura, 1998) most with ciliary projections into the tunic. Several sensory neurons, called papillar neurons or anchor cells, are present in each palp, which constitute the adhesive organ; they can be classified as chemosensory. Moreover, three groups of epidermal sensory neurons have been localized in the rostral trunk (RTEN), apical trunk (ATEN) and in the tail (DCEN, VCEN) (Takamura, 1998; Hotta et al., 2007). *C.intestinalis* genome is available (Dehal et al., 2002); it is composed of 160 Mb and contains approximately 16,000 protein-coding genes, a genome size and a gene number quite compact as compared with those of vertebrates, in fact this genome contains a basic set of genes for the chordate body plan with less genetic redundancy than the genomes of vertebrates. *POU* genes are transcription factors involved in important steps of early embryogenesis

and especially in the neural differentiation and patterning in both vertebrates and invertebrates. One of these genes, *Ci-POU IV*, is expressed in all the sensory cells of the peripheral nervous system (PNS), and in some cells of the visceral ganglion in the central nervous. As stated in the chapter 2, *Ci-POU-IV* expression has been characterized by Candiani *et al*., (2005).

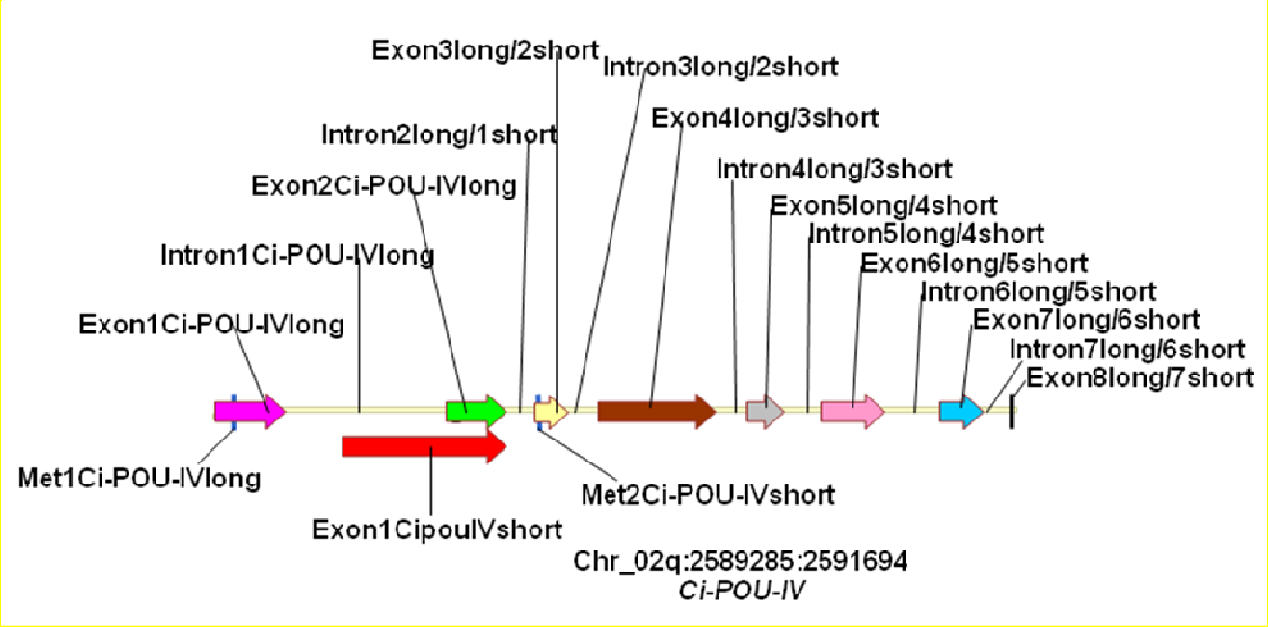

Fig. 3.2.1. Genomic organization of *Ci-POU-IV* with the two isoforms evidenced.

The existence of two alternative transcripts was then evidenced (Zega *et al*., 2007). In particular there is a short isoform, whose length is 294 and a long isoform 412 bp long (fig. 5.1). The POU-IVbox is present only in the long isoform while the POU homeodomain and the POU specific domain are shared features between the two isoforms. This situation is also present in the mammalian gene *Brn3a* and in the zebrafish gene *Brn3b* (Latchman, 1999; DeCarvalho *et al*., 2004). In particular it has been shown that *Brn-3a* exists in two different forms which are generated by alternative splicing of its RNA (Latchman, 1999). Although both these forms contain the C-terminal POU domain, they differ in that the longer form of *Brn-3a* contains an additional 84 amino acids at the N-terminus which are absent in the shorter form. In mouse both the long and the short forms of *Brn-3a* were able to induce neurite outgrowth, but the neurite outgrowth could be also obtained simply by over expressing the isolated POU domain of *Brn-3a*, indicating that this region of the protein is sufficient for determining this effect (Latchman, 1999). In zebrafish the two *Brn-3b* alternative transcripts exhibited a different timing of expression during the development but shared overlapping expression domains in the adult visual system (DeCarvalho et al., 2004). The

Drosophila POU IV class gene *Acj6* is also expressed at the level of PNS and shows two alternative transcripts that display different activities *in vivo* (Certel et al., 2000).

The aims of this study are first of all to characterize the expression profile of the short isoform of *Ci-POU-IV* and to understand the alternative transcripts of the *Ci-POU-IV* gene in the development of the *C. intestinalis* PNS.

Materials and Methods

Probe for *Ci-POU-IV* short

The short isoform is a truncated version of the long isoform and lacks completely of the **POU-IV box**. The long and short isoforms encode for 412 and 294 amino acids, respectively (Fig. 3,2.2)

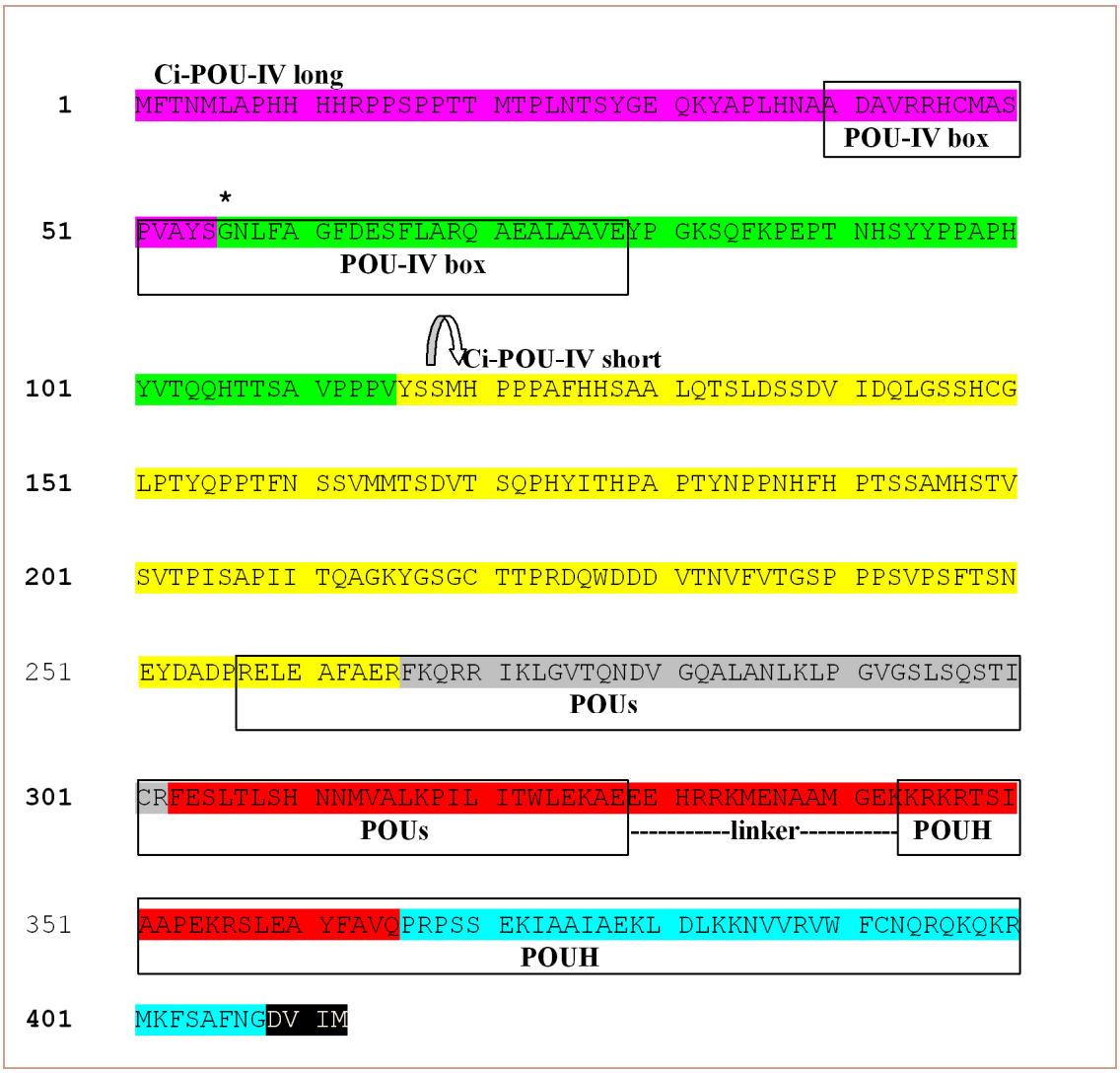

Fig. 3.2.2. Indication of POU-IV-box, POUs (POU specific domain), POUH (POU homeodomain) and the linker region. The different colours used correspond to the exons showed in the genomic organization. The arrows indicate the two possible codon start.The first amino terminal intron that interrupts the coding sequence at level of **POU-IV box**, is indicated with an asterisk.

To build the specific probe for the short isoform, we designed primers for PCR that aplified a region situated in the first intron of the long isoform and encompassing the 5'UTR of the short isoform, as indicated in figure 3.2.3.
The used primers were:
FORWARD: 5'TGAATAGAAACTTGAAGT3'

REVERSE: 5'ACCTGCACGGCGAAGTAAG3'

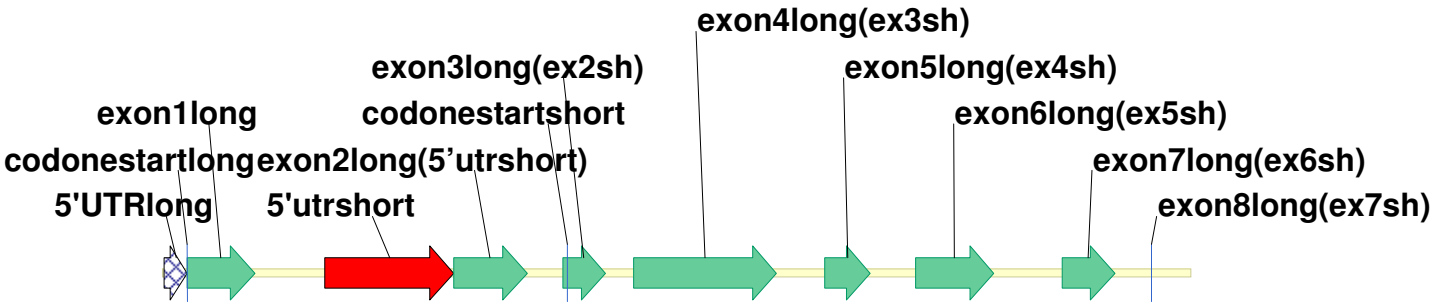

Fig. 3.2.3: The red arrow indicates the position of the sequence used to synthetize the short isoform specific probe.

The primers were used to amplified a cDNA library of C.intestinalis. The product of PCR was sequenced, inserted in TOPO vector and cloned in a *E.coli* strain as already described (see material and methods of chapter 3.1).

Gene-knockdown experiments

We designed Morpholino oligos to perform gene knock-down experiments for the different isoforms, the short and the long one. Injections were performed as described by Ristoratore et al., (1999) and Bertrand et al., (2003). In order to perform the microinjections, the eggs were previously dechorionated. Dechorionation was carried out by treating the eggs with a solution 1% sodium thioglycolate (Serva), 0.05% protease E (Sigma), in sea water, pH 10, for 5-10 minutes with

pipetting. After this treatment eggs were transferred onto plastic dishes coated with 0.9% agarose in milliporefiltered artificial sea water. Microinjections were carried out before fertilization with 10 µg/µl Fast Green (Sigma F7252) as a marker dye. Approximately 30 pl of solution was injected per egg. The sequences of the morpholino oligos purchased from Gene Tools, are as follow:

Ci-POU-IV short –MO: ATTGAACTGTAAACTGGTGGTGGTA;
Ci-POU-IV long- MO: TGTTAGTAAACATATCGTATCAAAA.

Ci-POU-IV short control: ATGGTGGTGGTCAAATGTCAAGTTA
Ci-POU-IV long control: AAAACTATGCTATACAAATGATTGT

Morpholino concentrations were 0.2mM for the short isoform and 0.7 mM for the long one. Control experiments were performed injecting the egg with a control inneffective Morpholino, at the same concentrations.
Injections were performed trough borosilicate glass capillaries GC 100 TF, Harvard Apparatus Ltd. UK, Harvard Part. No. 30-0038 1.0 mm O.D. x 0.78 mm I. D. Tip needles have been pulled using a Flaming/Brown Micropipette Puller Model P-97 (Sutter Instruments).
The injection apparatus was constituted by a Narishige Mcromanipulator MWS -2. Injections were performed in a 17°C cooled room.
Injected eggs were transferred to a fresh agarose dish kept at 17° C until fertilization after the required delay of incubation. Embryos were rared at 18°C in Filtered sea water until control reached the prehatching stage (16hpf)

In situ hybridization

Full-length cDNA was used as template for in vitro transcription by using Roche DIG RNA labeling Kit, according to the supplier's instructions, and stored in H_2O at -80°C.
Larvae and embryos were fixed with 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS (3-N-morpholino propansulfonic acid), pH 7.5, at room temperature for 90 minutes, dehydrated in 30%, 50% and 70% ethanol (10 minutes each) and stored in 70% ethanol at -20°C. Embryos were rehydrated and after several washes in PBT they spent 1 hour in prehybridization buffer (50% formamide, 5´ SSC, 50 mg/ml heparin, 50 mg/ml yeast tRNA, 5´ Denhardt's solution, 0.1% Tween 20) at 48°C, embryos were allowed to hybridize with 0.25 mg/ml.
Hybridization was carried out at 50°C for 16 hours. The specimens were washed in 50% formamide, 4´ SSC, 0.1% Tween 20 (2´ 15 minutes, 50°C), then in 50% formamide, 2´ SSC, 0.1%

Tween 20 (2´ 15 minutes, 50°C) and in solution A (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% Tween 20) (3´ 10 minutes, 37°C), then treated with 20 mg/ml RNase A in solution A (30 minutes, 37°C) and washed with 50% formamide, 2´ SSC, 0.1% Tween 20 (20 minutes, 50°C), 50% formamide, 1´ SSC, 0.1% Tween 20 (2´15 minutes, 50°C), 1´ SSC/PBST 1:1 (15 minutes), PBST (4´5 minutes). RNA hybrids were detected immunohistochemically. After blocking in 5% Normal Sheep Serum (NSS) in PBST (30 minutes), the specimens were incubated with 1:2000 alkaline phosphatase-conjugated anti- DIG antibody (Roche) in the above buffer (overnight, 4°C). The specimens were washed with PBST four times (20 minutes each) and alkaline phosphatase buffer (APB) (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0) (3´10 minutes). A pre-washing inAP buffer without Mg was performed twice for 10 minutes in order to prevent any precipitation from the previous solution of PBT. Signal detection was performed in AP Buffer containing 4.5 ml/ml NBT (nitroblue tetrazolium salt) and 3.5 ml/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) according to the supplier's instructions (Roche DIG RNA Detection Kit). When satisfactory signals over the background were obtained, the solution was replaced with PBST.

Results

Characterization of the expression profile of *Ci-POU-IV* short isoform

To verify if the alternative transcripts were expressed in different neuron populations, we synthesized a probe selective for the *Ci-POU-IV* short isoform. The *in situ* hybridizations, compared to the whole expression profile of *Ci-POU-IV,* showed a lack of expression of the "short" form in the sensory epidermal neurons of the trunk. The short isoform as the entire *Ci-POU-IV*, is first detected at the neurula stage in two pairs of epidermal cells in the posterior dorsal part of the embryo. As exposed in chapter 1 these cells could be the precursors of the tail epidermal sensory neurons (ESN) of the larva. Also in the case of *Ci-POU-IV* short, the early tail-bud embryos showed the expression that extends to some ventral epidermal cells of the tail, whose number gradually increases during development. The only detectable differences were in anterior part of the embryo where in the short isoform injected embryos the transcripts were not detected at the level of the rostral and apical neurons (Fig. 3.2.4).

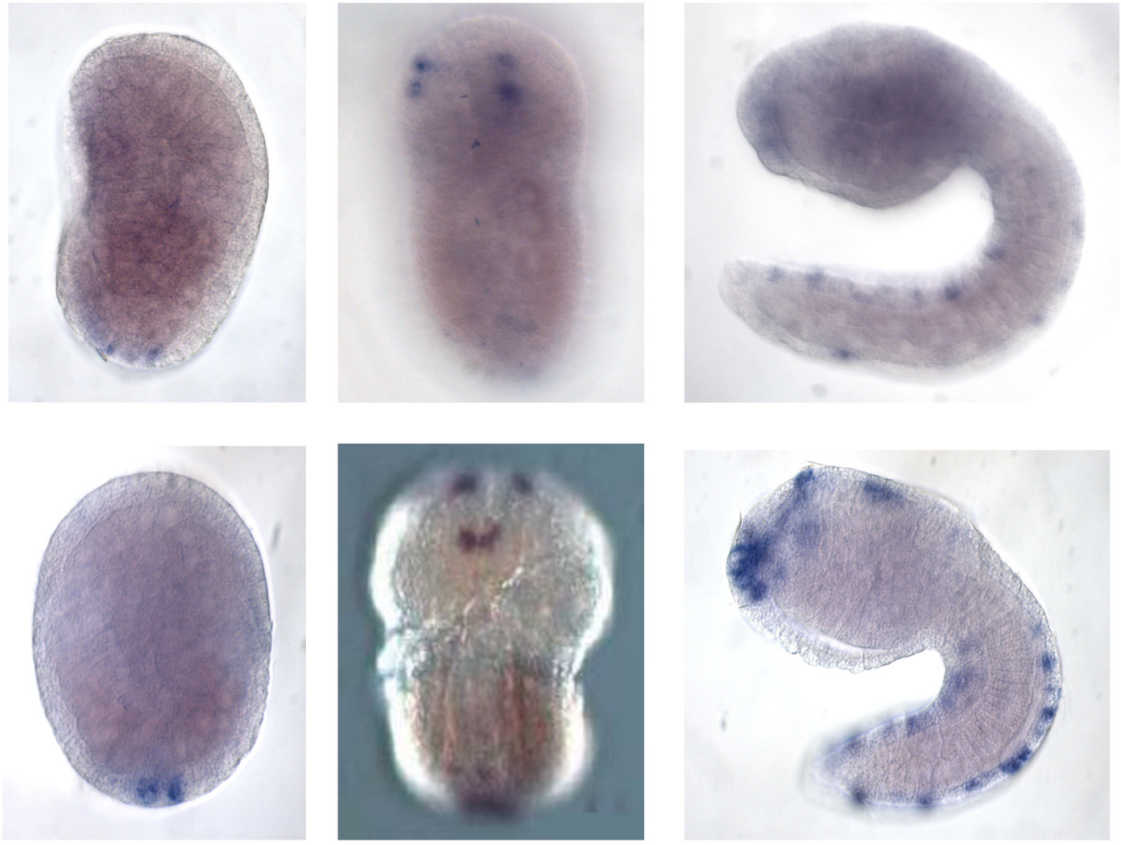

Fig. 3.2.4. Expression profile of the short isoform compared to that of *Ci-POU-IV*. The upper line refers to *Ci-POU-IV* short; from left to right in the upper line we see a late neurula, an initial tailbud and a mid tailbud. The lower line refers to *Ci-POU-IV*; as above from left to right in the upper line we see a late neurula, an initial tailbud and a mid tailbud.

Gene knock-down experiments

Results from these experiments revealed differences in the expression in al the three genes observed. In particular *Ci-Pans* was affected in the embryos injected with the morpholino against the *Ci-POU IV* long isoform. In these specimens there was a clear difference between controls and treatments. In the latter the development seems to be delayed with a staining still recorded in the CEN, while in the controls the CEN were not yet marked. On the other hand in the embryos injected with the morpholino against the short isoform there was no detectable difference between the control and treated ones (Fig. 3.2.5; 3.2.6)

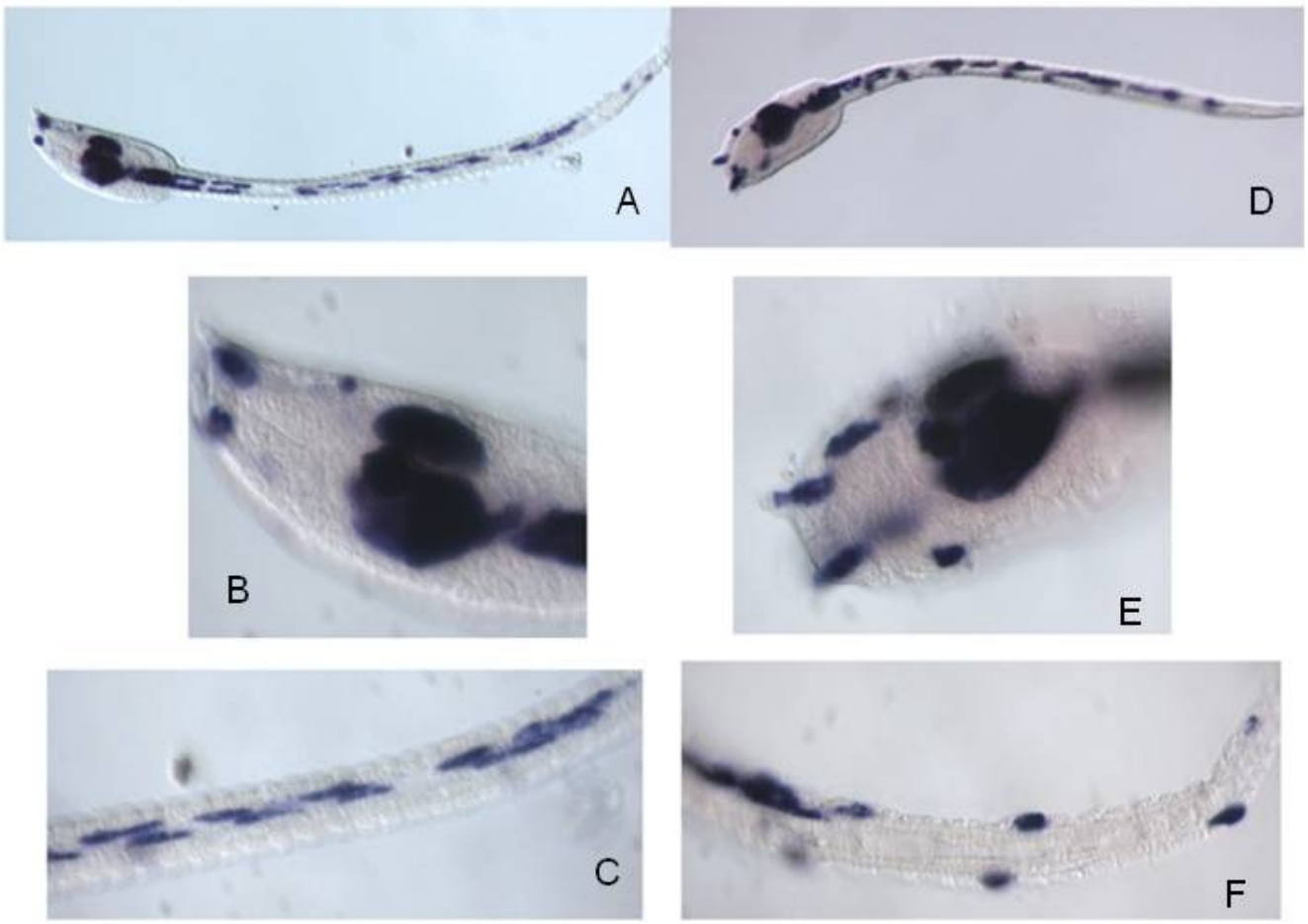

Fig. 3.2.5. Effects of *Ci-POU-IV* long Morpholino 0.7 mM on *Ci-Pans* expression in larvae of *Ciona intestinalis*; A, B, C = controls; D, E, F treated specimens.

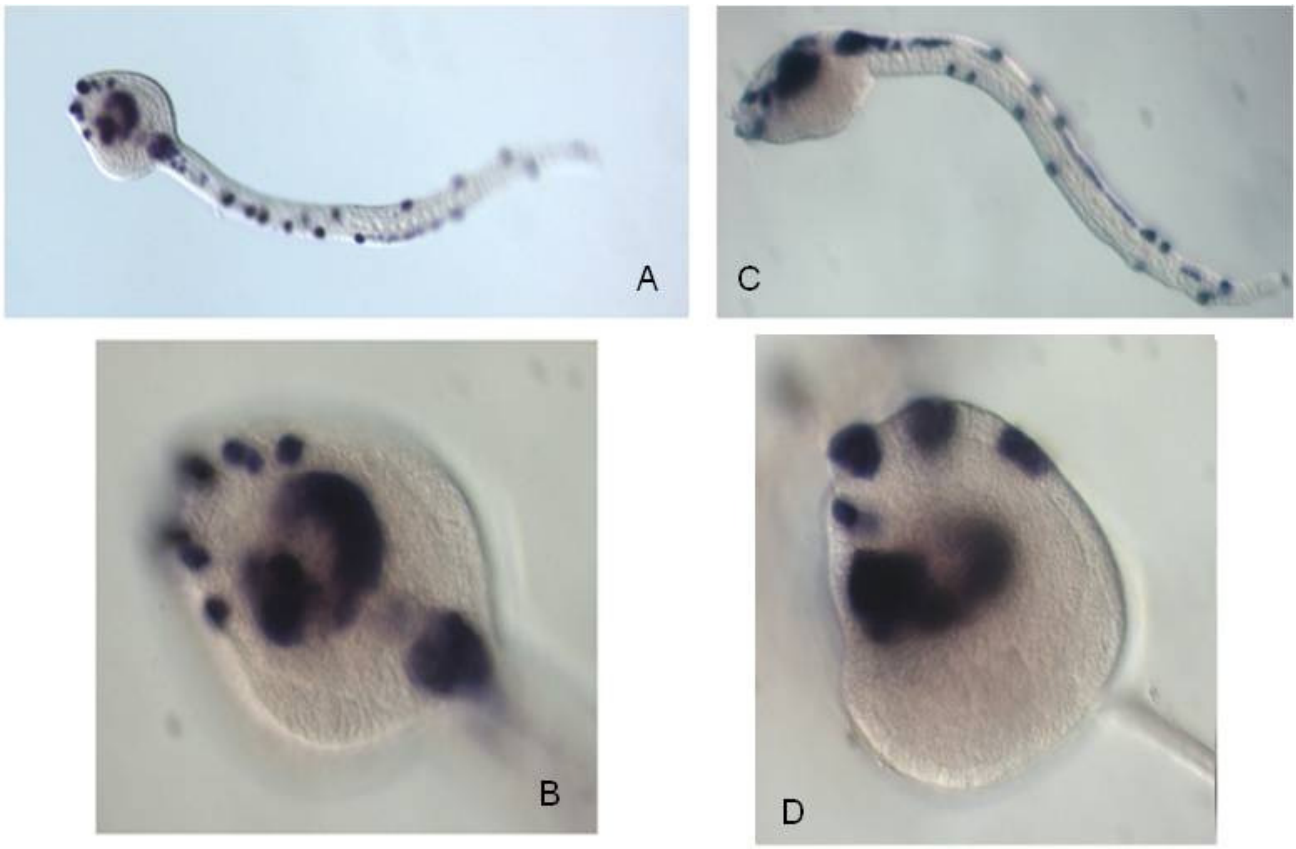

Fig. 3.2.6. Effects of Ci-*POU-IV* short Morpholino 0.2mM on the expression *Ci-Pans* in larvae of *C. intestinalis*. A, B = controls; C, D, = treated specimen.

Also the serotonin rate-limiting synthesis enzyme, tryptophane hydroxylase (*Ci-Tph*) is affected in injected embryos with the morpholino against the long isoform. In particular in the injected embryos there is a clear lack of expression at the level of the visceral ganglion (Fig. 3.2.7, A, B). The injection with the short isoform also affect *Ci-Tph* expression in the visceral ganglion and moreover seems to determine an overexpression of *Ci-Tph* in the tail (Fig. 3.2.7 C, D).

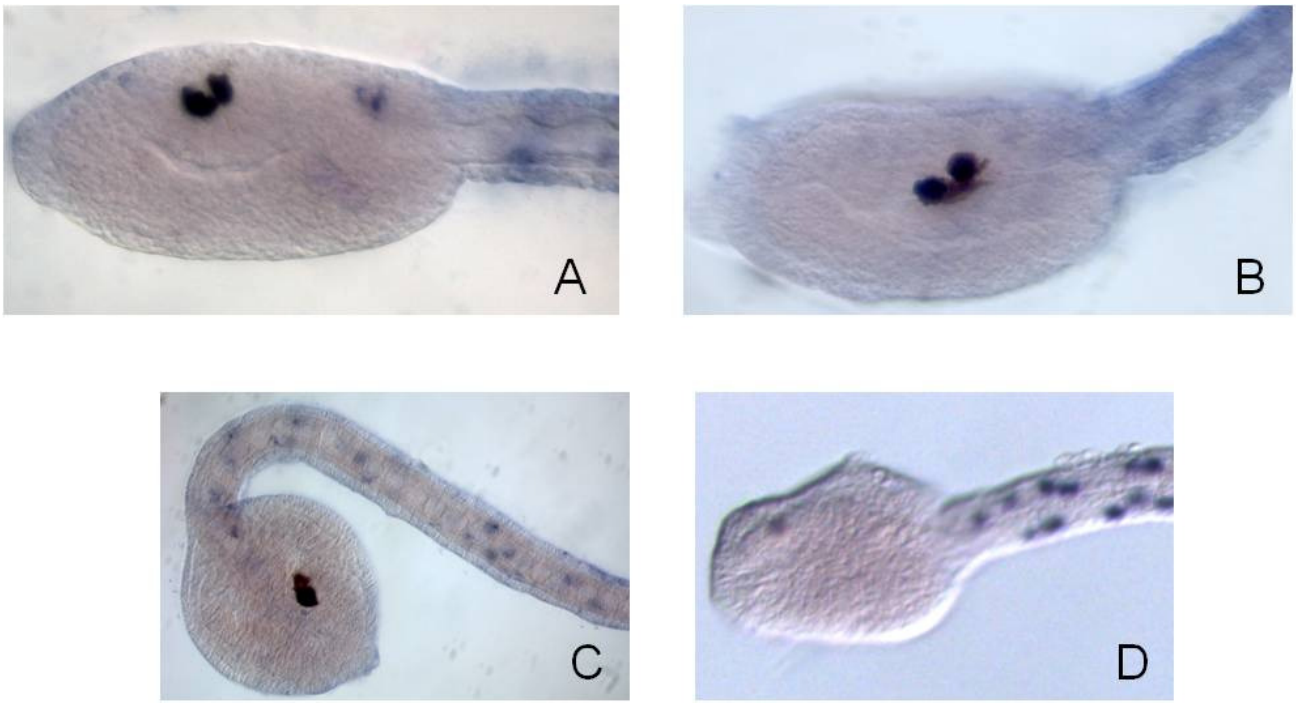

Fig. 3.2.7. Effects of *Ci-POU-IV* long Morpholino l 0.7 mM and *Ci-POU-IV* short Morpholino 0.2 mM on tryptophane hydroxylase (*Ci-Tph*) expression. A = control for *Ci-POU-IV* long Morpholino; B = specimen injected with *Ci-POU-IV* long Morpholino. C = control for *Ci-POU-IV* short Morpholino. D = specimen injected with control for *Ci-POU-IV* long Morpholino.

The glutamate transporter (*vGlut*) could be regulated by the long transcript. As a matter of fact the injection with the morpholino against the long isoform strongly affected its expression that was almost totally missed in treated embryos (Fig. 3.2.8). On the contrary the short isoform appeared not to influence *Ci-VGlut* expression (Fig. 3.2.9).

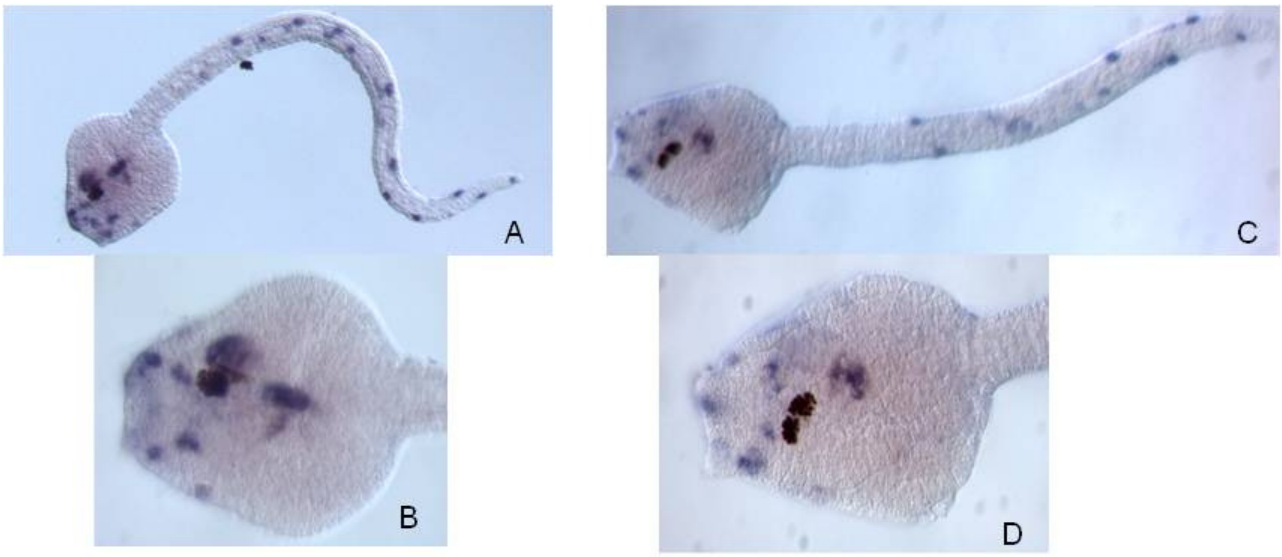

Fig. 3.2.8. Effects of *Ci-POU-IV* short Morpholino 0.2mM on the expression of *Ci-vGlut*. A, B = control embryos. C, D = injected specimens.

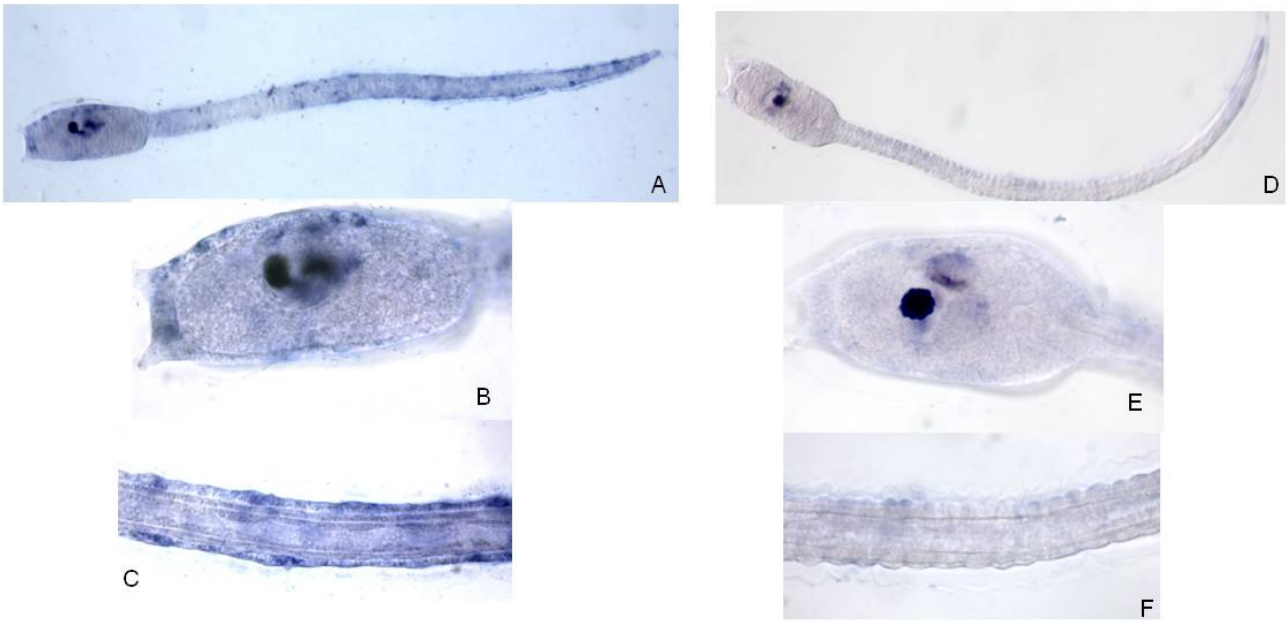

Fig. 3.2.9. Effects of *Ci-POU-IV* long Morpholino 0.2mM on the expression *Ci-vGlut*. A, B, C = control embryos. D, E, F = injected specimens.

Discussion

Results from this work allow to hypothesize that the two isoforms of *Ci-POU-IV* play different roles during nervous system differentiation and will help us to delineate the signal cascade of *Ci-*

POU-IV during PNS development. *Ci-POU-IV* play a role in the differentiation of both CNS and PNS. The effects of the long isoform knock-down underline the relationship existing between *Ci-POU-IV* and the differentiation of the sensory vesicle and of the pigment cells. Investigating the role of the bone morphogenetic protein (BMP) pathway during neural tissue formation in ascidian embryos, Darras & Nishida (2001) showed that a partial blocking of the BMP pathway mainly affect anterior pigment cell formation while a more inclusive blocking inhibits the formation of both pigment cells. In particular at the tailbud stage, when *Ci-POU-IV* is expressed by the pigment cell precursors, BMPb (the *dpp*-subclass *BMP*) induces the differentiation of expressly the anterior type of pigment cell, such as the otolith; while posteriorly, CHORDIN suppresses BMP activity andpromotes *ocellus* differentiation. Thus if *Ci-POU-IV* morfants develop two otoliths (anterior pigment cell) and no ocellus (posterior pigment cell) a role of *Ci-POU-IV* in affecting *Ci-Chordin* action could be hypothesized.

The importance of *Ci-POU-IV* in the CNS specification is also stressed by the results of the *in situ* hybridazion with the probe for *Ci-Tph*. In this case it is clear that the morfants lack the expression in the motoneurons.

The short isoform seem to play a major role in the regulation of the differentiation of the neuron of the PNS. In particular the in situ with the probes for *Ci-vGlut* shows the absence of the glutamatergic neurons in the morphants for the short isoform.

Different role played by two isoforms of a POU gene of class IV have already been described in mammals for the genes *Brn-3a* an *Brn-3b* (Latchman, 1999), in zebrafish for the gene *Brn-3b* (De Carvalho *et al*. (2004) and in drosophila for the gene *Acj6* (Certel et al., 2000).

The existence and the conservation of these alternative transcripts among different *taxa* is remarkable and suggests a distinct specific role for every isoform during development. Transactivation activity and mediation of protein–protein interaction can be performed by both the carboxy-terminal POU domain and the amino-terminal regions of POU proteins (De Carvalho *et* al., 2004). For example, the N-terminal region of *Brn-3a*, with the POU IV box, is fundamental for transactivation of Internexin promoter as reported by Budhram-Mahadeo et al., (1995). Observations *in vivo* of the alternative transcripts of *Drosophila Acj6* showed that they display different activities (Certel et al., 2000). In the same way, mouse *Brn-3b*(s) does not bind octamer-DNA target sequences: moreover it can form some hetero-oligomeric complexes together with other transcription factors that do not bind DNA (Theil et al., 1995). Liu et al., (2000) anyway suggested a functional redundancy between short and long human *Brn-3b* isoforms which are heterologously expressed in chick. The expression profiles of each isoform provide initial clues to their role in development. In zebrafish De Carvalho et al., (2004) showed a remarkable temporal difference in

the expression of the long and short *Brn-3b* isoforms. The long transcripts were detected by RT-PCR at 18 hpf, when a potential role in the early neural differentiation can be hypothesized. The short was however first detected much later at 36 hpf and recorded at low levels. Later in development, in the retina of larvae and also in the adult tectum both isoforms were present. In the rat cultured dorsal root ganglion cells, the *Brn-3b* short isoform is down-regulated, whereas the long isoform is up-regulated (Liu et al., 1996).

Our data suggest that the two isoforms play different roles also during nervous system differentiation of Tunicates. In particular the long isoform, which includes the POU-IV box, seems to have a role during the specification of the CNS, while the short isoform display a major role in the development of the PNS and in particular of the glutamatergic neurons.

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4. Does *Ci-POU IV* act downstream of the Delta/Notch pathway?

Abstract

Delta/Notch signaling is an evolutionarily conserved pathway that is used by metazoans to determine cell fates through local cell interactions. The aim of this work is to understand the relationship between the Delta/Notch pathway and the gene *Ci-POU-IV*. In particular it is interesting to understand if the neuron specifications by the pathway acts upstream the of *Ci-POU-IV* activity in the neural precursors. Results confirmed the increase in the tail sensory epidermic neurons after the inhibition of Delta/Notch. Interestingly *Ci-POU-IV* showed an ectopic expression in embryos and larvae in which the pathway was inhibited. Our results point out the act that *Ci-POU-IV* activity stays down-stream Delta/Notch pathway.

Introduction

Notch signaling is an evolutionarily highly conserved cell signaling system that is used by metazoans to control cell fates through local cell interactions. It is present in all the metazoans and in mammals four different Notch receptors that is Notch1-4 have been identified. The gene encoding the Notch receptor was discovered in the fly *Drosophila melanogaster* at the beginning of the 19 century, following the observation that partial loss of function (haploinsufficiency) resulted in notches at the wing margin (Moohr, 1919). Notch received its notoriety as a result of classic embryonic analyses of lethal loss-of-function mutations, that were conducted by Poulson (Artavanis-Tsakonas, 1999). Notch pathway plays a major role in cell-to-cell interactions in a number of animal *taxa* acting trough the well known mechanism of lateral inhibition (Fig. 4.1). From several studies both classic and recent, the basic mechanism is based the extracellular domain of the delta ligands, expressed on the surface of one cell, interacting with the extracellular domain of the Notch receptor on an adjacent cell. Following the interaction with Delta, the enzyme γ-secretase cleaves an intracellular fragment of the Notch molecule, called NICD (Notch IntraCellular Domain) which then moves into the nucleus and forms a complex with the transcription factor Suppressor of Hairless [Su(H)] (CBF1/RJBk in mammals, LAG-1 in *C. elegans*). As a result of receptor activation, Su(H) binds to regulatory sequences of genes of the *Enhancer-of-split* [E(spl)] locus, which encode nuclear basic helix-loop-helix (bHLH) proteins, The bHLH factors, in turn, affect the regulation of downstream target genes. One well-defined target is the Achaete- Scute complex, which contains proneural genes that encode proteins involved in the establishment of neuronal cells (see for example Gomezskarmeta et al., 1995; or the classic study of Campuzano et al., 1985). Thus, in the neuroectoderm, Notch signaling blocks the neural fate and promote the epidermal one. The first cell that produces Delta, the Notch ligand, promotes the surrounding cells

to become epidermal. In the absence of the Notch signaling there is an excessive formation of neuroblasts at the expenses of the epidermis, while on the other hand an extensive activation of the Notch itself determines a significant reduction in the neuroblast number (Fig. 4.2). A number of studies have shown that Notch signaling also functions at multiple steps in the development of various animals. In vertebrate neurogenesis, Notch signaling is also involved in some events such as asymmetric cell division (Knoblich 1997), neural differentiation and neural tube formation (Tanabe and Jessell 1996). It is also well known that Notch signaling is involved in somitogenesis of vertebrates (Maroto and Pourquié 2001). Recently Oda et al (2007) showed that Delta-Notch signaling is essential for caudal lobe formation in aracnids, preceding initiation of segmentation and revealed that the formation of mesoderm and caudal ectoderm is a single event that sets up a functional caudal lobe in the Chelicerate embryos.

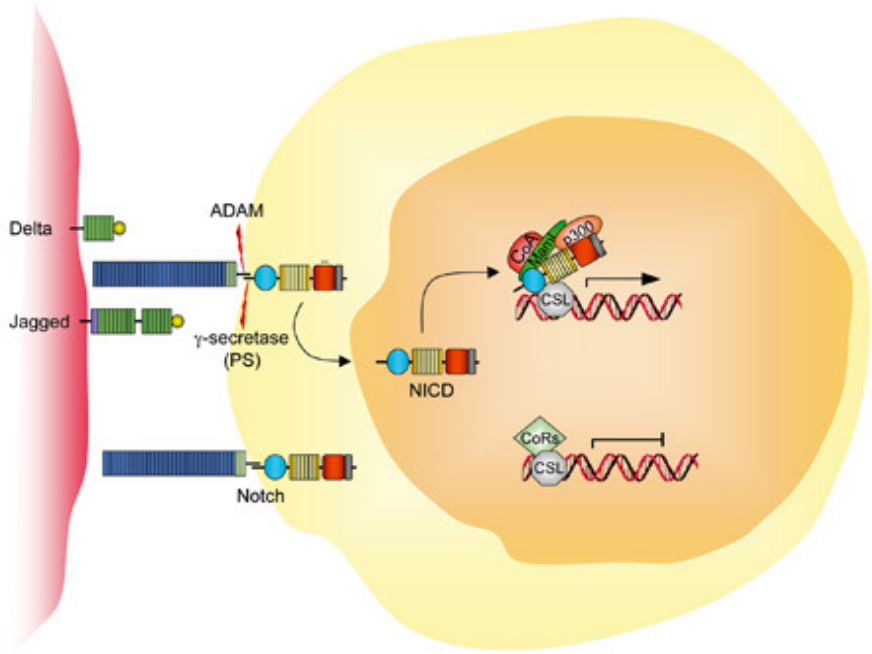

Fig. 4.1. the Delta/Notch pathway and its main components (modified from Campuzano et al, 1985).

As for ascidians, Akanuma et al.(2002) studied the *HrNotch*, that is a *Notch* homologue of the ascidian *Halocynthia roretzi* and is expressed maternally. Maternal transcripts are partitioned into the ectodermal lineage cells of the animal hemisphere (Horie et al., 1997). These authors showed that Notch signaling is fundamental for ascidian nervous system formation and that it affects the fate choice between palps and epidermis and between peripheral neurons and epidermis within the neurogenic regions of the surface ectoderm by suppressing the formations of palps and peripheral neurons and promoting epidermal differentiation. A more detailed picture in ascidians comes from

the work of Pasini et al. (2006). Analyzing the role of Delta/Notch pathway in PNS determination in C. *intestinalis,* they found that the midlines of the tail epidermis are neurogenic regions with the competence to generate both epidermal cells and caudal epidermal sensory neurons (CESNs). In their study the authors outline that the Notch pathway negatively affects the number of midline cells that become CESN. However the exact role of this mechanism should be further elucidated. In fact even if it is demonstrated that a Notch-dependent process of lateral inhibition works to prevent an excessive number of midline cells from adopting a CESN fate, it is still unclear if during the first step of the selection of CESN precursors Delta/Notch also leads the process through lateral competition among midline cells (Pasini et al., 2006). Anyway the expression profile of Notch ligand Delta2 is progressively turned on in scattered cells following a sort of caudo-rostral wave (Pasini et al 2006). This is similar to what happens in vertebrates where the expression of the Notch ligand Delta1 occurs in the nascent neurons (Adam et al., 1995).

In this context the aim of this chapter is to understand the relationship between the Delta/Notch pathway and the gene *Ci-POU-IV*. In particular it is interesting to understand if the neuron specifications by the pathway acts upstream the of *Ci-POU-IV* activity in the neural precursors.

Material and Methods

Animals and embryos

Adults of *Ciona intestinalis* were collected in the bay of Naples. Naturally spawned eggs were fertilized in vitro using a suspension of dry sperm in artificial sea water and 0.1 M TRIS pH9.5. The fertilized eggs were rainsed in filtered artificial sea water at 18 °C. Tadpole larvae hatched about 18–20 h after fertilization. Samples at appropriate stages were collected by low speed centrifugation and were fixed in MOPS Fix (4% PFA, 0.5M NaCl, 0.1M MOPS pH7.5) for whole-mount in situ hybridization and on PFA 4% for immunostaining.

Inhibition of Delta/Notch pathway

In order to inhibit the Delta/Notch pathway, two different strategies were used (Fig. 4.2). First, the pathway was blocked using DAPT, a γ–secretase inhibitor (Calbiochem, San Diego, California, United States). A 20mM stock solution was prepared in DMSO since DAPT is poorly soluble in seawater. The most consistent results were obtained by diluting the stock solution 200 times in seawater, followed by filtering to remove DAPT precipitate immediately prior to embryo treatment.

Delta/Notch pathway inhibition was performed also using transient transgenesis by electroporation. The plasmid electroporated is the construct **pFOG::VeSu(H)DBM,** generated by Dr. A. Pasini

using the Gateway system (Invitrogen, Carlsbad, California, United States). Detailed procedures are described in the adapted Gateway manual that is consultable at the web site of the IBDML of Marseille (http://www.ibdm.univ-mrs.fr/index_gb.php). It carries a dominant-negative mutant of Su(H) that inhibit its binding to DNA target sequences (DBM=DNA Binding Mutant). Electroporation was effectuated in *C. intestinalis* oocytes just after fertilization. Approximately one hundred microliters of fertilized *C. intestinalis* eggs were mixed with 250 µl of 0.96M mannitol containing 50 µg of purified plasmid DNA and electroporated in 4 mm cuvettes using a square pulse protocol (50V and 16ms per pulse). After the pulse, the zygotes were returned to clean artificial sea water and allowed to develop until the desired stage. When the embryos reached the larval stage, they were fixed in MOPS.

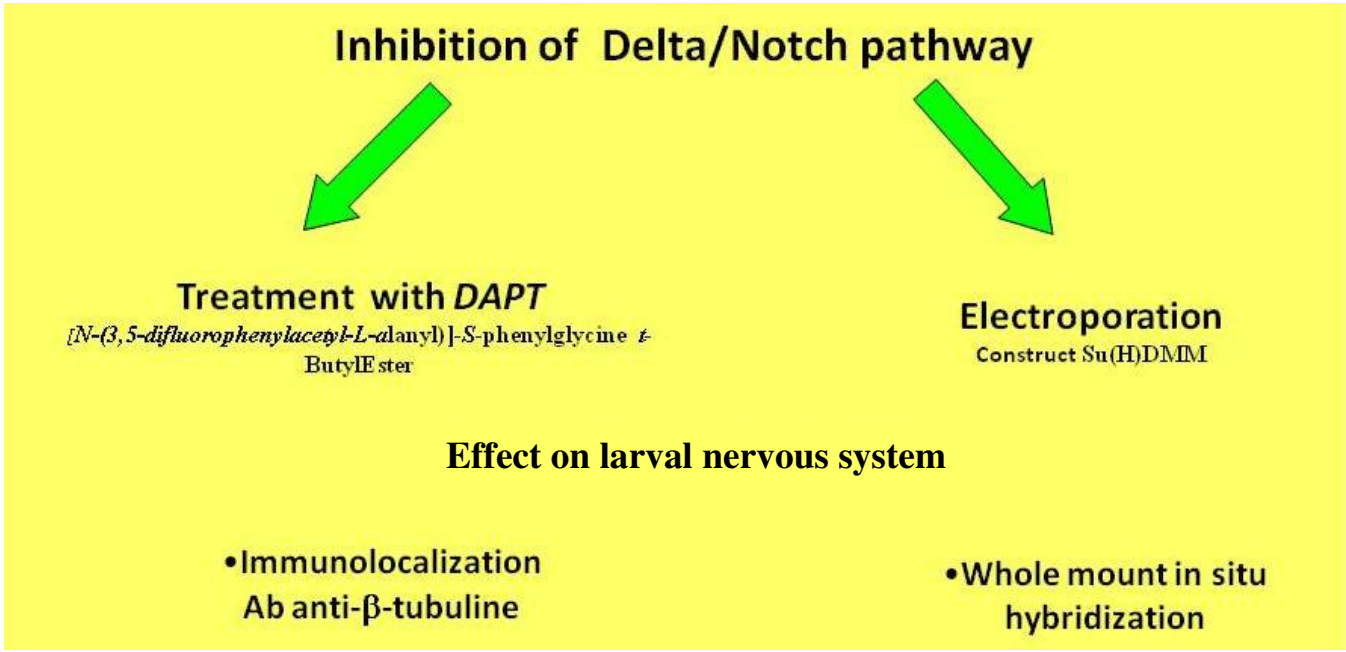

Fig. 4.2. Experiment of Delta/Notch pathway inhibition setting.

Whole-mount *in situ* hybridization was carried out according to Holland et al. (1992) with some modifications. A cDNA restriction fragment (nucleotide 115–445) was cloned in the *Eco*RI site of the pBluescript KS+. The plasmid was digested with *Bam*HI or *Xho*I and used as template for in vitro transcription by using the Boehringer Mannheim digoxygenin (DIG) RNA labelling kit, according to the supplier's instruction, and stored in H_2O at –20°C. Larvae and embryos were fixed MOPS Fix at room temperature for 90 min, dehydrated in 30, 50 and 70% ethanol (10 min each) and stored in 70% ethanol at –20°C. All further steps were performed at room temperature unless otherwise specified. Embryos were manually deprived of their envelopes after rehydration by successive incubation in 50 and 30% ethanol and in PBST (phosphate-buffered saline containing 0.1% Tween 20). The specimens were then treated with 20 µg/ml proteinase K in PBST (30 min,

37°C) and the digestion was stopped by washing with PBS three times (5 min each). The specimens were postfixed with 4% paraformaldehyde in PBS for 1 h, followed by washing with PBST three times (5 min each). Acetylation was carried out in 0.1 M triethanolamine, pH 8.0, supplemented with 0.25% acetic anhydride for 10 min and then the specimens were washed in PBST three times (5 min each). The specimens were incubated in prehybridization buffer (50% formamide, 5 × SSC, 50 µg/ml heparin, 50 µg/ml yeast tRNA, 5 × Denhardt's solution, 0.1% Tween 20) for 1 h at 60°C. Hybridization was carried out at 60°C overnight. After the hybridization, specimens were washed in 50% formamide, 4 × SSC, 0.1% Tween 20 (2 × 15 min, 45°C), then 50% formamide, 2 × SSC, 0.1% Tween 20 (2 × 15 min, 60°C) and solution A (0.5 M NaCl, 10 mM TRIS-HCl pH 8.0, 5 mM EDTA, 0.1% Tween 20; 3 × 10 min, 37°C); treated with 20 mg/ml RNase A in solution A (30 min, 37°C) and washed with 50% formamide, 2 × SSC, 0.1% Tween 20 (20 min, 45°C), 50% formamide, 1 × SSC, 0.1% Tween 20 (2 ×15 min, 60°C), 1 × SSC/PBST 1:1 (15 min) and PBST (4 × 5 min). RNA hybrids were detected immunohistochemically. After blocking in 5% normal sheep serum (NSS) in PBST (30 min), the specimens were incubated with 1:2000 alkaline phosphatase-conjugated anti DIG-antibody (Boehringer Mannheim) in the above buffer (overnight, 4°C). The specimens were washed with PBST (4 × 20 min) and alkaline phosphatase buffer (APB; 100 mM NaCl, 50 mM MgCl2, 100 mM TRIS-HCl pH 9.0; 3 × 10 min). Signal detection was performed in APB containing 4.5 µl NBT/ml (nitroblue tetrazolium salt) and 3.5 µl BCIP/ml (5-bromo-4-choro-3-indolylphosphate) according to the supplier's instruction (Boehringer Mannheim DIG RNA Detection Kit). When satisfactory signals over the background were obtained, the solution was replaced with PBST. Larvae were thus fixed 4% PFA, used for taking pictures at the microscope and then and conserved in glycerol solution at –20C°.

Immunofluorescence staining

Immunofluorescence staining was performed according to Pennati et al (2003). Embryos processed for immunostaining were fixed for 1 h at room temperature in 4% paraformaldheyde dissolved in phosphate-buffered saline (PBS; pH = 7.2), rinsed twice for 10 min in PBS and stored in 70% ethanol at 20°C. After rehydration, samples were dechorionated using a solution 1µM of Proteinase K warmed at 42 °C. Washed in PBS, 0.25% Triton-X100 for 20 minutes and incubated for 30 min with 50% normal goat serum (NGS) in PBS. Samples were incubated at 4°C for 48 h in a solution of 50% NGS, 50% PBS and different primary antibodies. After several washes in PBS, the samples were incubated in 1% bovine serum (BSA) in PBS for 1 h at room temperature, washed in PBS and incubated at 4°C overnight with the secondary antibody. β-tubulin staining were performed with the 6–11B-1 and 2-28-33 monoclonal antibodies (Sigma-Aldrich, St. Louis, Missouri, United

States). After washes, samples were mounted in 1,4-diazabicyclo octane (DABCO; Sigma) and observed with a Leica confocal microscope equipped with an argon/krypton laser.

Results

The immunofluorescence staining with β-tubulin antibody confirmed a significant increase of the neuron number after the treatment with DAPT (Fig. 4.3,4). The control larvae are characterized by a strong staining of the protruding neuronal cilia. The neurons are regularly distributed in dorsal and ventral tail epidermis (Fig. 4.3 A, B) in particular is well evidenced the fact that the cilia are coupled and largely spaced (Fig. 4.3 C). In the treated specimens the tail shows a higher number of CEN (Fig. 4.3 D,) that are identifiable by the higher number of cilia (Fig. 4.3 E). In particular neurons are not regularly coupled and spaced; in the tail we observe much more neurons that form dense and irregular masses. Every neuron has its cilium protruding. This result confirms that the concentration of DAPT used (0.1 mM) has successfully inhibited the Delta/Notch signaling. This concentration was tested both on early and late tailbud embryos. The samples were fixed at the initial larval stage.

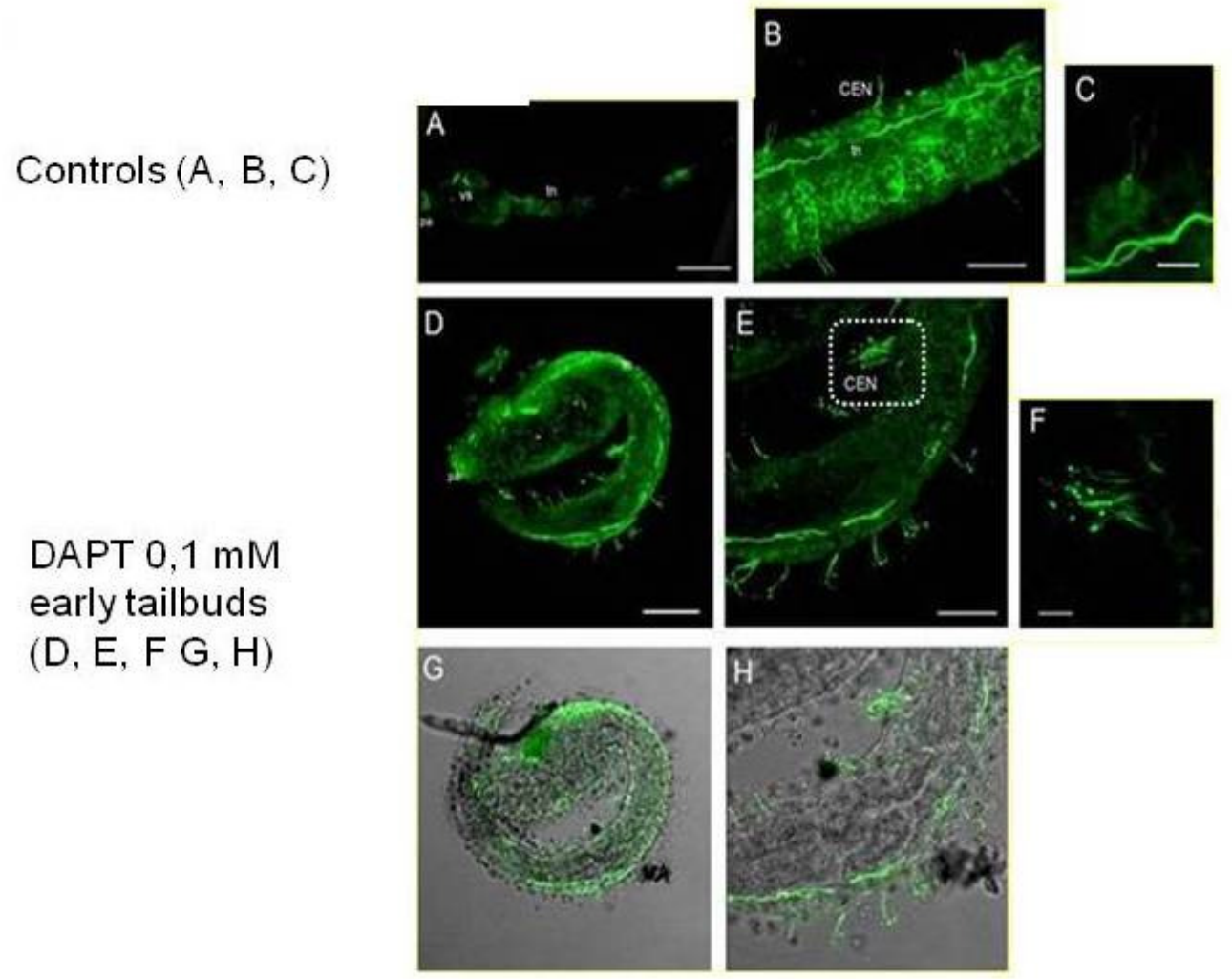

Fig. 4.3. Immunofluorescence with Ab anti β-tubuline. Pictures refer to embryos of *C. intesinalis* at the stage of early and late tailbud.

DAPT 0.05 mM early tailbud
(I, L, M)

DAPT 0,1 mM late tailbud
(N, O)

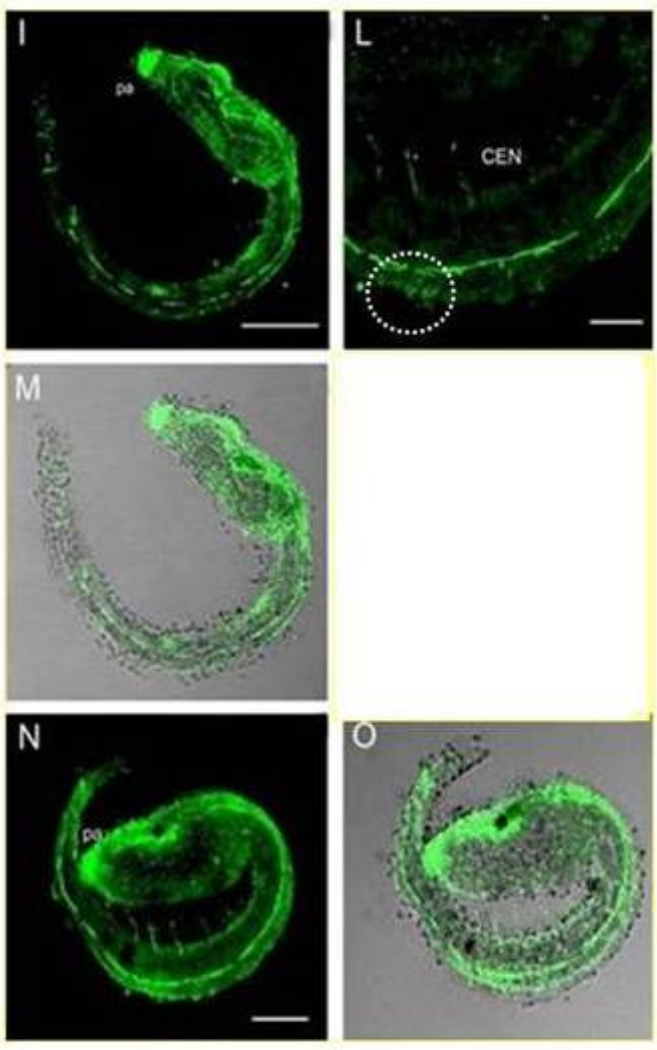

Fig 4.4. Immunofluorescence with Ab anti β-tubuline. Pictures refer to embryos of *C. intestinalis* at the stage of early and late tailbud.

Larvae from the same batches were used for in situ hybridization with the probe for *Ci-POU-IV*. Results showed an ectopic expression of *Ci-POU-IV* both in the trunk and in the tail (Fig. 4.5.).

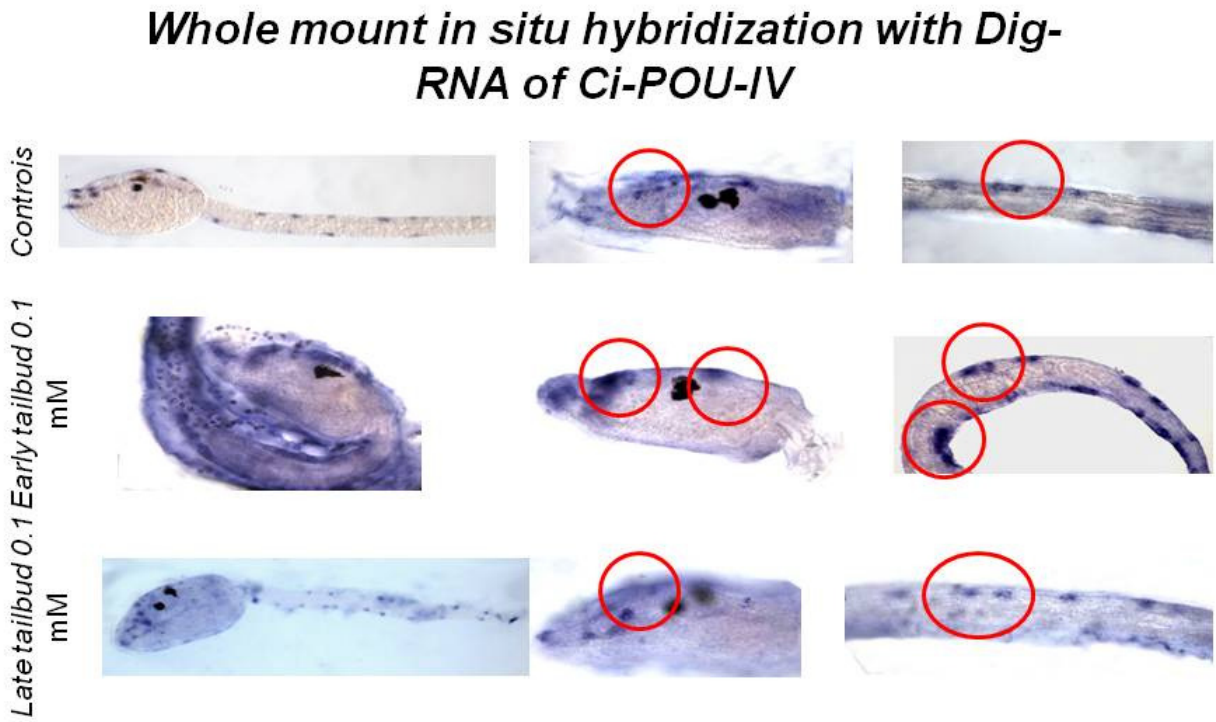

Fig. 4.5. Results of whole mount in situ hybridization with Dig-RNA of *Ci-POU-IV* performed in larvae treated at the early and late tailbud stages with 0.1mM DAPT.

The same ectopic expression of *Ci-POU-IV* is evidenced in embryos in which was carried out the electroporation of the construct pFOG::VeSu(H)DBM. The *in situ* results are shown in Fig. 4.6.

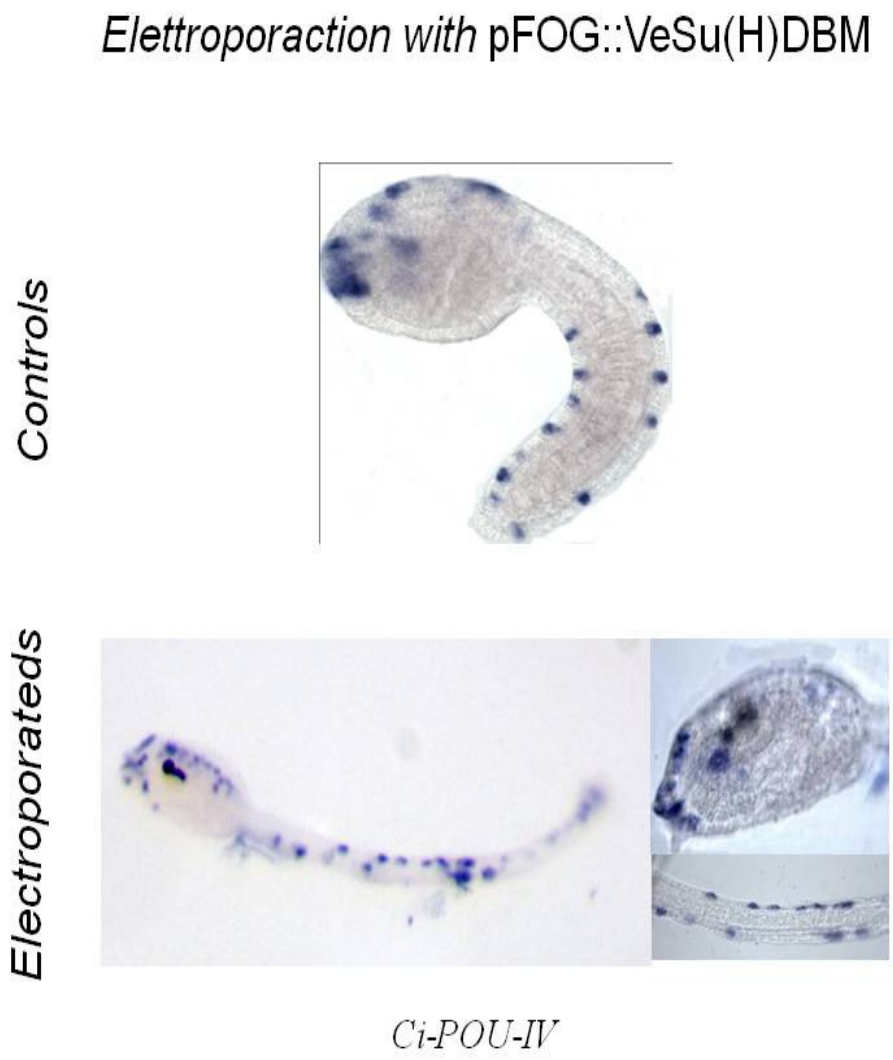

Fig. 4.6. whole mount in situ hybridization on controls and electroporated larvae showing the expression of *Ci-POU-IV*

Discussion

Delta/Notch signaling is an evolutionarily conserved pathway that is used by metazoans to determine cell fates through local cell interactions. The comprehension that this signaling mechanism controls an astonishingly broad variety of cell fates and developmental processes (in organisms ranging from sea urchins to humans) resulted in a true explosion of Notch-related studies in the past decade (Artavanis-Tsakonas, 1999). Pasini *et al.* (2006) studied the role of Delta/Notch pathway in determination of PNS features in the ascidian C. *intestinalis* and they found that the

Notch pathway controls the number of midline cells that become caudal epidermal sensory neurons (CESN). These cells are generated, together with the epidermal cells, by the tail midlines that are neurogenic regions. There are no studies that correlate the expression profiles of the *Ci-POU-IV* gene to the Delta/Notch signaling. *Ci-POU-IV*, as shown in the previous chapters, is specifically expressed in all the peripheral nervous system (PNS) territories and in some cells of the central nervous system during *C. intestinalis* development (Candiani et al., 2005). The results from this chapter showed an ectopic expression of *Ci-POU-IV* in the tail region of the larvae in which the Notch signaling has been inhibited. This fact allows to assess that the expression regulation of *Ci-POU-IV* is situated downstream the signaling activity. At the current status of the knowledge is not possible to say if there is a direct control of the activation of *Ci-POU-IV* by Delta/Notch pathway or if there is an interaction of intermediate factors related to neuron specification.

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5. Which are the genes controlled by *Ci-POU-IV*? An *in silico* approach.

Abstract

In this study we looked for the *Ci-POU-IV* targets by a bioinformatic approach. The possible consensus sequences were obtained by bibliographic research of those known for the POU IV family in both invertebrates and vertebrates. We used these sequences to build a matrix that was employed to perform a bioinformatic research in the whole *C. intestinalis* genome with a software elaborated by the Lemaire team of the IBDML of Marseille. With the most restrictive analysis we identified at least 18 possible targets of *Ci-POU-IV*.

Introduction

The members of the POU transcription factors family play critical role in regulating gene expression, in particular in nervous system cells. Although POU transcription factors were originally identified on the basis of their functions in particular system and then shown to contain a common POU domain, a number of other investigations subsequently isolated further members of this family on the basis of their containing a POU domain. Of particular interest are the neuron-specific class IV POU domain proteins including Unc- 86 from *C. elegans* (Finney et al., 1988), zebrafish Brn-3.1 (Sampath and Stuart, 1996), the mammalian Brn-3a/Brn- 3.0, Brn-3b/Brn-3.2 and Brn-3c/Brn-3.1 proteins (Collum, *1992;* Xiang et al., 1993), *Drosophila* Acj6 (Certel *et al*.,2000) and Ci-POU-IV of *Ciona intestinalis* (Candiani *et al*., 2005; Pennati *et al*., 2009). All of these proteins have been shown to perform essential functions in the differentiation of specific neuronal cell types (Erkman et al., 1996; Vahava et al., 1998; Xiang et al., 1997). The *C. intestinalis* genome has only three genes coding for the POU transcription factors. As described in the previous chapters, three *C. intestinalis* POU genes have been cloned and their expression profiles analyzed. The only one that seems to play an important role in the development of the peripheral nervous system is *Ci-POU IV*. At the larval stage, *Ci-POU IV* is expressed in all the sensorial cells of the peripheral nervous system and in some the visceral ganglion cells of the central nervous system. The aim of this study is to identify the targets of *Ci-POU IV* in order to increase the knowledge about the role played by this gene during the development of the ascidian PNS.

Some studies have already begun to focus on the transcriptional targets of the PouIV factors in the developing nervous system, with the use of expression arrays with partial genomic coverage to undestand global gene expression in the sensory ganglia and retina of mammals, in particular mice, lacking *Brn3a* and *Brn3b* genes, respectively (Eng et al., 2004; Mu et al., 2004). In these cases, the

majority of regulated transcripts belonging to gene families with known or potential roles in neurodevelopment, although only a few shared targets were identified (Lanier *et al*., 2007).

The results of Eng *et al*., (2004) and of Mu *et al*. (2004) suggest that the PouIV factors regulate on the order of 100 downstream transcripts in a single tissue type at a given developmental stage, including both direct and indirect targets.

Moreover, there is a recent study of Lanier et al. (2007) in which the use of a real-time chromatin immunoprecipitation assay in embryonic sensory neurons adds new data on the relationship existing between Brn3a and its transcriptional targets. They showed that Brn3a is a direct repressor of some genes, such as *bHLH* genes *NeuroD4* (*Math3*) and *NeuroD1*; this action is mediated trough a consensus *Brn3a* binding site. In this case is interesting to know that *Brn3a* can also act as a repressor. A major point is that *Brn3a* also interacts directly with its own autoregulatory enhancer via relatively weak sites. In all three loci, most occupied *Brn3a* recognition sites occur in regions that are conserved in both human and mouse genomes (Lanier et al., 2006).

Biochemical and transfection studies have shown that the vertebrate and invertebrate members of the PouIV subclass bind to a consensus sequence consisting of ATAATTAAT and minor variants thereof (Gruber et al., 1997). *In vitro* studies have shown that all of the PouIV factors have very similar DNA recognition properties, binding with high affinity to the motif ATAATTAAT or very similar sequences (Gruber et al., 1997; Turner, 1996). Presumably, the *Brn3* factors regulate specific genes required for sensory neural development via such sites.

Trieu *et al.,* (2003) showed that *Brn3a* regulates its own expression *in vivo*, and that this regulation is mediated by an immediate interaction between *Brn3a* and its recognition elements that are placed within the *Brn3a* sensory enhancer region. Also in that case comparison of the mouse and human *Brn3a* loci revealed that the regulatory region that they described is remarkably conserved across the two mammalian species (Trieu et al., 2003). These authors indicated that the most suitable recognition site contains a core ATAATTAAT sequence and exhibits 1,000-fold higher affinity for *Brn-3.0* than the previously described *Brn-3* family consensus recognition elements. The optimal DNA recognition site for *Brn-3.0* and other several minor derivatives of this site that they obtained mediate activation of transcription from reporter gene constructs at levels which correlate well with their *in vitro* binding affinities (Trieu et al., 2003). The DNA binding properties of *Brn-3.0* are extremely similar to those of *Brn-3.2* and the *Caenorhabditis elegans Brn-3* homolog *Unc-86*, indicating that the evolutionary divergence of the POU-IV class proteins has occurred for reasons other than to permit distinctive DNA recognition (Trieu et al., 2003).

The aim of the study is to identify *Ci-POU-IV* targets. This is a major point of interest to understand the key mechanisms of SNP formation in which *Ci-POU-IV* is involved. In this way it is possible to

increase the knowledge of the role played by this transcription factor and especially to delineate its signal cascade during PNS differentiation.

Materials and Methods

In order to localize the possible targets of *Ci-POU-IV*, we crossed the information coming from two different methodological approaches.

First of all we used a bioinformatic approach to identify and localize in the whole genome of *Ciona intestinalis* the possible consensus sequences for *Ci-POU-IV*. In a successive step, we crossed the resulting possible target genes to their corresponding expression profiles selecting those that share at least a part of expression territory with *Ci-POU-IV*. The latter were obtained by the bibliographic research of the known consensus sequences for the PouIV family in both invertebrates and vertebrates. Thus we analyzed the *C. intestinalis* genome with the aim of identify the regions containing a possible consensus sequence. In order to indentify these sequences within the *C. intestinalis* genome, we used an informatic program developed by Pierre Khoueiry of the Patrick Lemaire equipe at the IBDML institute of Marseille (Khoueiry *et al.,* 2010) .

This program is called SECOMOD (Search for Evolutionary Conserved MODules, http://crfb.univmrs.fr/secomod/public/). This program identifies the regions containing a particular sequence repeated in a certain window and conserved between *C. intestinalis* and the congeneric species *C. savignyi*. The algorithm searches for clusters of binding sites in *C. intestinalis* according to a number of sites and a cluster size specified by the user. It then identifies the orthologous region in *Ciona savignyi* and checks whether this region also contains the required number of sites and whether it has the correct size. This cross species analysis allows to increase the probabilities to consider segments of DNA involved in important regulatory processes. As a matter of fact if a sequence is conserved among the two species there is the possibility that it has an effective role.

We performed the research using a matrix built thanks to the POU IV consensus sequences already known in both vertebrates and invertebrates. In particular we took sequences from the studies of Lanier et al. (2006), Trieu et al. (1999) and Gruber et al. (2001) in mice and we included also a variant belonging to the extensive study of Noyes et al (2008) on *Drosophila* transcription factors. At the sequence ATAATTAAT that according to Trieu et al., (1999) is the most suitable for the binding we added some little difference found in the other cited articles. The final matrix used is composed by the following sequence: ATWAWBDAT, where, following the official IUPAC nomenclature W means A or T, B means T, C or G and D means A, G or T.

We looked for the sequence occurring 3, 2 or 1 time within a window of 100 bp.

Regions with the occurring of a putative sequence and situated at a maximum distance of < 2Kb from both the start and the stop codon of a gene were selected and placed in a specific database.
Once performed the bioinformatic research we crossed the genes who have a possible consensus sequence in their surroundings to the rich database of ANISEED, (http://aniseed-ibdm.univ-mrs.fr/) containing a large number of data of expression profiles (Tassy *et al.*, 2010).

Results

Throughout the bioinformatic search we used different and increasing levels of selectivity. The less selective sequence was ATWAWBDAT. The maximum times of repeating of the putative sequence in the window of the research was 3 times. It was present 1 time around 3575 genes. Two times it occurred around 124 genes and 3 times only around the gene *Ci-Connexin*-44 at a distance from its ATG of 4239 bp. We then took into consideration only the sequences placed at a maximum distance of 2 Kb form any ATG. In this way the sequence ATWAWBDAT was found around 820 possible target genes. It occurred two times around 28 genes. In the other cases the sequence occurred only one time. The most selective sequence was that already described, such as ATAATTAAT. At a maximum distance of 2 Kb form any ATG it occurred around 18 possible targets. Then all these data were compared with the expression profiles contained in the database of ANISEED. On the basis of the fact that they share a more or less extend part of expression territory with *Ci-POU-IV*, we selected 6 possible target genes:

- *Ci-Pans*, a pan-neural marker;
- *Ci -Tph*, the serotonin synthesis enzyme;
- *Ci-β-Thymosin*, neural actin binding protein;
- -*Ci-AAKG2,* activated protein kinase, gamma-2 subunit
- -*Ci-WDR36,* containing Trp-Asp repeat activating T cells
- -*Ci-PCDH10,* putative protocadherin-10 precursor .

These genes have been selected to plan different experiments *in vivo* in order to verify if the identified regulatory sequences effectively drive their expression within the *Ci-POU IV* expression territory of *C. intestinalis,* meaning that the selected regions are effectively active at larval stages. A further electroporation of the same regions carrying mutations in the putative *Ci-POU-IV* consensus binding sites will permit to evidence if these genes are indeed *Ci-POU-IV* targets. These experiments are now ongoing and results are not provided here.

Moreover we investigated the regulatory region of *Ci-POU-IV* that has been described by Sierro and colleagues (2005).

We found the putative target sequence repeated four time in this region; two times occurs the sequence ATAATTAAT described by Trieu et al. (2003).

Discussion

The high similarity of tunicates and vertebrates during their development, coupled with the transparency of tunicate larvae, their well-studied cell lineages and the availability of simple and efficient transgenesis methods makes members of this subphylum ideal systems for the investigation of vertebrate physiological and developmental processes. Recently, the sequencing of two different *Ciona* genomes has lead to the identification of numerous genes. In order to better understand the regulation of *Ci-POU-IV* we used a bioinformatics approach to identify *in silico* its putative targets.

Different studies have shown that both invertebrate and vertebrate members of the PouIV subclass bind to a conserved consensus sequence (and related minor variants) such as: ATAATTAAT (Gruber et al., 1997).

In mammals the targets of *Brn3* are thought to be genes required for sensory neural development (Turner, 1996). An interesting data that the bioinformatic approach pointed out on *Ciona Ci-POU-IV* is the fact that in the regulatory region of the gene (Sierro et al., 2005) there is the suitable consensus sequence used in the research of the targets. Interesting this sequence is repeated 4 times. And two times occur exactly the sequence ATAATTAAT. This point could suggest the possibility of an autoregulation of *Ci-POU-IV in vivo*. As a matter of fact already Trieu et al., (2003) in mammals showed that *Brn3a* negatively regulates its own expression in vivo. It could be a confirmation of the fact that also the functional mechanisms of the POU IV regulatory could be remarkably conserved across different *taxa* as already observed in mammals (Trieu et al., 2003).

An interesting information comes also from the fact that among the putative targets there is the gene *Ci-Tph*. This gene is not really expressed at the level of the peripheral nervous system (but in the muscle cells of the tail at the level of neuro-muscular junctions, Pennati et al., 2007). From tailbud stage it is detected in the precursor cells of the visceral ganglion and in the tail. In the larva anyway there are *Ci-Tph* expressing neurons that form two clusters in the anterior central nervous system at the level of the visceral ganglion. Only these neurons, as described in the previous chapter, are affected by the experiments of gene knockdown for *Ci-POU-IV*.

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Conclusions and perspectives

This thesis has the aim of describe important mechanisms involved in the development of the larval peripheral nervous system in the ascidian *Ciona intestinalis*. After a review of what is already known about the molecular and cellular mechanisms that control the formation of the peripheral nervous system in Tunicates, both early patterning phenomena, controlled by RA, FGF and WNT, and more late mechanisms, under the control of the POU transcription factors have been analyzed.

It was first investigated if RA, FGF, and WNT have effectively a role in SNP determination as in vertebrates. The results obtained showed an antagonism between RA from a side and FGF/WNT from the other that involves a complex web of direct and indirect mechanisms. The second hypothesis tested was if the POU transcription factors, that are important in neuronal development of several *taxa,* have a role in SNP formation of *C. intestinalis*. To answer at this question the expression profiles of the three *POU* genes present in *C. intestinalis* were characterized. In particular the genes *Ci-POU-2* and *Ci-POU-like* that were not described yet were characterized. These two genes seem not to be involve in SNP formation, while the role of *Ci-POU-IV* in this process is assessed even if not totally understood. Thus the functions of two isoforms of *Ci-POU-IV* were studied in details. Results from these experiments revealed that the expression of the serotonin rate-limiting synthesis enzyme, *tryptophane hydroxylase* (*Tph*), could be regulated by the long transcript. Morphants for this isoform lack motoneurons. The knock down of the short isoform induced the loss of the glutamatergic neurons identified by *vesicular glutamate transporter* (*vGlut*) expression.

The thesis investigated also the relationship existing between *Ci-POU-IV* and the Delta/Notch pathway in order to understand if the activity of the gene is upstream or downstream the Notch signaling. Results showed that Delta/Notch inhibition induced an ectopic expression of *Ci-POU-IV* evidencing that its activity is downstream the Notch signaling.

Finally, in this study we tried to recognize the *Ci-POU-IV* targets. Throughout an *in silico* approach were identified at least 18 putative targets that could give important hints to delineate the signal cascade of *Ci-POU-IV* during PNS differentiation.

Altogether these results open interesting perspective; especially about the evolutive significance of the antagonism between RA and FGF/WNT. The fact that it occurs in a basal Chordate open the question if it is a Chordate innovation or if it appeared earlier in Metazoan evolution. Moreover there are interesting perspectives on a functional study of *Ci-POU-IV* that could describe in details the signal cascade by *in vitro* and *in vivo* experiments.

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