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by

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Thyroid-Like Function in the urochordate *Ciona intestinalis*

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Introduction

1.1 Iodine as driving force in the evolution of the thyroid.

The Iodine atom, with its 53 electrons and 126,9 g/mol molecular weight, is the heaviest and richest in electrons among the elements present in living systems. This element is necessary both for vegetal and animal cells thanks to its antioxidant activity. Indeed Iodine, mainly in its Iodide form (I^-), is able to act as electron donor in particular conditions, as for example in the presence of hydrogen peroxide and of an enzyme with peroxidase activity (Crockford, 2009). More than 800 million of years ago Cyanobacteria were the first living organisms capable to produce Oxygen, through the biochemical pathway known as photosynthesis, a process occurring in the chloroplasts or on intracytoplasmatic membranes, where the I and II photosystems are located (Grotjohann & Fromme, 2005). It is generally agreed that the earliest photosynthetic activity was carried out by bacteria that did not produce oxygen. These so-called anoxygenic photosynthetic bacteria most probably used other reductants than H_2O, as H_2S, H_2, H_2O_2, combined with carbon dioxide, to produce sugar. Oxygenic photosynthesis apparently developed later, approximately 3 billion years ago, in an ancestor of cyanobacteria (Buick, 2008; Ting, Rocap, King, & Chisholm, 2002). At that time, however, the oxygen, produced by the photosynthetic pathways, resulted very dangerous. Indeed, the lack of an ozone layer, surrounding the earth, permitted the UV rays to come down to the earth surface and use the "photosynthetic" oxygen for the formation of reactive oxygen species (ROS), as H_2O_2, or the superoxide radical (O_2^-), that resulted very dangerous for cells, causing

Figure 1.1 Iodine atom and its properties. The high molecular weight, the large number of protons and the electronic configuration, with its incomplete outer electron shell confer its unique antioxidant characteristics.

DNA/RNA damage, lipid peroxidation and protein injury. The organisms were thus compelled to find a mechanism or a compound or a molecule as defense against ROS (Venturi, 2000). The Iodine, with its high electro negativity value and its high availability in seawater, responded perfectly to these requirements. The antioxidant ability of Iodine, present in the seas as iodide (I^-), along with Iodate (IO_3^-) (Truesdale, 1994; Truesdale and Upstill-Goddard, 2003), is mainly due to the possibility, once it reacts with an organic compound, to release one electron. The sequential steps of the whole reaction involve the peroxidase enzyme that, in presence of H_2O_2, produces a compound "X", which oxidizes the iodide (I^-). The iodide (I^-), thus transformed into a highly reactive radical form, is in turn neutralized by coupling with amino acids or lipids (Crockford, 2009). Thanks to this process, in the very early life forms appeared, for the first time, iodine containing organic compounds, as Iodomethane (CH_3I), iodo-amino acids and iodinated forms of many lipids (Collen, 1994). The most used amino acid as scaffold for iodination was the tyrosine, besides hystidine and membrane lipids which were iodinated to a lesser extent. Tyrosine can go through different degrees of iodination and modifications. Tyrosine mono-iodination lead to the formation of the MIT (Monoiodotyrosine or 3-iodo-L-tyrosine), whereas the addiction of two iodine atoms gives rise to the DIT (Diiodotyrosine or 3,5-diiodo-L-tyrosine). These compounds have been found in both microalgae and macroalgae (Mairh et al., 1989; Saenko et al., 1978; Wong et al., 2002), where Iodine concentration is really high, representing more than 1% of total weight (Colin et al., 2003). The synthesis of more complex iodinated molecules as the Thyroid Hormones (THs), starting from the high available MIT and DIT, still requires the enzymatic peroxidase activity. The presence of a Vanadium dependent peroxidase thus allowed macroalgae, such as *Laminaria sp.* (Leblanc et al., 2006) and also some marine invertebrate species belonging to *Porifera* (Baden, 1979) and *Cnidaria* phyla (Mydlarz

and Harvell, 2006), to synthesize the Thyroxine, also known as the T4 thyroid hormone. It is about 700 million of years ago that in marine organisms appeared the THs that, at the beginning, most probably represented simply a reservoir of iodine, ready to be used, without having any further function. Subsequently, the algae containing iodinated tyrosines, *via* the food chain, contributed to the diffusion of iodinated tyrosine also in marine species that were not able to endogenously synthesize the TH. As proposed by Gorbman in 1955, many marine invertebrates became accustomed to a supply of organic iodine compounds that were then coopted as signaling molecules in different processes involving physiology, development and reproduction (reviewed in Eales, 1997). Later, some clades evolved the ability to fix iodine to organic molecules and produce THs by themselves. However, even in the animals owing the complete enzymatic machinery for iodine uptake and THs production, the food represents still the most effective way to provide them with the right amount of iodine-containing molecules.

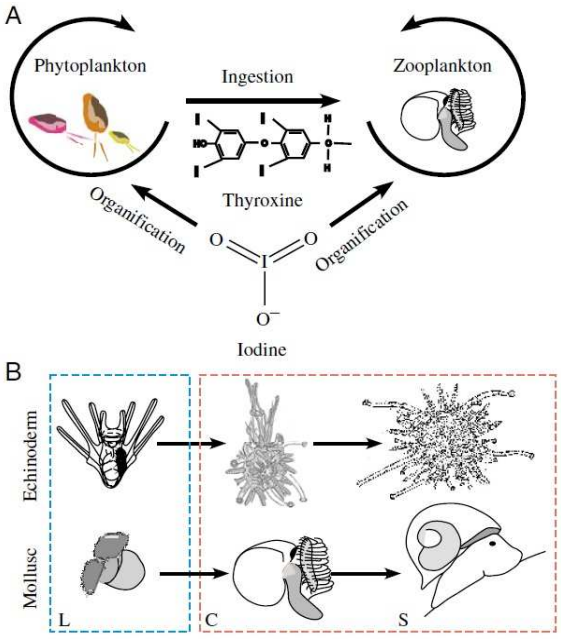


Figure 1.2 (A) Cross-kingdom communication in marine ecosystems. Marine organisms incorporate and fix Iodine into organic matrix, synthesizing Thyroid Hormones. THs can acquire signaling features when they can pass through the food chain from a specie to another. (B) Exogenous THs have also the ability to act as developmental signaling

molecules. In echinoderms and mollusks there are many evidences about the use of exogenous molecules to trigger developmental processes as, for instance, metamorphosis. Adapted from (Andreas Heyland & Moroz, 2005).

As the evolution proceeded, the animals moved from the sea to the freshwater and then to the land, where they had to face a strong reduction in iodine availability, that dropped down from 60 µg/L in seawater to 5µg/L in freshwater and to 0.1-13 mg/Kg in soil (Heyland, 2004).

Those pioneer organisms devised the right solution to this problem. Some of them used parts of organs, mainly devoted to other functions, as for example digestion, to fix iodine as efficiently as they could. This is the case of the basal Chordates, for instance amphioxus and ascidians, where the endostyle, an organ involved in mucus secretion necessary for food particles entrapment, gained this ability. Other organisms developed a new organ, the follicular thyroid, embriologically derived from the endoderm, whose main function was to accumulate iodine, to fix it to the amino acid tyrosine and synthesize THs, thanks to the presence of an efficient peroxidase enzyme.

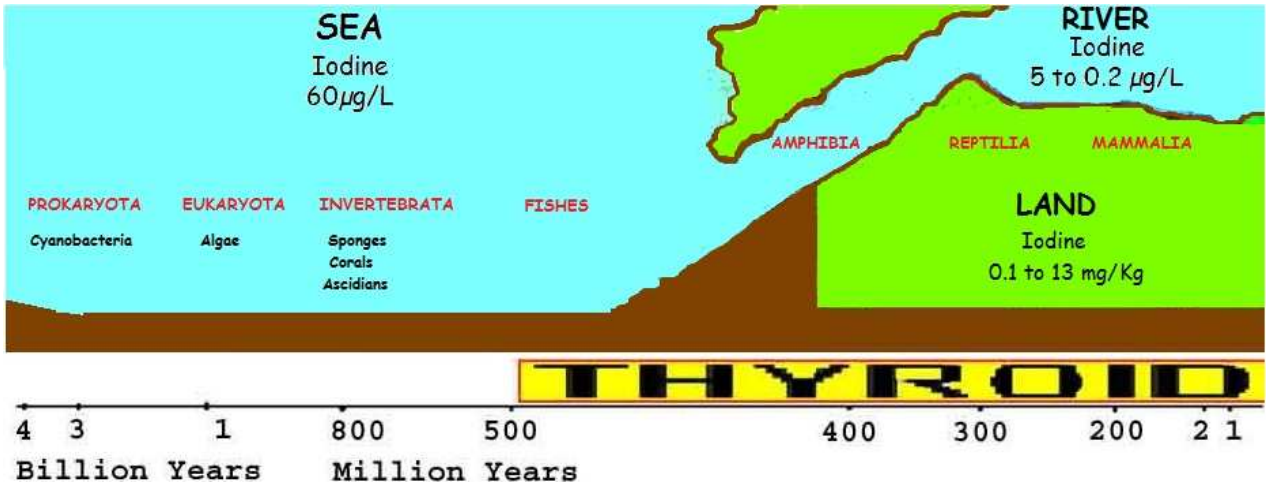


Figure 1.3

Iodine average concentration from seawater to land. The decrease in iodine content is related to the evolution of the very specialized follicular thyroid. In red there are different steps of evolution. Cyanobacteria and algae were the first

species in which the ability of taking Iodine from the water and linking it to organic compounds appeared. From fishes (500 million years ago) the follicular thyroid started to appear. Adapted from (Venturi, 2006).

1.2 From the Endostyle to the Thyroid: lessons from Protochordates and Agnata.

The thyroid is one of the largest glands in vertebrates. It plays a fundamental role in the regulation of energy production, in metabolic activity and growth. The thyroid is able to exert this large plethora of functions through the production of tyrosine-based, iodine-containing hormones. Thyroxine (T4) and 3,5,3'-triiodothyronine (T3) are the main form of hormone produced by a specialized type of thyroid cells, the follicular cells, which are devoted both to iodine uptake and hormone synthesis (J. T. Dunn & a D. Dunn, 1999). As suggested by the name, the follicular cells are organized into follicles, structures almost spherical, full of colloid which is a mixture of proteins, principally thyroglobulin, that represents the scaffold used, in Vertebrates, for THs synthesis (Taurog, Dorris, & Doerge, 1996). Two other types of cells are present in the thyroid: the thyroid epithelial cells, necessary to give mechanical support to follicles; the parafollicular cells, which secrete calcitonin. The embryology of thyroid gland has been well characterized in mammalians (De Felice & Di Lauro, 2007; R Di Lauro et al., 1995) and fishes, where thyroid primordium develops as an out-pocket of the floor of the pharynx (Tan, 1981). The detached primordium then migrates ventrally to reach its final position, at the anterior wall of the trachea, where it differentiates into the follicular thyroid gland. Although the ontogeny of this gland is almost well understood, especially for the jawed vertebrates, its phylogeny is still under investigation. From this perspective, the agnate (jawless vertebrates) as lampreys, represent the keystone, being the first organisms showing,

exclusively at the adult stage, a follicular thyroid gland (Barrington, 1966). Interestingly, Barrington and co-workers, in 1966, described a thyroid-like function also in lamprey larvae (ammocete) and ten years later, in 1976, Wright and Youson (Wright & J H Youson, 1976) observed an extremely interesting phenomenon, studying lamprey metamorphosis. During this process, they identified the endostyle, an organ present only in the ammocetes and absent in the adults, that directly transformed into the thyroid gland of the adult.

It is intriguing to note that in non-vertebrate chordates, cephalochordates (amphioxus) and urochordates (Tunicates, ascidians), is present exclusively the endostyle, that does not transform into a thyroid, while in vertebrates is present exclusively the thyroid gland and a structure as the endostyle, usually, does not form at any stage of their life.

Given these remarks, most efforts have been directed towards the demonstration of an homology between thyroid gland of vertebrates and the endostyle of non-vertebrate chordates. In Cephalochordates and Tunicates the endostyle is a rod shaped organ involved in producing mucus, crucial for the filter-feeding function of the pharynx. The endostyle is located ventrally to the pharyngeal floor and is structured into functional regions, organized ventro-dorsally (Barrington & Thorpe, 1965a). Already in the sixties, it was demonstrated that specific endostyle cells, both in amphioxus (regions 5a, 5b, 6, L Fig. 1.4) and ascidians (regions 7, 8, 9 Fig. 1.4), were able to concentrate radioiodine from sea water and to produce monoiodiotyrosine, diiodiotyrosine and tyroxine (Roche, Salvatore, & Rametta, 1962; Roche, Salvatore, Rametta, & Varrone, 1961). (A. D. Dunn, 1980) (Barrington & Thorpe, 1965b), thus suggesting that a complete enzymatic machinery, able to uptake iodine from the environment and to fix it to organic compounds, was present in these phyla (Tong, Kerkof, & Chaikoff, 1961).

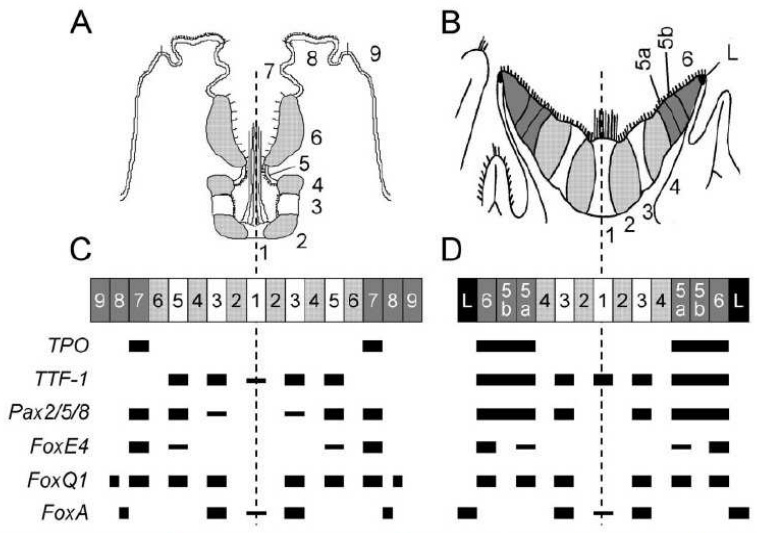


Figure 1.4 Comparison of hystological components and gene expression patterns in the endostyles of protochordates. In A ascidian endostyle with its classical division into 9 regions. From 1 to 6 glandular region, involved in mucus secretion for food particles trapping. From 7 to 9 thyroid equivalent regions. In B histology of amphioxus endostyle. 5a, 5b, 6 and L are the thyroid equivalent regions. In C and D thyroid related genes expressed in ascidian and amphioxus endostyle. Adapted from (Hiruta, Mazet, Yasui, P. Zhang, & Michio Ogasawara, 2005a)

Besides the physiological data, different molecular evidences have been accumulated in the last years strongly supporting a phylogenetic relationship between endostyle and thyroid. Orthologous genes of vertebrate thyroid specific markers, as TTF-1 (Thyroid Transcription Factor 1) TTF-2 (Thyroid Transcription Factor 2, also known as FoxE) , and Pax2/5/8 (reviewed by Di Lauro and De Felice in Endocrinology, 2001) are expressed in amphioxus, ascidian and lamprey endostyles, even though some of them are not present specifically in the iodine-binding zones (Hiruta, 2005) (Ristoratore et al., 1999), (Michio Ogasawara & Satou, 2003), (Cañestro, Bassham, & Postlethwait, 2008)

Members of the enzymatic complex acting in the thyroid gland have been found also to be expressed in the thyroid-equivalent part of the endostyle. It is well known that

Thyroid-peroxidase (TPO) is the enzyme involved both in tyrosine iodination and coupling to form Thyroxine (T4), the most abundant specie of thyroid hormone present in the thyroid; DUOX (a NADPH oxidase/peroxidase) is instead the enzyme that produces the hydrogen peroxide (H_2O_2) used for Iodine metabolism by TPO in vertebrate thyroid. In the ascidian endostyle both the orthologs of TPO (M Ogasawara, R Di Lauro, & N Satoh, 1999) and DUOX (Hiruta, Mazet, & Michio Ogasawara, 2006) have been detected, as well as in amphioxus (M Ogasawara, 2000) and in lamprey endostyle (Tsuneki, Kobayashi, & Ouji, 1983) .

The identification, in the endostyle, of thyroid-specific transcription factors and thyroid-specific enzymes, along with all the morphological and biochemical data, represents the key evidence that a thyroid-like function lie in the endostyle. Thyroid gland exerts a wide range of physiological effects, as will be discussed in the next section. Sometimes, phyla specific thyroid related effects have been found, although the common features of thyroid functions are shared among all vertebrates. To better understand the role of Thyroid gland and Thyroid Hormones, the next two sections will be focused on the biosynthesis of Thyroid Hormones and their mechanisms of action, mainly through the interaction with their receptors.

1.3 Biosynthesis of Thyroid Hormones.

Even though in the first section some details about the biosynthesis of Thyroid Hormones are provided, it is necessary to deeply analyze these mechanisms considering them as the first steps in the general definition of Thyroid function. The biochemical mechanism at the base of thyroid hormone synthesis has been well characterized in vertebrate, whose genomes code for all the members of these well coordinated pathways. An overview of the whole process is the following.

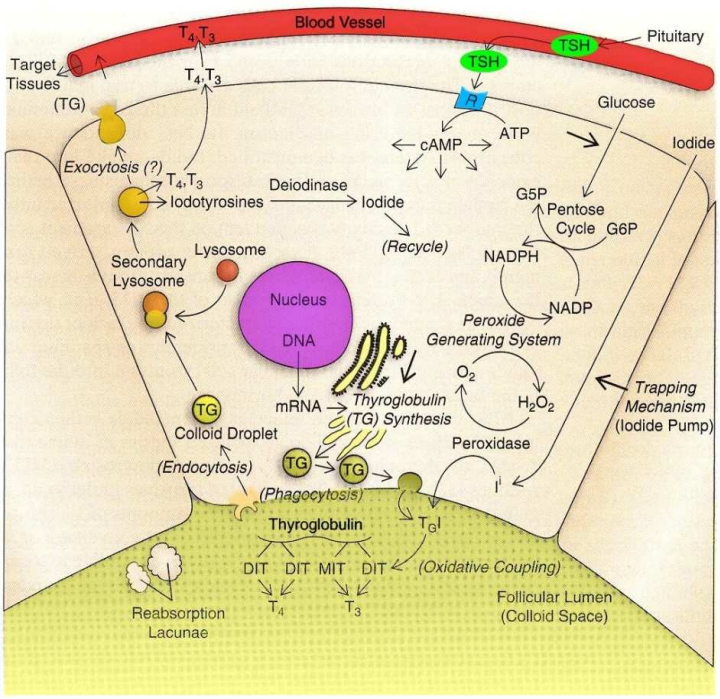


Figure 1.5 Thyroid follicular cell and thyroid hormone biosynthesis. The pituitary signal, the TSH (Thyroid Stimulating Hormone), activates a cascade leading to the production of cAMP (Cyclic Adenosine Mono Phosphate). The cAMP is involved into different pathways, one of them leads to the production of NADPH (reduced Nicotinamide-Adenin Dinucleotide). NADPH is used as electron donor for the reaction catalyzed by a Peroxide Generating complex, that produces H2O2. The thyroperoxidase (TPO) uses the H2O2 to oxidize the Iodine ions. The highly reactive Iodine is then bound to specific tyrosine residues of thyroglobulin. Iodinated tyrosines are then coupled together to form the thyroid hormones T4 and T3 by the TPO. THs containing thyroglobulin is then secreted through the apical membrane into the colloid where it is stored. Once necessary the TG-THs is uptaken from the colloid, degradated by a lisosomial complex and THs are released into blood circulation through the basal membrane. Adapted from trinity.edu.

The uptake of Iodide (I^-) is the first phase; it occurs through the activity of a ionic pump that, using the energy deriving from Sodium uptake, is able also to co-transport Iodide ions. The Na/I simporter has been identified and studied in vertebrates, although also in the basal chordates *Ciona intestinalis* and *Branchiostoma floridae* the ability to uptake Iodide from the seawater has been well documented (Paris et al., 2010). In the follicular cells, the Thyroperoxidase (TPO), in presence of H_2O_2 oxidizes Iodide and attaches it to particular tyrosine residues of the protein used as scaffold for Thyroid Hormone

synthesis: the thyroglobulin (Tg). The thyroglobulin, now containing Monoiodotyrosines, Diiodotyrosines, thyroxine (T4) and Triiodothyronine (T3) is then secreted in the colloid in the central part of the follicles. When needed, the Tg, with the attached hormones, is taken up from the follicular cells apical membrane and, in the cells, is broken down to release T4 and T3 in blood circulation through the basal membranes (Reviewed by Dunn JT, in Endocrinology, 2001). The thyroglobulin is a fast evolving protein whose main, and probably unique function is to be the scaffold on which THs are synthesized. Ortologous of the gene coding for thyroglobulin have been found in Vertebrates, while a clear Tg like gene has not been identified in tunicates and amphioxus genomes (Hiruta et al., 2006; M Ogasawara et al., 1999; Paris et al., 2010).

The thyroglobulin (Tg) is one of the most represented molecule in Vertebrate thyroid follicles. It is found mainly as 660 kDa dimers. The Tg polypeptide chain is characterized by three main parts. The N-terminal region which shows the repetition of 10 CWCVD motifs, called Tg type I domain, shared by many proteins with unrelated functions (Molina, Pau, & Granier, 1996). The second part does not show any homology with other proteins, even though it contains one hormonogenic tyrosine residue. At the C-terminal is present an the Acetyl-cholinesterase domain that anchor this protein to cell apical membrane where the Tg is iodinated. Probably this domain is also capable of proteolytic activity, perhaps promoting its own degradation. About the Iodine, in physiologic condition 60 iodine atoms can be found per Tg dimer: 30% of Tg iodine is in T4 and T3 hormone whereas the rest is part of the inactive precursors MIT (monoiodotyrosine) and DIT (diiodotyrosine).

After the synthesis, occurring on polyribosomes anchored to the ER (Endoplasmic Reticulum), Thyroglobulin goes through different phases of maturation and folding, mediated by chaperones, necessary for its correct tridimensional structure and for its sorting to the follicles lumen.

The essential steps in THs synthesis are:

- Oxidation of Iodide in a form capable of iodination
- Iodination of Thyroglobulin tyrosyl residues
- Coupling of two iodotyrosyl residues in order to produce T4 and T3 hormones
- Thyroglobulin proteolytic degradation, necessary to release the free form of the thyroid hormones.

The first three phases take place at the apical membrane, where iodide, TPO, H_2O_2 and thyroglobulin meet. After tyrosine iodination, leading to the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT), that preferentially occurs at specific residues in Thyroglobulin, the coupling reaction is fundamental for the production of T4 (two DIT coupling) and T3 (MIT and DIT coupling). The polypeptidic chain of Tg, after iodination and coupling, is stored in the colloid; when needed, thyroglobulin molecules are degraded by lysosomal proteases, so that THs can be released into the blood circulation.

1.4 General mechanism of action of Thyroid Hormones.

It is likely that all cells in the body are targets for thyroid hormones, thyroxine (T4) and triiodothyronine (T3), since they have profound effects on many physiologic processes, such as development, growth, metabolism, and deficiency in thyroid hormones is not

compatible with normal health. The mechanism of TH action is mediated through the interaction with TRs (Thyroid Hormone Receptors) (Sap et al., 1986; Weinberger et al., 1986) which are members of the nuclear receptor superfamily and are ligand-activated transcription factors (M. Robinson-Rechavi, 2003). The nuclear receptor superfamily is composed by phylogenetically related proteins; 21 members have been found in *Drosophila melanogaster* genome, 48 in humans and more than 250 in *Caenorhabditis elegans* (Bertrand, F. G. Brunet, Escriva, Parmentier, Laudet, & Marc Robinson-Rechavi, 2004a). As part of this superfamily, thyroid hormone receptors share some common features, such as the presence of different domains, each one with a precise function. The N-terminal part of the TRs, known as A/B domain, is very variable among species and is involved in transcriptional regulation, owing an Activation Function 1 (AF1) and through the interaction with co-activator and co-repressor proteins. At the C-terminal is present the DNA binding domain (DBD), the region of TRs with the highest degree of conservation all along the Chordate phylogeny. The DBD is characterized by the presence of 8 cysteine residues organized in two zinc fingers motifs. Between the two zinc finger domains there is a sequence, the P Box, important for DNA binding specificity of TRs on target promoters (Kumar & Thompson, 1999; reviewed in Nuclear Receptor Factsbook by Laudet & Gronemeyer, 2001). The DNA binding sites for different NRs, although specific, share common features and are collectively called Hormone Response Elements (HRE). Usually a HRE is characterized by two repetition of a consensus sequence (the half sites). These sequences can be organized as direct repeats (DR), inverted repeats (IR), everted repeats (ER) or palindromic (Pal). The most diffused binding sites are DRs, recognized by Thyroid hormone receptors, Retinoic Acid Receptors and Vitamin-D Receptors.

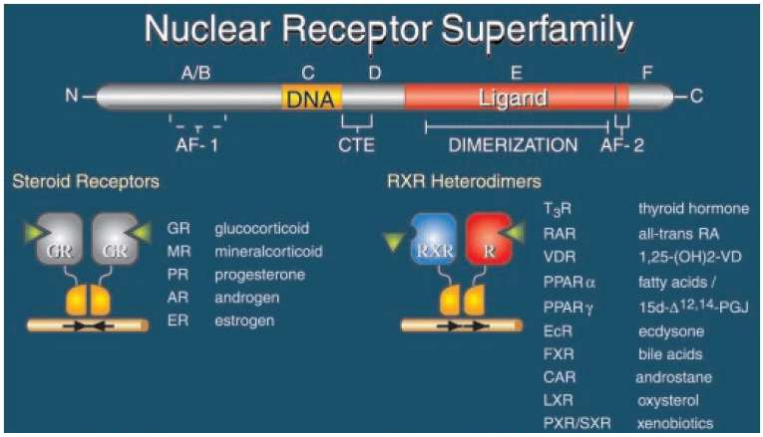


Figure 1.6 Organization of Nuclear Receptors. The six domains (A-F) of nuclear receptors are presented in the upper part of the figure. The C domain is the DNA Binding Domain whereas the E domain is the Ligand Binding Domain. The D domain is the hinge region, a very variable domain. In the lower part of the figure different mechanism of DNA binding are showed. Homodimerization in the case of Steroid Receptors, RXR heterodimerization in the largest case. TRs for instance binds to DNA as RXR heterodimers.

The mode of action of TRs is schematically divided in three parts. The first part, is the repression phase, in which, in absence of ligand, the TR is bound to the DNA but the co-repressor complex, recruited along with an HDAC (Histone Deacetylase), blocks the transcription of downstream genes. Once the ligand is present, the co-repressor complex dissociates from the TR and an HAT (Histone Acetyl Transferase) is recruited in order to acetylate the histones and de-condense the chromatin. At the same time a co-activator complex, composed by different proteins, such as TRAP, DRIP and ARC, is assembled on the site and establish contacts with transcription machinery, leading to the transcription of target genes [reviewed by (Santos, Fairall, & Schwabe, 2011)].

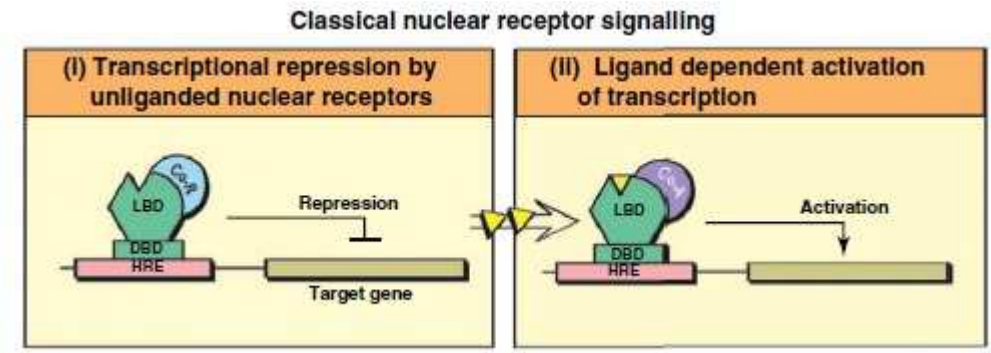


Figure 1.7 Model of signaling bu Nuclear Receptors. (i) Transcriptional repression through corepressor recruitment in absence of ligand. (ii) In presence of ligand (yellow arrows), the switch in LBD conformation leads to corepressor complex release and coactivator complex recruitment.

Orthologs of thyroid hormone receptors have been found in many species, although for some of them an activity, as thyroid hormone receptor, has not been proved. This is the case of ascidian nuclear receptor CiNR1 that, although possessing an high degree of homology with TRs family, seems not to be able to bind thyroid hormone (Carosa et al., 1998).

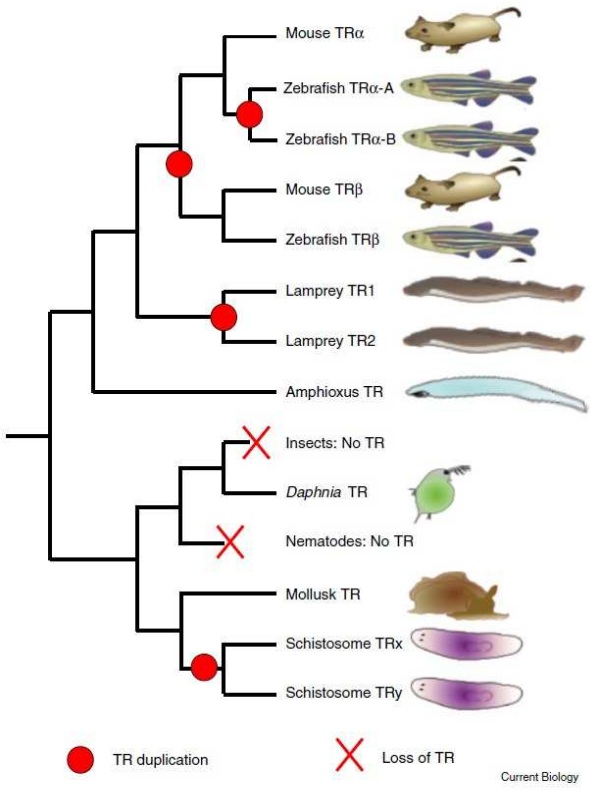


Figure 1.8 Phylogeny of Thyroid Hormone Receptors. The red circles are located in correspondence of gene duplications. One duplication occurred at the base of vertebrate leading to the formation of TRα and TRβ, since in the invertebrate chordates only one TR is present. Other duplications are lineage specific, as for fishes, lampreys and Platyhelminthes. No TRs have been found in insects and nematodes (Adapted from Laudet, 2011).

The classical "genomic" mechanism of TH action thus include its translocation in the cell interior and then in the nucleus where, thanks to the interaction with the nuclear receptor, TH can influence specific gene transcription. However in the past decade it has been demonstrated that TH can have a faster way of action, called "non-genomic" mode, in a variety of cells, including those of central nervous system. This mechanism involves TH interaction with a plasma membrane receptor or a nuclear receptor located in the cytoplasm. The plasma membrane receptor is located on integrin αvβ3, at a specific TH recognition site, and after the interaction the signal is, usually, transduced by the MAPK cascade via the ERK proteins (P. J. Davis, Leonard, & F. B. Davis, 2008).

Acting in a "genomic" or "non-genomic" mode, it is clear that THs play pivotal roles in a huge number of physiological, metabolic, pathological and developmental phenomena, including the fascinating process of chordate metamorphosis.

1.5 Thyroid functions throughout Metazoan evolution: a particular look to the metamorphosis.

At the time when, Gudernatsch came to the Zoological Station "A. Dohrn" in Naples, in 1910, to study embryonic development and growth in frogs, nothing was known about thyroid and its ability to influence development of living organisms. He fed frog tadpoles with extracts from different vertebrate tissues or organs that he collected during his travel to Naples (Gudernatsch, 1912). Surprisingly the extract from thyroid gland was able to anticipate the onset of amphibian metamorphosis. The theory, according to which the

thyroid has a role in frogs metamorphosis, was confirmed 15 years later, when Allen inhibited metamorphosis just removing tadpole thyroid gland. Thyroid Hormone (TH) can thus be considered the first developmental morphogen ever discovered.

Metamorphosis, a post-embryonic phenomenon in which coexist pathways leading to growth and differentiation, but also to apoptosis and death, strongly affects life styles of many species, by inducing changes in their morphology, physiology, ecology and behavior (Bishop et al., 2006). It is probably one of the most dramatic events during the life of amphibians, as well as insects, fishes, lampreys and invertebrate chordates (Cephalochordates and Tunicates). Sometimes the morphological changes are not that spectacular as in zebrafish, while in other fishes, as flatfish, the modifications are very profound since a symmetric larva, with eyes at both sides of the head and with a well-formed dorsal fin, transforms into a completely different, asymmetric adult having both eyes on the same side. The examples of metamorphosis with appreciable or low morphological changes are not limited to fishes but include many different classes. The figure 1.12, adapted from Laudet (2011), shows a simple metazoan phylogeny, with a particular look to the ability of species to go through metamorphosis, in the panel A. In the panel B are collected pictures of larvae and adult of different species where the changes during metamorphosis are more or less visible.

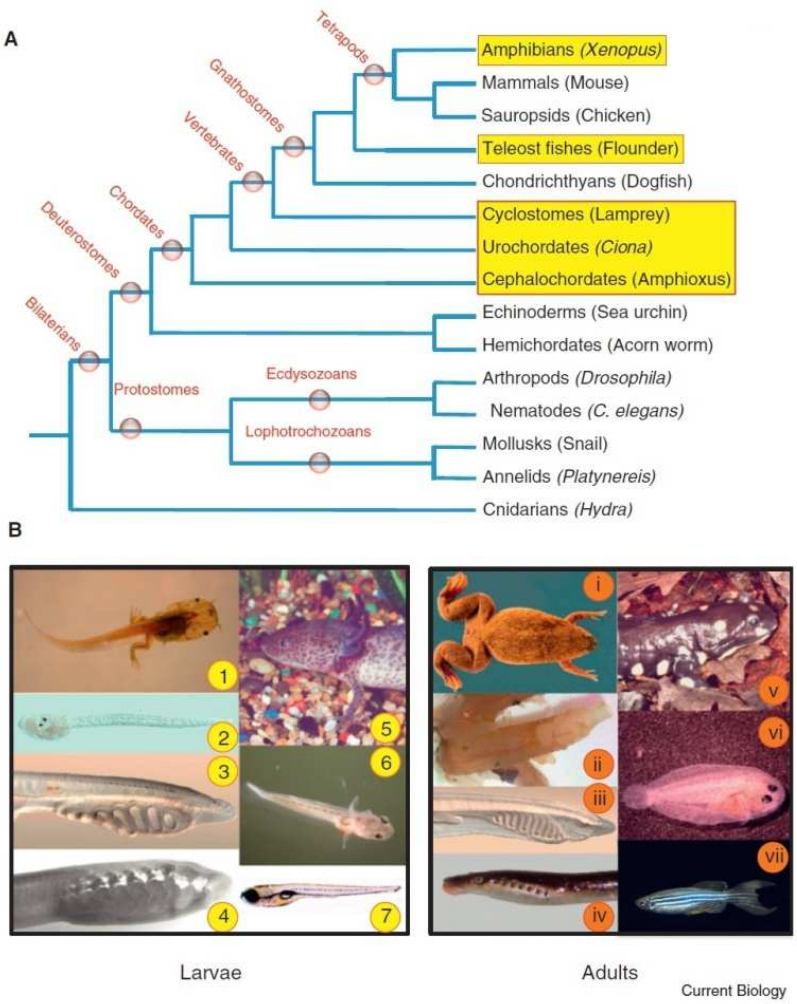


Figure 1.9 Distribution of metamorphosis in Metazoan. (A) Simple Metazoan tree of life. All the clades reported are known to contain many species that go through metamorphosis. In yellow are highlighted the chordates for which a role of Thyroid Hormones in metamorphosis has been well established and documented. (B) Larvae and adults of metamorphosing chordates. 1/I Xenopus laevis; 2/II *Ciona* intestinalis; 3/III Amphioxus; 4/IV Lamprey; 5/V Ambystomatids salamander; 6/VI Flatfish; 7/VII Zebrafish. Adapted from (Laudet, 2011)

Cephalochordates (amphioxus) show a TH/TR regulated metamorphosis, where a pelagic larva almost asymmetric is transformed in a benthic juvenile. In lampreys a tight regulation of thyroid hormone levels are extremely important to the accomplishment of the larva to juvenile transition. In this species, however, a drop in THs is observed at metamorphosis climax, differently than in other chordates, in which a raise in circulating TH levels triggers the changes typical of this stage (R G Manzon, Eales, & J H Youson,

1998; Richard G Manzon & John H Youson, 2002; J H Youson & Sower, 2001). According to the literature, amphibians are the organisms in which the involvement of THs in metamorphosis and its molecular regulation has been better described. Developmental strategies in amphibians vary from direct development of some anurans, as *Eleutherodactylus coqui* (Singamsetty & Elinson, 2010), where the metamorphic changes are really mild, to the spectacular tadpole to frog metamorphosis of others anurans. Until 1986, THs were thought to act by altering the metabolism. Following the discovery of TRs (Sap et al., 1986; Weinberger et al., 1986) and their ability, after the interaction with THs, to influence gene expression, all the changes occurring at metamorphosis were considered as effects of gene transcription variations. The discoveries of Gudernatsch, Evans and Vennstrom, taken together indicate that the accumulation of THs, during the embryonic development, directly activate the transcription and the autoregulation of the thyroid hormone receptor beta, necessary for triggering the wide array of events taking place before, after and at metamorphosis climax. These findings, reported in the figure 1.10 can be applied also to other chordate taxa (cephalochordates, fishes).

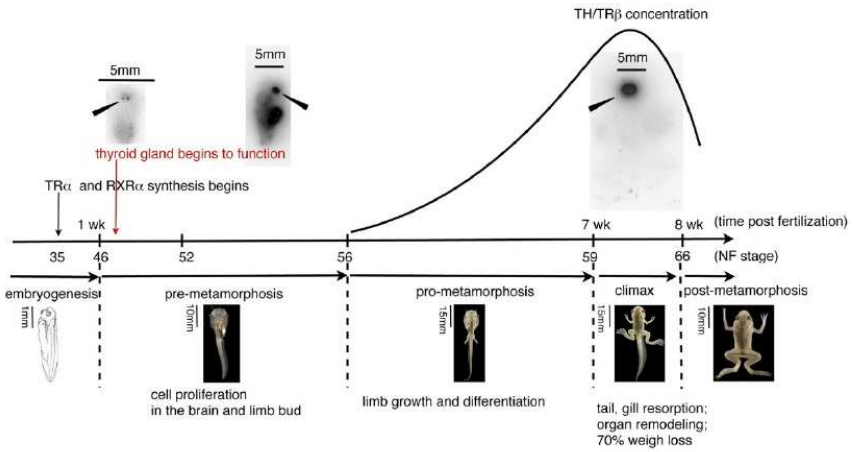


Figure 1.10 Amphibian metamorphosis. The developmental table of Xenopus laevis shows that 7-8 weeks after fertilization there is a peak in TH, followed by a peak in TRβ contemporary with metamorphosis climax. The

appearance of the thyroid gland is almost 6 weeks before the climax, suggesting the necessity of accumulation of TH until a threshold. Adapted from (Donald D Brown & Cai, 2007)

From these observations a new definition of metamorphosis arose: it is a postembryonic developmental stage characterized by a thyroactive compound (THs or derivatives)-dependent morphological remodeling (Paris & Laudet, 2008). Under this perspective we can consider that weaning, in mammalian, represents the homologous to classical metamorphosis, because it is a period of tissue remodeling, driven by THs (Fraichard et al., 1997), of intestine morphological changes and chondrogenesis. These modifications occur in correlation with a peak of THs production (Hadj-Sahraoui, Seugnet, Ghorbel, & Demeneix, 2000).

1.6 Tunicates: key organisms in developmental and evolutionary biology.

The presence of a cellulose-containing tunic, covering their bodies, is the common feature of Tunicates, one of the three subphyla in which the Chordata phylum could be subdivided. The other two subphyla are the Cephalochordates (Amphioxus) and the Vertebrates.

The subphylum Tunicata, also known as Urochordata is divided into three classes: Ascidiacea (sea squirts), Thaliacea (salps) and Appendicularia or Larvacea (larvaceans).

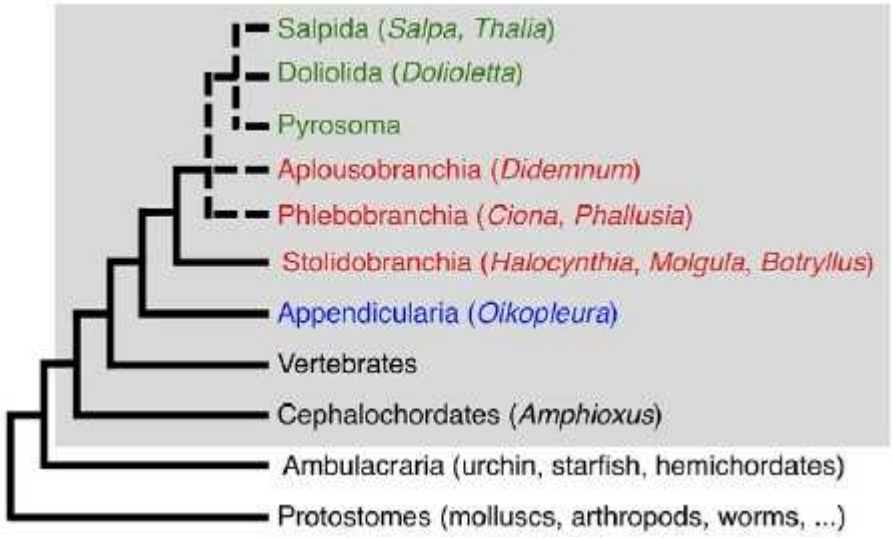


Figure 1.11 Simple phylogeny of Tunicates. In red are highlighted the Ascidiacea class. In green the Thaliacea class. In blue the Larvacea class or Appendicularia. Adapted from (Lemaire, 2011)

Among these, the first class includes the solitary or colonial ascidians, whose life cycle is characterized by a planktonic phase, up to the tadpole larval stage, and a sessile phase after the metamorphosis. Both the other two classes comprise small pelagic organisms: salps, that after embryonic development metamorphosize into a barrel-shaped animals, whereas larvaceans, as Neotenic species, reach sexual maturity without losing larval features. Tunicates, especially adults, do not show any distinctive feature of the chordate body plan, but, analyzing the larval stages of ascidans and salps, it is possible to recognize some of the Chordate specific landmarks as:

- The notochord
- A dorsal hollow nerve cord
- Segmented muscles

In the last ten years the Chordata tree of life has been drawn and re-drawn many times, according to the continuous availability of new molecular and genomic data from different species.

1.6.1 The Chordate phylogeny: relationships among Tunicates, Cephalochordates and Vertebrates.

Traditional studies tended towards a Chordate phylogeny in which Cephalochordates, and not Urochordates, were the closest living relatives to vertebrates, given the presence of some Vertebrate-specific features, as a post-anal tail and segmented muscles all along the bodies in cephalochordates absent in tunicates. Besides the morphological characteristic, the phylogenetic reconstructions have been made also by comparing the sequences of set of genes considered hortologues among species belonging to different phyla and different classes. More recently, the release of genomic sequences of many different species, lead to a re-analysis of the phylogeny. With the data obtained from one urochordata specie, *Oikopleura dioica*, belonging to the appendicularia class, the chordate phylogeny has been thus rewritten in 2006 (Delsuc, Brinkmann, Daniel Chourrout, & Philippe, 2006).

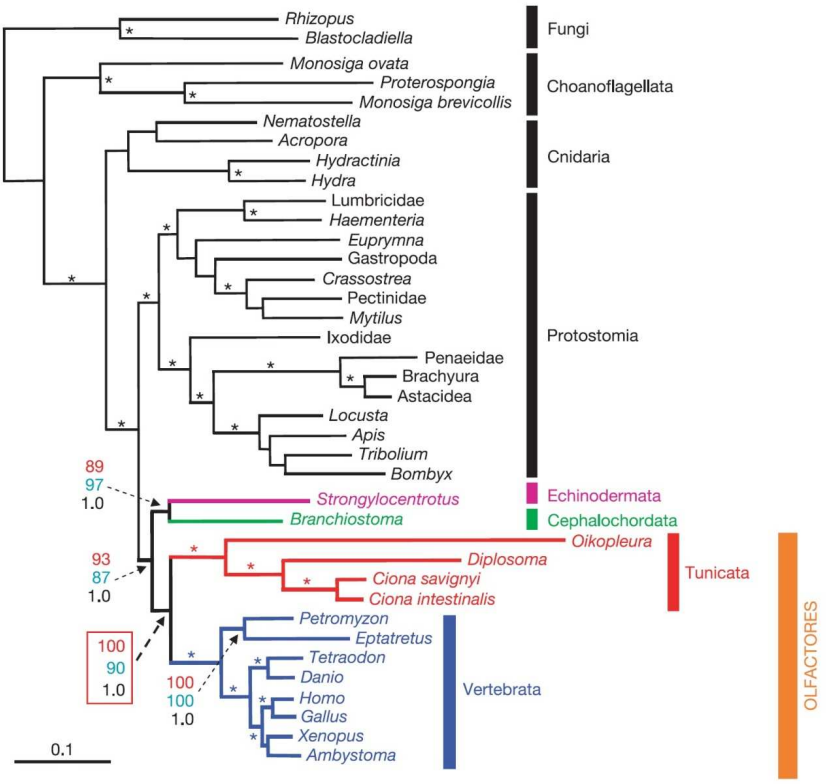


Figure 1.12 New Metazoan Phylogeny. Phylogenetic analyses of genomic data strongly support the grouping of tunicates and vertebrates into Olfactores. ML tree obtained from the analysis of 33,800 aligned amino acid positions under a WAG substitution matrix plus a four-category gamma rate correction (a ¼ 0.5) using two independent reconstruction algorithms. Bootstrap proportions obtained after 100ML (red) and 1,000 MP replicates (blue), as well as bayesian posterior probabilities (black) are shown for selected branches. A star indicates that all three values are maximal (100%, 100% and 1.0). Scale bar indicates number of changes per site. Adapted from (Delsuc et al., 2006)

Delsuc and co-workers (2006) performed indeed a comparative phylogenomic analysis using a wide dataset. Applying different methods of analysis, they could infer that "Tunicates and not cephalochordates are the closest living relatives to vertebrates". These results also prompted a reinterpretation of the phylogeny previously influenced by those morphological characteristics as the metameric muscles segmentation and the post-anal tail. They hypothesized, therefore, that those vertebrate-like structures should have be ancestral features of the common chordate ancestor, lost in Tunicate, due to their fast genome evolution rate. Tunicates should no longer be considered "primitive" but highly diverged species with specialized lifestyles and developmental modes. This chordate phylogeny confirm also the role of Tunicates and cephalochordates as complementary models for studying the origin of vertebrate developmental programs.

1.6.2 The model organism: the ascidian *Ciona intestinalis.*

Among the various tunicate species used as model organism in basic research as well as in environmental toxicology, *Ciona intestinalis*, is one of the most suitable systems in a wide range of biological studies. The ascidian *Ciona intestinalis* (Linnaeus 1767) is a marine invertebrate chordate belonging to the subphylum Urochordata or Tunicata. Among the three classes of Urochordata, *Ciona intestinalis* belongs to the Ascidian class. Most of the chordate characteristics of this animal, discussed in the first part of this section, are exhibited clearly in the larval stage and gradually lost during metamorphosis. The adult

Ciona sp. is a simple vase like filter feeder and sessile animal. During their life cycle they produce the motile form only as the tadpole larva which displays its chordate affinities: a dorsal hollow nerve cord with underlying notochord, firstly discovered by Alexander Kowalevsky in 1866, the first researcher thinking ascidians as members of the Chordata phylum.

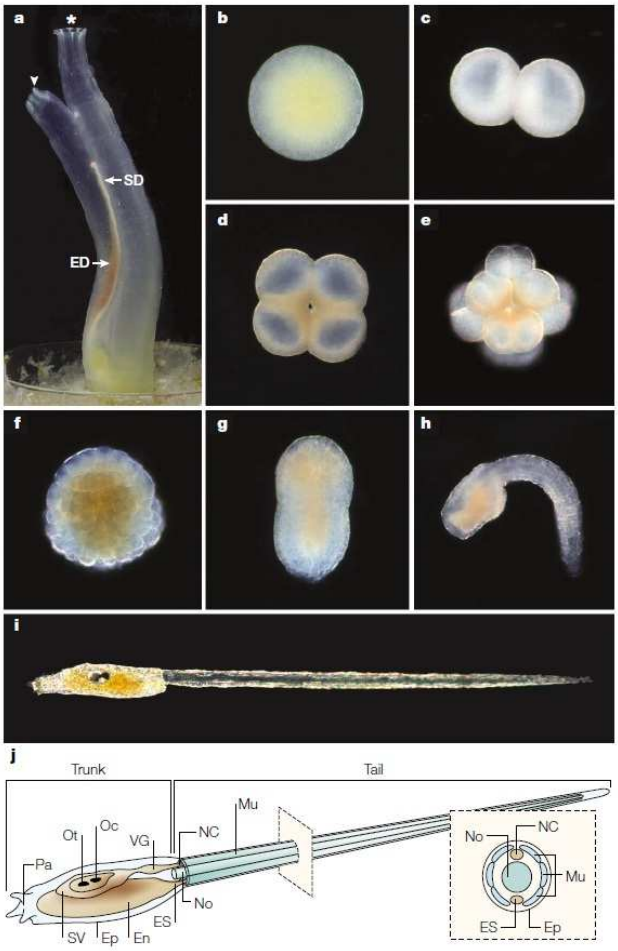


Figure 1.13 Ciona intestinalis adult and during embryonic development. (a) Adult sample of Ciona intestinalis. The oral siphon is indicated with the * whereas the atrial siphon with an arrowhead. SD is the Sperm Duct and ED is The Egg Duct. Beneath the gonads there is the digestive system with the stomach and part of the intestine, and the heart. Underneath the tunic in the central-upper part of the body there is the branchial basket with the endostyle lying along it from the oral siphon to the bottom. (b-i) Embryonic development. (b) fertilized egg, (c) 2-cell stage, (d) 4-cell stage, (e) 16-cell stage, (f) gastrula, (g-h) early and middle tailbud, (i) tadpole larva. (j) schematic representation of tadpole larva: Pa Palps, SV Sensory Vesicle, Ot Otolith, Oc Ocellus, Ep Epidermis, En Endoderm, ES Endodermal Strand, VG Visceral Ganglion, NC Nerve Cord, No Notochord, Mu Muscle. Adapted from (Nori Satoh, 2003)

Given its extremely simplified structure, *Ciona* tadpole fascinated embryologists and molecular biologists to study the chordate development mechanisms using *Ciona* as a model organism.

Ascidians were the first metazoan model organisms in which the experimental embryology was carried out. Due to their simple and unique developmental process they

have been serving as classic experimental systems for the study of developmental mechanism for more than a century [reviewed by (Nori Satoh, 2003)]. Their unique phylogenetic position and their compact genome (170 Mb) offer it as an useful model system for the analysis of chordate developmental program at both morphological and molecular level.

1.6.3 Ascidian genomes.

Some of the tunicate members contain the smallest bilaterian genomes (Holland *et al*, 2003). Up to now the whole genome has been fully sequenced for two tunicates, *C. intestinalis* and *C. savignyi*. The draft genome of *C. intestinalis*, sequenced by whole-genome shotgun method, was announced in December, 2002 (Dehal et al., 2002; Putnam et al., 2008). This is the third complete genome sequenced of an invertebrate, after *C. elegans* (*C. elegans* Sequencing Consortium, 1998) and Drosophila (Adams *et al*., 2000) genomes, and the first of a non-vertebrate in the deuterostome branch of Metazoa (Putnam et al., 2008). More genome sequences have been completed recently from two non-vertebrate deuterostomes, as the sea urchin *Strongylocentrotus purpuratus* (Sodergren et al., 2006) and the amphioxus (Putnam et al., 2008), and from two ascidian species, as *Ciona savignyi* (Vinson et al.,2005 and Small et al., 2007) and *Oikopleura dioica* (Seo et al., 2001). The increasing number of sequenced genomes provides a huge opportunity, through comparative analyses, to resolve a number of outstanding evolutionary questions. The size is ~153-159 Mb, one-twentieth the size of mouse genome, since the ascidians diverged before the gene duplication event (Holland et al., 1994) and contains a total of 15,852 protein coding genes distributed over 14 chromosomes (Nori Satoh, Satou, Davidson, & Michael Levine, 2003). On average, there is one gene in every 5 kb of DNA (Dehal et al., 2002) due to the compact organization. *C*

intestinalis genome has been evolving very rapidly (L. Z. Holland & Gibson-Brown, 2003), with a loss of intergenic region together with entire genes, as in the case of some members of Hox gene family (Hox 7, Hox 8, Hox 9, Hox 11) (Spagnuolo et al., 2003). A comprehensive study of *Ciona* genome revealed that ascidians contain, in many cases, a single representative of multiple paralogous vertebrate genes (Dehal et al., 2002). This is the case for many of the gene families involved in developmental signaling and regulatory processes such as, LEF/TCF (Arce *et al*., 2003), SMAD or T-box genes (Nori Satoh et al., 2003). This characteristic offers the unique opportunity to study gene function without the genetic redundancy seen in many vertebrates.

Another important advantage of the compact *Ciona* genome is that key *cis*-regulatory DNAs tend to map near the core promoter, within the first 1.5 kb upstream of the transcription initiation site (Corbo, M Levine, & Zeller, 1997; Takahashi, Mitani, G. Satoh, & N Satoh, 1999). This feature of the ascidian genome, together with the electroporation method, that permits the analysis of thousand of embryos contemporaneously (Corbo et al., 1997), has made them particularly useful to study the function and the transcriptional regulation of developmental genes.

1.6.4 Developmental biology of Ascidians.

Ascidians, with the exception of *Oikopleura dioica*, are hermaphrodites, i.e. each individual produces both gametes which provides a better opportunity of reproduction both for sessile and planktonic species, although some of them are partially or totally self-sterile (Marikawa, Yoshida, & N Satoh, 1994). General time for life cycle depends on species (Table 2) which is in *Ciona* less than 3 months but can reach up three years in the case of *Halocynthia roretzi*.

Species	Genome (Mb)	Asexual reproduction	Season of sexual reproduction in the wild	Life cycle (egg to egg)	Distribution	Eggs per adult	Egg diameter (µm)	Embryo electroporation	RNA interference
Ciona intestinalis	160	No	Most of the year‡	2-3 months	All temperate seas	Up to 10,000	140	Yes	Yes*
Ciona savignyi	190	No	Most of the year‡	2-3 months	Temperate Pacific	Up to 10,000	160	Yes	?
Phallusia mammillata	<160	No	March to December	<1 year	Europe	Up to 1 million	120	Yes	?
Halocynthia roretzi	~160	No	November to January	3 years	North East Asia	Up to 30,000	280	Yes	?
Botryllus schlosseri	~700	Yes	March to December§	<3 months	Worldwide	<5 per zooid	220-250	?	Yes†
Molgula oculata	?	No	July/August	1 year	North West Europe	Up to 5000	90	No	No
Molgula occulta	?	No	July/August	1 year	Europe	Up to 5000	110	No	No
Oikopleura dioica	70	No	All year	4 days	Warm and temperate seas	~150	65-75	?	?

*Single report.
†Only tested in adults.
‡*Ciona* reproductive season is largely determined by water temperature and so varies between regions.
§In Monterey Bay, CA, USA. Reproductive season varies with location.
?, Unknown or untested.

Table 2. Characteristics of different Tunicates used in research. The size of the genome, the type of reproduction, the seasonality, the length of the life cycle, the features of the eggs and the molecular tools available for those species are illustrated. Notice that tunicates are very heterogeneous, with life cycles from months to years, with genomes with a maximum of 700Mb (B.schlosseri) and a minimum of 70Mb (O.dioica).

Ascidian embryonic development is very simple, fast and easy to follow. Soon after fertilization an invariant and bilaterally symmetrical cleavage program starts which is quite rapid and the embryos develop in a stereotyped fashion. Each division in the embryo produces pretty large and recognizable cells. So, the developmental stages of early ascidian embryos are named according to the number of cell like 8-, 16-, 32-, 64-, and 110-cell stages, instead of the morula and blastula stages (figure 1.14) (Nori Satoh, 2003).

Moreover, each blastomere is distinguishable with a specific and predictable lineage which are named according to Conklin, 1905 (Conklin, 1905). Gastrulation starts at only 110 cell stage when the fates of most blastomeres have already become restricted to a specific tissue type (Lamy & Lemaire, 2008). So, each type of tissue can be traced back at the cell level in the early embryo. It is fascinating as ascidian species phylogenetically far, such as *Ciona intestinalis* and *Halocynthia roretzi*, whose genomic sequences are quite different, especially in the regulatory regions, undergo such similar embryonic development. Indeed, for instance, the cell lineage data can be transferred from one specie to another, almost completely.

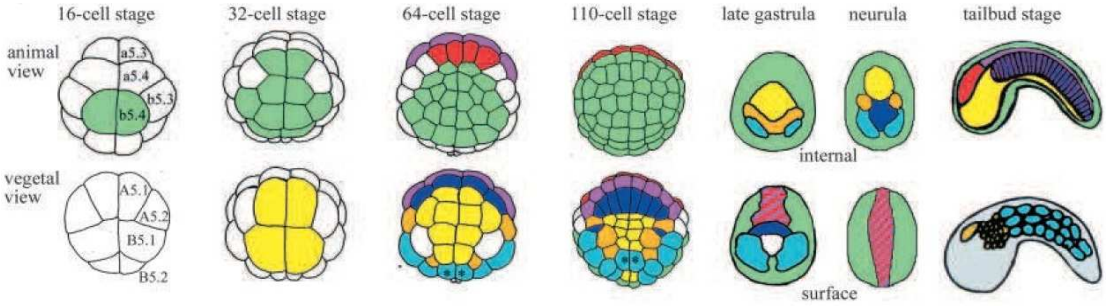


Figure 1.14 Schematic representation of developmental fate restriction in ascidian embryos. Blastomeres whose fate is restricted are colored in yellow for the endoderm, green for the ectoderm, dark blue for the notochord, light blue for the muscles ad red for the Central nervous system. The data obtained by Nishida in Halocynthia can be easily transfer to *Ciona* embryonic development. Adapted from (K. S. Imai, Hino, Yagi, Nori Satoh, & Satou, 2004)

18-24 hours post fertilization the non feeding swimming larvae hatch out at 18°C (Marikawa et al., 1994). *Ciona intestinalis* larva is composed of approximately 2600 cells organized to form few tissues and organs as epidermis, the central nervous system (CNS), endoderm, mesenchyme, notochord, and muscles.

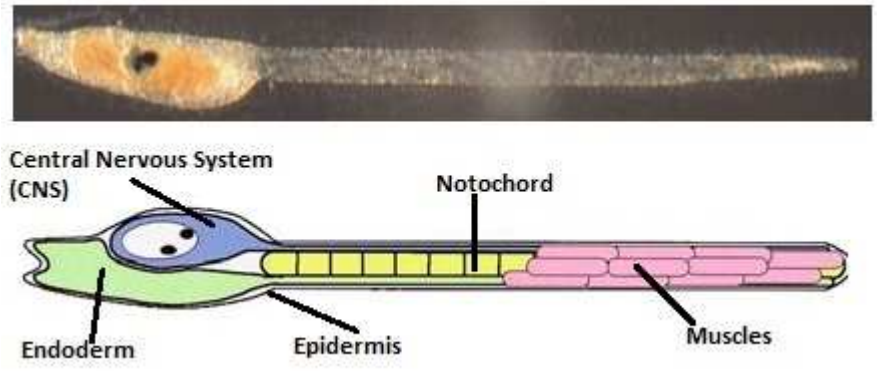


Figure 1.15 Picture and schematic representation of *Ciona* intestinalis larva. In the scheme are presented the main tissue composing the tadpole larva. In green the endoderm, in violet the central nervous system (CNS) and the dorsal nerve cord. In yellow is presented the notochord and in pink the tail muscles. The epidermis, surrounding the entire larva is presented in white.

In *C .intestinalis*, the notochord is composed of 40 cells arranged in a single column which is surrounded by 30 muscle cells in tail region. There are 500 endodermal cells and ~900 mesenchymal cells, which, after metamorphosis, give rise to most of the adult tissues, ~800 epidermal cells (Marikawa et al., 1994), while the CNS contains less than 130 neurons and 230 glial cells (J. H. Imai & Meinertzhagen, 2007).

At the rostral end the larvae bear some adhesive papillae through which they attach themselves to a suitable substrate and start metamorphosis usually within few hours after hatching. It takes 1 or 2 months for the juvenile to become adult with reproductive capability, depending on the temperature of the environment (Marikawa et al., 1994).

1.6.5 Ascidians post-embryonic development: the metamorphosis.

During the swimming period, lasting from hours to several days, the larvae prepare for the onset of metamorphosis: for instance they alter their response to light and gravity. They are negatively geotactic and positively phototactic just after the hatching and for the whole swimming phase, whereas they escape from light and go down to the bottom of the seas when they are ready to go through metamorphosis. As in other metazoan species, the metamorphosis in ascidian represents a strong change in morphology, physiology,

metabolism, ecology and behavior that transforms swimming non feeding larvae into sessile, filter feeding adults. Ten separate phases have been distinguished in the ascidian metamorphosis (Cloney RA, 1982).

The first event is the secretion of adhesive materials by the papillae necessary for the attachment to the substrate (**Adhesive papillae**); soon after, the tail begins to retract (**Tail Resorption)**. Other crucial events in the ascidian metamorphosis are the **Loss of outer cuticular layer, Blood-cells migration** from the hemocoel to the epidermis of the tunic, where they can move actively, and **Body axis Rotation** through an arc of 90° causing a re-collocation of siphons and viscera in the positions found in the adults. As metamorphosis proceeds and the juvenile structures are built, a new body part appears, the **Ampullae**, necessary for a better attachment to the substrate. This organ is of epidermal origin, full of glands secreting sticky materials. The last phase of metamorphosis lead to the **Destruction of transitional larval organs.** The sensory vescicle retraction and an extensive phagogytosis, against the visceral ganglion, sensory organs and cells of the axial comples, are typical events at the end of metamorphosis (Cloney 1961 and Satoh in Developmental Biology of Ascidians,1994).

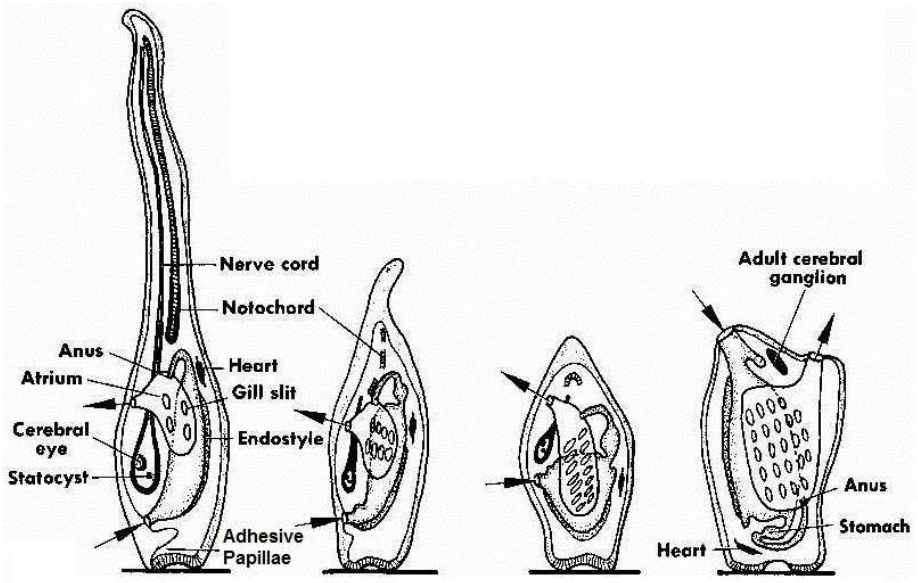


Figure 1.16 Schematic representation of ascidian metamorphosis. After the attachment of the larva to the substrate due to the adhesive papillae a plethora of events starts. Tail retraction is one of the first stages of ascidian metamorphosis followed by destruction of larval tissues and formation of adult organs. It is possible to observe a 90° body axis rotation leading to the opening of siphons upwards. Adapted from the web.

As previously mentioned, pre-metamorphic ascidian tadpole larvae are mainly formed by 6 different tissues, amongst which the endoderm and the mesenchyme are the sole territories made of undifferentiated cells that give rise to most part of adult tissues after metamorphosis. Hirano and Nishida (2000) by intracellular microinjection of HRP (Horseradish Peroxidase) have been able to follow the fate of mesenchymal and endodermal blastomeres from 110-cell to larval and up to the juvenile stage. The Mesenchyme, at the larval stage, is constituted by about 900 cells and is organized in two main groups: the Trunk Ventral Cells (TVCs) and the Trunk Lateral Cells (TLCs). Hirano and Nishida studies have demonstrated that the TVCs originates body-wall (atrial siphon and latitudinal mantle) muscle, heart, and pericardium of juvenile while the TLCs give rise to blood (coelomic) cells as well as body-wall (oral siphon and longitudinal mantle) muscle of juvenile. The Endoderm in the larva is formed by 500 cells derived from 8 blastomeres of the 64-cell stage embryos. *Ciona* or *Halocynthia* larvae, as other ascidian species, do not feed, since they are not provided of functional digestive organs as pharynx, stomach, intestine, endostyle, that differentiate only after metamorphosis just from trunk endoderm that, at the larval stage, appears as a mass of undifferentiated cells rich in yolk granules. Although no sign of organogenesis is evident in the larval endoderm, the studies of Hirano and Nishida have demonstrated that, after metamorphosis, developmental fates of these cells are almost fixed, indicating that a certain degree of regionalization, in this tissue, already exists. These lineage tracing analyses are supported also by molecular evidences as, for example, the expression profile *of Cititf1* gene, the *Ciona* orthologue of *Titf1* (thyroid specific transcription factor 1), a gene involved in the early morphogenesis of vertebrate

thyroid gland. Cititf1 in *Ciona*, besides delineating early endoderm specification, is present, at the larval stage, in the anterior-ventral part of head endoderm, a region that forms, after metamorphosis, the endostyle, the organ considered the homologue of the vertebrate Thyroid gland, where *Cititf1* is still present (Ristoratore et al., 1999a). Interestingly, *Cititf1*, with its expression profile, while assessing the regular projection from the larval endodermal regions to the adult stage digestive organs, represents also, together with Ci-FoxE (TTF-2) and Ci-FoxQ, as previously noticed, one of the molecular evidences linking the thyroid gland to the ascidian endostyle.

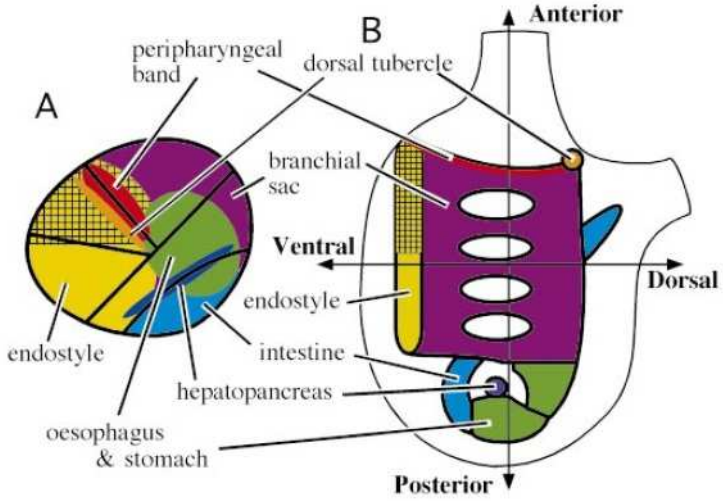


Figure 1.17 Fate map showing the position of each prospective region in larval endodermal tissue. (A) Trunk larva endoderm; different colors to indicate adult tissues. The anterior part of the trunk endoderm gives rise to the endostyle. (B) adult tissues colored to explain their pre-metamorphic correspondence. Adapted from (Hirano & Nishida, 2000)

1.7 Aim of the research.

The studies reported here started with the aim to give insights in the evolution of the Thyroid function in the chordate lineage.

To this end the ascidian *Ciona intestinalis* offers as a valuable model system, given some peculiar characteristic of this organism that include:
a metamorphosis process that previous evidences indicate to be slightly sensitive and influenced by Thyroid Hormones; the presence of the endostyle, organ whose homology with the thyroid gland is supported both by physiological and molecular evidences.

Furthermore *Ciona intestinalis* genome has been fully sequenced and it encodes almost all the proteins and all the enzymes involved in the biosynthesis of THs. Among all, a putative TH receptor, that previous studies failed to functionally characterize as a classical thyroid hormone receptor, has been found. Surprisingly no homolog of vertebrate thyroglobulin has been identified in *Ciona* genome, as well as in amphioxus, suggesting that another protein(s) is employed as substrate, by these organisms, for iodotyrosine synthesis and thus for Thyroid Hormones production.

My Ph.D. project, taking advantage of all the characteristics previously mentioned of *Ciona* as model system, has been focused on the evaluation of the role of Iodine and Thyroid Hormones on metamorphosis. Furthermore I tried to determine the impact of an impaired thyroid hormone biosynthetic pathway on this developmental process. In particular, by using goitrogens, drugs able to interfere at different levels with TH biosynthesis, I studied their effect on metamorphosis and on the ability of the endostyle to produce THs.

To fill the gap in the delineation of the complete molecular machinery underlying *Ciona* thyroid function, during my studies I tried also to identify the protein(s) used, in the

endostyle, as scaffold for thyroid hormone synthesis. I used an antibody able to recognize the thyroid hormone Thyroxine (T4) to isolate the proteins that putatively contain this hormone and afterwards I identified them by two different mass spectrometry assays.

To define in the best way this thyroid-like function, the third goal of my Ph.D work was the study of the putative *Ciona* thyroid hormone receptor (CiNR1). I studied its ability, as transcription factor, to bind to specific DNA sequences by EMSA assays. Furthermore by means of transient transfection in mammalian cells I tried to characterize its TH-dependent transcriptional activity.

Methods

2.1 In vivo Assays.

2.1.1 *Ciona* eggs and embryos.

C. intestinalis adults were collected in the bay of Naples. Eggs and sperm were collected from the gonoducts of several animals, used for *in vitro* fertilization. Embryos were also used in *in situ* hybridization experiments. Embryos were raised in Millipore-filtered seawater at 18-20° C. Samples at appropriate stages of development were also collected by low speed centrifugation and used for protein extraction or fixed for *in situ* hybridization.

2.1.2 Artificial seawater preparation.

Ciona intestinalis eggs were fertilized in different seawater conditions: natural seawater (MFSW, Millipore Filtered Seawater) and Artificial seawater (ASW).

Two different types of Artificial seawater were prepared:

- Complete Artificial seawater (ASW)
- Iodine free seawater (ASW-I)

The salts used in the preparation of the ASW are divided into 4 groups (A, B, C, D) and are listed in the following tables.

Group A

A1	NaCl	26 g/L
A2	$MgSO_4x7H_2O$	6.58 g/L
A3	$MgCl_2x6H_2O$	4.88 g/L

A4	$CaCl_2x2H_2O$	1.46 g/L
A5	KCl	0.675 g/L
A6	$NaHCO_3$	0.184 g/L

Stock solutions 10X for A1 and 100X for A2-A6 were preparated.

Group B

B1	KBr	100 mg/L
B2	$SrCl_2x6H_2O$	24.2 g/L
B3	NaH2PO4xH20	2.25 mg/L
B4	LiCl	1.09 mg/L
B5	$Al_2(SO_4)_3x18H_2O$	0.042 mg/L

Stock solutions were prepared as follow: B1-100X, B2-200X, B3 to B5 1000X.

C1	H_3BO_3	25.4 mg/L
C2	Na_2EDTA	9.44 mg/L
C3	$FeCitratexH_2O$	3.83 mg/L
C4	$Na_2MoO_4x2H_2O$	24.2 µg/L
C5	$MnSO_4xH_2O$	169 µg/L
C6	$ZnSO_4x7H_2O$	22.9 µg/L
C7	$CuSO_4x5H_2O$	9.98 µg/L
C8	KI	79.1 µg/L
C9	$CoSO_4x7H_2O$	13.4 µg/L
C10	$NaVO_3$	4.876 µg/L

Stock solutions were prepared as 1000X for C1, C2, C3 and 10000X for C4 to C10.

Solutions C3, C5, C6, C7 and C9 were also subjected to a chelation step through the addition of 2 molar equivalent of Na_2EDTA.

Group D

D1	Thiamine HCl	1.953 mg/L
D2	Cyanocobalamin	0.997 µg/L

Group D solutions were prepared as 10000X stocks.

After the preparation of the stock solutions, group A solutions were mixed first, along with distilled water. Once dissolved, group B solutions, previously mixed together, were added. A mix of group C solutions was added to the solution composed by Group A + Group B + ddH_2O. The solution prepared in this way was then filtered through a 0.22 µm Millipore filter and kept at 4°C maximum for one week. When necessary in the amount of artificial seawater needed was added the group D.

All the salts listed in the tables are necessary to produce the complete Artificial SeaWater (ASW). To make the Iodine-free ASW it is enough to avoid, during the preparation of the ASW, the salt C8 (KI, Potassium Iodide).

2.1.3 Iodine concentration in seawater. The Sandell-Kolthoff reaction.

It is known that is possible to estimate small amount of iodine (as Iodide ion I^-) by measuring its catalytic effect on the reaction between quadrivalent cerium and trivalent arsenic in sulphuric acid solution. The reaction, known as Sandell-Kolthoff reaction:

$2Ce^{IV} + As^{III} \rightarrow 2Ce^{III} + As^{V}$

proceeds with extreme slowness in dilute sulphuric acid at room temperature in absence of a catalyst. The addition of iodide cause the decolourizations of the solution from yellow (due to Ceric cerium ions) to white. With the exception of Osmium and Ruthenium, no elements have been found that markedly catalyse the reaction.

To check iodine content in natural and artificial seawater an assay based on Sandell-Kolthoff reaction has been used.

Four solutions were prepared as follows:

- 0.01M KI (Potassium Iodide)
- Sodium Arsenite [250mg of Sodium (meta)arsenite dissolved in 1.5mL of 97% Sulfuric acid and 8.5mL of milliQ water]]
- Ceric cerium stock solution [500mg of Ammonium cerium(IV) sulfate dehydrate dissolved in 1mL of 97% Sulfuric acid and 4mL of milliQ water]
- Ceric cerium working solution was prepared with 300µL of Ceric cerium stock solution + 200µL of 97% Sulphuric acid and 14.4mL of milliQ water.

The solution containing concentrated Sulphuric acid were prepared in glass beckers, put on heater and sealed with 4 layers of Parafilm® to avoid sulphuric acid vapours emission.

Dilutions of the KI solution were made from 0.1 to 1 nanomole equivalent of Iodide ion, were used to build a standard curve.

To perform this assay series of 14 samples were prepared in acid resistant cuvettes and analyzed. The volume in each cuvette was at the beginning 200µL. The Blank was prepared with 200 µL of milliQ water. The standards were prepared with the amount of KI solution needed and milliQ water to 200 µL. The seawater samples were prepared

with 200 µL of MFSW (Millipore Filtered Natural SeaWater), 200 µL of ASW (complete Artificial SeaWater) and 200 µL of ASW-I (Iodine-free Artificial SeaWater).

100µL of concentrated sulphuric acid was added to the cuvettes with a delay of 1 minute between a cuvette and the next one. After the addition of sulphuric acid to the 11th cuvette of the series, 40 µL of Br_2 was added to the first cuvette. Then sulphuric acid was added to the 12th cuvette and Br_2 to the second cuvette and so on until the end of the series.

After the addition of Br_2 to all the cuvettes the absorbance of the samples was read in a spectrophotometer at 410nm.

Before the reading, to each cuvette were added:

- 750 µL of Ceric cerium working solution
- 100 µL of Sodium Arsenite solution

The content of the cuvette was mixed well and immediately the absorbance was read at time 0, 10, 20 and 30 seconds, to evaluate the decrease in the colour of the mixture.

The decrease in the colour of standard solution, where the iodine content was known, was used to construct a standard curve. Based on this curve the iodine concentration in the samples [Natural SeaWater (MFSW), Artificial SeaWater (ASW) and Iodine free artificial SeaWater (ASW-I)] was inferred.

2.1.4 Treatment of *Ciona intestinalis* larvae with L-Thyroxine (T4).

A stock solution of L-thyroxine (T4) sodium salt (Sigma-Aldrich) was prepared each day by dissolving 5 mg of hormone in 100mL of 0.001M NaOH, which was then diluted with seawater (MFSW and ASW±I). *Ciona* intestinalis just hatched larvae, in MFSW and ASW±I were treated with L-thyroxine stock solution to obtain a final thyroxine

concentration ranging from 25 to 32 µg/mL. The onset of metamorphosis, in treated and untreated larvae, was followed each hour for the first 10 hours and then at 24 hours post treatment.

2.1.5 Treatment of *Ciona intestinalis* with Goitrogens.

Ciona intestinalis eggs were fertilized in MFSW (Millipore Filtered Seawater) containing different goitrogens, dissolved directly at the final concentration in the MFSW.

These drugs, all provided by SIGMA-ALDRICH, were used at the following concentrations:

Methimazole 500-1000 mg/L

Thiourea 2500-5000 mg/L

Potassium Perchlorate ($KClO_4$) 2500-4000-5000-6000-7000 mg/L

All the treatments started at the fertilization. The effect on the onset of metamorphosis was followed in the first 10 hours post hatching. The phenotypes of treated animals were checked 24 hours and 48 hours post fertilization.

2.1.6 Rescue of metamorphosis with L-Thyroxine.

At Potassium Perchlorate ($KClO_4$) concentration higher than 5000mg/L, a block in *Ciona intestinalis* metamorphosis was observed. Larvae retained the tails and their swimming ability for several days after hatching. Rescue of metamorphosis was followed after the addition, 5 hours post hatching, of 32 µg/mL L-Thyroxine (T4).

2.1.7 Immunohistochemistry with anti-thyroxine (anti-T4) antibody on *Ciona* endostyle sections.

Adult *Ciona* specimens were incubated for 4 days in MFSW in presence of 500 mg/L and 1 g/L of Potassium Perchlorate. Endostyles from treated and control animals were dissected and fixed overnight in a mixture containing: 4% paraformaldehyde, 0.1 M MOPS pH 7.5, 0.5 M NaCl.

The endostyles were then washed twice in PBS 1X for 15 minutes and then dehydrated as follows:

- 1h in 70% Ethanol
- Overnight in 95% Ethanol
- 3 x 45 minutes in 100% Ethanol

They were then incubated

- 3 x 45 minutes in 100% Xylene
- Overnight in 50% Xylene, 50% Paraffin at 65°C
- 3 x 1h in 100% Paraffin at 65°C

After the third change in Paraffin, the organs were placed with the right orientation in a plastic mold filled with fresh paraffin, and kept at room temperature to allow the paraffin to solidify.

Endostyle 6 µm cross section were done using the Leica RM2245 Microtome and placed on Menzel-Glaser SuperFrost® slides.

On the slides an Immunohistochemistry was performed as follows:

I day

1) 3 x 10 minutes in 100% xylene
2) 2x 5 minutes in 100% Ethanol
3) 2 minutes in 95% Ethanol
4) 2 minutes in 90% Ethanol
5) 2 minutes in 80% Ethanol
6) 2 minutes in 70% Ethanol
7) 2 minutes in 50% Ethanol
8) 2 minutes in 30% Ethanol
9) 3x 5 minutes in PBS
10) 30 minutes in PBS containing 3% H_2O_2
11) 3 x 10 minutes in PBS
12) Incubate slides in a humid chamber at room temperature for 10 minutes in PBS containing 10% goat serum;
13) Incubate slides in a humid chamber at 4°C overnight in PBS containing 1% BSA and a 1:1300 dilution of the anti-T4 primary antibody.

II day

1) 3 x 10 minutes in PBS
2) Incubate slides in a humid chamber at room temperature for 1 hour in PBS containing a 1:250 dilution of the secondary antibody (Anti-Rabbit-HRP Conjugated);

3) 4 x 8 minutes in PBS
4) Prepare 30 min before use the following solution: 5 ml PBS + 1 drop of A reagent and 1 drop of B reagent.
5) Incubate in a humid chamber at room temperature for 30 minutes in solution of point 4
6) 4 x 8 minutes in PBS
7) To make DAB: dissolve 1 DAB tablet and 1 tablet urea tablet in 5 ml PBS, filter and use (in the dark);
8) Incubate the slides in DAB solution until the appearance of the signal
9) Stop with water.

2.2 Thyroid Hormones precursor protein investigation.

2.2.1 Protein Extraction from *Ciona intestinalis* endostyles.

Endostyles were dissected from adult, sexually mature, *ciona* specimens. Just after the dissection the organs were dried, frozen in Liquid Nitrogen and stored at -80°C. Protein were extracted each time from 300mg of tissues (ca. 30 endostyles) by grinding in liquid nitrogen with pestle and mortar. After 5 cycles of homogenization the tissue powder was collected into a 1.5mL Eppendorf tubes and 400µL of RIPA protein extraction buffer was added.

The composition of the RIPA buffer 1.25X is:

- 50mM Tris-HCl pH 7.5
- 150mM NaCl
- 0.1% SDS

- 5mM $MgCl_2$
- 0.5% Deoxycholic acid (as Sodium Dehoxycholate)
- 1% Triton X-100

The RIPA buffer prepared as a 1.25 stock is stored at 4°C. At the moment a complex of Protease and Phosphatase Inhibitors was added. In particular RIPA buffer was completed with:

- 0.05M NaF
- 0.5mM Na_3VO_4
- 0.5mM PMSF
- 0.001M DTT
- 1X Protease Inhibitors mix (Complete™ Protease inhibitor cocktail tablets, Roche)

After the addition of the protein extraction buffer, the samples were left for 20 minutes in agitation at4°C. Debris were discarded by centrifugation at 15000g at 4°C. Protein concentration was determined by Bradford assay. The supernatant is then transferred to a clean 1.5mL Eppendorf, precipitated with 4 volumes of cold acetone (-20°C), vortexed and put at -20°C overnight. The samples are then centrifuged for 30 minutes at 15000g and the acetone (supernatant) was discarded from the tube. The protein pellet was air-dried and then resuspended in 2-DE solubilisation buffer (...of chapter methods).

2.2.2 2-DE (two-dimensional gel electrophoresis).

Protein extracted from *Ciona* intestinalis endostyles were separated by 2-DE. Proteins were analysed in two replicates, one to be silver stained (...of chapter Methods), the other to be transferred to PVDF filters for immunoblotting (...of chapter methods). 300µg of Acetone Precipitated endostyle proteins were resuspended in 2-DE solubilisation buffer:

- 8M Urea
- 2M Thiourea
- 0.1% Triton X-100
- 10% 2-Propanol
- 4% CHAPS
- 50mM DTT
- 0.5% IPG Buffer pH 3-10 (GE Healthcare)

Once resuspended proteins were subjected to subjected to IEF (isoelectrofocusing) in immobilized, linear pH gradient using 11cm-long, 0.1mm thick, pre-cast Immobiline Dry Strips pH3-10L (GE Healthcare). Strips were rehydrated with 200µL (300µg of protein) of resuspended proteins for 10 hours at room temperature. IEF was conducted at 20°C using the following voltage program, set up for our purpose, considering a maximum current of 50mA/strip:

- 6 h at 50V (Step and Hold)
- 1h at 200V (gradient)
- 1h at 500V (gradient)
- 1h at 1000V (Step and Hold)
- 30 min at 8000V (gradient)
- 6h at 8000V (Step and Hold)

Proteins in the DryStrips were reduced and alkylated in 10mL in SDS-PAGE suitable equilibration buffer. Reduction was performed for 10 minutes at RT in Buffer 1:

- 6M Urea
- 0.375M Tris/HCl pH 8.8
- 2% SDS
- 20% Glycerol

- 2% (w/v) DTT

Alkylation was conducted for 10 minutes at RT in Buffer 2:

- 6M Urea
- 0.375M Tris/HCl pH 8.8
- 2% SDS
- 20% Glycerol
- 2.5% (w/v) IAA (Iodo Acetammide)

After the equilibration DryStrips were gently rinsed in SDS-PAGE running buffer (0.025M Tris base, 0.2M Glycine and 0.1% SDS) and layered onto a 5-15% total acrylamide vertical gradient gel in 0.375M Tris/HCl pH 8.6 and 0.1% SDS, mounted in a Hoefer SE600 vertical electrophoresis unit. SDS-PAGE was conducted in 0.025M Tris base, 0.2M glycine and 0.1% SDS at the constant current of 10mA per gel for 16h at 15°C. Broad range molecular mass standards were from Bio-Rad. Analytical gel to be silver stained were fixed 50% methanol, 5% Acetic acid at least for 20 minutes.

2.2.3 Western blotting of proteins separated on 2-DE.

Protein separated by 2-DE were transferred onto PVDF (Polyvinylidene fluoride) membrane (Amersham Hybond-P, GE Healthcare) by semi-dry electrophoretic transfer in 0.025M Tris and 0.01M Glycine. Membranes were blocked at least for 2h in PBS-Tween with 5% Blotting grade blocker Non Fat Dry Milk (Bio-Rad) and incubated with a 1:1000 dilution, in PBS-Tween + 5% Non Fat Dry Milk, of a Rabbit Anti-thyroxine (Anti-T4) serum (MP Biomedicals) overnight at 4°C under continous agitation. After 3 rinses in PBS-Tween (PBS + 0.1% Tween-20) membranes were incubated with a 1:10000 dilution of HRP (horseradish peroxidase)-conjugated, secondary anti-rabbit IgG antibodies for 1h

at room temperature. Immunoreactive spots were then detected by chemiluminescence (ECL® Plus, GE Healthcare).

2.2.4 Silver staining of SDS-PAGE gels.

Analytical and preparative gels were silver stained using a Mass Spectrometry compatible protocol (Shevchenko et al., 1996). Gels were fixed for 20 minutes in 50% methanol, 5% acetic acid. After rinsing for 10 minutes in 50% methanol and for 10 minutes in milliQ water, gels were sensitized with 0.02% sodium thiosulphate for 1 minute, and then rinsed quickly twice in milliQ water. The gels were then incubated in 0.1% Silver Nitrate for 20 minutes at 4°C in the dark, and then rinsed twicw in milliQ water. Staining was developed in 0.04% formalin, 2% sodium carbonate. The developing solution has to be always clear and has to be changed when yellow. The gel were then blocked in 5% acetic acid. Gels were stored at 4°C in 15 acetic acid until peaking of protein spots for MS analysis.

2.2.5 Identification of Anti-T4 reactive proteins by Mass Spectrometry.

Silver stained 2-DE gels of the *Ciona* intestinalis endostyle proteome were aligned with the chemiluminescent detection maps of the corresponding Immunoblots with anti-T4 antibodies, in order to locate the protein that reacted with the anti-T4 antibodi within the 2D separation patterns. Protein spots of interest were excised from the gels with a scalpel, placed in Eppendorf tubes, washed twice with 50µL of milliQ water and destained with 0.050M NH_4HCO_3 in 50% aqueous acetonitrile. Destained spots were dehydrated by submersion into acetonitrile, dried under vacuum and incubated in 20 µL of 0.050M NH_4HCO_3 containing 12ng/ µL of trypsin on ice. After 45 minutes of digestion, the

supernatants were removed and incubated overnight at 37°C. The triptig digests were extracted in 40 µL of acetonitrile, 2.5% formic acid, desalted and concentrated, using a Poros Oligo R3 microcolumns, for analysis by MALDI-TOF-MS (matrix-assisted laser-desrption ionization-time-of-flight-Mass Spectrometry).

MALDI-TOF analysis were performed by Dr. Gianfranco Mamone at the CNR in Avellino, Italy.

Peptide assignment were performed using the GPMAW software (www.gpmaw.com). MS data were searched against the NCBInr protein sequence databases, using the Mascot server (www.matrixscience.com).

Ciona intestinalis Nuclear receptor 1: structural and functional studies.

2.3 CiNR1 DNA binding assay.

2.3.1 Radiolabeling of oligonucleotide by 5' terminal phosphorylation.

Oligonucleotides were radiolabeled at 5' end by means of Polynucleotide kinase (PNK). This enzyme catalyses transfer of γ-phosphate residues from [γ-^{32}P]ATP to the 5'-hydroxyl termini of dephosphorylated oligonucleotide. The reaction mixture contained:

- 1µL of Forward oligonucleotide [10µM]
- 1µL of PNK buffer A
- 1µL of γ-^{32}P-ATP
- 1µL of PNK

- 6µL of H_2O

The mixture was incubated at 37°C for 1 hour.

After an excess of Reverse primer was added:

- 1µL of Reverse primer [100µM]

The mixture was then heated at 100°C for 2 minutes and let cool down at least for 30 minutes at room temperature to allow the formation of the double strands. The probe was then purified on illustra ProbeQuant G-50 Micro Columns (GE Healthcare) following manufacture's instruction. The solution was then loaded on a pre-run 5% polyacrilamide gel made as follow:

- 5mL of 40% Acrylamide (Acry/Bis 19:1)
- 2mL of 10X TBE (final concentration 0.5X)
- 33mL H_2O
- 40µL TEMED
- 400µL of 10% APS

After 40-60 minutes the run was stopped and the gel was put in a cassette and a film was exposed for 30 minutes to localize the probe. Once localized the piece of gel containing the probe was cut and put in a DNase free 1.5mL Eppendorf tube. The probe was then eluted from the gel by adding 300µL of water and smashing the gel piece with a scalpel. After an overnight incubation the tube was centrifuged and the supernatant, with the probe, was transferred to a clean DNase free 1.5mL Eppendorf tube.

2.3.2 *In vitro* transcription and translation.

In vitro transcription and translation of the full length cDNA of *CiNR1 and CiRXR*, contained in the pBluescript SK II vector, was carried out using the Kit (PROMEGA) "TNT® Coupled Wheat germ extract System". The reaction was carried out using this reaction mixture:

- 25µL of wheat germ extract
- 2µL of TNT Buffer
- 0.5µL of amino acid mix (minus Methionine)
- 0.5µL of amino acid mix (minus Leucine)
- 1µL of T3 polymerase
- 1µg of DNA template
- To 50µL of water

The reaction was incubated for 90 minutes at 30°C.

6µL of CiNR1 and 6µL of CiRXR were used for an EMSA assay (section ...of Methods).

2.3.3 Band-shift assay.

Band shift assay was performed by in presence of 20mM Tris-HCl pH 8.0, 50mM KCl, 10% glycerol, 50mM NaCL, 2mM $MgCl_2$, 2mM DTT. The mixture was incubated 10 minutes at RT with 5 µl of *CiNR1 or* 5 µl of *CiRXR or* 2.5 µL *CiNR1+2*.5 µl of *CiRXR* in vitro translated proteins before the addition of radiolabeled DNA probe (10^5 cpm) in a final volume of 20 µl in the presence or absence of specific or random double strand cold competitor oligonucleotides corresponding to a 10 to 100x molar excess. The sequence of the double strand random oligonucleotide (R) was: 5' -atagagtaagccgattattg-3'. After addition of the labeled DNA, the samples were placed 30 minutes at RT and successively

separated by electrophoresis at 180 mV 2 hours on 5% polyacrylamide/0.5x TBE gel. Run was stopped when bromophenol blue run out of the gel. Gels were fixed in 10% Acetic Acid, 20% Ethanol for 20 minutes. After 2 hour drying at 70° C by means of a Bio-Rad 583 gel dryer, the gel was analyzed by autoradiography by exposing overnight on a Phosphor-imager screen and by analyzing it on a Fuji fla5100 Phosphor-imager.

2.4 CiNR1 Whole Mount In Situ Hybridization (WMISH).

2.4.1 Embryo fixation and storage.

Embryos of the desired developmental stages were collected and fixed overnight at 4ºC/ 1 hour at RT in the Fixing solution (4% formaldehyde, 0.1 M MOPS pH 7.5, 0.5 M NaCl.). The embryos were washed three times in 1XPBT (Phosphate Buffered Saline, 0.1% Tween-20) and immediately used or dehydrated with a series of 30%, 50% and 70% Ethanol and stored in 70% ethanol at -20ºC. These fixed embryos were further used for WMISH or Immunohystochemistry.

2.4.2 Plasmid linearization.

To synthesise sense and antisense mRNA corresponding to the cDNA fragment of CiNR1, isolated from the *C. intestinalis* cDNA library, the plasmid containing the promoters T3 and T7 respectively upstream and downstream of the cloning sites was first linearised by digestion with suitable restriction enzyme. When the plasmid was digested with an enzyme cutting at the 3’ end of the insert, RNA polymerase recognised T3 promoter and a sense mRNA was obtained, whereas, if an enzyme cuts the 5’end the T7 promoter was recognised and an antisense mRNA was synthesised.

2.4.3 Linearized plasmid purification.

In order to eliminate protein contamination, the linearised plasmid was treated with Proteinase K (0.2 µg/µl), Tris 1M pH 7.4 (0.1), SDS (0.5%), in a total volume of 200 µl, using DiEthylPyroCarbonate (DEPC) treated H_2O. The reaction was then incubated for 30 minutes at 50ºC. Purification was done by adding 1 volume of phenol : chloroform : isoamylic alcohol (25:24:1), vortexed vigorously and centrifuged at 14000 rpm for 10 minutes at 4°C. The aqueous phase was collected and 1 volume of chloroform : isoamylic alcohol (24:1) was added; the sample was vortexed vigorously and centrifuged at 14000 rpm for 10 minutes, at 4°C. The aqueous phase was recovered and the DNA was precipitated adding 3 volumes of ethanol 100%, 1µl of glycogen (20 mg/ml), and 1/10 volume of Sodium acetate 3M pH 5.2, stored over night at -20°C or 1 hour at -80°C. Then, it was centrifuged at 14000 rpm for 15 minutes, at 4°C. The precipitated DNA was washed with ethanol 70% (DEPC-treated), centrifuging at 14000 rpm for 15 minutes at 4°C. The ethanol has been removed and the DNA pellet was air-dried at R.T. At the end, the DNA pellet was resuspended in a suitable volume of H_2O (DEPC-treated). Aliquots were analysed on 1 % agarose in TBE 1x and quantified in Nanodrop (1000, Thermo Scientific).

2.4.4 Synthesis of riboprobe.

Both sense and antisense mRNAs were obtained by using a Digoxygenin RNA labeling kit (Roche) in the following reaction mixture: 1µg linearized plasmid DNA, 10xNTP mix, 2 µl; 10xtranscription buffer 2 µl, 20 U/µl RNase inhibitor 1 µl, 20 U/µl T7 RNA polymerase 2 µl or 20 U/µl T3 RNA polymerase 2 µl,DEPC H_2O up to 20 µl. Synthesis reaction was conducted at 37 °C 2 hours. To remove DNA template, 2 µl of 10 U/µl DNase I RNase free were added and incubated at 37 °C 10 minutes. The reaction was

stopped by adding EDTA pH 8 to a final concentration of 25 mM. The unincorporated nucleotides the RNA probes were removed using the Mini Quick Spin RNA Columns G-50 Sephadex (Roche), following manufacturer's instructions.

To check the quality and the quantity of the synthesized riboprobe gel electrophoresis and Dot Blot analysis were done respectively. The riboprobe was immediately stored at -80 °C until use.

2.4.5 Quantification of riboprobe.

For each newly made Dig-labelled riboprobe, the concentration was evaluated by dot-blot immunostaining with anti-Dig antibody AP conjugated (Roche) against a known standard, Dig-labelled RNA control (Roche). RNA dilutions were prepared using the dilution buffer [DEPC H2O: 20X SSC: formaldehyde (5:3:2)]. Typically, dilutions of the Dig-labelled riboprobes were blotted to Hybond N membrane (Amersham) along with serial dilutions of standard Dig-labelled RNA (Roche) and UV-crosslinked to the membrane, with Stratalinker for 30 seconds. The filter was first incubated for 30 minutes in blocking solution (5% BSA in 0.1 M maleic acid pH 7.5) and then incubated, 1 hour at RT, in the same solution containing the anti-Dig alkaline phosphatise (AP) antibody (0.15 U/ml). To remove unbound antibodies, two washes in a solution containing 0.1 M maleic acid pH 7.5 and 0.15 M NaCl were done. The filter was equilibrated in the detection solution (100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM MgCl2) and then incubated in dark in the same solution in which 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (50 mg/ml) and nitroblue tetrazolium (NBT) (50 mg/ml) were added. The AP enzyme produces an insoluble blue precipitate in the presence of these 2 enzymatic substrates. The colored compound starts to precipitate in few minutes. The reaction was blocked after 10 minutes by washing the filter with H_20. The concentrations of experimental

riboprobes were estimated by comparing spot intensities of the standard control and the experimental dilutions.

The concentration of the ribonucleic probes were estimated making serial dilutions 1:1, 1:4, 1:16, 1:64, 1:256, of the sample ribonucleic probes and comparing with the same dilutions of a control RNA of reference (1:1 100 ng/µl; 1:4 25 ng/µl; 1:16 6.25 ng/µl; 1:64 1.56 ng/µl; 1:256 0.39 ng/µl) (Roche).

2.5 Transient Transfection assays.

Molecular cloning.

2.5.1 DNA digestion with restriction endonucleases.

Plasmid DNA was digested with appropriate restriction endonuclease/s containing 5 units of enzymes/ 1µg of DNA in a final volume at least 20 times larger than the enzyme volume. In the presence of 1/10 volume of its respective buffer the mixture was incubated at least four hours/over night in a specific temperature suggested by the manufacturer's instruction.

2.5.2 DNA dephosphorylation.

DNA dephosphorylation has been conducted as follows: a convenient amount of DNA has been incubated with 1U of Calf Intestinal Alkaline Phosphatase enzyme (CIP) (Roche) per 1 pmol 5' ends of double stranded digested DNA, in 1X CIP dephosphorylation buffer (Roche), at 37º C for 30 minutes. After this time, a second aliquot of CIP as been added, and the reaction has been carried on for another 30 minutes, at 37ºC.

2.5.3 DNA gel electrophoresis.

To check the length and the quality of DNA samples such as PCR products, genomic DNA, plasmid DNA and restriction digestions were run on 1% agarose gel (containing 0.5 µg Ethidium Bromide) prepared in 0.5 X TBE buffer (Tris Borate EDTA). Before loading into the well DNA samples were mixed with 6 X loading buffer. After running for appropriate time nucleic acid bands were observed under UV and pictures were taken using GelDoc 2000 (BioRad).

2.5.4 DNA extraction from agarose gel.

DNA fragments from PCR product or enzymatic reaction of plasmid were extracted from gel using QIAGEN® QIAquick Gel Extraction Kit according to the manufacture's instruction.

2.5.5 DNA ligation.

Each ligation reaction was carried out in 20 µl reaction mixture containing 1X T4 Ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, pH 7.5); and 1 µl of T4 DNA Ligase (New England Biolabs) at 1 unit/µl conc. The proportion of plasmid vector and insert DNA was usually kept 1:3, and the total amount of DNA was kept within 50-100 ng. T4 DNA ligase was used to catalyze the formation of a phosphodiester bond between juxtaposed 5'-phosphoryl and 3'-hydroxyl termini in duplex DNA. It is also able to repair single-stranded nicks in duplex DNA and joins both blunt-ended and sticky-ended restriction fragments of duplex DNA potentially.

2.5.6 Bacterial cell electroporation.

This method permits introduction of foreign DNA in bacterial cells via circular plasmid. Briefly, circular plasmid containing the gene of interest and the competent bacterial cells

(prepared by the Molecular Biology service of the 'Stazione Zoologica, Anton Dohrn', Naples) were placed in a 0.2cm electrocuvette. The electrocuvette was then subjected to an electric pulse at constant 1.7 V using a Bio-Rad Gene Pulser™ electroporation apparatus. The transformed E.coli cells were allowed to recover for one hour at 37ºC in 1ml LB medium (NaCl 10g/l, bactotryptone 10g/l, yeast extract 5g/l,). An aliquot was spread on a prewarmed LB solid medium (NaCl 10g/l, bactotryptone 10g/l, yeast extract 5g/l, agar 15g/l) in the presence of specific selective antibiotic grown at the same temperature overnight.

2.5.7. PCR screening.

Transformed bacterial colony were checked for the right insert by PCR where half of each single colony was placed for PCR where as the rest half was grown in 3 ml of LB liquid medium in the presence of suitable antibiotic (50 g/ml) O/N shaking at 37C. The PCR reaction was set in a total volume of 20 l ,the reaction mixture was as follows: 1X PCR buffer containing $MgCl_2$ (Boehringer Manneheim, Monza, Italy); 40 mM dNTP mix (dATP, dTTP, dCTP, dGTP); 20 pmol of each forward and reverse suitable oligonucleotides; and 1U Taq DNA polymerase (Roche; Biogem). PCRs were carried out with the following protocol:

First step (one cycle)

First denaturation: 5 minutes at 95ºC.

Second step (repeated for 25 cycles)

Denaturation: 1 mintute at 94ºC

Primer annealing: 1 minute at 45ºC-65ºC (according to the melting temperature of oligo)

Extension: at 72ºC for a suitable time, (depends on the amplified fragment length that is 1min/kb).

By gel electrophoresis analysis, the samples presenting a band of expected size have been identified and plasmid DNA has been purified from the corresponding bacterial colonies.

2.5.8. DNA sequencing.

The DNA sequences were obtained using Beckmen CEQ 2000XL NA analysis system Apparatus by the (SBM) Molecular Biology Service at the SZN.

2.5.9 Plasmid DNA isolation: DNA mini and Maxi-Preparation.

A single bacterial colony containing the plasmid DNA of interest was grown overnight in suitable volume of LB in the presence of appropriate antibiotic shaking at 37ºC for plasmid DNA purification. The culture was then centrifuged at top speed to pellet the bacterial cell. The pellet was resuspended in respective buffer and manufacturer's handbook instructions were followed. For Mini-prep Qiagen and for Maxi-prep Qiagen and Sigma Maxi-Prep Kits were used. Before starting the protocol an aliquot of 750 µl of the overnight culture was mixed with 250 µl of sterile glycerol and stored at -80ºC as stock.

2.5.10 Purification on CsCl of plasmid DNA.

Plasmid DNA was resuspended in 1xTE and for each ml of DNA, 1.2 g of CsCl and 100 µl of EtBr (10 mg/ml) were added. The samples were then transferred in Beckman Quick Seal ultracentrifuge tubes and centrifuged in a VTi-65 rotor 16 hours at 55,000 rpm and at 25 °C in a Beckman L8-70M ultracentrifuge.

By this technique, circular plasmid DNA was separated by contaminant bacterial DNA and RNA. The separation occurred in virtue of different density acquired by plasmid DNA in the presence of EtBr compared to chromosomal DNA. Two distinct bands were formed on the gradient, the upper one contained nicked bacterial plasmid and chromosomal DNA, whereas, the lower one corresponded to supercoiled plasmid DNA. The band containing the DNA of interest was collected using a 21-gauge needle.

The EtBr was removed by adding 1 volume of isoamyl alcohol and by centrifuging 10 minutes. The extraction was repeated several times until EtBr was eliminated from the alcoholic phase. Finally, removal of CsCl was obtained by precipitation of plasmid DNA. For precipitation, the sample was incubated on ice for 15 minutes after the addition of 3 volumes of H_2O and 2 volumes of 100% ethanol and then centrifuged at 10,000 rpm 20 minutes at 4 °C. The pellet was washed in 70% ethanol and resuspended in sterile H_2O. The plasmid purified were then used for the transfection in KEK cells.

2.5.10 Preparation of transfections contructs.

I cloned the full length *Ciona* Nuclear Receptor 1 by polymerase chain reaction (PCR), on the plasmid pBS-CiNR1 isolated from the Satoh's cDNA collection, into a pcDNA 3.1(+) vector between the *Bam*HI/*Xho*I sites. The rat TRα, and rat TRβ cloned into pSG5 vector, were a gift from Vincent Laudet. Chimera comprising the GAL4 DNA-binding domain fused with the LBD of amphioxus TR (residues 121 to 431) was a gift from Vincent Laudet.

The LBD of CiNR1 (resdues 177 to 376) was cloned by PCR into the vector pAS2.1 (Invitrogen) in the *Nco*I site. The Chimera Gal4-DBD-CiNR1-LBD was then excised using *Hind*III sites and cloned in pcDNA 3.1(+) in the *Hind*III site.

The reporter plasmid used in the transient Transfection assays are:

- DR4 Luciferase (gift from K.Gauthier)

- UAS Luciferase, pGL 4.31 (gift from G.Benoit).

2.5.10 Cell culture and Transfection.

Human Embryonic Kidney 293 cells (HEK) were cultured in DMEM, containing 10% Fetal Bovine Serum (FBS) and antibiotics. The day before the transfection HEK cells were seeded in 96 wells multiwell (30000 cells/well). HEK cells were transfected with ExGen500 transfection reagent according to manufacturer's recommendations. A total amount of 125ng of DNA was transfected per well. In particular the transfection was done with the following amounts of DNA:

- 12.5 ng/well of CMV-β galactosidase, to normalize the transfection

- 12.5 ng/well of reporter (DR4-Luciferase or UAS-Luciferase)

- 12.5 ng/well of thyroid hormone receptor encoding plasmid

- 75 ng/well of empty pSG5 vector

48 hours post transfection transfected cells were Lysed by adding 50µL of 1X Passive Lysis Buffer (Promega).

The Luciferase activity was tested 48 hrs post transfection on in a Glomax Luminometer, and the standardization was made on 25 µL of the lysates by testing β-galactosidase activity in a spectrophotometer at 420nM.

Results

3.1 Iodine effect on *Ciona intestinalis* metamorphosis.

The effect of thyroid hormones on vertebrate and invertebrate (amphioxus) has been well documented. For tunicates there are few information about the roles that thyroid hormone can play during embryonic and post-embryonic development. The data collected so far indicate that THs, as in other species, are able to influence the metamorphosis. In the tunicates, *Ascidia malaca* and *Ciona intestinalis*, THs are able to anticipate the onset of metamorphosis allowing the animals to reach the young adult stage in a shorter time. Treating hatched larvae with 15 to 50µg/L Thyroxine (T4) cause an acceleration of the metamorphosis leading to tail retraction in ≈ 40% of the larvae treated.

Given these preliminary data my first approach aimed to discover whether the inhibition of TH synthesis could interfere with the advancement of metamorphosis in *Ciona intestinalis*. The THs are characterized by the presence of 4 iodine atoms, in T4 and 3 iodine atoms in T3. I prepared an artificial seawater deprived of Iodine (ASW-I) and an artificial seawater where the iodine content was comparable to natural seawater (ASW+I). The procedure to prepare the artificial sea water, where the iodine content was undetectable, took a long time since iodine is usually present as contaminant in one or more of the 30 salts used to prepared the ASW. I thus checked all the salts for the presence of Iodine, also in traces. To prepare the ASW+I the Iodine content (as Potassium Iodide) was adjusted by checking, drop after drop, the Iodine concentration through the Sandell-Kolthoff reaction (section 2.1.3 chapter Methods).

Ciona intestinalis eggs, taken from three specimens were separated into three Petri dishes each reared in: natural seawater Millipore filtered (MFSW), artificial seawater without

Iodine (ASW-I) and artificial seawater with Iodine (ASW+I). Eggs where then in vitro fertilized and embryos were cultured at 16°C. The day after the fertilization, about 18 hours post fertilization, I started to follow the onset and the advancement of metamorphosis. Animals grown in MFSW were viable and well grown as well as in ASW+I and ASW-I.

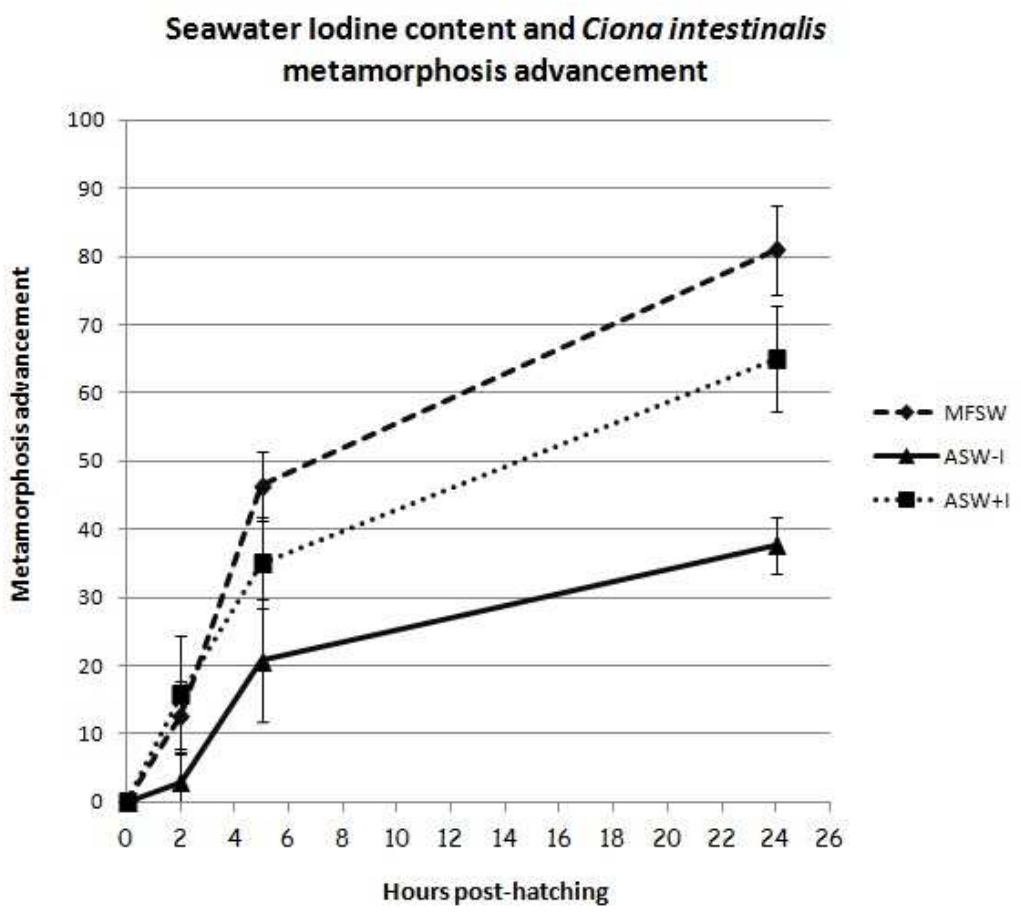


Figure 3.1 *Ciona intestinalis* metamorphosis in different seawater conditions. *Ciona* eggs were fertilized in natural seawater (Dashed line, MFSW, Millipore Filtered Sea Water), in complete artificial seawater (Dotted Line, ASW+I) and in Iodine free artificial seawater (Full line, ASW-I). The advancement of metamorphosis in those different conditions was followed by counting the number of tail retraction (2 and 5 hours) and body axis rotation (24 hours).

It was very striking the effect that the lack of Iodine in the seawater had on the onset of metamorphosis. Larvae grown in ASW-I appeared to be at the same developmental stage compared to the control, since they hatched synchronously; the difference was that, the

onset of metamorphosis was delayed. 2 hours post hatching, when the larvae acquire the competence to sense the endogenous and environmental stimuli to trigger the metamorphosis, the behaviour of larvae in MFSW and ASW+I was almost the same. Indeed 15-20% of larvae started the tail retraction whereas in the ASW-I less than 5% were able to go through metamorphosis. 24 hours post hatching, (about 40 hours post fertilization), more than 80% of larvae, n the MFSW, and 65%, in the ASW+, completed the metamorphosis and were at the late rotation stage, while in ASW-I the larvae that started the metamorphosis were retarded and still at the early rotation stage (Figure 3.3).

3.2 The role of Thyroid Hormones in *Ciona* metamorphosis.

The Iodine is the fundamental component of Thyroid hormones, and for this reason I tried to check the effect of the Thyroid hormone T4 (Thyroxine) on animals grown in Iodine deficiency. I treated larvae grown in MFSW, ASW+I and ASW-I with 25µg/mL T4 and its effect on metamorphosis was checked.

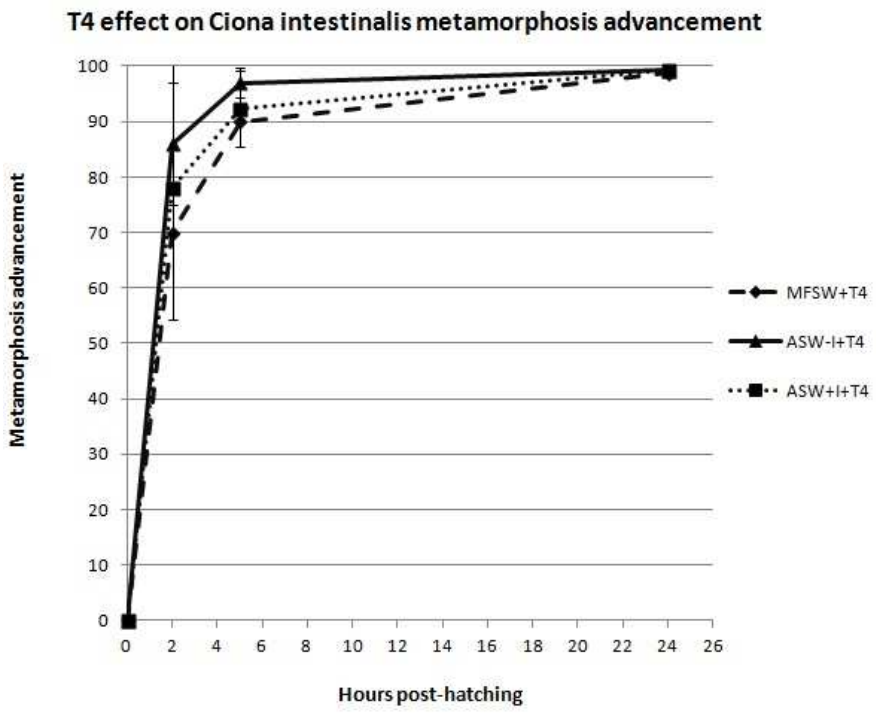


Figure 3.2 *Ciona intestinalis* metamorphosis in different seawater conditions after T4 treatment. *Ciona* eggs were fertilized in natural seawater (Dashed line, MFSW, Millipore Filtered Sea Water), in complete artificial seawater (Dotted Line, ASW+I) and in Iodine free artificial seawater (Full line, ASW-I). At hatching, swimming larvae were treated with 25µg/mL T4. The advancement of metamorphosis in those different conditions was followed by counting the number of tail retraction (2 and 5 hours) and body axis rotation (24 hours).

The effect of T4 in triggering the metamorphosis was striking. 1 hour after the addition of the hormone to the Petri dish, more than 60% of swimming larvae started to retract the tail. This percentage, obtained for animals grown in MFSW and ASW+I, was even higher for the animal grown in ASW-I (see the graph in figure 3.2 and the Table below).

		MEAN % (-)	ST.DEV (-)	MEAN % T4 30µM	ST.DEV T4 30µM
MFSW	1 hph	12,7	4,9	70	13,2
	5 hph	46,3	5,2	90	8,7
	24 hph	81	6,6	98,7	2,3
ASW+I	1 hph	15,7	8,7	78	7,9
	5 hph	35	6,7	92	6,8
	24 hph	65	7,8	99	1,2
ASW-I	1 hph	3	4,3	86	5,5
	5 hph	20	8,9	97	2,6
	24 hph	37	4	100	1,2

Table 3.1 *Ciona intestinalis* metamorphosis in different seawater conditions after T4 treatment. In the table are reported the data used for the constructuion of the graph in figure 3.1 and 3.2.

T4 was not only able to anticipate, in such a striking way, the metamorphosis, but it accelerated the whole developmental program.

Indeed, by comparing the phenotype of animals grown and gone through metamorphosis it is possible to appreciate the effect on the thyroid hormone T4. This effect was more evident on animals grown in the ASW-I. Indeed 24 hours post hatching more than 60% of the animals kept in ASW-I remained as tadpole larvae, whereas the 100% of the animals treated with 25µg/mL T4 were phenotipically comparable to the control. In the

larvae treated with T4, besides the acceleration of the metamorphosis, it was possible to observe that the organogenesis, taking place at metamorphosis, was initiated in the proper way (figure 3.3, left panel).

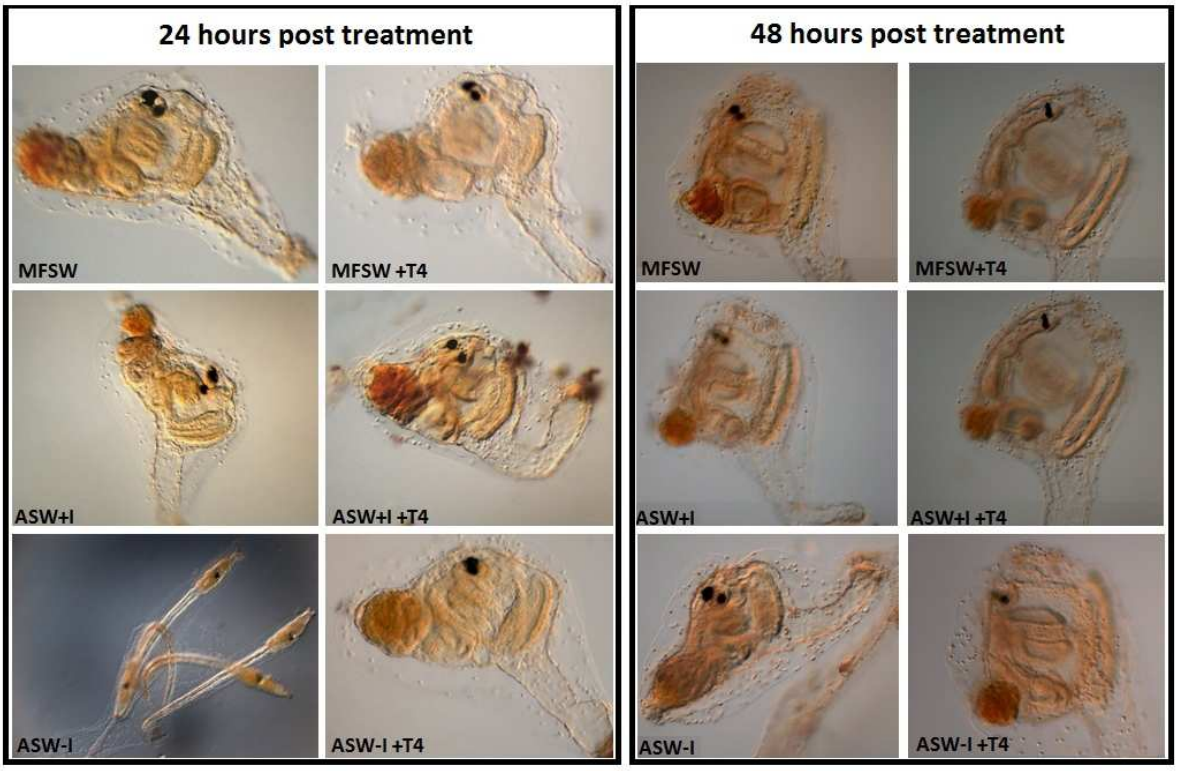


Figure 3.3 Phenotypes of *Ciona* intestinalis grown in different seawater conditions. Animals cultured in natural seawater (MFSW, Millipore Filtered Sea Water), in complete artificial seawater (ASW+I) and in Iodine free artificial seawater (ASW-I). At hatching, swimming larvae were treated with 25µg/mL T4. 24 and 48 hours post hatching (post-treatment) animals, treated and not, were photographed to compare the differences in the phenotype.

Two days after the addition of T4 the differences between the treated and untreated animals become less evident in animals grown in presence of Iodine. The differences were still evident in absence of iodine, where the animals were retarded, at least of 20hrs, compared to the same animals treated with the hormone. If the ASW-I animals seemed to be at a stage comparable to the beginning of a late rotation stage (24-36 hours post fertilization), the addition of T4 caused the development until the end of late rotation

stage (figure 3.3 right panel) (48-60 hours post fertilization)(Chiba, Sasaki, Nakayama, Takamura, & Nori Satoh, 2004).

3.3 Interfering with the thyroid function.

The availability of a large number of drugs known to interfere, at different levels, with the biosynthetic pathway leading to TH synthesis (Goitrogens), allowed me to check in an indirect way the role that THs play in *Ciona intestinalis* metamorphosis.

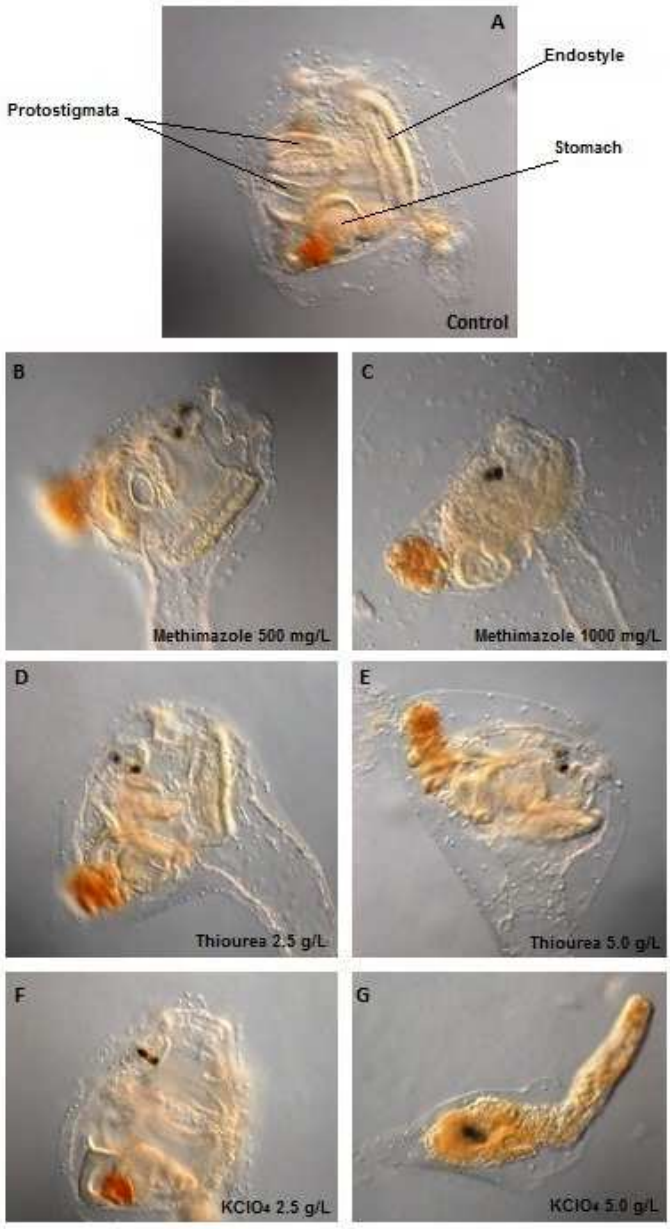


Figure 3.4 Phenotypes of *Ciona intestinalis* grown in seawater and treated with goitrogens. *Ciona* intestinalis embryos were cultured in seawater with the addition of goitrogens. 72 hours after hatching the phenotype of control juveniles (A) was compared to that of methimazole (B and C), Thiourea (D and E) and Potassium Perchlorate (F and G) treated animals. Organs and structures utilized to compare the advancement of the organogenesis are reported in the figure 3.4A.

The goitrogen used in these assays were the methimazole, the thiourea and the potassium perchlorate.

The methimazole and the thiourea are known to be drugs inhibiting the activity of thyroid-peroxidase, the enzyme involved in iodination and coupling of tyrosines in order to form the Thyroid Hormones. In figure 3.4 it is evident that animal treated with these two drugs showed a defect in the development. In particular, besides the effects on the metamorphosis advancement, the animals that, somehow, started the metamorphosis did not develop normally. In comparison with the control, animals treated with methimazole showed a defect in the organogenesis. In particular the whole digestive system seemed to be affected. The stomach, whose localization is indicated in the fig 3.4A, looked normal whereas the endostyle and the gill slits were absent or non differentiated properly. As the concentration of both goitrogens (Methimazole and Thiourea) increased, the phenotypes become more severe and also the rotation of the body axis was impaired.

The potassium Perchlorate ($KClO_4$) is a salt able to block the uptake of iodine. It is a well known antagonist of the ionic channel that allows the iodine to enter the cells. In *Ciona intestinalis* genome it has been found a gene encoding a ionic channel of the family of the Sodium Symporter, even though its role in iodine uptake has not been determined. By using this drug I tried to block the uptake of Iodine to inhibit the synthesis of TH at its first step. In figure 3.4F at the lowest $KClO_4$ concentration tested (2.5 g/L) animals looked normal. The endostyle was formed and also the gill slits were open. The

organization of digestive system seemed to be pretty normal, although the dimension of the stomach was smaller than the control; the siphons were already open. By increasing the concentration (figure 3.4G), a complete block of the metamorphosis was observed.

3.4 T4 rescues the block of metamorphosis induced by $KClO_4$.

$KClO_4$ is also a compound with an intrinsic oxidant power. To understand whether its effect on metamorphosis was due to the hypothetical lack of TH synthesis, derived from the inhibition of iodine uptake, or to a non-specific effect, I tried to rescue the effect of $KClO_4$ by adding exogenous T4.

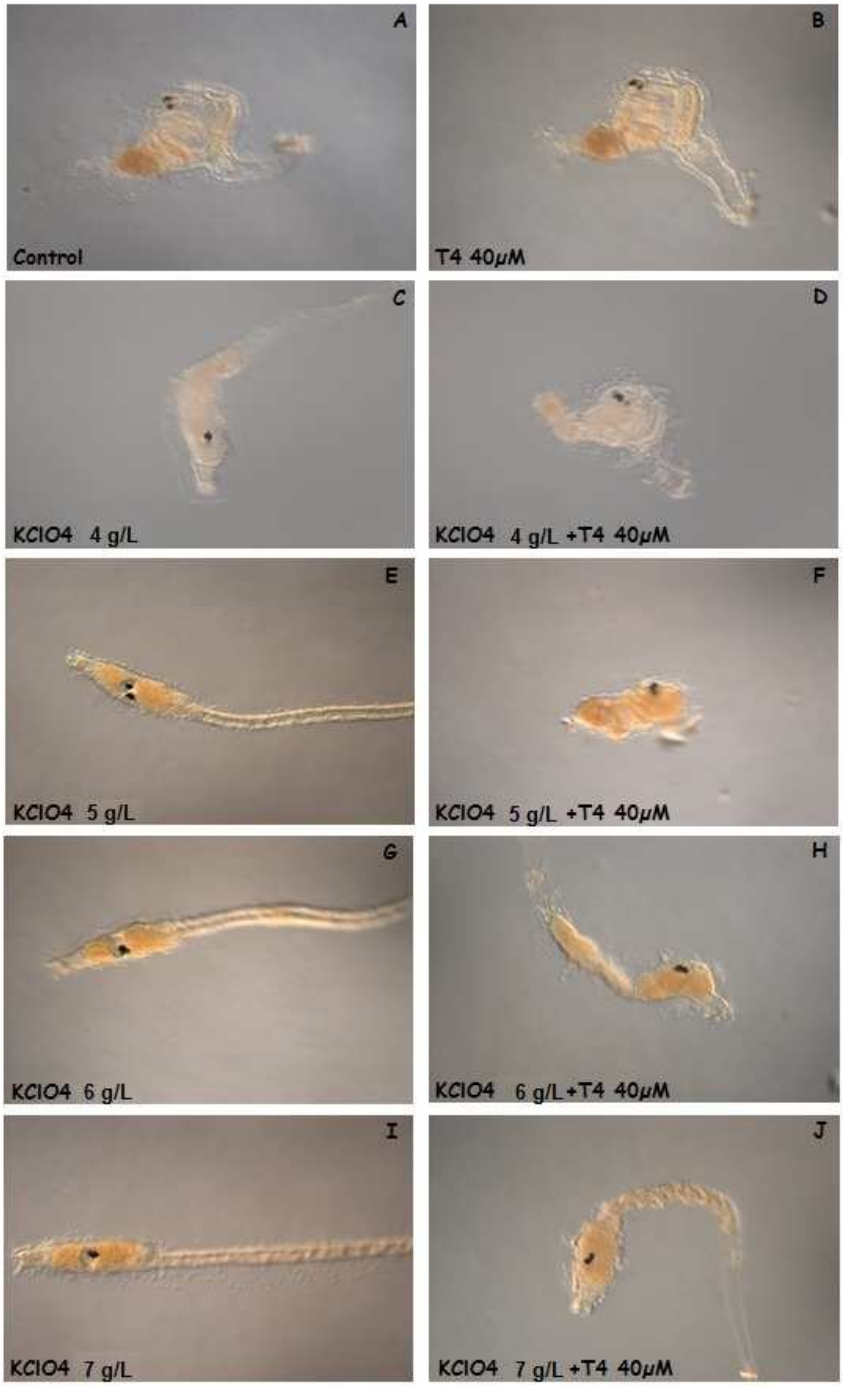


Figure 3.5 T4 rescues the metamorphosis in Potassium Perchlorate treated animals . *Ciona* intestinalis embryos were cultured in seawater with the addition of different concentrations of Potassium Perchlorate ($KClO_4$). At hatching larvae were treated with 40μM T4. 24 hours after hatching the phenotype of KClO4 treated animals (C, E, G, I) was compared to KClO4 + T4 treated animals (D, F, H, J). In fig 3.5A a control juvenile and a T4 treated juvenile is presened in fig.3.5B

The concentration of $KClO_4$ tested were compatible with the life of larvae and were able to induce an effect. In particular *Ciona* embryos were cultured in seawater with $KClO_4$ whose concentration was in the range 4-7 g/L. $KClO_4$ from 6 to 7 g/L completely inhibited the ability of larvae to start metamorphosis (figure 3.6 lanes 7 and 9) , whereas at 4 and 5 g/L $KClO_4$ more than 70% of larvae were blocked although some of them went through metamorphosis at different extent (figure 3.6 lanes 3 and 5). The addition of T4 rescued the capability of the larvae to bypass the block and initiate the metamorphosis, when the concentration of $KClO_4$ were not too high. Indeed only 30% of larvae treated with 4 g/L $KClO_4$ had the ability to go through metamorphosis, while the presence of 40µM T4 rescued the metamorphosis almost in all of them (figure 3.6 lane 4). Almost the same was for larvae treated with 5 g/L $KClO_4$ and 5 g/L $KClO_4$ plus 40µM T4 (figure 3.6 lane 6). Higher concentrations of $KClO_4$ were detrimental since larvae were almost unable to initiate the metamorphosis also in presence of T4 (figure 3.6 lanes 8 and 10).

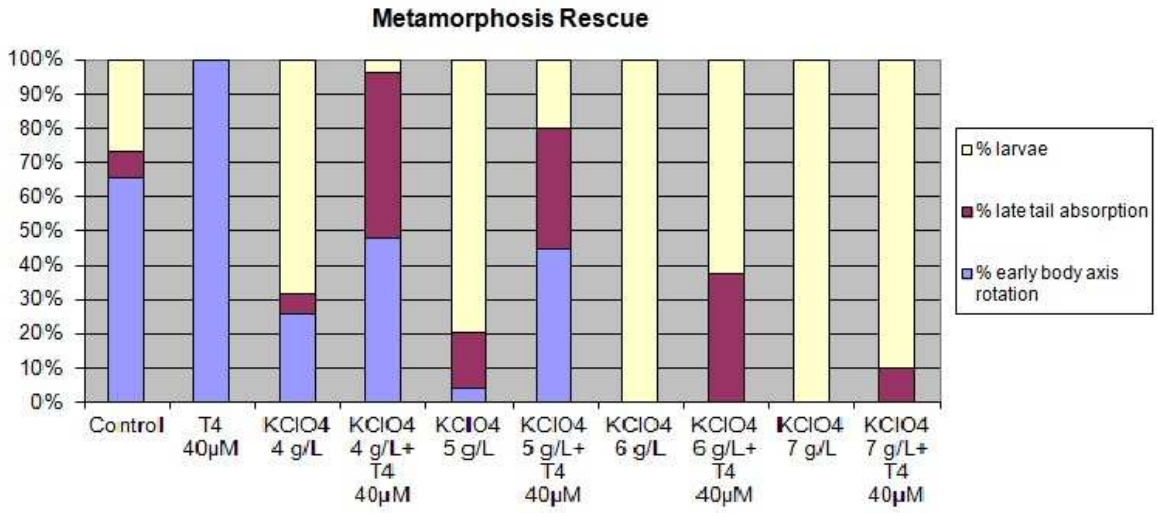


Figure 3.6 Statistic analysis of the T4 rescue of metamorphosis in Potassium Perchlorate treated animals. *Ciona* intestinalis embryos were cultured in seawater with the addition of different concentrations of Potassium Perchlorate ($KClO_4$). At hatching larvae were treated with 40µM T4. 24 hours after hatching the advancement of metamorphosis

was reported as follows: Yellow (swimming larvae, no metamorphosis); Red (late tail resorption, early metamorphosis); Violet (early body axis rotation, late metamorphosis).

3.5 KClO4 effect on endogenous THs synthesis in the endostyle of adult Ciona.

The differentiated endostyles in ascidian, amphioxus and lamprey larvae are, as already noticed, histologically divided in "zones" containing different cell types (Hiruta, Mazet, Yasui, P. Zhang, & Michio Ogasawara, 2005b). In the ascidian *Ciona intestinalis*, these iodination centers are present in zones 7, 8 and 9 at the tip of the endostyle. By probing cross sections of adult *Ciona* endostyles with an anti-T4 antibody, we localized an immunostaining in the region 8 (figure 3.7, Control). To check if the treatment with $KClO_4$ had an effect on endogenous thyroid hormone synthesis, I looked for the presence of THs, by T4 immunostaining, in the endostyles from adults specimen of *Ciona intestinalis* treated with $KClO_4$. Since the $KClO_4$ concentrations I used for larvae treatment (2.5 to 7 g/L) were highly toxic, aready 12 hours post treatment, for adult *Ciona*s, I decided to decrease the concentrations to 500 mg/L and 1 g/L, After 4 days of treatment, the animals were sacrified and the endostyles were fixed, sectioned and probed with anti-Thyroxine antibody in an Immunohistochemistry (IHC) experiment in comparison with controls (figure 3.7).

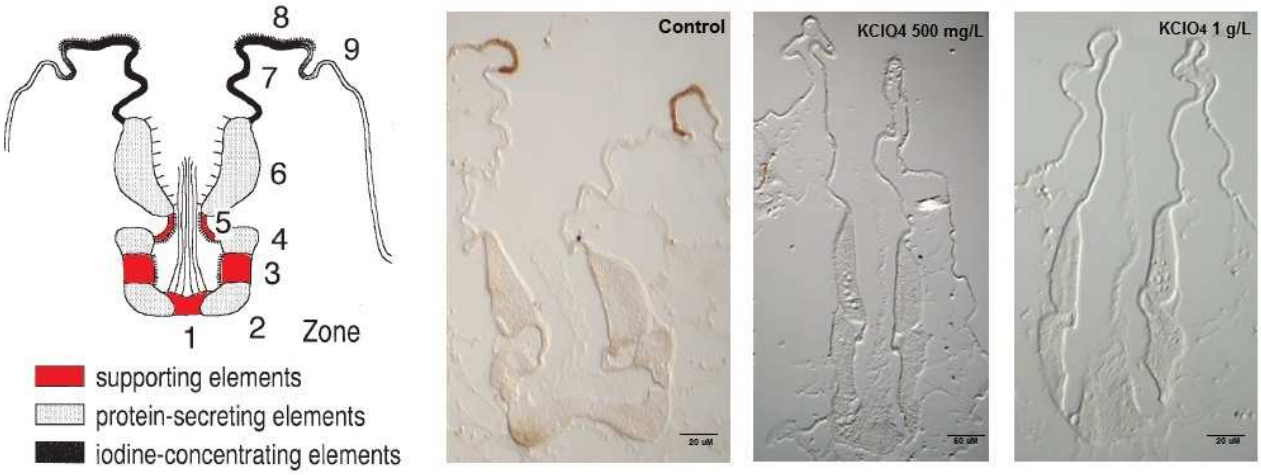


Figure 3.7 $KClO_4$ effect on T4 biosynthesis *in Ciona* endostyle. *Ciona intestinalis* endostyles from animals incubated in natural seawater and in presence of 500 mg/L and 1 g/L $KClO_4$ were dissected and fixed. Immunohistochemistry (IHC) was performed with an anti-T4 antibody on endostyle cross sections and the staining of the endostyles from KClO4 treated animals was compared to the control. It is evident, by comparing the IHC with the scheme, that the anti-T4 antibody stains the region 8 of the endostyle.

In figure 3.7 are reported the results of the IHC. In the control the positive staining was localized in the region 8 of the endostyle, that corresponds to the ciliated region in the scheme. By contrast endostyles from animals kept in seawater with the goitrogen, did not present any immunoreactivity in the presumed region indicating that Potassium perchlorate represents an efficient goitrogen also in the ascidian *Ciona intestinalis*. It is, furthermore, interesting to note that the immunological localization of T4 is in the region 8 of the endostyle. Surprisingly, the enzyme involved in TH synthesis, the Thyroid-Peroxidase (TPO) is expressed in the region 7 (figure 3.7).

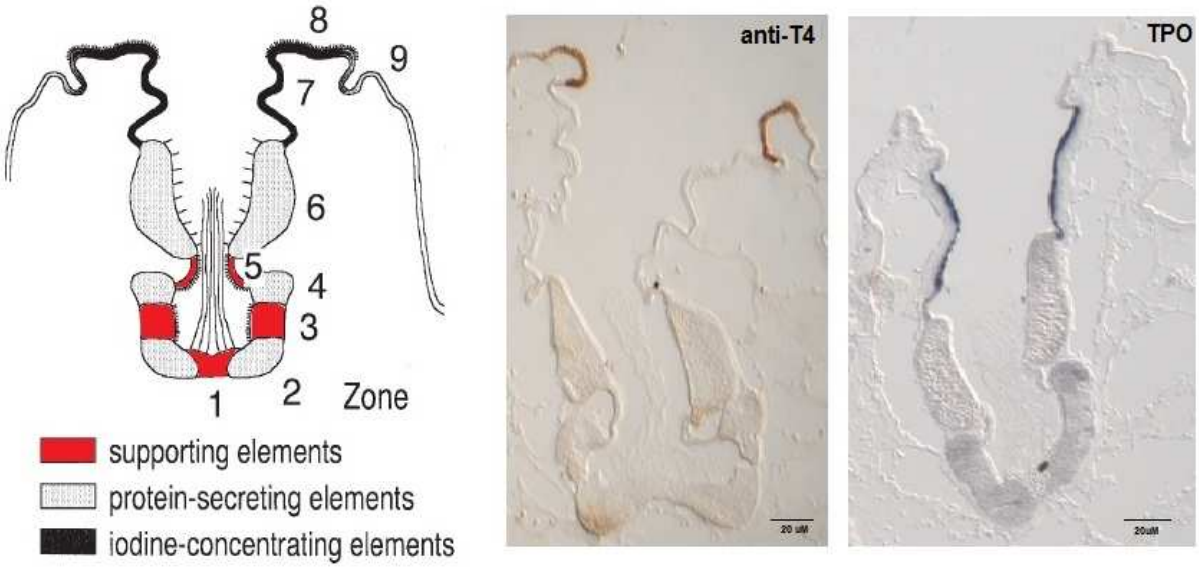


Figure 3.7 T4 and Thyroid-peroxidase do not co-localize in *Ciona* endostyle. *Ciona* intestinalis endostyles from animals incubated in natural seawater were dissected and fixed. Immunohistochemistry (IHC) with the anti-T4 antibody and In situ hybridization with a *Ciona* thyroid-peroxidase (CiTPO) antisense probe were performed on endostyle cross sections. The anti-T4 antibody stains the region 8 of the endostyle whereas it is clear that the CiTPO transcript is localized in the region 7.

3.6 Looking for the precursor of Vertebrate Thyroglobulin.

The protein precursor of thyroid hormones in vertebrates is the thyroglobulin. No orthologs of thyroglobulin have been found so far in the genome of *Ciona intestinalis* as well as amphioxus indicating that these organisms use a different protein as scaffold for thyroid hormone synthesis. I thus devoted my attention to the identification of this protein, using a proteomic approach.

A total protein extract was obtained from endostyles dissected from adult *Ciona*s. The proteins were precipitated and resuspended in a buffer suitable for a bidimensional denaturing polyacrylamide gel electrophoresis (2-DE, section 2.2.2 of the chapter Methods).

Just after the 2-DE, the proteins were transferred by western blot onto a PVDF membrane. The membrane was probed with the anti-T4 antibody used also in the immunohistochemical assay presented in the previous section. Different proteins showed immunoreactivity to this antibody by western blot (figure 3.8).

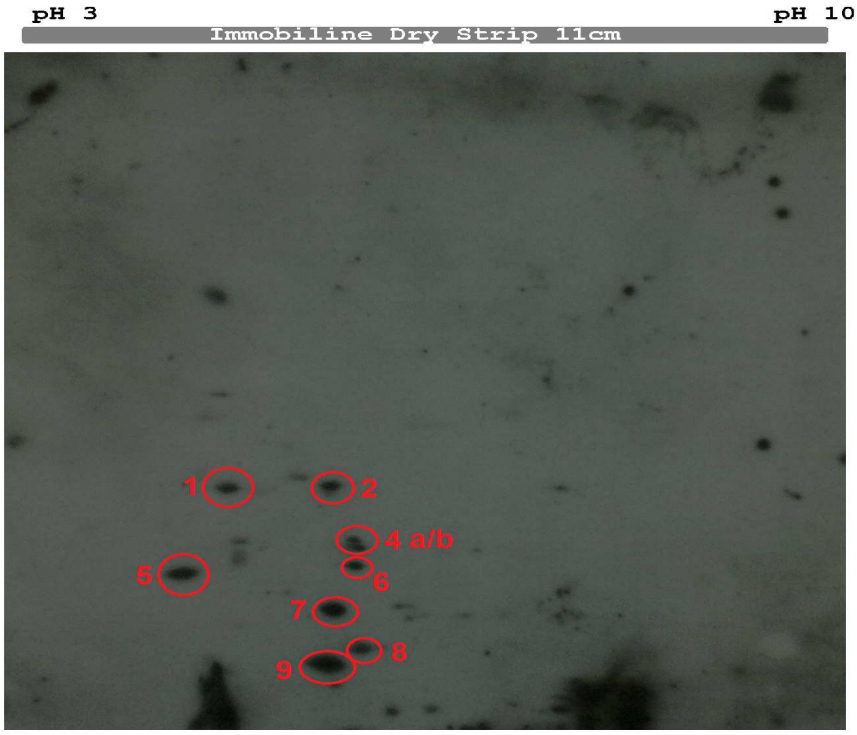


Figure 3.8 Western blot with anti-T4 antibody on *Ciona* endostyle proteome. Protein were extracted from *Ciona* intestinalis endostyles and separated by 2-DE. Then they were transferred onto a PVDF membrane and probed with the anti-T4 antibody. Highlighted in red the most reactive spots to the antibody used.

At least 9 main proteins presented a positive reaction to the anti-T4 antibody, although several others spot seemed to react. The spots highlighted in the figure 3.8 were chosen as the fastest in appearing during the chemiluminescence detection. A correspondence was determined between each spot on the film and the relative on the gel.

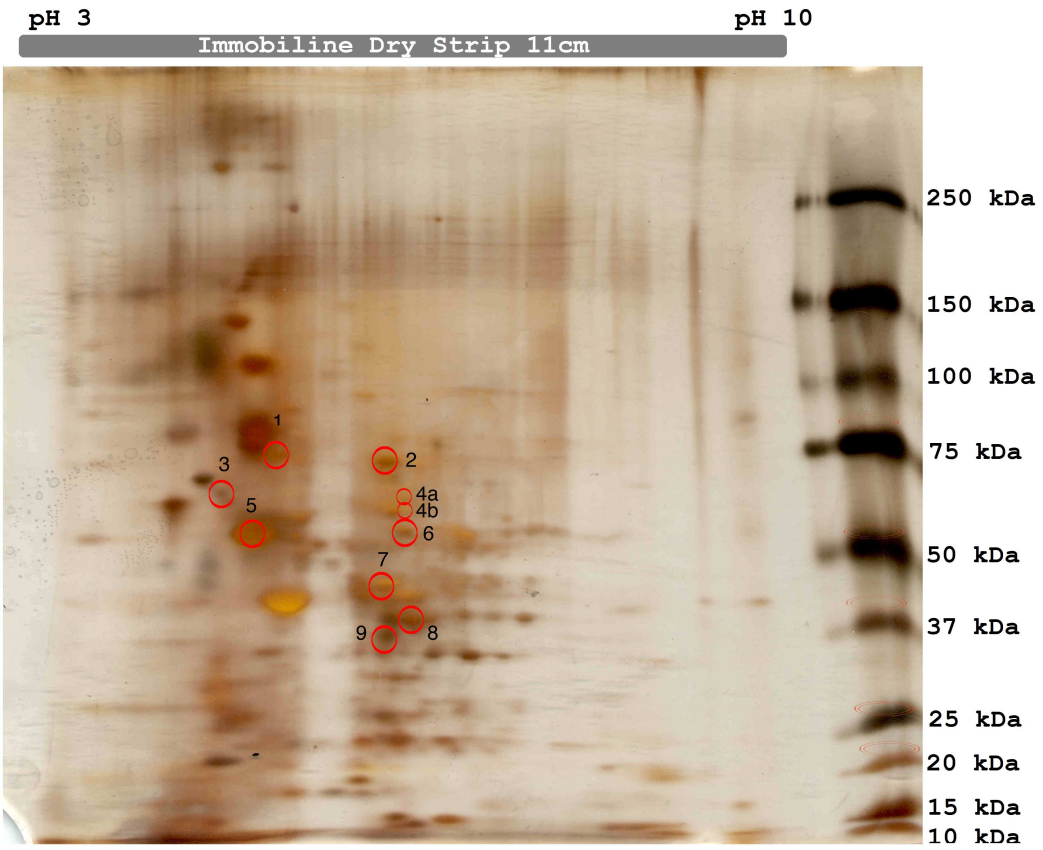


Figure 3.9 *Ciona* intestinalis endostyle proteome. Protein were extracted from *Ciona* intestinalis endostyles and separated by 2-DE in a pH range from 3 to 10. Highlighted in red the most reactive spots to the anti-T4 antibody.

Furthermore another spot, the number 3, was added to the analysis as a non reactive control.

The spots were then taken from the polyacrylamide gel and sent for mass spectrometry analysis by MALDI-TOF.

Through those experiments I obtained the sequences of the proteins that reacted with the anti-T4 antibody.

Spot[a]	General index (NCBI)	Protein name	Score Mascot	Coverage (%)[b]	Number of peptides[c]	E value[d]	Theoretical[e] MW;pI
1	gi|198429034	*similar to* cortical granule lectin-like [Ciona intestinalis]	199	36%	14/19	2.3e-14	45935; 5.56
2	gi|198415631	*similar to* prolyl 4-hydroxylase, beta polypeptide, partial [Ciona intestinalis]	265	39%	19/27	5.7e-21	42750; 7.68
4b	gi|198430555	similar to glutamate dehydrogenase [Ciona intestinalis]	198	37%	17/27	2.9e-14	61167; 6.34
5	gi|198420709	*similar to* LOC495074 protein [Ciona intestinalis]	296	40%	22/28	4.5e-24	50571; 4.63
6	gi|198423786	*similar to* dihydrolipoamide dehydrogenase [Ciona intestinalis]	198	39%	14/24	2.9e-14	54028; 6.65
7	gi|198413081	*similar to* citrate synthase [Ciona intestinalis]	185	26%	13/16	5.7e-13	52570;6.48
8	gi|198420523	*similar to* cystathionine gamma-lyase inhibitor, partial [Ciona intestinalis]	195	48%	11/18	5.7e-14	27483; 6.25
9	gi|198423876	*similar to* transferrin [Ciona intestinalis]	265	39%	18/25	5.7e-21	38064; 5.92

Table I: Identification of proteins by MALDI-Tof MS analysis.
[a] Spots numbers corresponded to gel 2DE;
[b] Percentage of sequence coverage based on the peptides identified;
[c] number of matched peptides (number of unmatched peptides);
[d] E value obtained from MASCOT search results;
[e] Theorical MW(kDa) and pI values obtained from MASCOT search results.

Some of the identified proteins are associated to a function, although it is an *in silico* assignation. The spot 5, by contrast, codes for a protein whose identity has not been determined yet.

To analyze deeper the proteins that reacted with the antibody, I decided to perform a deeper mass spectrometry analysis. In particular the proteins present in the positive spots were completely hydrolysed by using Pronase and Amminopeptidase, in order to release, if present, the thyroid hormones.

Then the hydrolysates were analyzed through LC-MS (Liquid Chromatography-Mass Spectrometry). This technique permits the identification of the single components of the amino acids mixture and eventually of other small molecules contained in it.

The LC-MS analysis conducted so far lead to the discovery that the protein in the spot n.5 actually contained low amount of T4 and its precursor DIT. Spot 5, indeed, appear to be a good candidate for our purpose. By consulting the *Ciona* intestinalis Protein database

(CIPRO) at the URL http://cipro.ibio.jp I discovered that the protein underneath the spot n.5 is a CRELD like protein. CRELDs are Cystein Rich with EGF-Like domain proteins that in vertebrates have been determined to be involved in atrioventricular septal defects (Maslen, 2004).

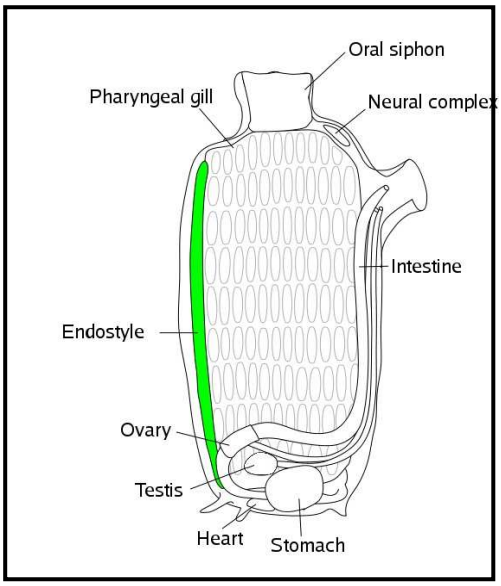


Figure 3.10 CRELD localization. Creld protein is specifically localized in the endostyle (Green). Adapted from **http://cipro.ibio.jp**.

Interestingly CRELD is exclusively expressed in the endostyle, suggesting that it exerts its specific role in that organ.

3.7 *Ciona intestinalis* Nuclear receptor 1 (CiNR1). A phylogenetic analysis.

Ciona intestinalis Nuclear Receptor 1 is a member of the steroid/thyroid hormone receptor family (Carosa et al., 1998). The features of nuclear receptors consist into a conserved zinc finger DNA-binding domain (DBD) and a ligand-binding domain (LBD). The evolutionary combination of these domains led to the generation of diverse families of ligand-activated transcription factors that regulate gene expression in response to ligand binding. Some members of this superfamily have hydrophobic ligands, such as thyroid hormones, Vitamin D or Retinoic acid, while other nuclear receptors are -called "orphan" since the identity of their ligand, if any, is unknown. The phylogenetic study of gene families provides insights in the evolution of genes and contributes to the understanding of genomes evolution. To reconstruct the origin of CiNR1 I thus carried out a phylogenetic analysis using MEGA 5.05 software; as shown in figure 3.11 the Neighbour Joining tree, obtained from the alignment of the DNA Binding Domain and the Ligand Binding Domain of Thyroid hormone receptors from Invertebrate and Vertebrate representatives, located CiNR1 at the base of Vertebrate TR phylogeny. CiNR1 grouped with both Vertebrate TRα and TRβ, suggesting that the events of duplication, leading to the appearance of at least two copies of TRs in vertebrates, was subsequent to their radiation from the Tunicates.

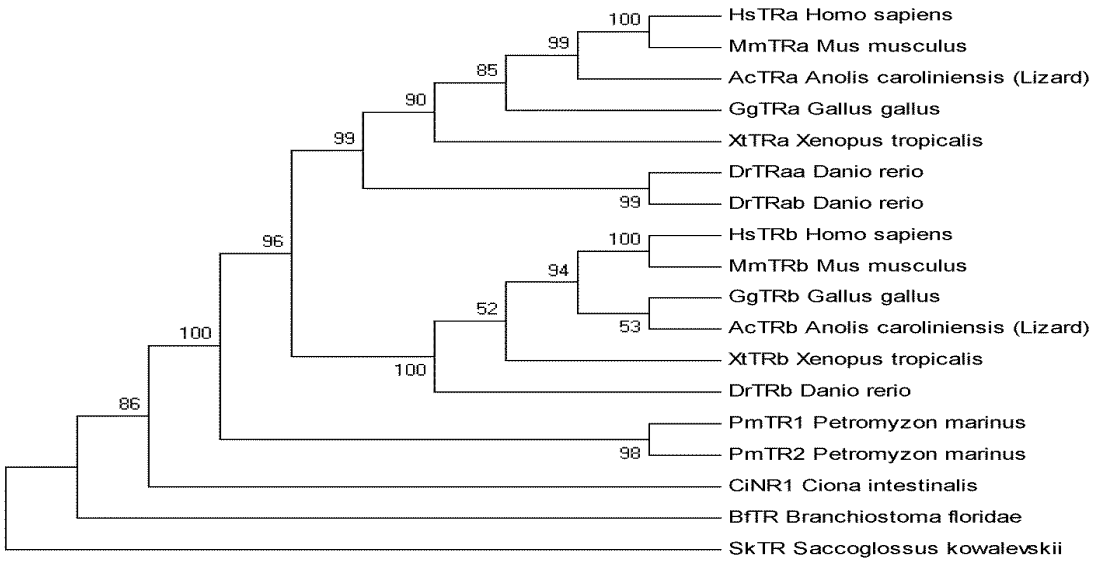


Figure 3.11 Phylogenetic reconstruction of chordata TRs. A neighbor-joining (NJ) was obtained on the basis of the amino acid sequences of the DBD and LBD of several deuterostomes TRs by using the MEGA 5 software. Bootstrap values are obtained after 5000 replicates. The tree was rooted with the hemichordate S.kowalevskii TR.

In the lineage of vertebrates, thus, at least three independent gene duplication events occurred. One at the base of vertebrates, after the radiation of lampreys, giving rise to TRα and TRβ. Furthermore, in lamprey an apparently independent duplication gave rise to two receptors, called TR1 and TR2, grouping with both TRα and TRβ. The presence, in fishes, of supplementary copies of TRs is correlated to the round of whole genome duplication which occurred in teleost lineage, followed, in some cases, by gene loss. Besides Tunicates, one TR has been found also in amphioxus and in the hemichordate *Saccoglossus kowalevskii*. The origin of members of steroid/thyroid hormone receptor family is very ancient since TRs have been found also in flatworms and molluscs (Wu, Niles, & LoVerde, 2007). Interestingly, no TRs have been found in Insects, where so far no Thyroid hormones or a Thyroid related function have been described. More than 270 Nuclear receptors have been found in *C.elegans* but no one of them seems to be related to TR family (Bertrand, F. G. Brunet, Escriva, Parmentier, Laudet, & Marc Robinson-Rechavi, 2004b). The phylogenetic analysis, based on the comparison of deduced protein

sequences, has however to be supported by functional data attesting that they act as a classical thyroid hormone receptor. A lot of information are available for Vertebrate TRs, whereas few studies have been conducted on invertebrate TRs, mostly on amphioxus TR that has been demonstrated to be acting as a classical thyroid hormone receptor, despite its high sequence divergence from vertebrate TRs. Concerning the ascidians, previous studies on CiNR1 failed to demonstrate its TR activity and thus it has been defined as an inactive thyroid hormone receptor (Carosa et al., 1998). The presence, however, of a thyroid like function in Tunicates, the presence in their genomes of almost all the members of THs synthesis pathway and the ability of Thyroid Hormones to influence their metamorphosis, prompted me to reconsider the studies on the functionality of CiNR1 as thyroid hormone receptor. From this perspective CiNR1 should have, at least, two main abilities:

- Specific DNA binding

- Activation of gene transcription, mediated by the binding to a Ligand.

The study of those properties is presented in the following sections.

3.8 CiNR1 spatial expression pattern.

Once isolated the CiNR1 full length cDNA from the Satoh's cDNA collection available in our laboratory (http://ghost.zool.kyoto-u.ac.jp)I started my study by analyzing its spatial expression through whole mount in situ hybridization experiments. As previously reported (Carosa et al., 1998) , CiNR1 is expressed in the endodermal territories starting from the neurula stage.

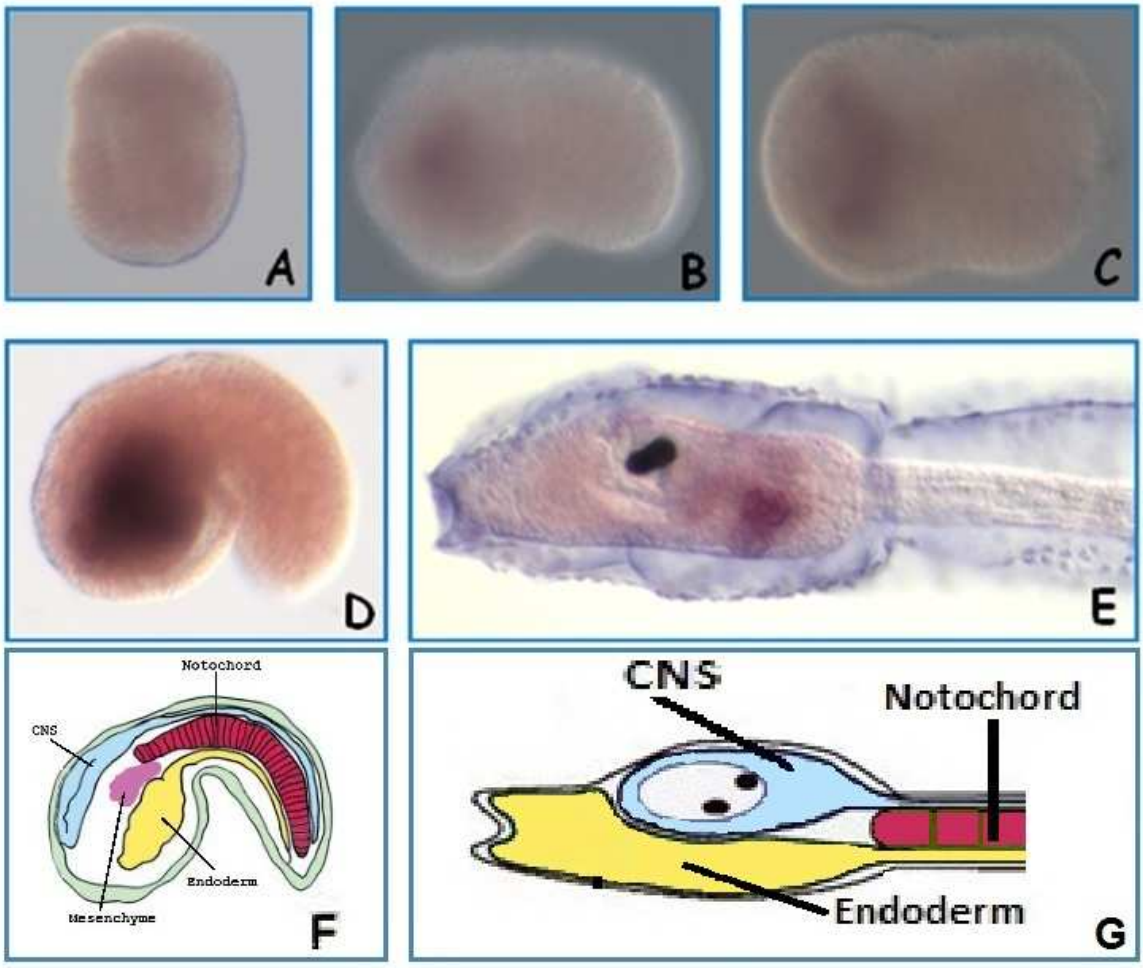


Figure 3.12 Spatial expression of CiNR1. At neurula (A) stage the expression is restricted to the endoderm precursor cells. (B and C) lateral and dorsal view of early tailbud embryos; CiNR1 is expressed in the head endoderm. Also at late tailbud stage (D) the gene is expressed in the whole head endoderm. At larval stage (E) the expression is confined to the posterior part of the trunk endoderm. (F) schematic representation of a *Ciona intestinalis* larva with its tissues: Central Nervous Sysyem (CNS) in light blue, mesenchyme in pink, notochord in red and endoderm in yellow.

At the early (figure 3.12 B-C) and late (figure 3.12 D) tailbud stages the expression of CiNR1 is kept in the endoderm and at the larval stage (figure 3.12 E) CiNR1 becomes restricted to the posterior part of the trunk endoderm.

3.9 CiNR1 DNA binding ability.

As members of the Nuclear receptor superfamily, Thyroid Hormone Receptors are Ligand activated transcription factors. They are, therefore, able to interact with specific sequences of DNA (target site), that, in the case of hormone receptors, are named Hormone Response Elements (HRE). The HRE bound by Thyroid Hormone Receptors is the canonical DR4 sequence, also known as TRE (Thyroid Response Element) where two direct repetition of the sequence AGGTCA are separated by 4 nucleotides (DR4). Furthermore the main way of interaction of TR with its recognition sequence is through the heterodimerization with a RXR (Retinoic X Receptor). I thus tested the ability of CiNR1 to bind the canonical DR4 sequence as homodimer or heterodimer with RXR through an EMSA assay. The data indicated that CiNR1 showed a very weak binding to the canonical DR4 as homodimer, whereas, when incubated in presence of CiRXR, the binding was stronger. The specificity of the binding was assessed by demonstrating that none of the combinations, CiNR1, CiRXR or CiNR1/CiRXR, was able to interact with an unrelated probe (NS).

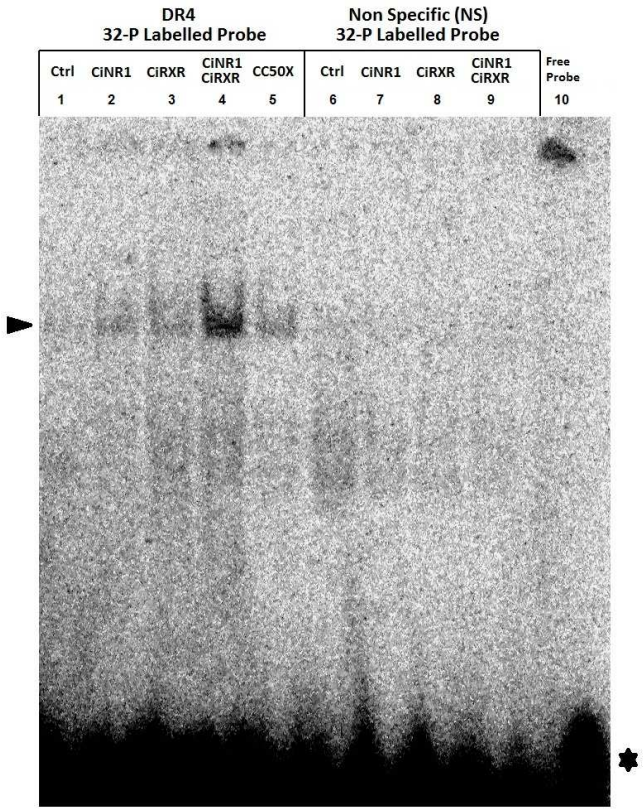


Figure 3.14 EMSA with DR4 and non related probe. CiNR1 DNA binding ability was tested by EMSA on a DR4 (thyroid hormone response element) labeled probe. The homodimer binding of both CiNR1(lane 2) and CiRXR (lane 3) was very weak compared to the heterodimer CiNR1/CiRXR (lane4). The presence of a specific cold competitor (lane 5, CC50X) decrease the efficiency of the band-shift. From lane 6 to lane 9 CiNR1, CiRXR and CiNR1/CiRXR were assayed

for the binding with a non related probe. In lane 10 the free probe is highlighted with an asterisk. The Protein/DNA complex are indicated with an arrowhead.

The heterodimer CiNR1/CiRXR was assayed also for the binding to others canonical DRs, having the nucleotide spacer length in the range of 0 to 5. It is known, indeed, that the length of the spacer is fundamental for binding specificity. of different Nuclear receptors.

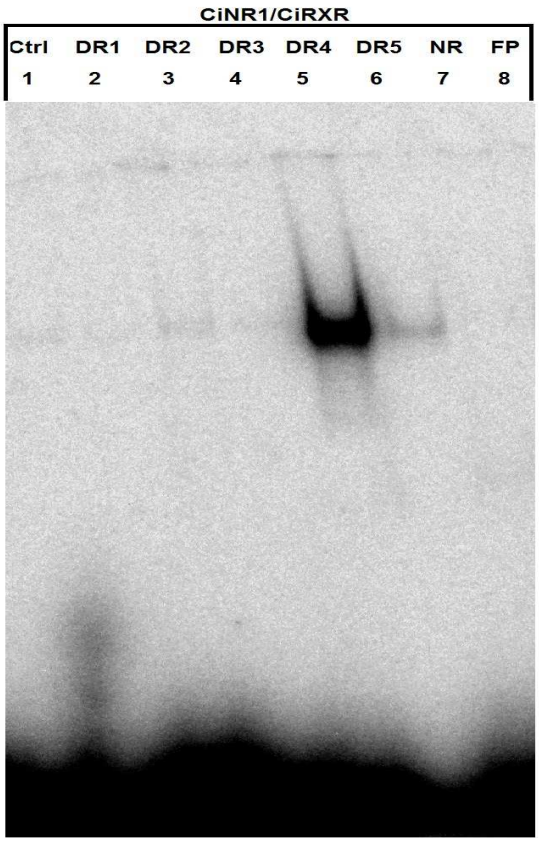


Figure 3.13 EMSA with different DRs and non related probe. CiNR1/CiRXR DNA binding ability was tested by EMSA on a DRs from DR1(lane 2) to DR5(lane 6) labeled probe.

For instance DR3 is bound by VDR (Vitamin-D Receptor) whereas DR5 is bound by RAR (Retinoic Acid Receptor).

In vertebrates and also in the cephalochordate amphioxus (Paris et al., 2008; Tata, 2000), TRs are subjected to transcriptional autoregulation. TRs are able to regulate their own

expression by binding to specific TRE on their promoter. This mechanism is characteristic of the TRs isoforms regulated during development. Vertebrate TRα is expressed during development at a constant level. TRβ, instead, is finely regulated during postembryonic development. In amphibian metamorphosis TRβ shows an expression that has a peak in correspondence of the climax of metamorphosis, thanks to its autoregulation. By using a software, available on line at the URL www.nubiscan.unibas.ch (NUBISCAN), it is possible to identify any putative Nuclear receptor binding sites in a nucleotide sequence. The Nubiscan analysis of a 2500 bp sequence, upstream of CiNR1 transcription start site, revealed the presence of two putative Thyroid Hormone Receptor binding sites (DR4) (resumed in figure 4) using a sequence identity threshold of 65%. The putative DR4 were located at -1050 (CiDR4.2) and -1657 (CiDR4.1) bp from the transcription starting site of CiNR1.

Repeat	Position (strand)	score	z-score	p value	E value	half-site E value	site sequence
DR4	1050(-)	0.637764	n/a	0.248033	n/a	0.356254	TGGTCAtgacAGTTTA
DR4	1657(-)	0.657459	n/a	0.190699	n/a	0.320435	AGTTCCggtcCGGTCA

Figure 3.15 In silico analysis of putative TR binding sites. Nubiscan analysis of CiNR1 promoter reveals the presence of two putative thyroid hormone receptor binding site (DR4).

The two putative DR4, CiDR4.1 [1050 (-)] and CiDR4.2 [1657 (-)], were used as probes in an EMSA assay, for their ability to interact with the heterodimer CiNR1/CiRXR, in comparison with canonical DR4 (see figure 3.16). The results indicated that, while CiDR4.2 did not show any capacity of interaction (data not shown), CiDR4.1 instead appeared to behave as a canonical DR4 Thyroid Response Element, since it was recognized and bound by CiNR1/CiRXR heterodimer (Figure 3.16, lane 10).

CiNR1/CiRXR binding to the consensus DR4 probe (lane 2) seemed to be weaker compared to the binding to the CiDR4.1 probe (lane 10). The competition of CiDR4.1 over the binding of CiNR1/CiRXR to the DR4 probe is very low (lanes 5 and 6). By contrast when CiNR1/CiRXR is bound to CiDR4.1 the competition with a cold 10 and 100X molar excess of DR4 (lanes 11 and 12) is very effective. It is however intriguing that also for CiNR1 a possible auto regulative mechanism is presumable, although it is necessary to support the in vitro data with some in vivo evidences.

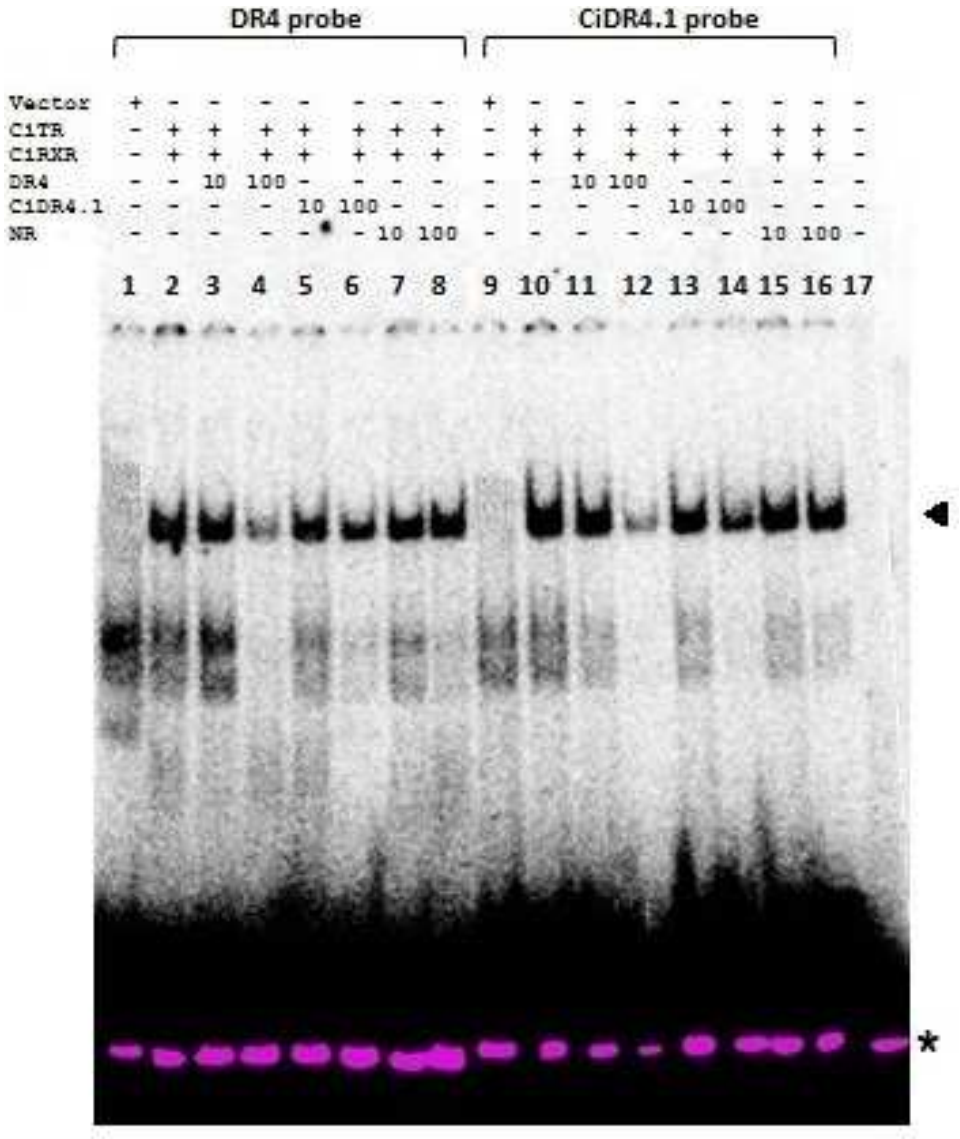


Figure 3.16 EMSA with DR4 and CiDR4.1 probe. CiNR1/CiRXR DNA binding ability was tested by EMSA on a DR4 (thyroid hormone response element) labeled probe and on the CiDR4.1 probe. From lane 1 to 8 the binding of the heterodimer CiNR1/CiRXR was tested for the binding with the canonical DR4 probe. DR4 10X and 100X cold competition was assayed in lane 3 and 4. CiDR4.1 competition over CiNR1/CiRXR binding to DR4 was assayed in lane 5 and 6. In lane 8 and 9 non-specific cold competition. From lane 9 to 16 the heterodimer CiNR1/CiRXR was tested for the binding with the putative DR4 site found in CiNR1 promoter (CiDR4.1). In lanes 11and 12 is presented a 10X and 100X cold competition of DR4 over the binding of CiNR1/CiRXR to the CiDR4.1 probe. CiDR4.1 10X and 100X cold competitions were assayed in lanes 13 and 14. Non-specific cold competition in lanes 15 and 16. Lane 17, free probe.

3.10 Ligand dependent transcriptional activity.

Thyroid Hormone receptors, as already noticed, are members of the Nuclear receptor superfamily. They are, thus, ligand activated transcription factors. They have the ability to stimulate the transcription of regulated genes when bound to their relative ligand. To test whether CiNR1 is an active TR, I assayed its transactivation capability in a transient transfection experiment.

To this end, I cloned the full length cDNA of CiNR1 in a plasmid suitable for in vivo eukaryotic expression, under the control of CMV (Cytomegalovirus) promoter to permit the transcription of an high yield of the CiNR1 mRNA. This plasmid was transfected in Human Embryonic Kidney (HEK) cells, along with the reporter plasmid (DR4Luc), in which the reporter firefly luciferase cDNA is under the control of three repetition of the Thyroid hormone receptor specific binding site (3X-DR4) linked to a Tk minimal promoter. A scheme of the transfection experiments is shown in figure 3.17.

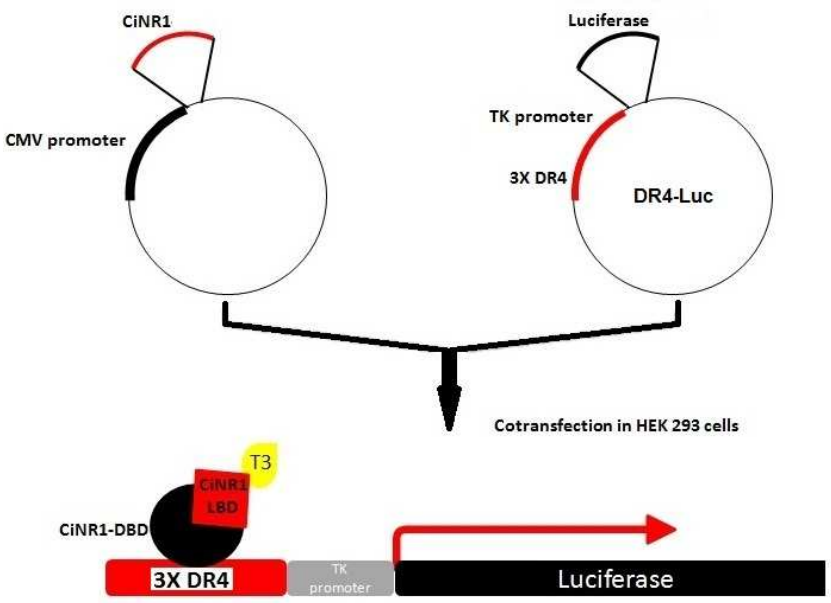


Figure 3.17 Schematic representation of the transient transfection assay. HEK 293 cell are transfected with a plasmid encoding TRs under the control of CMV promoter along with a reporter encoding Luciferase under the control of 3

repetition of the DR4 consensus sequence. In presence of T3 (triiodothyronine) the receptor is supposed to activate the transcription of the reporter gene under the control of the DR4 element.

As control, I compared the capacity of CiNR1, to transactivate Luciferase expression, with that of *Rattus norvegicus* TRβ (rTRβ). These constructs were used in a series of experiments aimed at the demonstration of:

1) the ability of CiNR1 to interact with 3X-DR4 and activate the expression of Luciferase, upon Thyroid Hormone stimulation.

As shown in figure 3.18, in presence of 10nM T3 (Triiodothyronine, the most active among THs in Vertebrates) CiNR1 was able to induce Luciferase expression (lane 2), although at less extent than ratTRβ (lane 1). As further control I transfected HEK cells with the sole DR4Luc plasmid, revealing that almost 30% of the total activity depended on endogenous TRs (lane 3)

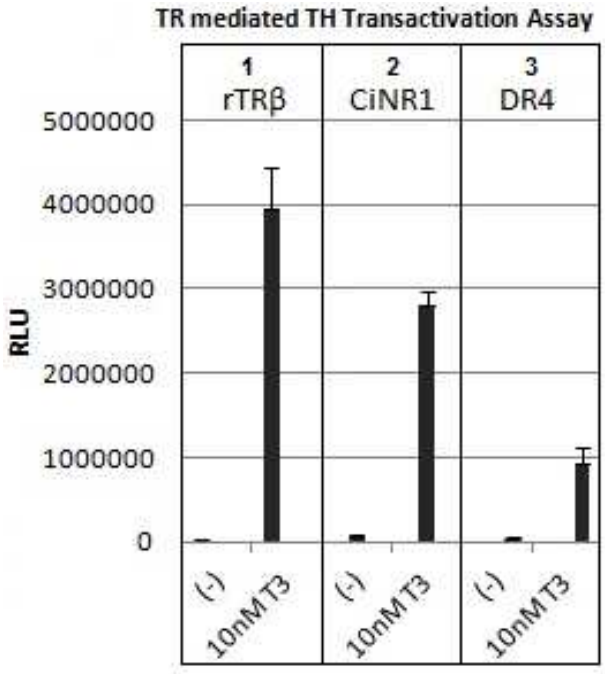


Figure 3.18 CiNR1 is a T3 dependent activator of transcription. Full length construct of rat TRβ and *Ciona* NR1 were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of 10nM T3. In the column DR4 only

the reporter plasmid was transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

2) The activity of CiNR1 in presence of increasing amount of T3, since the Ligand dose-dependence is a common feature of nuclear receptors.

Figure 3.19 shows the result of the dose-response assay. Taking into account the contribution of HEK endogenous TRs (lane 3), the data indicated .that the increase of T3 concentration, in a range from 0.1nM to 100nM, caused an increase in CiNR1 activity (lane 2), although at less extent compared with ratTRβ (lane 1).

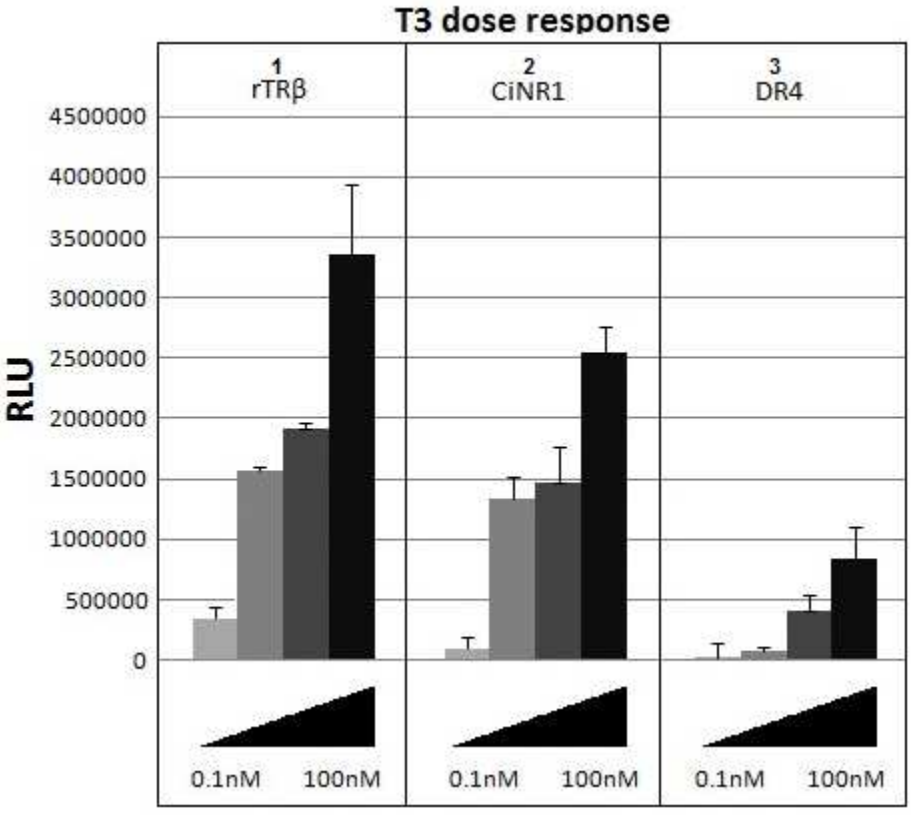


Figure 3.19 T3 dose-dependent transcriptional activation. Full length construct of rat TRβ and *Ciona* NR1 were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of increasing concentration of T3. The range was from 0.1nM to 100nM T3. In the lane 3 only the reporter plasmid was transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

3) The activity of increasing amount of CiNR1 in presence of a constant concentration of T3.

As shown in figure 3.20, the increase in the concentration of CiNR1 corresponded to an increase in luciferase activity (lane 2), although at less extent compared with ratTRβ (lane 1). This data thus represent a robust demonstration that the increased luciferase expression was dependent on the presence of more CiNR1, able to transactivate DR4Luc, and was not related to the activity of endogenous TRs, present in HEK cells, since their concentration were constant (lane3).

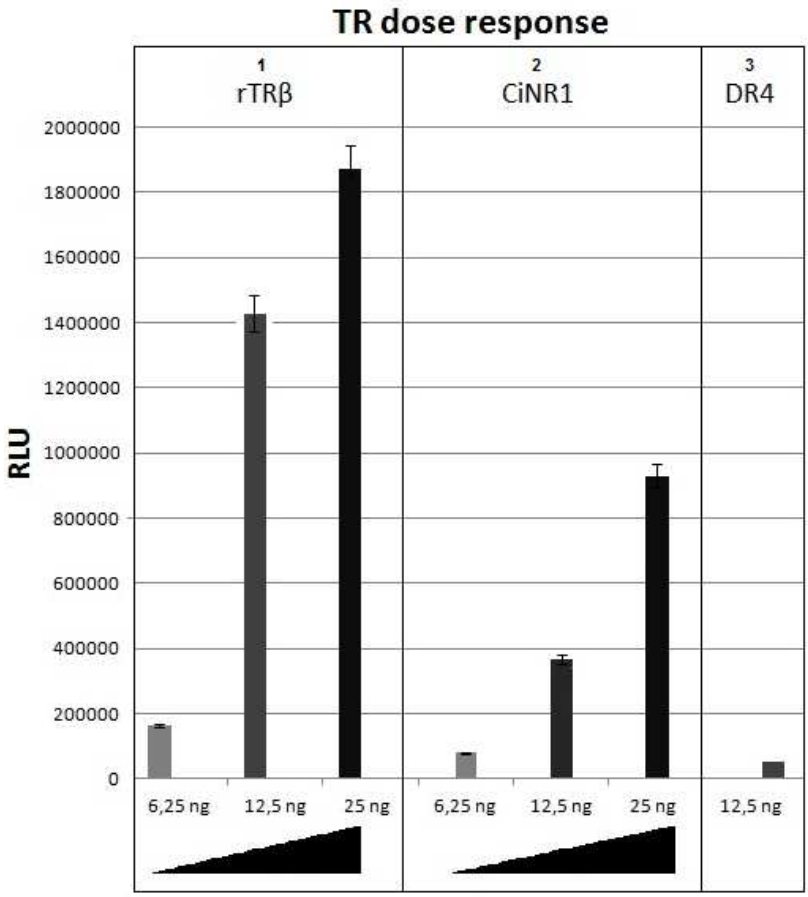


Figure 3.20 TR dose-dependent transcriptional activation. Increasing concentration of the full lengh construct of rat TRβ and *Ciona* NR1 were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of 10nM T3. HEK 293 cells were seeded in 96-wells multiwell and the amount of receptor transfected was 6.25 ng, 12.5 ng and 25 ng per well. In the lane 3 only the reporter plasmid was

transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

3.11 Thyroid Hormone antagonist (NH3) effects on CiNR1.

In the last decade many efforts have been done to design and synthesize antagonists and agonists of thyroid hormone receptors to better define the TH/TR signalling pathway.

In 2002 a new molecule, named NH3, was synthesized by Scanlan's group. NH3 is a TRβ selective antagonist that competes with T3 for the ligand binding pocket (figure 3.21).

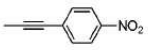
	5'-substitution	$K_D \pm SE$ (nM)[a]		% $TR\beta_1$ activation[b]	% $TR\alpha_1$ activation[b]
		$hTR\beta_1$	$hTR\alpha_1$		
T3		0.10 ± 0.03	0.10 ± 0.03	100	100
GC-1[d]	**H**	0.10 ± 0.02	1.8 ± 0.2	100	100
NH-3		20 ± 7	93 ± 29	3	10

Figure 3.21 Thyroid Hormone Receptor agonists and antagonists. In the table are presented the natural TR agonist (T3) and two synthetic compounds:GC1 and NH3. GC1 is a β-selective agonist whereas NH3 is a β-selective antagonist.

To check if the functional features of CiNR1 were comparable to that of ratTRβ, I tested the effect of the antagonist NH3 on CiNR1 activity, in comparison with ratTRβ.

In a first series of experiments, HEK cells transfected with full length CiNR1 or ratTRβ were treated with NH3 and its effect was evaluated in a competition assay with T3.

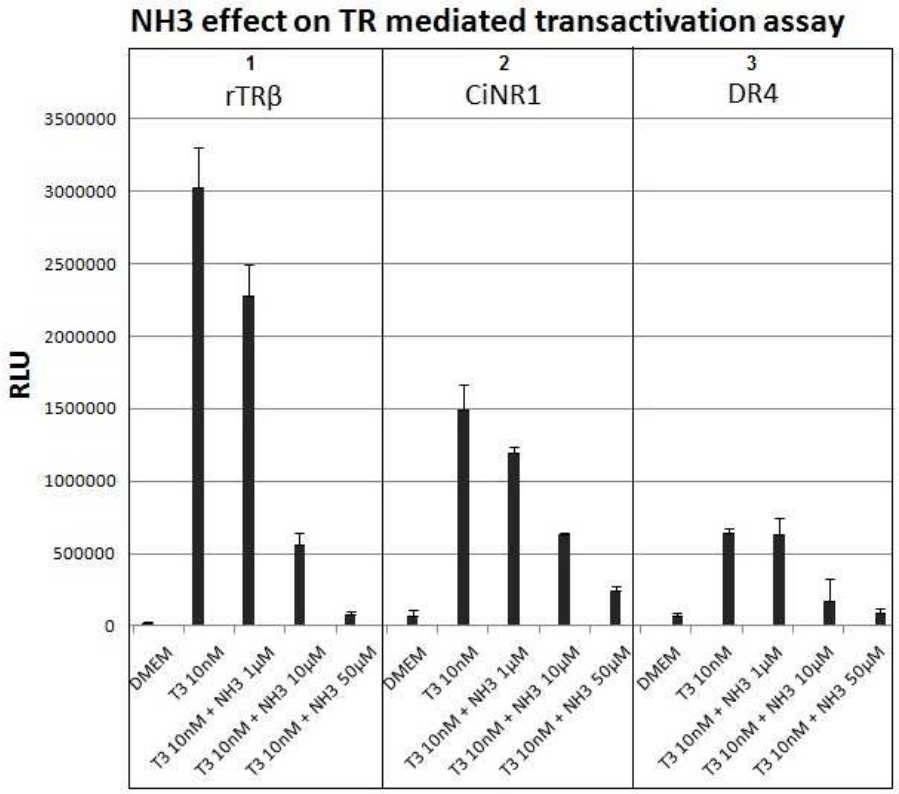


Figure 3.22 NH3 acts as a CiNR1 antagonist. Full length construct of rat TRβ and *Ciona* NR1 were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of 10nM T3 and increasing doses of NH3 (1µM to 50µM). In the lane 3 only the reporter plasmid, DR4-Luc, was transfected to test the effect of NH3 on HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

As shown in figure 3.22, CiNR1 was sensitive to the antagonistic effect of NH3 (lane2), in a dose dependent manner. Indeed, the increase in the concentration of NH3, while keeping constant the amount of T3, corresponded to a decrease in Luciferase activity .

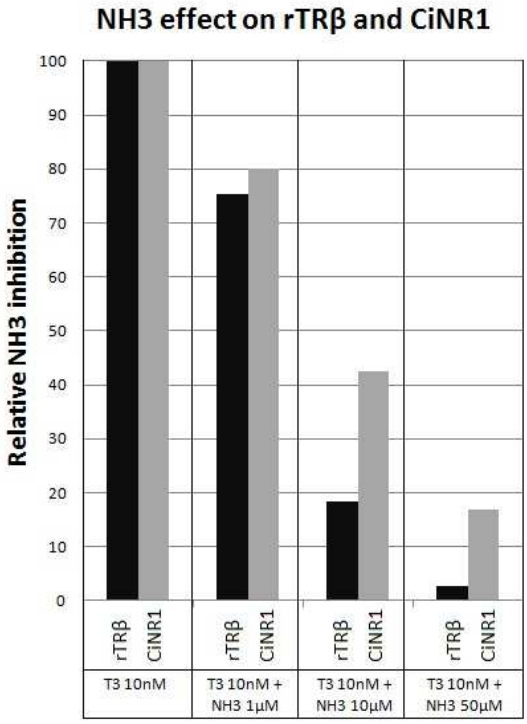


Figure 3.23 NH3 is a better ratTRβ antagonist. Relative inhibitory effect of increasing doses of NH3 (1µM to 50µM). The inhibitory effect of NH3 is reported as percentage decrease, considering the T3 activation as 100%.

As expected, the efficiency of NH3, to inhibit T3 activation, was higher towards rat TRβ (lane1 figure 3.22 and figure 3.23), and this does not represent a novelty, since NH3 has been designed specifically for Vertebrate TRs.

In a second series of experiments I evaluated the effect of NH3 on increasing concentration of CiNR1. The rationale behind this experiments consisted in the fact that in HEK cells are present endogenous TRα and TRβ receptors. While endogenous TRβ is inhibited by NH3, the TRα is not affected. Thus, the treatment with increasing amount of CiNR1 could permit to find a saturation level above which the residual activity, not affected anymore by NH3, was due to CiNR1 and not to the endogenous TRα.

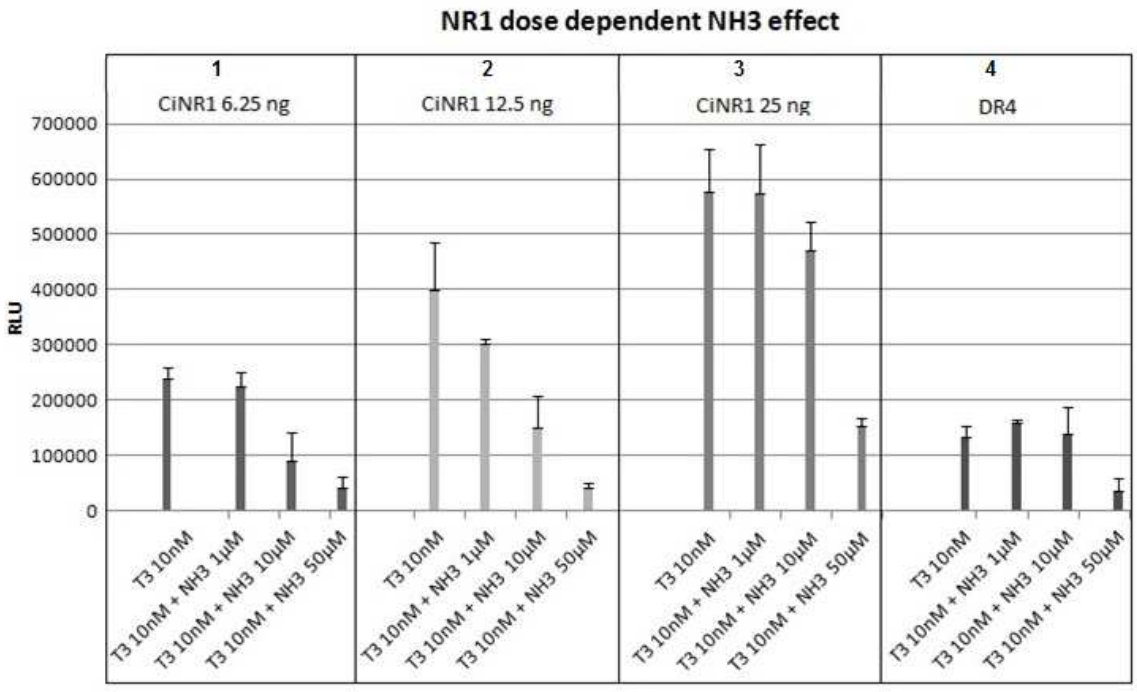


Figure 3.24 NH3 antagonist activity is dependent on CiNR1 concentration. Increasing doses of the full length construct coding for *Ciona* NR1 was tested for its ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of 10nM T3 and increasing doses of NH3 (1µM to 50µM). HEK 293 cells were seeded in 96-wells multiwell and the amount of receptor transfected was 6.25 ng, 12.5 ng and 25 ng per well. In the lane 3 only the reporter plasmid, DR4-Luc, was transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

Even though the effect of NH3, at the highest concentration tested (50µM), was still appreciable, it was evident that when cells were transfected with a 4X amount of CiNR1 (25ng/well in a 96-wells multiwall), the effects of 1 and 10 µM NH3 was almost absent. This means that when the amount of available receptor was increased, more free (NH3 unbound) receptor was available for the binding with T3, giving rise to the high luciferase activity we observed. Since the endogenous TRα, present in HEK cells, was at a constant concentration, the increased luciferase expression could be assigned to more CiNR1 activity. When different concentration of ratTRβ were used to test the effect of NH3 (data

not shown), the luciferase activity remained low, due to the high specificity of NH3 for ratTRβ.

3.12 THs and TH derivatives effects on the transcriptional activity of CiNR1.

The *Ciona intestinalis* Nuclear Receptor 1 is able to bind to T3 and activate the expression of target genes in a transient transfection assay. Since the luciferase activity is evaluated 48 hours after the transfection, one can suppose that, during this time, T3 could be metabolized and that this derived form of TH, and not just T3, is the right ligand for CiNR1. To better delineate its Ligand binding properties I tested the full-length CiNR1 in transient transfection assays treating the transfected cells with different THs and their derivatives as follows,.

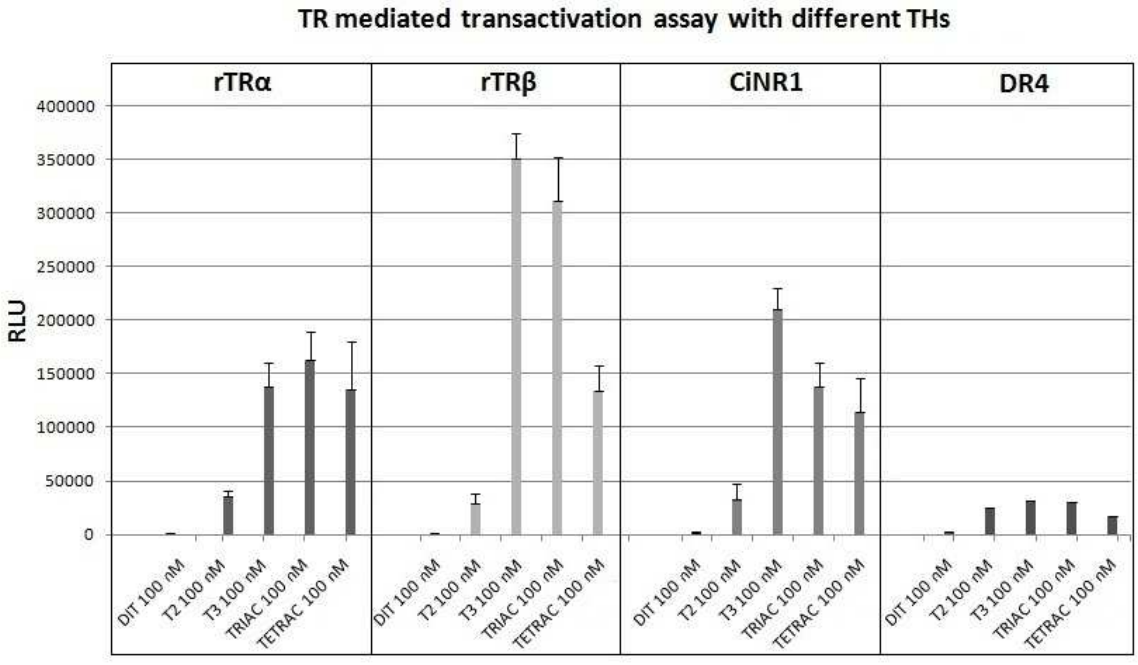


Figure 3.25 THs, their metabolites and synthetic compounds were tested as CiNR1 ligands. Full length construct of rat TRα, rat TRβ and *Ciona* NR1 were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of

DIT, T2, T3, TRIAC and TETRAC (100nM) and GC1 10nM. In the column DR4 only the reporter plasmid was transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

The results shown in figure 3.25 indicated that the TH precursor DIT (Diiodotyrosine) failed to activate ratTRα, ratTRβ and CiNR1. T3 and rT3 (reverse T3, an inactive T4 metabolite) undergo further deiodination, predominantly to the common metabolite 3,3'-diiodothyronine (3,3'T2), which is generated by Inner Ring Deiodination (IRD) of T3 and by Outer Ring Deiodination (ORD) of rT3 (1-3). Thus, ORD is an activating pathway by which the pro-hormone T4 is converted to active T3, whereas IRD is an inactivating pathway by which T4 and T3 are converted to the metabolites rT3 and 3,3'T2 (3,3'-diiodothyronine), respectively. The metabolite 3,3'T2, reported in the figure 3.25 as T2, is mostly inactive on TRs. Further metabolites are generated by side-chain metabolism of iodothyronines,presumably, by action of aromatic L-amino acid decarboxylase (AADC). In this pathway T3 and T4 are converted into iodothyronamines which in turn generate the iodothyroacetic acid metabolites Tetrac (from T4) and Triac (from T3 and T4), through the action of monoamine oxidases, MAO-A or MAO-B. Triac has significant thyromimetic activity and its affinity for ratTRα is equal to that of T3 while for ratTRβ is even higher than that of T3. The affinity and the effects of Tetrac on vertebrate TRs is still unknown but some data indicate that Tetrac seems to be not really active both on vertebrate TRα and TRβ. The activity of Triac and Tetrac on CiNR1 is almost comparable. The effect of the agonist Tetrac was further analyzed testing it in a dose-response experiment.

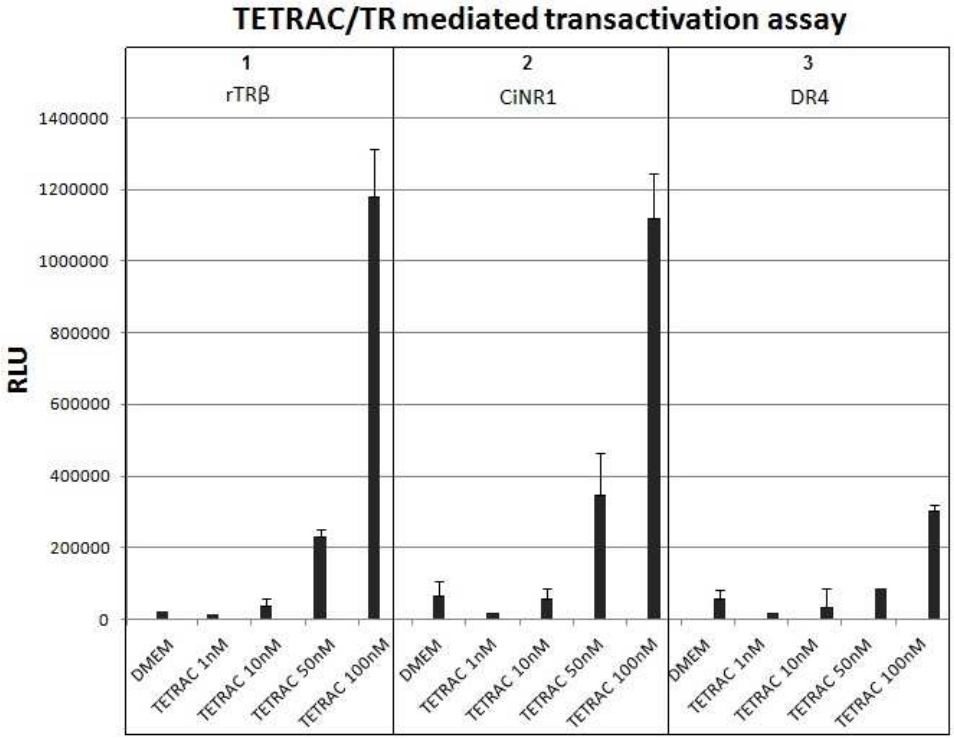


Figure 3.26 TETRAC dose-dependent transcriptional activation. Full length construct of rat TRβ (lane 1) and CiNR1 (lane 2) were tested for their ability to activate a 3xDR4-Tk-Luc reporter plasmid in presence of increasing concentration of TETRAC. The range was from 1nM to 100nM T3. In the lane 3 only the reporter plasmid was transfected to test the effect of HEK 293 endogenous TRs. Error bars represent the standard error of the mean (SEM).

It is worth to note that the response of both ratTRβ (lane 1) and CiNR1 (lane 2) to Tetrac was dose-dependent. It is also interesting that in this assay, the activation of CiNR1 was clearly higher than the control (lane 3), indicating a very low contribution to the total activity of ratTRβ or CiNR1 by the endogenous TRs of HEK cells.

3.13 Mammalian two-hybrid assay. Gal4-LBD constructs to test the transactivation activity of Nuclear receptors.

To avoid definitively the possible interference of HEK endogenous TRs, that could lead to a wrong evaluation of CiNR1 activity, I performed a mammalian two-hybrid assay. In particular I prepared a construct in which I fused the Ligand Binding Domain (LBD) of

CiNR1 with the DNA Binding Domain of Gal4, a yeast activator of the transcription. The chimera so formed is thus able to bind to the Gal4 recognition sequence, the Upstream Activation Sequence (UAS), but the activation has to be mediated through the CiNR1 Ligand Binding Domain. The plasmid coding for the chimera Gal4-DBD/CiNR1-LBD was transfected in HEK 293 cells along with a reporter plasmid in which the luciferase coding gene was under the control of a UAS sequence (figure 3.27).

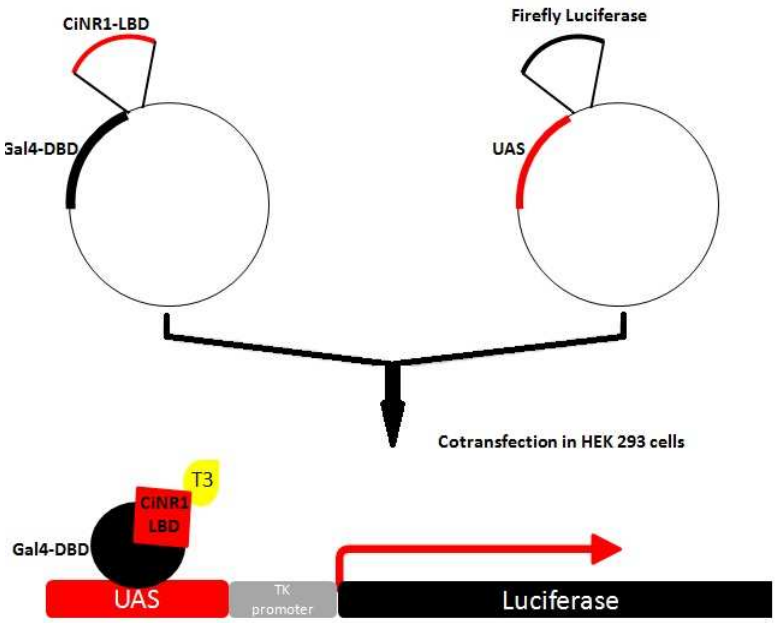


Figure 3.27 Schematic representation of the two-hybrid transient transfection assay. HEK 293 cell are transfected with a plasmid encoding a chimeric gene composed by the Gal4 DNA Binding Domain (Gal4-DBD) and the TR Ligand binding domain (amphiTR-LBD and CiNR1-LBD) along with a reporter encoding Luciferase under the control of Upstream Activation Sequence (UAS) recognized by the Gal4-DBD. In presence of T3 (triiodothyronine) the receptor is supposed to activate the transcription of the reporter gene under the control of the UAS element.

Since the luciferase gene is under the control of the UAS promoter, only the chimeric protein Gal4-DBD/CiNR1-LBD could influence the expression of this gene. HEK

endogenous TRs do not have the ability to bind to the UAS sequence so they can not interfere with the assay.

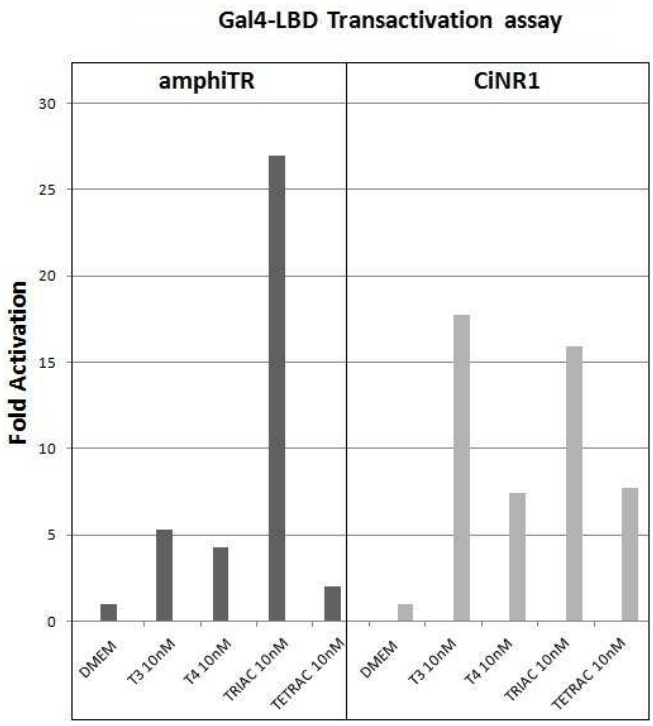


Figure 3.28 THs and their metabolites were tested as amphiTR and CiNR1 ligands. Gal4-LBD constructs for amphioxus TR and *Ciona* NR1 were tested for their ability to activate a UAS-Luciferase reporter plasmid in presence of T3, T4, TRIAC and TETRAC (10nM). Values are presented as fold activation referred to the basal luciferase activation observed in non treated cells.

In these experiment I compared the activity of CiNR1 with amphioxus AmphiTR (*Branchiostoma floridae* Thyroid Hormone Receptor), This experiment confirmed that AmphiTR is activated by Triac (Paris et al., 2008) and not by T3, whereas CiNR1 was activated by T3 as well as by Triac Tetrac.

CiNR1 is, in conclusion, able to bind to specific DNA sequences (DR4, Direct Repeats with a 4 nucleotides spacer) and it is also able to activate gene expression when it is bound to T3 or Triac. Furthermore CiNR1 is subjected, as vertebrate TRs, to the

antagonist activity of the synthetic compound NH3. The effect exerted by TRβ selective antagonist NH3, together with the ability to bind to T3 and Triac, thus suggest a similarity of CiNR1, at functional level, with vertebrate TRβs.

Discussion

4.1Thyroid-like function: the ascidian case.

The ability to concentrate iodine from the environment and the possibility to bind it to a substrate are the first and fundamental phases of the biosynthetic pathway leading to thyroid hormone formation. These functions can be exerted even in the absence of a thyroid organ, as in the case of ascidians, amphioxus and lamprey larvae, organisms which use for this purpose the endostyle, an organ mainly devoted to digestive function.

The endostyle, in the adult *Ciona*, is a ciliated, mucus-secreting groove in the ventral surface of the pharynx that aids in transporting food to the esophagus. Specific endostyle cells have been shown to concentrate radioiodine from sea water ((Barrington, 1957), and to produce monoiodiotyrosine, diiodiotyrosine and tyroxine (Dunn, 1974) (Kennedy, 1966) (Roche et al., 1959). So, early in chordate evolution, the secretion of iodine compounds began in the endostyle while it was still a part of the feeding mechanisms. The thyroid gland, like the endostyle, arises as an unpaired evagination from the midventral pharyngeal floor. Thus, it has been proposed that the history of the thyroid gland, in phylogeny, is one in which a feeding structure is transformed into a gland of internal secretion.

Several lines of molecular evidence, such as the prominent expression of the thyroid transcription factor-1 Ci-TTF1, Ci-FoxE and Ci-FoxQ in *Ciona* intestinalis endostyle and expression of the thyroid peroxidase (TPO) gene in the thyroid-equivalent region of the endostyle, support the scenario that the endostyle is homologous to the vertebrate thyroid gland (Ogasawara et al., 1999;Ristoratore et al., 1999;Ogasawara and Satou, 2003). Furtehrmore, the presence, in *Ciona* genome, of almost all the components of the battery involved in THs metabolism and modification, included a putative thyroid hormone

receptor, coupled with preliminary data, available in Literature, on a potential effect exerted by THs on *Ciona* metamorphosis, represented for me a starting point for my PhD thesis work.

Studying the thyroid-like function in an experimental model such as *Ciona intestinalis* is, indeed, a great opportunity to decipher how this function appeared, evolved and how it influences some phases of post-embryonic development along the chordates lineage.

With the aim to clarify different aspects and give a whole and complete view of the thyroid-like function in the urochordate *Ciona intestinalis*, my PhD work had three main goals: the determination of the role of iodine and THs on metamorphosis; the identification of the protein used as scaffold for THs synthesis and the characterization of the thyroid hormone receptor CiNR1.

4.2 Iodine and THs play an important role in ascidian metamorphosis.

When I started this part of the study, only few evidences in Literature indicated that exogenous thyroid hormone, T4, slightly anticipated the onset of ascidian metamorphosis, the post-embryonic process consisting in the transformation of a non-feeding swimming larvae into a sessile filter-feeding juvenile. By growing *Ciona* embryos in artificial seawater deprived of Iodine, I firstly tried to assess the role that this element could play on embryonic and post-embryonic development. One of the first evidence from my in vivo assays concerned a toxic effect exerted by high concentrations of iodine. Indeed, while I was setting up the protocol for the production of artificial seawater, with a iodine concentration similar to that occurring in natural seawater, I observed that, at iodine concentration 1.5 times higher than that present in natural sea water (ca. 60µg/L), the embryonic development stopped before the gastrulation, suggesting a toxic activity

exerted by iodine. The lack of iodine, in contrast, did not have any effect on embryonic development. The animals developed synchronously and were well-formed embryos comparable, although at a lower percentage, to the controls. The most prominent difference between the animals grown with and without iodine was related to their ability to go through the metamorphosis. *Ciona intestinalis* larvae kept at 18°C begin the metamorphosis 4-9 hours post hatching, while. a slight reduction in the developmental time table is observed when animals are kept at 21°C. As shown in figure 3.1, animals incubated at 21°C in different seawater conditions had diverse behaviours. The lack of iodine strongly affected the ability of the larvae to go through metamorphosis. This effect was more visible with the advancement of development. In particular, 24hours post hatching, when more than 80% of control larvae had already retracted the tails and started the body axis rotation, most of the ASW-I embryos remained as swimming larvae and only 37% of them were able to initiate the metamorphosis. 48 hours post hatching these ASW-I metamorphosed larvae were however delayed in development compared to the MFSW or ASW+I juveniles. Indeed the MFSW or ASW+I animals were at the 1st ascidian juvenile stage, whereas in ASW-I the animals were at the early rotation stage, thus with a delay of more than 12 hours. However the animals grown in ASW-I, after one week, recovered the delay and appeared morphologically similar to MFSW and ASW+I juveniles. Another interesting finding was that some ASW-I larvae tentatively initiated metamorphosis 48 hours after hatching but the process stopped almost immediately, after tail retraction. In more than 30% of the larvae grown in ASW-I the metamorphosis was instead completely blocked and they remained viable and swimming for more than a week. This diversified approach to metamorphosis by the ASW-I larvae can be interpreted in two different ways. The larvae, that in a batch are healthier can use the traces of iodine, still present in the ASW-I (since it is quite impossible to produce a

seawater completely deprived of iodine) to endogenously synthesize THs. Alternatively one can suppose that the non-feeding larvae could have a storage of Thyroid hormones, maternally inherited, that they use to allow the accomplishment of metamorphosis in case that endogenous TH biosynthesis cannot start. Usually the species with a non-feeding larval stage are characterized by the production of eggs rich in nutrients, to allow the development until a feeding phase. Zebrafish larvae, for example, do not feed until 5 days post hatching (7 days post fertilization) and have a source of maternally derived thyroid hormones in the yolk (D D Brown, 1997), which are involved in many aspect of development and could eventually compensate the lack of zygotic thyroid hormones when their synthesis is compromised. *Ciona* shows the peculiarity to have eggs full of yolk and not feeding larvae; thus one can speculate that in *Ciona*, as in zebrafish, the yolk represents a source of nutrients and a storage of maternally derived thyroid hormones which could exploited to trigger the start of metamorphosis and, eventually, supply for the impairment of zygotic TH synthesis due to low amounts of available iodine. To this end it would be really informative to look for the presence of TH in *Ciona* eggs and embryos, at different developmental stages, either by immunocytochemistry, using the T4 antibody, or by RIA (Radio Immuno Assay). One cannot exclude also the involvement of additional factors, besides TH, in controlling ascidian metamorphosis, that could help to bypass the impaired synthesis of THs induced by low iodine concentration in the sea water.

It has been previously reported indeed that treatment of ascidian larvae cultured in MFSW with 50µg/mL T4 anticipates the onset of metamorphosis (Patricolo, Cammarata, & D'Agati, 2001; Patricolo, Ortolani, & Cascio, 1981). I thus decided to check whether it would be possible to rescue the ability of the swimming larvae to go through and complete the metamorphosis by treating them with an exogenous source of THs (T4,

thyroxine, in this case). What emerged from the data I obtained was a striking effect of T4, even at a concentration of 30µg/mL. Indeed, when I treated just hatched larvae with 30µg/mL T4 already 1 hour post hatching I observed an extensive tail retraction (>70%). It is interesting to note that larvae grown in a iodine deprived seawater (ASW-I) were slightly more sensitive to T4 addition than the control and the larvae grown in ASW+I. 1 hour after hatching indeed 85% of ASW-I larvae treated with 30µg/mL T4 had retracted the tail and had thus begun the metamorphosis, compared to the MFSW (70%) and ASW+I (78%) larvae. It seemed that larvae, in which the endogenous TH synthesis was, in some way, negatively affected, were more sensitive and receptive to the inducing effects of exogenous Thyroxine. One can suppose that the whole molecular machinery underneath the metamorphosis was ready and just waiting for the triggering factor, which allow the process to start and to proceed even in an extremely faster fashion, compared to the normal behaviour, in ASW-I, as well as in ASW+I and MFSW larvae. The lack of iodine seems to cause a delay in metamorphosis that could be correlated to an impaired synthesis of thyroid hormones. Besides the deprivation of iodine, a colinear way to interfere with TH is by using some substrances, named goitrogens, already demonstrated to affect thyroid hormone synthesis in vertebrates. Those drugs were thus tested on fertilized eggs and the metamorphosis advancement was followed after the hatching (16-18 hours post fertilization). The goitrogens I used are able to interfere with TH biosynthesis at two different levels. Thiourea and methimazole inhibits the activity of thyroid-peroxidase, thus blocking the iodination and coupling of tyrosine residues on the substrate protein, whereas the Potassium Perchlorate ($KClO_4$) is a known specific competitor of the ion channel involved in iodine uptake. The results obtained using these drugs confirmed and strengthened the role of THs in controlling *Ciona* metamorphosis. In particular methimazole and Thiourea did not seem to affect the onset of metamorphosis,

but rather the organogenesis and the growth which were highly impaired. Indeed animals treated with these two drugs presented an aberrant organization of the endoderm-derived organs; the whole gastrointestinal system was affected, including the formation of the endostyle. It is not surprising that the stomach and the intestine are the organs suffering for the partial or total lack of THs. Thyroid hormones, via the interaction with their receptors, have been, indeed, demonstrated, in *Xenopus laevis*, to be involved in the morphological changes occurring, during the metamorphosis, at level of intestine. The couple TH/TR triggers two parallel pathways; one leading to the apoptosis of tadpole intestine epithelial cells, and another whose effect is the proliferation of the cells forming the adult intestine (Ishizuya-Oka, 2011). The expression of *Ciona intestinalis* Nuclear Receptor 1 in the posterior part of larval endoderm (figure 3.12) a region that fate map studies of Hirano and Nishida (Hirano & Nishida, 2000) have indicated to give rise just to the intestine and stomach (see Fig. 1.17), could let suppose that a TH/TR mechanism is involved, in *Ciona* too, in the correct formation and organization of the gastrointestinal system. From this point of view, it is interesting that the only data available in literature have indicated the localization, in *Ciona* larvae, of T4 immunoreactivity at the level of mesenchymal cells, which are located in the trunk and are scattered in the body cavity all around the endoderm. It is tempting to imagine a mechanism by which T4 present in mesenchymal cells is used to instruct the developmental fate of the endodermal cell which express the CiNR1 thyroid hormone receptor.

The goitrogens I used, namely the thiourea (TU) and the methimazole (MMI), belong to the family of compounds, collectively named thioamide, known to specifically interfere with thyroid-peroxidase function. They have been demonstrated to be extremely effective, even at low concentration, in inhibiting the thyroid function and thus in impairing some developmental processes in vertebrates (fishes) (Brown et al., 1997). The

goitrogen effects of thiourea and methimazole have been extensively studied in zebrafish. When embryos are cultured in presence of increasing amount of methimazole, a delay in the hatching success of developing zebrafish is observed, starting from the concentration of 170 mg/L. At higher concentrations, ranging from 350 mg/L to 1.7 g/L, the development is almost completely impaired and an high mortality is observed. The thiourea, is even more active than methimazole in inhibiting the development of zebrafish embryos. At 300 mg/L all the treated embryos die, whereas at lower concentrations (50-100 mg/L) the embryos could not hatch properly and the phenotypes start to be aberrant.

In *Ciona* larvae, treated with 500-1000 mg/L methimazole or 2.5-5.0 g/L thiourea, concentrations higher than those utilized in zebrafish, no interference with the hatching success was observed. However, aside from a delay in the onset of metamorphosis and in the metamorphosis advancement, the juveniles, at 72 hours after hatching, were viable but showed defects in the organogenesis, mostly at the level of endoderm-derived organs. The differences in the efficiency of goitrogens between *Ciona* and zebrafish can be related to the environment surrounding these two species. The thioamide drugs (TU and MMI) are known to impair iodination of tyrosine residues of the protein used as scaffold for TH synthesis, probably by competing with iodine, as substrates for thyroid-peroxidase (TPO), and thus preventing the oxidation of iodide by this enzyme. *Ciona* lives in the seas, where Iodine concentration is much higher than in freshwater and consequently more iodine is available as substrate for the TPO. To compete with the iodine, larger amounts of thioamide goitrogens could thus be needed in order to affect the synthesis of TH and in turn interfere with *Ciona* development.

On the other hand we cannot exclude that the specificity of thioamide drugs (TU and MMI) is dependent on the TPO primary structure (amino acid sequence). *Ciona*

intestinalis thyroid-peroxidase (CiTPO) shares 40% identity with vertebrates TPO. Even though the highest degree of conservation has been found in TPO functional domains, it could be possible that the divergence in other regions of the protein is responsible for the different sensitivity to TU and MMI.

The third goitrogen I used, the Potassium Perchlorate ($KClO_4$), has been reported to be less active in zebrafish, compared to thiourea and methimazole. To observe an effect on zebrafish development it is necessary to reach concentrations ten times higher (2.5 g/L) than TU and MMI. In *Ciona,* $KClO_4$ resulted effective in inhibiting the metamorphosis, at 5g/L, while between 2.5-4 g/L it caused a graded delay in the onset of metamorphosis and in the metamorphosis advancement; the juveniles, however, were viable and did not show any grossly defect in the organogenesis and in the overall development. The data on goitrogens thus indicate that in *Ciona* the effects of 5g/L $KClO_4$ were stronger compared with 5g/L thiourea, since, at this concentration, with $KClO_4$ I observed a complete block of metamorphosis, while with thiourea the larvae were able to start metamorphosis, although the whole process was somehow impaired. Unfortunately, I could not collect data for methimazole, at concentrations greater than 1g/L, since it precipitates in sea water. The higher efficiency, in *Ciona*, of $KClO_4$ compared to thiourea, could be related to the action it exerts in blocking sterically the iodine specific ion channel (NIS, Sodium/Iodide Symporter). In vertebrates NIS expression is localized at the level of thyroid and salivary glands, where the most of iodine uptake occurs; while no information are available in literature about the expression of NIS in *Ciona*. One can speculate that in *Ciona* larvae, given the absence of a specific organ devoted to TH synthesis, a wide tissue localization of NIS is implied in the effect exerted by $KClO_4$ in inhibiting the metamorphosis. Obviously the answer relies on the delineation of NIS expression pattern in *Ciona* larvae.

The Potassium Perchlorate is also an oxidant compound. The block of metamorphosis I observed could thus be a consequence of this activity, rather than the outcome of an impairment of TH biosynthesis. To verify whether $KClO_4$ exerted its effect by inhibiting TH synthesis I checked the ability of KClO4–treated animals to endogenously produce THs, by means of an immunological approach. The control endostyle showed a positive T4 immunoreaction staining, in the regions devoted to iodine binding, while this staining disappeared in the endostyles of $KClO_4$ treated animals. The data strongly indicate that KClO4 is thus an efficient inhibitor of TH biosynthesis in *Ciona* as it does in vertebrates. The correlation between $KClO_4$ and THs was further corroborated by the findings that the external addition of T4 was able to rescue, at some extents, the developmental block to which the larvae were subjected in presence of.$KClO_4$.The concentration of 5g/L. $KClO_4$ represents the threshold level above which the addition of 40µM T4 was almost unable to recover a normal metamorphic behaviour in *Ciona* larvae. One can imagine that at concentration greater than 5g/L, $KClO_4$ has side effects, derived from its oxidant activity, affecting in an irreversible way *Ciona* developmental program.

4.3 Looking for the homolog of Thyroglobulin.

When in 2002 the draft of the genome of the urochordate *Ciona* intestinalis was published (Dehal et al., 2002), and the annotation of genes started, it was clear that *Ciona* had almost all the molecular pieces of the system controlling the correct accomplishment of THs biosynthesis and function. They include orthologous genes of iodine transporters, devoted to iodine uptake, of Duox, the enzymes involved in the oxidation of Iodine, of Thyroid-peroxidase, the enzyme responsible of iodination and coupling of tyrosine residues. Furthermore the genome contains the sequence of a putative thyroid hormone receptor, CiNR1, while a clear ortholog of thyroglobulin seems to be absent. In

vertebrates thyroglobulin is the most abundant protein present in thyroid follicles and some of its specific tyrosine residues are iodinated and coupled in order to form the thyroid hormones. None of the *in silico* approaches, based mainly on the comparison of amino acid sequences, succeeded in fishing a putative homolog of thyroglobulin gene in *Ciona intestinalis* genome. Given these remarks, I used a proteomic-based approach to try to discover the protein that in *Ciona* represents the scaffold for THs synthesis. To this end I exploited the anti-T4 antibody, which has been instrumental in revealing an immunoreactive signal in the endostyle regions previously demonstrated to be devoted to TH synthesis, as a probe to fish any positive reacting protein, amongst the proteins extracted from *Ciona* endostyles and separated by 2-DE (Bidimensional SDS-PAGE). This method revealed the presence of 9 reactive spots (proteins) and the analysis by Mass Spectrometry (MALDI-TOF) revealed that 8 out of the 9 proteins were orthologous of vertebrate proteins having a precise and assessed function. The remaining protein, specifically present in the spot 5, was orthologous of vertebrate CRELD a family of proteins recently discovered and suggested to have widely diverse biological roles in both developmental events and subsequent cell function. However, up to now only for CRELD1 it has been demonstrated that mutation in this gene causes atrioventricular septal defects. CRELD encodes members of a subfamily of epidermal growth factor-related proteins characterized by a cysteine-rich with epidermal growth factor-like domain. Needless to say that its function in *Ciona* in unknown but it is interesting to note that it is specifically expressed in the endostyle (http://cipro.ibio.jp/), suggesting a specific role of this protein in this organ. Furthermore, by means of a deeper mass spectrometry analysis, it has been possible to discover that only the spot n.5-CRELD protein, amongst the 9 spots analysed, contained traces of the thyroid hormone T4 and an appreciable amount of its precursor DIT. In vertebrates CRELD proteins are supposed to have a

membrane location. This feature could be functional whether CRELD would play a role in TH biosynthesis. In vertebrates thyroid follicles the thyroid-peroxidase, the enzyme that iodinates and couples the tyrosine residues of thyroglobulin, is located and exert its function close to the plasma membrane. The thyroglobulin should thus be translocated towards the membrane to be subjected to iodination. The presence, at the plasma membrane, of a protein ready to accept iodine atoms could be extremely useful for the purpose. The dynamic situation occurring in the filter feeding *Ciona,* where the iodine contained in the seawater is only transiently in contact with the endostyle regions able to uptake it, could necessitate of a more efficient and rapid system to take and fix it to a protein substrate. A protein already close to the change interface (seawater/endostyle), could permit the whole process to proceed in a faster way.

4.4 Thyroid Hormone Receptors in Tunicates: functional studies on *Ciona* intestinalis Nuclear Receptor 1 (CiNR1).

In 1998 Carosa and co-workers identified and isolated, in *Ciona* intestinalis, a gene coding for an ortholog of vertebrate thyroid hormone receptors. The gene, named CiNR1 (*Ciona intestinalis* Nuclear Receptor 1), codes for a protein of 588 amino acids with a molecular weight of 67 kDa with a nuclear localization. It is characterized by a DNA binding domain (DBD) constituted by two cystein rich, zinc fingers motifs and a Ligand Binding Domain (LBD) spanning for more than 400 residues. The DBD shares more than 70% identity with the DBDs of vertebrate TRs. By contrast the less conserved LBD has only the 30% of identity. As previously determined, and confirmed also by the phylogenetic analysis showed in figure 3.11, CiNR1 is close to the common ancestral gene of TRs, originated before the divergence between ascidians and vertebrates. Some previous studies showed that CiNR1 seemed not to be an active thyroid hormone receptor

since it did not show any DNA binding ability and failed to interact with the thyroid hormone T3 (Carosa et al., 1998)

CiNR1 is expressed at the level of endoderm starting from the neurula stage (figure 3.12). The fact that, in *Ciona*, many of the organs found in the adult originates from the larva endoderm, suggests that CiNR1, expressed specifically in this territory, may trigger a specific pathway involved in the development and the organogenesis occurring at metamorphosis.

The demonstration that the biosynthesis of Thyroid Hormones occurs in the region 8 of the endostyle, along with the evidences suggesting that THs exert biological effects on *Ciona* metamorphosis, represented the basis of this part of my studies. I focused my attention on CiNR1 since the demonstration of its functionality as thyroid hormone receptor in *Ciona* could further support a thyroid hormone action in tunicates that is exerted, as in vertebrates, through the interaction with their specific receptor. I studied CiNR1, mainly through in vitro approaches, to give a complete functional description. The action of TRs necessitate three main features:

- the ability to bind to specific DNA target sequences
- the ability to bind to a ligand (thyroid hormone)
- the ability to activate, in a ligand dependent manner, gene expression

The first series of experiments I carried out were thus devoted to the determination of the capability of CiNR1 to bind to specific DNA sequences. In particular I assayed whether CiNR1 was able to bind to the canonical thyroid-hormone response element (TRE). In vertebrates the consensus sequence specifically recognized and bound by TRs is AGGTCAnnnnAGGTCA and it is known as DR4. It is composed by two half-sites

(AGGTCA) separated by 4 nucleotides. The ability to bind specifically to the DR4 is necessary for TRs action. I first assayed by EMSA the binding of CiNR1 to different DRs, ranging from DR1 to DR5, differing only for the length of the spacer and specifically bound by different nuclear receptors. Interestingly CiNR1 was able to bind to DR4 but only in heterodimerization with CiRXR .

CiRXR encodes the *Ciona* homolog of the Retinoic X Receptor, the partner in DNA binding, also in vertebrates, of the TRs. It is worth to note that in previous experiments (Carosa et al., 1998) CiNR1 failed to bind to the canonical DR4, a data that suggested it was an inactive thyroid hormone receptor. My results clearly have demonstrated that the binding capacity of CINR4 is strictly linked to the presence of CiRXR. This data thus represent the first step to define CINR4 as a TR with an activity comparable to that of vertebrates TRs.

As thyroid hormones also thyroid hormone receptors, in particular TRβ, are among the main regulators of the vertebrate metamorphosis. It is known, indeed, that during amphibian metamorphosisa peak in THs level is followed by a peak of TRβ. The couple TH/TRβ regulates the expression of downstream genes leading to the completion of the metamorphosis. The mentioned peak in TRβ expression is obtained through a positive loop guaranteed by the presence, in TRβ promoter, of DR4 elements. Those DR4s permit the binding of TRβ to its own promoter in order to trigger its own expression. This phenomenon is very conserved among vertebrates and this kind of regulation seems to be present also in amphioxus (Paris et al., 2008). By using the software NUBISCAN (http://www.nubiscan.unibas.ch/), I found two putative DR4s in the promoter of CiNR1. This is intriguing, since it may suggest that also CiNR1 can go through a kind of positive loop of regulation. I thus tried to demonstrate that CiNR1 was able to bind to those DRs.

The two *Ciona* DRs, named CiDR4.1 and CiDR4.2 were tested for the binding by the heterodimer CiNR1/CiRXR. The CiDR4.2, the closest to the transcription start site, failed to show any binding.

CiNR1/CiRXR interacted instead with high affinity to CiDR4.1, although this binding was subjected to a stronger competition by the canonical DR4 (figure 3.16) compared to the cold CiDR4.1 oligo. The presence of a specific DR4 in CiNR1 promoter, however, does not necessarily mean that it is bound, in vivo, by CiNR1. Further experiments involving 1) studies of CiNR1 transcriptional regulation together with 2) a deep analysis of any change in the expression of CiNR1 during different metamorphosis stages and 3) assays to reveal if CiNR1 expression is sensitive to thyroid hormones, will be instrumental to assess if a positive CiNR1 self-regulatory mechanism exist also in *Ciona*.

The second common feature of TRs is the ability to stimulate, in presence of its ligand, the expression of downstream genes under the control of DR4 elements. Previous experiments carried out on CiNR1 failed to demonstrate its ligand-dependent ability to trigger the expression of a reporter gene (Carosa et al., 1998). In my studies CiNR1 was tested, by means of a transient transfection in mammalian cells, for its ability to stimulate the expression of the Luciferase reporter gene under the control of a DR4 element. The cell line used for these experiments was the HEK (Human Embryonic Kidney cells), that, in a first series of experiments, were transfected with a plasmid encoding the full length CiNR1, under the control of an eukaryotic specific promoter (CMV), along with a reporter plasmid coding for the luciferase, under the control of three DR4 elements (DR4Luc). Once produced in the cells, CiNR1 is supposed to bind to the DR4 sequences and trigger the expression of the luciferase, when Thyroid Hormones are added to the medium.

An appreciable increase of luciferase activity was unexpectedly observed when CiNR1/DR4Luc transfected cells were treated with T3, the most active TH in vertebrates. This suggest that CiNR1 was activating gene expression in a TH dependent way. It was thus behaving as a thyroid hormone receptors. By analyzing the TRs phylogeny reporter in figure 3.11 is evident the divergence of CiNR1 before the duplication that led to the appearance, in vertebrates, of TRα and TRβ. The comparison of the CiNR1 amino acid sequence with those of vertebrate TRs, did not provide any cue to determine whether CiNR1 is more similar to TRα or TRβ and discern which one, of the two vertebrate TRs, is closer, at functional level, to the common ancestor. I thus decided to test, on CiNR1, a synthetic compound, NH3, designed to be TRβ specific and acting as an antagonist competitor of T3 for the binding to TRβ. Interestingly CiNR1 resulted to be sensitive, although at lesser extent compared to TRβ, to NH3, indicating a ligand binding similarity between CiNR1 and TRβ. TRβ is known to be the TR that in vertebrates is involved in the metamorphosis and whose expression is finely regulated during the development. So it would not be surprising that CiNR1, if potentially involved in ascidian development and metamorphosis, is much similar to TRβ than TRα. From the results obtained on the effects that thyroid hormones exert on *Ciona* metamorphosis, T4 emerged to be the most efficient in eliciting an effect, while in vitro assays indicated that, in contrast, T3 was the most active on CiNR1, amongst the different thyroid hormones and their derivatives tested. This discrepancy can be explained by the metabolic pathway to which THs are subjected, once taken up by the cells. Thus, it could be that neither T3 or T4, represent the real ligand of CiNR1 and that the real form of TH, active in *Ciona*, is one of their metabolites. Some of these derivatives were tested for the ability to activate CiNR1 and two of them resulted to be active on CiNR1: TRIAC and TETRAC. The wide range of compounds to which CiNR1 can respond, compared to the highly specialized vertebrate

TRs, could be related to the high sequence divergence. It could also be seen as a strategy aimed to improve the effects of THs. If we suppose that, in *Ciona*, the TH synthesis efficiency is lower, compared to the species with a follicular thyroid, the ability of the single receptor (CiNR1) to be activated both by THs and their metabolites could be explained as a way to amplify the response to those morphogenetic hormones.

To bypass the limitations of the cell system used (HEK cells), namely in relation to the presence in these cells of endogenous TRs that could interfere with the interpretation of my results, I tried a different approach.

It is reported in literature and widely accepted that the two domains of TRs, DBD and LBD, can exert their functions also when isolated from the rest of the protein. I thus decided to take the LBD, from residue 177 to 366, and used it to produce a chimeric protein with the DNA binding domain of the yeast transcription factor Gal4. I tested, then, whether the chimera Gal4-DBD/CiNR1-LBD was able to trigger the expression of luciferase, under the control of Gal4 target sequence. By this approach I prevented any possible interference due by the HEK endogenous TRs. The results indicated that once again CINR1 was activated by T3 and TRIAC.

One can suppose that the reason why previous experiment failed in showing any activity of CiNR1 derives from some differences in the amino acid sequences between the clone I used, from Satoh's cDNA collection, and the clone used in the previous assays (Carosa et al., 1998). In particular I noticed a substitution, in one of the three residues, considered to be directly involved in TH binding. In the clone used by Carosa and coworkers the first residue, supposed to interact with the ligand, is a glutammine (Q), whereas in amphioxus is a lysine (K) and in vertebrate TRs is an arginine (R). In the clone isolated from the Satoh's cDNA collection in place of a glutamine, I found a tryptophan. No further amino

acid variations have been detected between the two clones in the other two positions. Interestingly no substitution between the two clones have been found in the DNA binding Domain (DBD). The failure of CiNR1 in binding to DNA, reported in previous experiments (Carosa et al., 1998) was most probably due to a limitation of the technique they used. They indeed assayed the binding of CiNR1 to the DR4 consensus sequence without taking into account the lack of RXR, its partner, that in my experiments resulted fundamental for CiNR1 DNA binding ability. Concerning instead the substitution, in one of the three residues, considered to be directly involved in TH binding I analyzed the sequences of CiNR1 present in the different *Ciona* available databases (http://genome.jgi.doe.gov/Cioin2/Cioin2.info.html, http://www.aniseed.cnrs.fr/, http://www.ncbi.nlm.nih.gov/). I detected an high degree of heterogeneity that is linked to the high grade of polymorphism which characterizes natural populations of this specie. Obviously, a comparative analysis of the ligand binding capacity between the two clones will be instrumental to assess if these substitution are irrelevant or could affect the receptor functionality.

It is noteworthy that in chordate phylogeny CiNR1 is the first receptor able to activate gene expression when stimulated with T3. Amphioxus TR has been demonstrated to bind and to be activated by TRIAC (Paris et al., 2008). The ability of vertebrate TRs to bind to TRIAC is also well documented. This feature could be a characteristic that vertebrate TRs share with the common ancestor, while the binding of Thyroid hormone receptors to T3 could represent an innovation appeared after the divergence of amphioxus from other chordates. *Ciona* intestinalis thus seems to be the first chordate in which T3 could have acquired the function as it has in vertebrates.

4.5 Conclusion and future perspectives.

In sum, the results I'm reporting in my thesis represent a step forward in the comprehension of the biological effect that THs exert in the induction and accomplishment of *Ciona intestinalis* metamorphosis and organogenesis. In addition with the demonstration of a T4 immunoreactivity in the endostyle regions, previously demonstrated to concentrate the iodine and to produce THs, I further supported the phylogenetic link between thyroid gland and the endostyle organ. The anti-T4 antiboby has been instrumental also for the identification, amongst the proteins extracted from *Ciona* endostyle, of a protein, CRELD, apparently not related to vertebrate thyroglobulin, that hypothetically could represent the scaffold for TH synthesis in *Ciona intestinalis*. To continue with this study I already organized a number of experiments to demonstrate that CRELD represents, for certain, the precursor of THs in *Ciona* and thus find the evolutionary link for a function so fundamental for body metabolism. The approach I used could then be extended to other invertebrate species in which, up to now, no thyroglobulin has been detected in the genome, to reveal if, outside the vertebrates, the scaffold for TH synthesis is conserved or if each specie use a different thyroglobulin-like protein. My present studies have also demonstrated that the *Ciona* CiNR1 is a functional thyroid receptor, further strengthening the idea that a thyroid function, comparable to that of vertebrates, is present also in tunicates. To continue these studies I already planned some experiments to demonstrate the role that CiNR1 could play during the development and metamorphosis, with the final aim to definitively assess that the mechanism of TH synthesis/TH signalling pathway, through a specific receptor, is of ancestral origin in chordates, if not in deuterostomes, although with specific elaborations in each lineage.

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