The evolution of bilaterian body-plan: perspectives from the developmental genetics of the Acoela (Acoelomorpha)

Marta Chiodin

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MARTA CHIODIN

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UNIVERTSITAT DE BARCELONA

Facultat de Bilogía
Departemento de Genética
Programa de Doctordado: Genética

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'La evolución de los eje corporales: perspectivas de la genética del desarrollo de los acelos (Acoelomorpha)'

Memória presentada por Marta Chiodin para acceder al título de Doctor por la Univerisdad de Barcelona, bajo la direccón del Doctor Pedro Martínez Serra

Marta Chiodin
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EL DIRECTOR DE LA TESIS
Dr. Pedro Martínez Serra
Investigador ICREA
Facultad de Biología
Universidad de Barcelona

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Introduction

1. The Acoela.

1.1 Habitat and Ecology

Acoels are slender, acoelomate, bilateral symmetric worms that live predominantly in benthic marine habitats, although two fresh water species have been described. Their adult body size and shape can vary considerably, reflecting adaptation to *e.g.* habitat and diet (Achatz, Chiodin et al. 2012).

The two species of this study *Symsagittifera roscoffensis* (von Graff 1891) and *Isodiametra pulchra* (Smith and Bush 1991) as well as several other species live in the mud or sand of shallow marine waters, although species, prevalently tropical, that live among corals and/or algae or under the rocks are also known, *e.g.* species of the genus *Convolutriloba*, which have recently gained attention for their potential in developmental and regeneration studies (Sikes and Bely 2008; Bely and Sikes 2010; Sikes and Bely 2010).

Acoels can be predators, usually feeding on various small marine invertebrates (e.g. *Convolutriloba*) whereas others feed on bacteria and or microscopic algae (e.g. *Isodiametra pulchra*).

Species of the genus *Symsagittifera* and *Convolutriloba* ingest symbiotic algae when they reach their adult stage a time when these and some species stop feeding (Adam, Balzer et al. 2004; Shannon and Achatz 2007; Bourlat and Hejnol 2009). Such algae confer to the worms a brilliant green color, whereas brownish, red and white color patterns are due to the presence of pigments (Hirose and Hirose 2007; Hooge, Wallberg et al. 2007).

1.2 Morphology

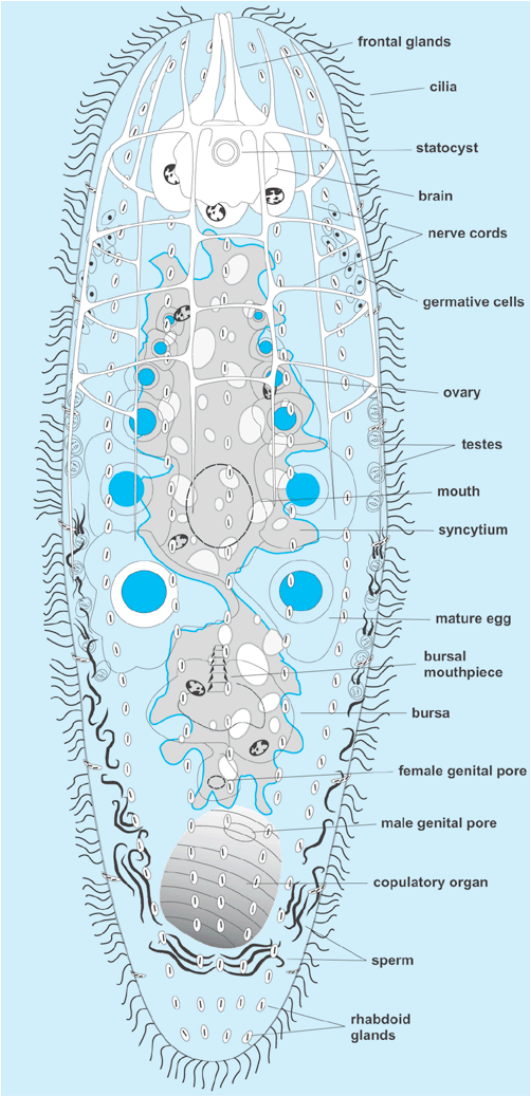


Fig.1 Schematic drawing of an acoel body plan.
From http://www.accessscience.com/search.aspx?rootID=790053.

The first stricking feature of the acoel body plan is the absolute absence of body cavities, features that also conferred the name to the group. The body space between the digestive system (often syncytial) and the body wall is completely filled by cells, mostly parenchymal cells but also the soma of other cell types including glands and epidermal cells which are often sunken within the body (Smith and Tyler 1985)(see Fig.1 for general diagram of an acoel body plans and below for a more detailed description).

The body wall consists of an outer multiciliated epidermis, with cells that show a unique ciliary rootlet system and special truncated ciliary tips. These are the

uniting traits of the phylum Acoelomorpha *sensu* Haszprunar (1996), *i.e.* (Xenoturbellida+(Nemertodermatida+Acoela)) (Haszprunar 1996).

Glands are distributed all over the body, most likely to help the worms moving by ciliary gliding. An anterior concentration of glands makes up for the frontal organ, which opens at the anterior most tip of the worm. The cell bodies of the frontal organ are deeply sunken in the body, in close contact with the anterior nervous system (Rieger, Tyler et al. 1991).

Another distinctive anterior structure of the acoel body plan is the statocyst, a sensory organ formed by a central lythocite containing the statolith and two parietal cells(Bery, Cardona et al. 2010; Achatz, Chiodin et al. 2012). This organ is deeply embedded in the brain and it confers to the worm the capacity of orientation with respect to the gravity force.

The dorsal ventral axis is defined by the presence of a ventral mouth and ventral gonopores/copulatory organs that, however, exhibit a very variable position along the anteroposterior axis in different acoel taxa, ranging from terminal/ posterior (*Diopisthoporus*) to anterior (*Hofstenia*) (Hooge, Wallberg et al. 2007; Jondelius, Wallberg et al. 2011).

1.2.1 Digestive system

The mouth opens into the digestive system, either directly or through a muscular ciliated pharynx (Todt and Tyler 2006; Todt and Tyler 2007; Todt 2009). Although this organ presents great morphological variability among different acoel taxa, it seems now that the presence of a pharynx is the plesiomorphic status for the Acoela (Jondelius, Wallberg et al. 2011).

The digestive system is blind, *i.e.* an anal opening is absent, and it usually exists in the form of a digestive syncytium, which can be present from very early developmental stages or be formed only transiently for digestion (Smith and Tyler 1985; Rieger, Tyler et al. 1991; Smith and Bush 1991; Hejnol and Martindale 2008; Hejnol and Martindale 2008). In the latter species the syncytium is formed from digestive parenchymal cells, which in some cases (*Paratomella rubra*) do not fuse at all. Regardless of the presence of a syncytium, an epithelial digestive system is never present, and a digestive lumen is always lacking, perhaps with the only exception of *Paratomella rubra* (Smith and Tyler 1985; Rieger and Ladurner 2003) .

1.2.2 Nervous system

All acoels have a nervous system that consists of an anterior neuronal concentration, or 'brain' and a group of paired longitudinal neurite bundles (formerly called nerve cords), which can vary in number depending on the species (Rieger, Tyler et al. 1991; Raikova, Reuter et al. 1998; Reuter, Raikova et al. 1998; Raikova, Reuter et al. 2004; Hejnol and Martindale 2008; Bery, Cardona et al. 2010; Semmler, Chiodin et al. 2010; Achatz, Chiodin et al. 2012). The acoel central nervous system is sunken below the body-wall (there is no basal lamina between the epidermis and muscles) that coexists with a supramuscular irregular nerve plexus (Rieger, Tyler et al. 1991; Achatz, Chiodin et al. 2012; Achatz and Martinez In press).

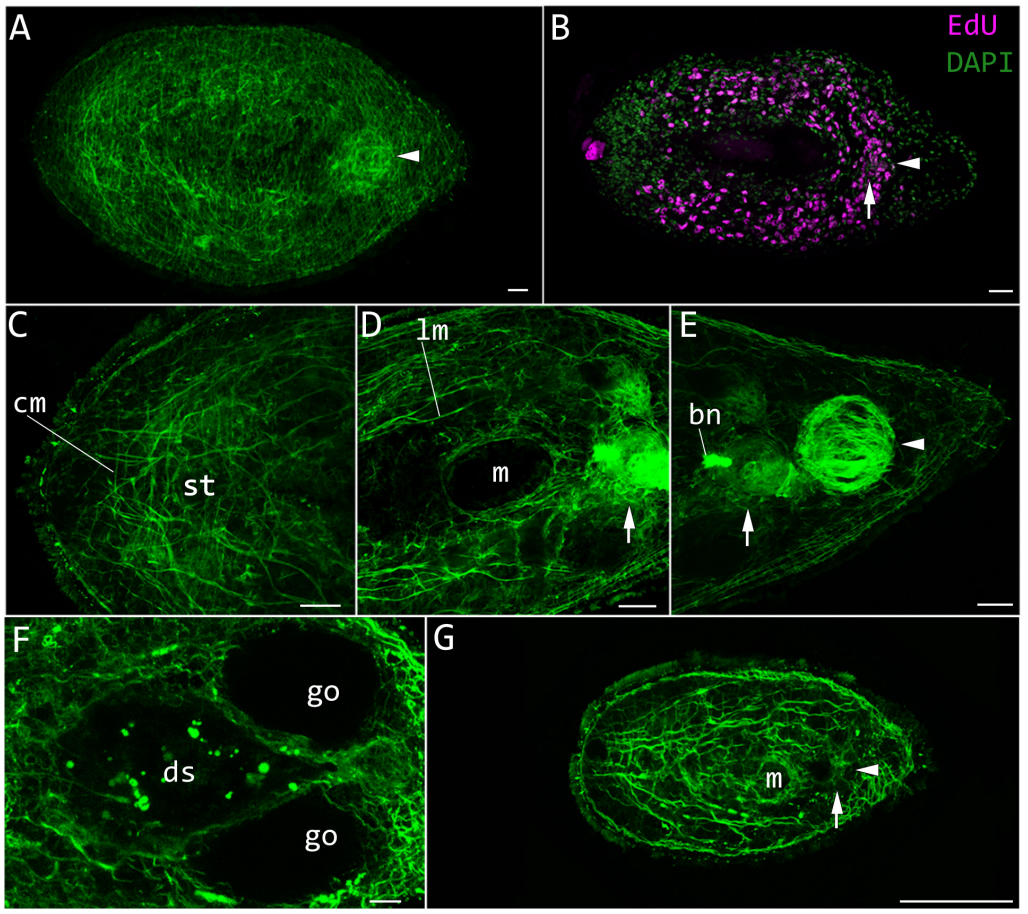
Whereas there is an obvious antero-posterior gradient of neural structures along the antero-posterior body axis, there is no clear displacement of the neurite bundles towards the dorsal or the ventral side of the animal. The longitudinal

bundles are connected by transversal rectangular commissure regularly distributed along the anteroposterior axis (Raikova, Reuter et al. 1998; Reuter, Raikova et al. 2001; Bery, Cardona et al. 2010; Semmler, Chiodin et al. 2010). There have been some debates on whether the anterior neuron concentration of the acoels should be termed as a true brain or not. One major problem is related to the absence of an extracellular matrix, which does not insulate the brain from the surrounding tissues. Moreover the sunken cell bodies of glands or epidermal cells intermingle with neural tissue and some muscular fibers penetrate the brain (Raikova, Reuter et al. 1998; Tyler and Rieger 1999; Bery, Cardona et al. 2010). However most of the ultrastructural investigations have shown that a pretty compact neuropile surrounded by the neurons' somata (cortex) is present (Bery, Cardona et al. 2010), justifying the use of the term "brain". The morphology of the brain can vary considerably, from a ring shaped commissural brain to a more compact symmetrical bilobed brain, usually present in more derived acoel families. Nonetheless, the value of the brain morphology as a phylogenetic significant character has not yet been ascertained.

Fig.2 *Isodiametra pulchra* body plan organization. ►

Mainly mesodermal structures are shown. Muscle are in green (except in B), marked by phalloidin. Neoblasts in magenta, marked with EdU that detects proliferating cells. The arrow points the female copulatory organ, the arrowhead points the male copulatory organ. Anterior is to the left in all aspects. Scale bar 50 μm.

A. Dorsal view of an adult. B. Ventral view of a old juvenile. The nuclei are green whereas the magenta nuclei belong to the neoblasts. The arrow and the arrowheads indicate the developing copulatory organs. C. Magnification of the 'head muscles'. cm, circular muscle; st, statocyst. D. Magnification of the ventral mouth region. lm, longitudinal muscle; m, mouth. E. Magnification on the copulatory organs. bn, bursal nozzle. F. Dorsal cross muscles (used for feeding and egg laying). G. Juvenile specimen with anlage of the copulatory organs.



1.2.3 Musculature

The body wall musculature consists of an orthogonal grid of thick inner longitudinal muscles and thinner outer circular ones (Hooge 2001; Rieger and Ladurner 2003; Achatz, Chiodin et al. 2012) (Fig.2A). Diagonal muscles that often cross each other at the dorsal body midline are frequently located between the layers of longitudinal and circular muscles. Additionaly, numerous muscles cross the acoel body dorso-ventrally at different angles, and they are usually

termed as parenchymal muscles. (Ladurner and Rieger 2000; Hooge 2001; Semmler, Bailly et al. 2008).

In those acoels that lack a pharynx, the ventral body wall musculature presents usually a more complex pattern of fibres arrangement than its dorsal counterpart (Tyler and Rieger 1999; Hooge, Haye et al. 2002; Gschwentner, Mueller et al. 2003).

The most prominent difference between the dorsal and ventral side of these animals, is the presence of modified longitudinal ventral muscles in the form of U-shaped muscles that encircle the mouth at its posterior rim. Occasionally, in some species other modified accessory diagonal or longitudinal muscles associated to the mouth exist (Fig.2F). These modified, mostly ventral muscles are suspected to act as a functional pharynx, given their close association to the mouth opening and their absence in those acoel families with a pharynx (Tyler and Rieger 1999; Hooge 2001; Semmler, Bailly et al. 2008; Todt 2009). Additional ventral accessory muscles are the ring muscles encircling the mouth opening and the special muscles of the copulatory organs (Fig.2D-E).

Usually acoelomorphs muscles are mononucleated and of the smooth type (Rieger, Tyler et al. 1991), although pseudostriation patterns, *i.e.* regularly arranged Z-bodies not forming Z-discs, have been described in some species (Tyler and Rieger 1999; Todt and Tyler 2006)

1.2.4 Reproductive system

Acoels are hermaphroditic, and their gonads morphology is very variable among different species. Acoel gonads can be diffuse or compact, mixed or not, paired or unpaired but at least they always lay in the parenchyma, in most cases

not being lined by any tissue, thus more similar to the cnidarians and ctenophores gonads than to those of any other bilaterian (Rieger, Tyler et al. 1991; Boone, Willems et al. 2010; Jondelius, Wallberg et al. 2011; Achatz, Chiodin et al. 2012).

The germinative regions of the female gametes (oogonia) are usually located quite anteriorly in the parenchyma. Mature oocytes are most commonly found in close proximity to the mouth from which the fertilized eggs are released, although in some taxa the eggs are laid through the disruption of the body wall. Most commonly a dorsoventral distinction exists between the location of ovaries and testes within the same animal. The male germ cells (spermatogonia) are not regionalized along the antero-posterior body axis. At least in *Isodiametra pulchra* the process of spermatocytes maturation occur in a proximo-distal direction, with the mature spermatids being found closer to the body midline (Boone, Willems et al. 2010). Acoels have uniquely biflagellate sperm cells, whose axoneme ultrastructure has proven to be a very valuable character to resolve internal phylogeny (Hooge, Haye et al. 2002; Petrov, Hooge et al. 2004; Tekle, Raikova et al. 2007; Achatz, Hooge et al. 2010). In addition to the gonads, acoels have specialized female and male copulatory (or genital) organs. The female copulatory organ most commonly consist of a bursa used for sperm storage, often complemented by a bursal nozzle that allow selection of the sperm, a vagina and a female gonopore (Petrov, Hooge et al. 2006; Achatz, Chiodin et al. 2012). The male copulatory organ, instead, consists of seminal vescicles (but false seminal vescicles exist too), and an antrum (Fig.2D-E). The wall of the seminal vescicle is usually strongly muscular and in some cases harbours a penis which is a distinctive taxonomic character in certain families (Hooge and Tyler 2005; Petrov 2005; Semmler, Bailly et al. 2008). The male

copulatory organ is a highly muscular structure easily recognizable in live and phalloidin preparations (Fig.2E).

1.2.5 Stem cells system

The acoel somatic stem cells, also called neoblasts, lay in the parenchyma in close proximity to the gonads and they are the only dividing cells present in the worm's body (Fig.2B)(Gschwentner, Ladurner et al. 2001; De Mulder, Kuales et al. 2009). These cells are usually small but have a big nucleus and a thin rim of cytoplasm. On the ultrastructural level, they can be recognized by their poorly condensed chromatin and the lack of endoplasmic reticulum. BrdU pulse/chase experiments with subsequent maceration showed that neoblasts can differentiate in all cell types, including germ cells, thus suggesting their likely pluripotency (Gschwentner, Ladurner et al. 2001; De Mulder, Kuales et al. 2009). However, since differentiation of all cell types from a single neoblast (Wagner, Wang et al. 2011)has not yet been shown in acoels, the existence of "pre-patterned" (or commited) subpopulations of stem cells cannot be excluded (article R2). So far, a preliminary molecular characterization of the acoel neoblasts has been carried out exclusively in the species *Isodiametra pulchra,* showing the the gene *piwi,* a bilaterian germ cells marker, is expressed in a subpopulation of neoblasts (De Mulder, Kuales et al. 2009).

It is only acoels, rhabditophoran flatworms and non-bilaterians (sponges and cnidarians) tha share this unique feature, namely *piwi* expression in somatic stem cells (De Mulder, Kuales et al. 2009; Egger, Steinke et al. 2009).

1.3 Regeneration, reproduction and life cycle.

The presence of numerous neoblasts within the acoel body confers to the worms the ability of regenerating damaged tissues or entire missing parts of their body.

This capacity is used by some species for asexual reproduction (fission), *e.g.* by the species *Convolutriloba longifissura,* which is able to generate three whole animals from a single one, though other asexual reproductive mechanisms such as budding are known (Shannon and Achatz 2007; Sikes and Bely 2008).

Yet, sexual reproduction is most common amongst acoels, given that even those species that can reproduce asexually do also mate.

The fertilization is internal and reciprocal and zygotes are laid as single or grouped in cocoons, depending on the species. The developmental time is also very variable among species, however after an initial period of growth inside the cocoon a juvenile starts spinning inside the eggshell until hatchling (Ladurner and Rieger 2000; Semmler, Bailly et al. 2008). Juveniles have the exact same bauplan than their adult stage although the reproductive system is not yet formed. Neoblasts and primordial germ cells are present already in freshly hatched worms, at least in the two species we have studied (personal observations). In *I. pulchra,* and probably in other species, the formation of the copulatory organs anticipates the differentiation of mature gonads (Fig.2G and manuscript R2).

1.4 Development.

Acoels have an early embryonic cleavage that is unique in the animal kingdom. At the second cleavage, *i.e.* when 2 cells-embryo divides to generate a the 4 cells stage, the 2 cells divide unequally and with the mitotic spindle oriented 45 degrees with respect to the animal vegetal axis. As a consequence, the plane that cuts through the two new-born cells with smaller in size (micromeres) appears shifted with respect to the plane that cuts though their bigger sisters

(macromeres) (Henry, Martindale et al. 2000). As development follows, new micromeres arise and always shifted in the same direction. Careful cell lineaging conducted in the species *Neochildia fusca* has shown that the first three duets of micromeres generate all ectodermal structures, *i.e.* the epidermis and nervous system, and that the third macromeres duet is committed to endo-mesodermal structures, *i.e.* the digestive system, the parenchyma and muscles (Henry, Martindale et al. 2000). Myogenesis, and most likely neurogenesis too, are initiated after gastrulation is completed at the anterior-animal pole (Ladurner and Rieger 2000; Semmler, Bailly et al. 2008) (Chiodin et al., manuscript submitted).

1.5 Phylogeny

Since they were first described, acoels and their relatives have taken a long journey through the bilaterian tree and to date, with the advent of modern tools for phylogenomic analyses, they might not have settled yet (Ruiz-Trillo, Riutort et al. 1999; Egger, Steinke et al. 2009; Hejnol, Obst et al. 2009; Philippe, Brinkmann et al. 2011).

Acoels, and their sister group the nemertodermatids (Ruiz-Trillo, Paps et al. 2002; Hejnol, Obst et al. 2009; Philippe, Brinkmann et al. 2011), were initially affiliated to the Plathelminthes, and considered to be their most basal representatives because of their lack of excretory organs and the presence of a poorly centralized nervous system. However this affiliation, based on the gross similarity of body plan organization, with characteristics such as a the presence of a blind gut, the lack of body cavities and neoblasts, was questioned by several authors who pointed out the lack of strong synapomorphies uniting the

group (Smith, Tyler et al. 1986; Haszprunar 1996; Carranza, Baguña et al. 1997). It was only in 2009 that Egger and colleagues showed that acoels and rhabdithophoran flatworms share the unique ability of replacing epidermal cells exclusively from neoblasts located in the mesoderm (or parenchyma, but see the discussion for the argument of using mesoderm) (Egger, Steinke et al. 2009). This unique feature might be a solid synapomorphy between acoels and rhabdithophorans, but would nevertheless exclude nemertodermatids and catenulids (they have stem cells in the epidermis), which have typically been nested inside the "classical" Plathelminthes too. The same group also approached the question of acoel affinities with molecular tools recovering the acoels as the earliest branching members of the Bilateria (Egger, Steinke et al. 2009). Interestingly, such an early branching position for the Acoela has been recovered in almost all molecular phylogenies published in the last fifteen years (Ruiz-Trillo, Riutort et al. 1999; Ruiz-Trillo, Paps et al. 2002; Egger, Steinke et al. 2009; Hejnol, Obst et al. 2009), and this was one major reason to attract the interest of molecular developmental biologists who wanted to investigate the early evolution of bilateral animals (Baguñà and Riutort 2004; Baguñá, Martinez et al. 2008; Hejnol and Martindale 2008; Hejnol and Martindale 2008; Wallberg 2009)

During the last few years several phylogenetic analyses have been carried out, using different genes and different species. Importantly one of the most comprehensive analyses, in terms of included gene loci and number of sampled species, has found strong support for the monophyly of the Acoelomorpha (Xenoturbellida+(Nemertodermatida+Acoela)) and has placed it as sister-group of all other Bilateria (Hejnol, Obst et al. 2009) (Nephrozoa, *sensu* (Jondelius, Ruiz-Trillo et al. 2002)). The inclusion of *Xenoturbella* within the

Acoelomorpha matched earlier propositions (Lundin 1998) and "hijacked" *Xenoturbella* from its affiliation with the deuterostomes (Bourlat, Nielsen et al. 2003; Telford 2008). In this scenario, features like an un-segmented acoelomate bodyplan, the lack of a centralized nervous system, the presence of a blind gut and the lack of excretory organs as well as a reduced *Hox* genes complement and paucity of microRNAs accounted for the Bilateria ancestral state (Cook, Jiménez et al. 2004; Sempere, Martinez et al. 2007; Baguñá, Martinez et al. 2008; Hejnol and Martindale 2008; Hejnol and Martindale 2008; Moreno, De Mulder et al. 2010; Nielsen 2010).

However, another recent phylogenomic study that uses less sequence data but include the analysis of mitochondrial genomes and adds *Xenoturbella*, nemertodermatids and more acoel species, keeps the Acoelomorpha (re-named by the authors Xenacoelomorpha) as a monophyletic clade but within the deuterostomes, therefore implying that their morphological, and probably molecular, simplicity is due to loss of characters (Philippe, Brinkmann et al. 2011).

In my opinion, it seems that only whole genome sequence data will clarify the phylogenetic status of the Acoelomorpha inside the Bilateria, although it seems to me that at least the monophyly of the group Acoelomorpha (Xenoturbellida+ (Nemertodermatida+Acoela)) can be already taken for granted.

1.6 Ancestral and derived traits within the Acoelomorpha

The uniting features of the Acoelomorpha are the overall similar body plan organization and a multiciliated epidermis with similar ciliary ultrastructure (both the complex rootlet system at the ciliary tips)(Fig.3Fig). Nevertheless, these morphological traits are not unique to the group, therefore at present there is not strong synapomorphy supporting the monophyly of the group. Another shared feature within the taxon is the lack of the PG3-class Hox gene orthologues (Cook, Jiménez et al. 2004; Moreno, Nadal et al. 2009; Nielsen 2010) and our own genome data, unpublished).

Even when *Xenoturbella* was excluded from the taxon Acoelomorpha, it was always clear to the morphologists that the Acoela exhibits more derived traits than its sister group the Nemertodermatida (Smith and Tyler 1985; Haszprunar 1996). These earlier observations are now corroborated by the placement of *Xenoturbella* within the Acoelomorpha (Fig. 3 and Fig. 4B). Consistent with these intra-group affinities, for example, an evolutionary trend can be traced for the nervous system, starting with the presence of an intraepithelial diffuse nerve net of *Xenoturbella* (Raikova, Reuter et al. 2000), leading first to the weak ring-like anterior concentration of neurons and weak neurite bundles in nemertodermatids (Raikova, Reuter et al. 2000; Raikova, Reuter et al. 2004) and later to the submuscular nervous system of the acoels with a commissural or bilobed brain, plus the neurite bundles (reviewed in (Achatz, Chiodin et al. 2012). Another striking feature, but in agreement with the more derived state of the Acoela, is the detection of a thick extracellular matrix in *Xenoturbella* (Ehlers and Sopott-Ehlers 1997), which is absent in the acoels (except a thin rim surrounding the statocyst). Again the nemertodermatids represent an

intermediate state, having small isolated islands of ECM scattered along the body (Rieger, Tyler et al. 1991).

Xenoturbella and the nemertodermatids have uniflagellate sperm cells (Boone, Bert et al. 2011; Obst, Nakano et al. 2011) and an epithelial gut, all plesiomorphic eumetazoan traits. Acoel sperm cells, though, are bi-flagellated (Tekle, Raikova et al. 2007) (Rieger, Tyler et al. 1991; Petrov, Hooge et al. 2004) and the digestive system most commonly consists of a digestive syncytium with wrapping parenchymal cells. However the digestive system in the basal acoel *Paratomella rubra* is not syncytial and might represent the intermediate state between the epithelial and syncytial gut (Smith and Tyler 1985; Rieger and Ladurner 2003).

Likewise, a line of increasing complexity can be traced when focusing on the genital organs. In *Xenoturbella* there are no genital organs; in the nemertodermatids there is only a simple male antrum, and finally the acoels display a large variety of strongly muscular, female and male, genital organs (Hooge and Tyler 2005; Petrov 2005; Petrov, Hooge et al. 2006). The lack of genital organs in *Xenoturbella* is most likely correlated with external fertilization, a character that is considered ancestral (Ehlers and Sopott-Ehlers 1997; Lundin 1998; Obst, Nakano et al. 2011). In the acoels instead, the genital organs have most likely evolved as an adaptation to optimize the internal fertilization (Achatz, Hooge et al. 2010).

In conclusion *Xenoturbella* retains traits that are apparently plesiomorphic and therefore features like the submuscular nervous system, the complex patterning of the body wall musculature and a reproductive system with mesodermally located gonads and genital organs must have been evolved independently in the

in lineage leading to the acoels, regardless of the phylogenetic position of the Acoelomorpha.

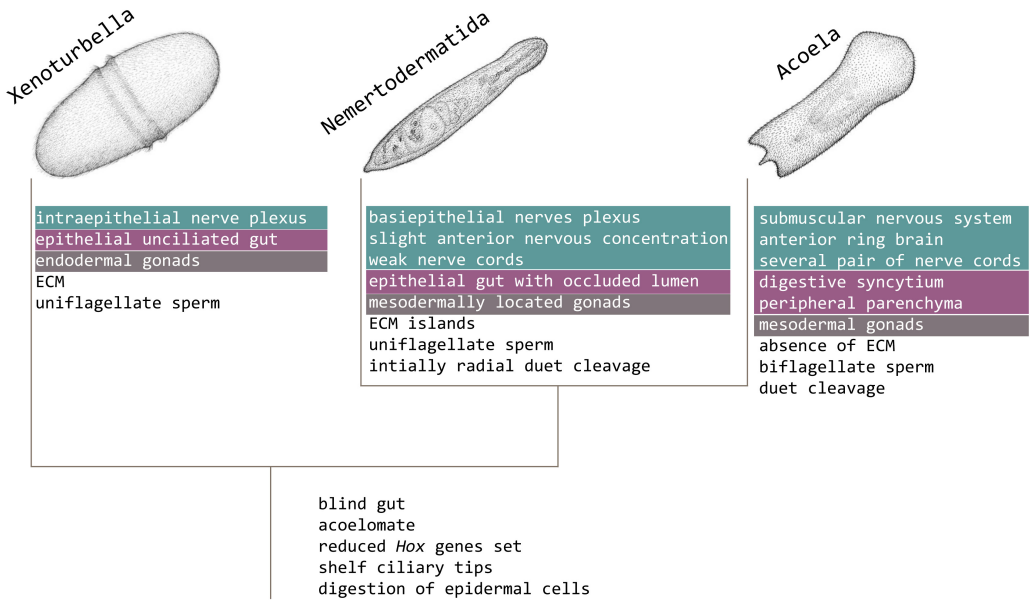


Fig.3 Acoelomorpha internal relationships.

An evolutionary trend can be drawn from Xenoturbella, which has the more ancestral morphological traits, to the more derived Acoela. The Nemertodermatida fits nicely the intermediate state linking *Xenoturbella* to the Acoelomorpha. The drawing of the worms are from (Nielsen, 2012).

1.7 Species included in this study

1.7.1 *Symsagittifera roscoffensis*

The species belongs to the family Sagittiferidae, but see Jondelius et al. 2011 for a recent synonymy of this family with Convolutidae (Jondelius, Wallberg et al. 2011). These animals are identified by the presence of green symbiont algae and sagittocyst. These are highly sclerotized structures, mostly used for copulation, defense and probably prey capture (Gschwentner, Baric et al. 2002). *Symsagittifera roscoffensis* lives abundantly in the north-east Atlantic coasts, having been described in locations from England to Portugal. During day light low tides, the worms move to the sand surface to allow the photosynthetic metabolism of the symbionts. In our experience a great number of specimens can be easily collected avoiding culture contamination with other organisms. They reproduce during the whole year; although the peak is reached between April to June (in the french population, Brittany). The fertilized eggs are laid in cocoons that during the 'reproductive season' can contain up to 30 embryos. Development lasts approximately 5 days, although this time can change during the warmest months. A white juvenile hatches and keeps feeding for about two to three weeks. After this time it ingests algae of the genus *Tetraselmis* (Semmler, Bailly et al. 2008) that will be kept as symbionts in the parenchyma, and starts developing the reproductive system. The gonads are paired; the testes are located dorsal to the ovaries. A female gonopore is present between the anterior mouth and the most posterior male gonopore, the latter a highly muscular structure that supports a simple antrum (Semmler, Bailly et al. 2008; Chiodin, Achatz et al. 2011). As most of the sagittocyst-bearing species, a penis

is absent. The sagittocysts are likely used to injure the body wall of the mating partner in preparation for the transfer of sperm. The ontogeny and the architecture of the muscular system have been described in great detail (Semmler, Bailly et al. 2008). Myogenesis is initiated at the anterior pole, where circular, longitudinal and diagonal muscles arise simultaneously. The muscular pattern is quite complex already at the juvenile stage, especially the dense net of parenchymal muscles. U-shaped muscles are present at the ventral side, most likely standing for the lacking pharynx (Semmler, Bailly et al. 2008). The nervous system has been thoroughly described by TEM and 3D resconstruction methodologies and also using immunological reactions (Bery, Cardona et al. 2010; Semmler, Chiodin et al. 2010). Expression studies are available for a handful of regulatory genes (Moreno, Nadal et al. 2009; Semmler, Chiodin et al. 2010). We have also developed the first species-specific molecular tissue markers (Chiodin, Achatz et al. 2011).

Last but not least, ESTs data and BAC libraries are available. Moreover, a whole genome sequencing project is about to be completed.

1.7.2 *Isodiametra pulchra*

This species lives abundantly in the mud of the west north Atlantic coasts, from which it is extracted through a laborious method which implies the use of magnesium chloride for anesthetization followed by separation of specimens, one by one, from the other interstitial organisms that share the same habitat. At present it is possible to keep *Isodiametra pulchra* cultures under standard at laboratory condition.

Adult specimens are about 500 µm long, they are easily cultured in glass Petri dishes kept at 18°, 12 hours light-dark cycle, and fed with diatomees. On average one worm lays one egg per day during the whole year.

The anatomy of the worm is well known as well as it is the stem cell system. The first molecular characterization of the acoel neoblasts has been carried out in *I. pulchra*, and, importantly, functional assays of gene activity can be carried out during homeostasis and regeneration (De Mulder, Kuales et al. 2009; Moreno, De Mulder et al. 2010). Moreover a working protocol for RNA interference during embryonic development will soon be available (Andreas Hejnol, personal communication).

As for *S. roscoffensis*, the nervous system has been described at the ultrastructural level (Achatz and Martinez In press) and the ontogeny and architecture of the muscular system in *I. pulchra* are known in detail (Ladurner and Rieger 2000). Like in other higher acoels, the brain is bilobed, the two lobes being connected by ring- shaped commissures anterior and posteriorly to the statocyst. Four pairs of strong longitudinal neurite bundles exit the brain towards the posterior rear where the CNS becomes much less prominent (Achatz and Martinez In press). The muscular pattern also reflects the quite derived state of the species. As mentioned above, the ontogenic process of myogenesis is known from the very early events. Initially single myocytes appear in a parallel arrangement at the anterior-animal pole. They subsequently elongate and join each other to form the first circular fibers. Although the posterior circular muscles arise later than the anterior ones, they are not built in a regular antero-posterior progression. The longitudinal muscles are formed later than circular ones following a similar mechanism (Ladurner and Rieger 2000). The adult musculature differs from the juvenile musculature by the

presence of the ventral components associated to the copulatroy organs and gonads. The female bursa has one, and sometimes more than one bursal nozzle, whereas the male genital organ consists of a muscular antrum and a penis (Tyler and Rieger 1999; Hooge and Tyler 2005). The gonads are paired, parenchymally located in close proximity to the region where most of the neoblasts are found. Primordial germ cells and neoblasts are present in freshly hatched worms suggesting an early embryonic segregation of these cell types (De Mulder, Kuales et al. 2009). Maturation of juveniles into adults takes about two weeks although the anlage of the copulatory organs is already observed in juveniles younger than one week (Fig.2G). At present, *I. pulchra* is the most promising acoel model system for developmental studies because of the possibility of keeping stable laboratory cultures and the availability of genetic functional tools for analysis.

2. Animals: bodyplans, molecules and phylogenetic hypothesis

2.1 Metazoa and Eumetazoa novelties

Animals (Metazoa) are multicellular, eterotrophic organisms that reach their body plan complexity through development from a single cell or zygote (Nielsen 2012). The majority of animals are bilaterally symmetric (Bilateria), *i.e.* they have an antero-posterior (AP) axis which define the plane of left-right symmetry and a dorso-ventral axis (DV); a smaller percentage of metazoans do not exhibit bilateral symmetry and are characterized by a lower morphological complexity than the Bilateria and are consistently recovered in more basal position in all molecular phylogenies (Pick, Philippe et al. ; Dunn, Hejnol et al.

2008; Hejnol, Obst et al. 2009; Edgecombe, Giribet et al. 2011). Despite their morphological simplicity, full genome sequencing of the principal non bilateral taxa have revealed quite complex genome architectures and the presence of most classes of transcription factors (Putnam, Srivastava et al. 2007; Srivastava, Begovic et al. 2008; Srivastava, Simakov et al. 2010), which by binding to *cis*-regulatory modules of downstream genes orchestrate development (Davidson and Erwin 2006).

The likely paraphyletic sponges are the earliest branching metazoans and have polarized cells forming layer although the cells are not sealed to each other by junctions thus they are not structured as epithelial tissue layers (Degnan, Vervoort et al. 2009; Srivastava, Simakov et al. 2010; Nielsen 2012).

A sealed epithelium is an apomorphy of the Eumetazoa. Placozoans must be considered eumetazoans because they have an epithelium, with digestive activities localized in its ventral side. It is settled that placozoans have diverged after the sponges and are the sister group to Cnidaria+Bilateria (Srivastava, Begovic et al. 2008). Placozoans and sponges lack contractile cells (muscles, but see below for different muscle types) and neurons, which were instead present in the last Cnidaria-Bilateria ancestor (Nielsen 2012). Ctenophores also possess muscles and neurons, but their phylogenetic position is controversial and therefore they are not discussed herein (Martindale and Henry 1999; Dunn, Hejnol et al. 2008; Hejnol, Obst et al. 2009; Nielsen 2012).

Cnidarians, which form the sister group to the Bilateria, are organized in two tissue layers and have a basal lamina, *i.e.* they are diploblastic. The inner endoderm (or gastrodermis) lines the gastric cavity, which corresponds to the archenteron (the embryonic and larval digestive system) and has a single digestive opening (the embryonic blastopore). The outer ectoderm contains

intraepithelial neurons that extend their projections basi-epidermally forming a diffuse nerve net (Ruppert, Fox et al. 2004; Nielsen 2012). Epithelial cells located in the ectoderm and in the gastrodermis can contract, as they bear contractile filament at their basal portion, and are therefore named epithelio-muscular cells (Ruppert, Fox et al. 2004) (Fig.6). This is the basic organization of basal cnidarians, although in the higher classes derived traits such as the presence of sensory organs and muscles have been reported (Stierwald, Yanze et al. 2004; Seipel and Schmid 2005; Steinmetz, Kraus et al. 2012). Recently most efforts have been put in developing genomic and molecular tools for the anthozoan species *Nematostella vectensis*, a representative of the less derived cnidarian class (Putnam, Srivastava et al. 2007; Renfer, Amon-Hassenzahl et al. 2010; Genikhovich and Technau 2011).

2.2 The Bilateria: the achievement of tremendously divergent body plans.

The origins of bilateral symmetry is one of the most important and debated topics in animal evolution. The lack of fossil evidences except trace records (Budd 2009) hampers the accurate reconstruction of the history of bilateral animals. Furthermore, calculations based on the use of molecular clocks suggest that bilaterians might have diverged much earlier than the Cambrian era (about 550 mya) (Peterson, Cotton et al. 2009), the time at which most of bilaterian body plans appear in the fossil record.

At present, there is general agreement in recognizing the Bilateria as a monophyletic group and their fundamental division into protostomes and the deuterostomes. Jondelius (Jondelius, Ruiz-Trillo et al. 2002) proposed the term Nephrozoa for the taxonomic group uniting protostomes and deuterostomes but

excluding the Acoelomorpha. The meaning of Nephrozoa (bilaterians with excretory organs) makes only sense when the acoelomorphs are interpreted as the sister group to all remaining Bilateria. However, given the present controversial placement of the Acoelomorpha, I will follow the terminology used by Nielsen (Nielsen 2012), *i.e.* Eubilateria instead of Nephrozoa. Thus, Eubilateria should be synonymised with Bilateria when the acoelomorphs are included within the deuterostomes (Philippe, Brinkmann et al. 2011), or to Nephrozoa when the acoelomorphs represent the earliest bilaterian offshoot (Hejnol, Obst et al. 2009).

Clear Bilateria synapomorphies are the obvious bilateral symmetry which also led to different grades of anterior concentration of neural structures and sensory organs, and triploblasty, *i.e.* including a third embryonic germ layer, the mesoderm. The AP staggered spatial expression of *Hox* genes and DV patterning mediated by *Bmp2/4* and *chordin* signalling also seems to have evolved in the stem Bilateria. A similar genetic patterning of the body axes appears to have evolved independently in some cnidarians lineage (Matus, Thomsen et al. 2006).

Other Eubilateria novelties are: the presence of a through gut with mouth and anus and a centralized nervous system (De Robertis and Sasai 1996; Arendt, Technau et al. 2001; Carroll, Grenier et al. 2005; Arendt, Denes et al. 2008; De Robertis 2008). Obviously, these might have been lost in the acoelomorphs if they are deuterostomes.

Whether segmentation of the body plan must be included in the list of ancestral eubilaterian traits, is not yet resolved (Erwin and Davidson 2002; De Robertis 2008).

Other key issues such as whether the digestive openings, the centralized nervous system and the mesodermal body cavities are homologous or not are central to the formulation of all hypothesis of bilaterian evolution (see sections below).

2.3 The Eubilateria

The protostomes form a quite heterogeneous group of animals characterized by the presence of a dorsal brain and paired ventral nerve cords. The group includes organisms as different as *e.g.* molluscs and insects. Gastrulation, *i.e.* the developmental process that leads to the separation of the three germ layers, occurs by a wide set of different mechanisms but it is usually assumed that there are two mesodermal sources, at least in spiralians: the ecto-mesoderm and the endo-mesoderm, which forms respectively from animal-ectodermal and vegetal-endodermal precursors (Boyer, Henry et al. 1996; Boyer, Henry et al. 1998; Technau and Scholz 2003; Lambert 2008). The fate of the blastopore is very variable among protostomes and does not reflect the etymology of the name (protostomes, mouth first) (Hejnol and Martindale 2009).

In the deuterostomes, instead, the blastopore always becomes the anus of the adult. Another, likely plesiomrphic character of the group, is the formation of gill slits, although this has been lost in echinoderms (Gilbert and Raunio 1997).

The nervous system is diffuse in the Ambulacraria (echinoderms +hemichordates, see below)(Lowe, Wu et al. 2003) whereas it arise as dorsal neural tube in the sister group Chordata (Gilbert and Raunio 1997). Other distinctive deuterostomes features are the enterocoelic formation of the mesoderm, by evagination of mesodermal pouches from the wall of the

archenteron, which leads to the archymeric body plan (Gilbert and Raunio 1997); and internal left-right asymmetry orchestrated by nodal signalling through the homeobox gene *Pitx* (Boorman and Shimeld 2002; Duboc, Röttinger et al. 2005). These characters, though, are not exclusive of the deuterostomes (Grande and Patel 2009).

2.4 Body cavities and segmentation

Clearly, there are variations from the aforementioned ground plan of Eubilateria, *e.g.* plathelminthes have a blind gut or echinoderms do not develop gill slits, however such traits are easily recognized as secondary modifications.
Segmentation and body cavities are most commonly present in the Eubilateria but it is difficult to make any homology statement on them.
Strikingly annelids, arthropods and chordates share a segmented body plan that consists in the serial repetition of mesodermal structures (coelomic sacs and somites, respectively) and neural structures (Nielsen 2012). The metameric bodyplan organization of these three phyla arises from a terminal posterior growth zone, and homologous genes direct molecular regulation of segmentation across the three phyla (Tautz 2004; De Robertis 2008; Saudemont, Dray et al. 2008).
Coeloms are, apparently, structures quite easy to develop(Clark 1964; Willmer 1990; Schmidt-Rhaesa 2007) and their multiple independent evolution is not difficult to imagine . However their development varies considerably across the Bilateria. There is, therefore, a common agreement that coelomic cavities are not homologous. The enterocoelic formation of coeloms is supposed to be a central step in bilaterian evolution according to the archycoelomate theory (see

below). According to this theory, the enterocoelic formation of coeloms might have great implications in our understanding of the evolution of segmented body plans (Tautz 2004), and although it has been neglected for quite some time I prefer to introduce its main concepts below.

Before moving into a more detailed description I need to illustrate old and new phylogenetic contexts in which the main proposals for bilaterians evolution have been developing.

2.5 Old and new phylogenies: internal revolutions of astonishing impact

Figure 4 illustrates old (left, A) versus new (right, B) phylogenetic schemes for metazoan relationships. One first obvious difference between the two topologies, is the increased taxon sampling of the new molecular-based phylogeny (Fig.4B) with respect to the laborious morphology-based one (Fig. 4A). The drop in the costs of DNA sequencing and the improved calculation capacity of modern super-computing centers offers the advantage of allowing the use of greater data sets that produce results (*i.e.* tree topologies) amenable to statistical testing (Dunn, Hejnol et al. 2008; Hejnol, Obst et al. 2009; Edgecombe, Giribet et al. 2011).

The traditional phylogenetic scheme, much influenced by the impressive work of Libbie Hyman (Hyman 1940), relies on the concept of "increasing complexity" and thus results in a progressive series of acoelomate-pseudocoelomate-coelomate taxa (although Hyman never awarded them with phylogenetic significance (Jenner 2004)). Accordingly, acoelomate plathelminthes (including acoelomorphs, and Hyman stressed their likely

ancestral traits within the plathelminthes) were placed at the base of the Bilateria, whereas the pseudocoelomates provided the missing link between the acoelomates and the 'higher coelomates'. As seen above, the present agreement on the independent evolution of coeloms regard as this gradual sequence as spurious.

Perhaps the greatest contribution of the modern molecular phylogenies was the introduction of the Spiralia (former Lophotrocozoa, but see (Hejnol 2010)) and Ecdysozoa groupings into the protostomes phylogeny, although several of their internal relationships remain obscure. Spiralia includes all spiral cleavaging taxa plus some others, *e.g*. lophophorates; Ecdysozoa includes all moulting protostomes (Adoutte, Belavoine et al. 1999).

Among the deuterostomes a new superphylum Ambulacraria (echinoderms +hemichordates) was erected, and the cephalochordates have replaced the urochordates as the most basal branch of the chordates (Swalla and Smith 2008). Brachiopods and phoronids, formerly included in the deuterostomes have found a stable place within the Spiralia (Edgecombe, Giribet et al. 2011). The affiliation of chaetognaths to the protostomes has finally gained some support, although its specific placement remains enigmatic (Matus, Copley et al. 2006; Edgecombe, Giribet et al. 2011). Finally, resolving the key acoelomorph position should prove crucial for the understanding of bilaterian evolution, as each of the two proposed acoelomorph positions lead to absolutely contrasting conclusions on the evolution of different traits.

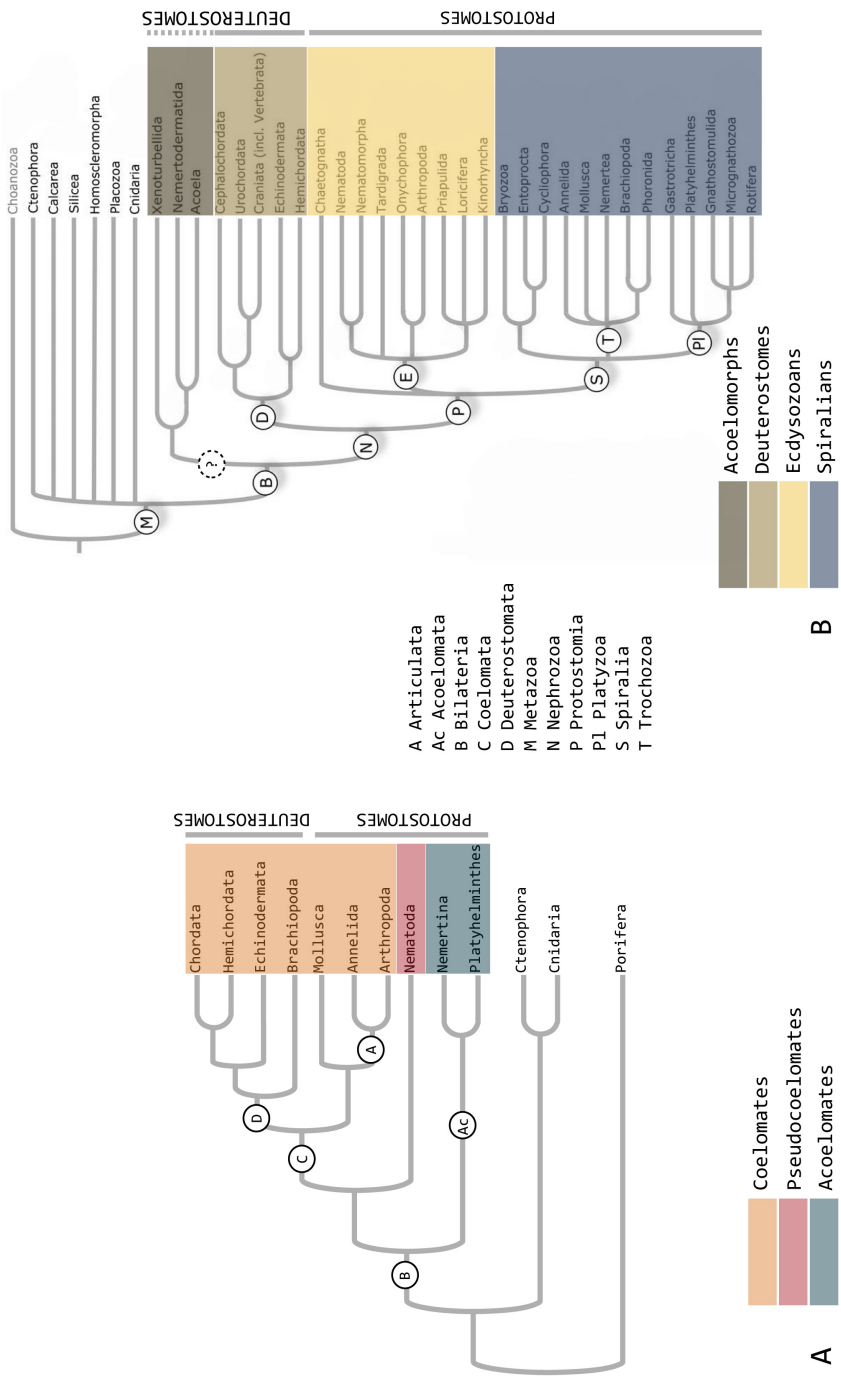


Fig.4 Comparison between old (A) and new (B) phylogenetic hypotheses.

A. Modified from (Adoutte, Belavoine et al. 1999). B. Modified from (Edgecombe et al. 2011)

3. Hypothesis on bilaterian evolution.

The planuloid/acoeloid and the archycoelomate theories of bilaterians evolution have much influenced authors interested in the subject and most of the alternative proposals can be fairly considered variations upon the central themes of these two hypotheses. I also consider these two theories as the most relevant in the interpretation of the data generated in my PhD thesis (Fig.5).

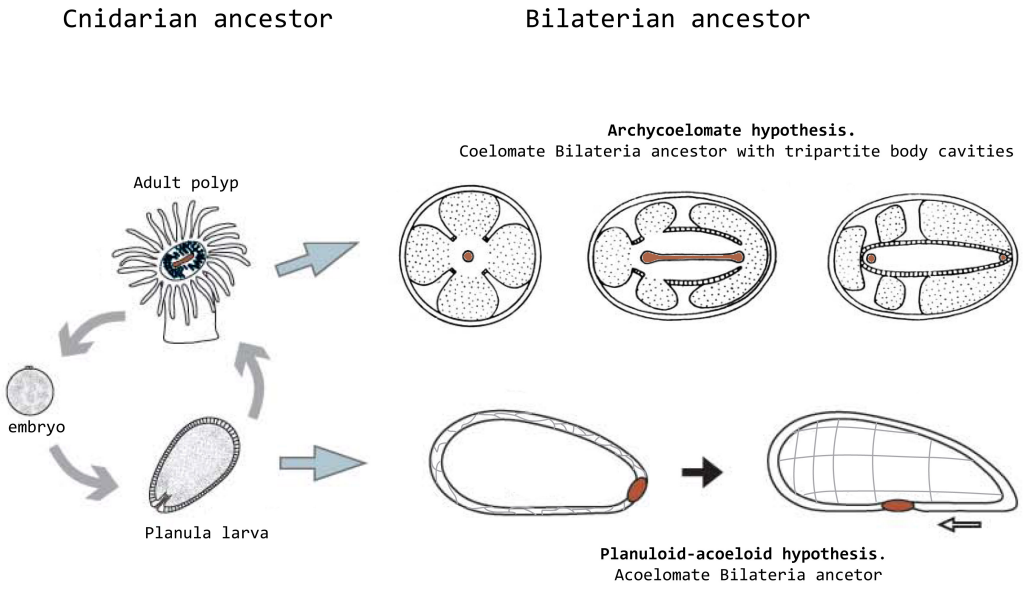


Fig.5. The two major hypotheses on bilaterian body plan evolution.

On the top the archycoelomate theory (modified from Tautz, 2004) and on the bottom the planuloid/ acoeloid theory (modified from Hejnol and Martindale, 2008). See text for details. The cnidarian digestive opening and the bilaterian blastopore-digestive openings are in red. Note that the planula has an intra-epidermal nervous system, whereas it is sub-epithelial in the bilaterian ancestor.

3.1 The acoeloid/planuloid theory

The acoeloid-planuloid theory proposes that the last common cnidarian/ bilaterian ancestor was an organism with a comparable morphological complexity to that of extant cnidarians larvae (planulae) (Fig.5, bottom) (von Graff 1891; Hyman 1951; Salvini-Plawen 1978). Planulae are diploblastic organisms, with a single opening, the blastopore, which is formed at gastrulation. They have a pelagic life style and swim by ciliary beating, although they also have epithelio-muscular cells and an intraepithelial diffuse nerve net. As a consequence of the adaptation to a creeping benthic life style, such planula-like ancestors would have evolved an anterior concentration of neural structures and shifted their blastopore to the ventral side of their body. Therefore, it was assumed that the organism derived from this process would have an astonishing similar complexity to that of extant acoelomorphs. As in the planulae, the acoelomorphs have a single digestive opening and lack a dorso-ventrally centralized nervous system, but in contrast to them the acoelomorphs present some more advanced features such as an anterior concentration of neural structure and the presence of muscles. Consequently, the supporters of the planuloid/acoeloid theory regard the acoelomorphs as the best proxies for the bilaterian ancestor.

The acoeloid/planuloid theory was initially proposed by von Graff (von Graff 1891)and developed by Hyman (Hyman 1951) who proposed the gradual transition from a bilaterian acoelomate to a bilaterian coelomate body plan organization . In her work, Hyman already proposed to use the acoelomorphs as models to infer the evolution of other bilaterian body plans, although she grouped them within the plathelminthes. Hyman's proposal has been initially

challenged by the new animal phylogeny (Fig.4B), which places the plathelminthes within the Spiralia, implying their secondary loss of morphological complexity. However, more recently, the interest in her thesis has been largely renewed by the placement of the Acoelomorpha at the base of Bilateria (Ruiz-Trillo, Riutort et al. 1999; Egger, Steinke et al. 2009; Hejnol, Obst et al. 2009).

3.2 The archycoelomate hypothesis and conflicting concepts to the acoeloid theory

The archycoelomate theory proposes that the bilaterian coelomic cavities have derived by the closure of the gastric pouches in an anthozoan-like diploblastic ancestor (Sedgwick 1884; Remane 1963) (Fig.5, top). By this process, a coelomate body plan, whit tripartite body cavities (archymeric), as observed in *e.g* hemichordates or brachiopods (Nielsen 2012), would have been present at the origins of the Bilateria radiation whereas the acoelomate body plan, like in the plathelmithes, would have arisen by secondary simplification (Rieger 1985). This process is indeed exemplified in *e.g.* extant acoelomate interstitial annelids (Fransen 1980; Rieger, Purschke et al. 2005).

Furthermore, the process of coelom formation in the anthozoan-like ancestor is linked to a shift of 90 degrees of the oral-aboral axis that in turn becomes the antero-posterior (AP) axis of extant Bilateria. Following this remodelling of the body plan, the digestive opening would have elongated along the new AP axis and subsequently closed medially, leaving two digestive openings, namely the anterior mouth and the posterior anus of extant bilaterians (reviewed in (Tautz 2004).

The advantages of this model are that both the coelomic pouching from the wall of the archenteron (enterocoely), and the closure of a slit-like blastopore (amphistomy) are observed in extant bilaterias, *e.g.* in ambulacraria and cephalochordates the enterocoely (Gilbert and Raunio 1997) and in polychetes the amphistomy (Arendt, Technau et al. 2001).

The evolution of the nervous system is not much contemplated in the archycoelomate theory per se. However it must be said that this theory predicts a complex bilaterian ancestor, with a coelomate and segmented body plan (Carroll, Grenier et al. 2005; De Robertis 2008), and although not integral part of the theory, their supporters also predict complexity in the organization of the nervous tissue, *i.e.* likely to be centralized already in the bilaterian ancestor.

The inverted expression of homologous dorso-ventral patterning genes, namely *Bmp* and *chordin* between protostomes and deuterostomes supports this notion (De Robertis and Sasai 1996; Arendt and Nübler-Jung 1997), but only when data from the hemichordates, which have a diffuse nerve net and differential *Bmp-Chordin* expression, are unregarded (Lowe, Terasaki et al. 2006).

4. The mesoderm

The three bilaterian embryonic germ layers, namely the endoderm, the mesoderm and the ectoderm segregate at gastrulation, and during further development they differentiate into all tissues and cells types of the organism. The endoderm mostly differentiate into the mid gut (fore and hindgut are ectodermal), and occasionally in few other organs; the ectoderm fate is giving rise to epidermal and neural structures (including sensory organs) whereas the mesoderm differentiate into a variety of structure including muscles, connective

tissues, body cavities, gonads, and in some taxa into inner organs (Technau and Scholz 2003).

The diploblastic Cnidaria and Ctenophores have muscular cells in between the endo and the ectoderm, and their homology to the bilaterian muscles has long been questioned (reviewed in Burton 2008). Initial evidences from molecular and developmental studies on the jellyfish *Podocoryne carnea* (class Hydrozoa) led some authors to the provocative conclusion that mesoderm might have originated before the divergence of Cnidaria and Bilateria (Seipel and Schmid 2005; Seipel and Schmid 2006).

Hydrozoan jellyfishes develop by budding out of the polyp trunk. During bud development, a tissue layer called entocodon detaches from the bud ectoderm and differentiates later on, into the musculature of jellyfishes. Interestingly the entocodon express a cassette of genes that are homologous to bilaterian mesoderm regulatory genes (Spring, Yanze et al. 2000; Spring, Yanze et al. 2002; Müller, Seipel et al. 2003). Similarly, the mature muscular fibers of the jellyfishes have the ultrastructure of bilaterian striated muscles with which they share the expression of key structural genes (Schuchert, Reber-Muller et al. 1993; Groger, Callaerts et al. 1999; Seipel and Schmid 2005). However, recent high throughput genomic level investigations for all molecular components of bilaterian muscles clearly show that these cell types evolved independently in the Cnidaria and Bilateria lineages, thus their ‘mesoderm’ (muscles) cannot be considered true homologues (Steinmetz, Kraus et al. 2012). The results of Steinmetz and colleagues (Steinmetz, Kraus et al. 2012) are very important because they show that muscular cells can evolve quite easily. In fact, only the most derived cnidarians classes have true muscles (Burton 2008), whereas the more basal anthozoans are bi-layered, and their contractile cells are exclusively

epithelio-muscular (Fig.6). Anthozoan cnidarians possess most of the genes that are known to specify the bilaterian mesoderm, and all these genes are expressed in the anthozoan endoderm (Fritzenwanker, Saina et al. 2004; Martindale, Pang et al. 2004; Magie, Pang et al. 2005; Genikhovich and Technau 2011). These evidences might indicate that the mesoderm arose from the endoderm, eventhough in several bilaterians double endodermal and ectodermal sources of mesoderm exist (see above).

4.1 Schizocoely and enterocoely: how does the mesoderm develop (and evolved)?

The bilaterian mesoderm develops according to two mechanisms: enterocoely and schizocoely (Technau and Scholz 2003).

Enterocoely is mostly observed in deuterostomes (mainly ambulacrarians and cephalochordates), and in the protostome chaetognaths. It usually gives rise to tripartite body cavities (the archymeric body plan). This organization is observed also in brachiopods and phoronids (Lüter 2000; Freeman and Martindale 2002), though they do not have an strict enterocoelic development (at least brachiopods develop the mesoderm from the larval archenteron, but not by evagination of its wall). Because the enterocoelic process involves outpouching from the embryonic intestine, the resulting coelomic cavities are lined by an epithelium, or coelothele.

Schizocoely involves proliferation of cells usually located at the blastoporal margin, and will eventually form an epithelial lined body cavity from an initial compact cell mass (Technau and Scholz 2003; Lambert 2008).

Perhaps, schizocelous mesoderm formation is better exemplified by the spiralian taxa. In Spiralia the early embryonic development is highly stereotyped and the whole endomesoderm derive from the proliferation of the single blastomere 4d, also called the mesentoblasts (Boyer, Henry et al. 1996; Lambert 2008; Hejnol 2010). The 4d progeny expands within the embryo and distributes in two bilateral bands, symmetrical with respect to the future AP axis of the organism. Eventually, fluid pressure between the mesenchymal cells forces the formation of a cavity whose surrounding cells regorganize themselves into an epithelium (Technau and Scholz 2003; Schmidt-Rhaesa 2007). These cavities are serially formed along the anteroposterior body axis leading, in annelids, to the formation of segmentally repeated coelomic sacs (reviwed in (Tautz 2004).

In spiralians, however, the mesoderm also can arise from the second and/or the third micromere quartets of the ABC quadrants. These micromeres are ecto-mesodermal precursors, and therefore the mesodermal structures that differentiate from them (mainly larval muscles) are called ecto-mesodermal.

To my knowledge there are only a few cases of ectomesoderm in Ecdysozoa (Cannon 1925), and none in the Ambulacraria. The vertebrates' neural crest differentiate into neural and head mesodermal structures (including face's muscles and bones) and therefore the latter should be, in part, regarded as ectomesodermal structures. The neural crest derive from the ectodermal neural plate, which they abandon to migrate to the prospective head region of the organism, among other locations (Gilbert and Raunio 1997).

In conclusion, although endomesodermal and ectomesodermal sources exist, there is a general agreement that the mesoderm has evolved from the endoderm. Indeed all bilaterians have endomesoderm whereas only few lineages also have

ectomesoderm which nevertheless differentiates into only a small subset of mesodermal structures.

The expression of bilaterian mesoderm orthologues in cnidarians' endoderm nicely corroborates the notion that the mesoderm evolved from the endoderm (Martindale, Pang et al. 2004). If the acoelomorphs remain as the earliest branching bilaterian lineage, the formation of acoel mesodermal structures exclusively from the endoderm will further support this notion (Henry, Martindale et al. 2000).

4.2 Body cavities, connective tissues and muscles.

There are primary and secondary body cavities. A primary body cavity can be considered as the remnant of the blastocoele, *i.e.* the hollow central space of blastula embryonic stage (Schmidt-Rhaesa 2007). A primary body cavity is not lined by any tissue but instead it is encircled by the basal lamina of the outer epidermis (ectoderm) and the inner gastrodermis (endoderm). As such, a primary body cavity cannot be considered a mesodermal derivative.

The secondary body cavities or coeloms instead are mesodermal derivatives, because they are surrounded by a mesodermal epithelium, also called coelothel (Schmidt-Rhaesa 2007). The coelothel can be a simple epithelium, or peritoneum, or a myo-epithelium. The body cavities are filled with fluid, incorporating gases and nutrients, and therefore they function as efficient systems for the transport of molecules to the periphery of the body. Indeed the development and evolution of effective circulatory system is intimately linked to the presence of body cavities. Fluid filled cavities however are also successfully used as supporting skeletons. The compression of the coelomic

fluid between two different compartments of the body allows the changes in body shape (Ruppert, Fox et al. 2004; Schmidt-Rhaesa 2007). Logically the evolution of body cavities allowed the evolution of larger body sizes as well as the evolution of new feeding and locomotory behaviours, resulting in successful occupation and adaptation to new ecological niches and consequently to the genesis of organisms' diversity. In several cases body cavities evolved into excretory system (metanephridia) or provided the space for the development of other organs, for example heart and gonads (Schmidt-Rhaesa 2007).

For all the above-cited reasons, it is therefore logical that body cavities have been a central theme in the discussions of the evolution and diversification of bilaterian body plans.

Besides forming the coelomic cavities, the mesoderm also forms connective tissues and muscles. Very different types of connective tissues exist across the Bilateria and more than one type can be present in the same organism; they are not discussed herein.

Muscles are instead present in all Eumetazoa; they play key roles in animals' evolutionary radiations and evolution and therefore they deserve special attention. They are briefly introduced in the next section.

5. The Musculature

Two basic types of contractile cells are present in the Eumetazoa. These are distinguished as epithelio-muscular cells and myocytes. The former are epithelial polarized cells, where a set of contractile filaments are arranged in the basal portion of the cells. These cells organize themselves forming tight epithelial layers. Myocytes instead are not polarized cells and they lack the epithelial apical portion (Fig.6). The myocytes usually aggregate into fibers and/or bundles, which indeed form what it is commonly known as a true muscle. Muscles can be divided in two main types according to their histological aspect. These are the smooth and the striated muscles, which most commonly are, respectively, mono or polynucleated. A third type, the oblique striated muscle, is an intermediate type between the smooth and striated muscles (Schmidt-Rhaesa 2007). Because the histological appearance of smooth and striated muscles strongly depends on the organization of their contractile elements, the common molecular architecture of all muscle types is here described first.

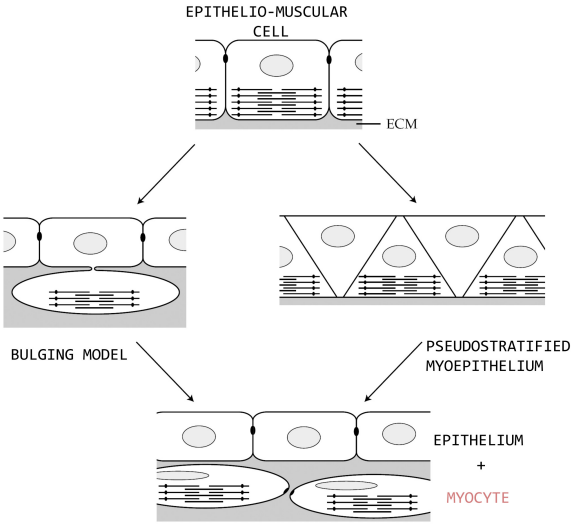


Fig.6 Models of myocyte evolution.
Myocytes arose from epithelio-muscular cells, either by separation of the contractile portion of the cell from its apical portion (bulging model), or through a pseudostratified myo-epithelium. Modfied from (Schimdt-Rhaesa, 2007).

5.1 Evolution of muscles

The epithelio-muscular cells are supposed to be the evolutionary precursors of the myocytes (Fig.6) (Schmidt-Rhaesa 2007; Arendt 2008). They are the main contractile cell types in cnidarians, actually the only one in basal cnidarians (Burton 2008), but also very spread in bilaterians, *e.g.* the amphioxus' notochord is a rope of piled up epithelio-muscular cells.

Two main models explain the evolution of myocytes through the separation of the contractile from the apical portions. In the first model, as observed in some extant cnidarians, the bulging basal portion of the epithelio-muscular cell would sink below this basal membrane (Schmidt-Rhaesa 2007) and (Fig. 6, left).

In the other model, the formation of a pseudo stratified myoepithelium, similar to that observed in the coelomic lining of extant echinoderms, precedes the formation of myocytes. The separation of the contractile cells would occur subsequently by their gradual secretion of extra cellular matrix that leads to the separation of the two layers (Rieger and Lombardi 1987). In this last model, a myo-epithelial lined coelom has necessarily to be formed prior to the formation of myocytes. The bulging model instead, does not imply the formation of any coelomic structure.

The evolution of myocytes by direct bulging of the contractile basal portion of epithelio-muscular cells below the basal membrane would suggest that myocytes represent the ancestral mesodermal cell type. However this hypothesis does not explain why this should have happened exclusively in the gastrodermis of the bilaterian ancestor, given that cnidarians have epitheliomusuclar cells in both the epidermis and the gastrodermis. The evolution of myocytes from the putative myo-epithelial coelomic lining of the

bilaterian ancestor does not contradict the endodermal origins of the mesoderm, because the coelom evolved from the endoderm itself. However this model is difficult to accept if the Acoelomorpha will settle as the sister group of Eubilateria.

5.2 The sliding filaments

The ability of nature in shaping forms perfectly adapted to their function is overwhelming. Muscles represent one of the most beautiful examples of functional adaptation. Furthermore, the growing improvements of technological sciences during the last century, has led us to a deep understanding of muscle physiology until its most subtle details.

The first observations of the muscular histology have been started in Holland almost 4 centuries ago, at the time when powerful optical lenses were being invented. Between 1670 and 1680, the microscopist van Leeuwenhoek observed that muscles are made of fibrous matter, and that this matter is composed of thinner and thicker fibres which formed units (we call them now sarcomeres) regularly arranged along the longitudinal axis of a muscle fibre. Almost 200 years later the English anatomist Bowman carried out a broad comparative histological analysis of musculature including several vertebrate and invertebrate species. He observed that in all these species, the regular units consisted of dark and light bands and that the light bands were obliterated during the contraction. These observations set the basis for the concept of the sliding filaments. Finally, in 1950 Huxley combined two powerful techniques that had been developing at the time: the electron microscopy and the X ray

crystallography, delivering a fine description of the muscular contraction (Huxley and Hanson 1954).

The clear bands of the sarcormere are bundles of thin actin filaments whereas the dark bands are bundles of the thick myosin filaments. An actin filament (F-actin) is a polymerous filament made of serially repeated actin monomers (G-actin). The ability of the G-actin to form polymers is intrinsic to its structure. A myosin molecule is instead a relatively more complex molecule (Fig.7A, top). It consists of two identical coil-coiled proteins each one having a globular head and a filamentous tail, therefore myosins are polarized molecules. In a sarcomere two myosin bundles mirror each other, *i.e.* their heads are always facing opposite directions (Fig.7A, bottom).

During the resting condition the myosin and the actin bundles only partially overlap.

In response to an increase of the intracellular calcium concentration (the stimulus for contraction), the head of a myosin can bind an ATP molecule and by hydrolizing it in a following step it undergoes a conformational change. As a consequence the myosin head interacts with the following binding site along the actin filament to, subsequently, return to its resting conformation, thereby provoking in this porcess a sliding of the thin filament upon the thick filament (Fig.7B)(Alberts, Johnson et al. 2008).

The actin filaments are firmly bound to proteic platforms, called Z bodies in smooth muscles or Z discs in the striated muscles (Fig.7C) (Schmidt-Rhaesa 2007). When the actin filaments are forced to slide on and by the myosin filaments, the Z bodies or discs get closer to each other and cause the shortening of the contractile unit (Fig.7C). The cycle of myosin-actin interaction is repeated several times during the muscular contraction and it affects all

contractile units in the cell, therefore causing an overall shortening of this cell (Alberts, Johnson et al. 2008).

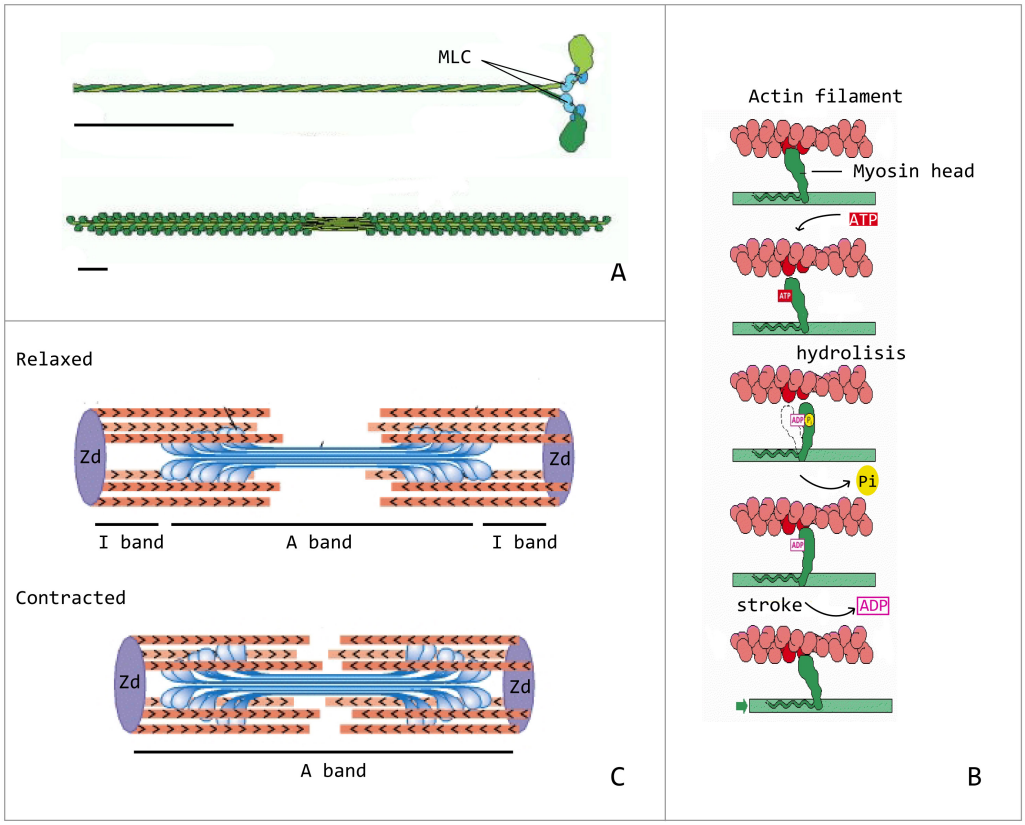


Fig.7 The sliding filaments mechanism.

A. A Myosin Heavy Chain protein (top), and a Myosin Heavy Chain bundle (bottom). MLC, Myosin Light Chain. B. Physiology of the sliding mechanism. C. Overall affect of the contraction on a sarcomere. Zd, Z-disc. Modified from (Alberts,Johnson et al. 2008)

5.3 Smooth and striated muscles: differences in the ultrastructure and in the molecular regulation of contraction.

The most obvious difference between smooth and striated (or skeletal) muscles relay in their ultrastructure. A shown in Fig.8A and B, the striated pattern of skeletal muscles is due to a highly ordered arrangement of thin and thick filaments, which lay parallel to the cell longitudinal axis. Furthermore, the proteic platforms for actin attachment (Z-bodies) are perfectly aligned with each other and distributed at regular intervals across the longitudinal axis of the muscular cell. They are so compactly organized that in fact assume the shape of a disc and therefore receive the name of Z-disc (Schmidt-Rhaesa 2007).

In smooth muscles instead, Z-bodies (so called because of their globular aspect) are sparse in the cytoplasm, as well are the thin and thick filaments (Fig.8D). Accordingly the regular striation pattern is not achieved (Schmidt-Rhaesa 2007).

As aforementioned, the contraction of muscles is triggered by the sudden release of calcium ions stored in the sarcoplasmic reticulum, into the cytosol. In skeletal muscles the synchronous activation of all sarcomeres is yielded by an intricate net of extension of the plasma membrane (T-tubules) that promptly distribute the action potential necessary to release the calcium (Alberts, Johnson et al. 2008).

The T-tubule system is not present in smooth muscles which by this mean and by the non-regular orientation of the contractile units, contract slower than skeletal muscles, although the contraction itself lasts longer.

Along the anatomical differences between smooth and striated muscles, the molecular mechanism that regulates the contraction depends on different proteins.

The tropomyosin is a key protein that lay in the furrow of the coiled actin filaments (Fig.8C) and that is common to the two muscle types (Lees-Miller and Helfman 1991; Lehman, Hatch et al. 2000). During resting conditions the tropomyosin blocks the myosin binding sites thereby inhibiting the sliding mechanism. The tropomyosin is instead displaced from its inhibitory configuration in response to the rise of cytosolic calcium. In the skeletal muscles, the calcium ions binds to a subunit (TnC) of the troponin complex provoking a conformational change that affects the other two subunits of the complex (TnI and TnT), and indirectly also the tropomyosin. Thus, when tropomyosin has been displaced free space becomes available to the myosin (Fig.8C) (Farah and Reinach 1995; Gordon, Homsher et al. 2000; Galinska-Rakoczy, Engel et al. 2008; Lehman, Galinska-Rakoczy et al. 2009).

In smooth muscles there are two core proteins that inhibit the myosin-actin interaction: the caldesmon (in vertebrates) acts on the thin filaments, by stabilizing actin and tropomyosin during resting conditions (Morgan and Gangopadhyay 2001); and the myosin light chain that instead inhibits the thick filaments. The caldesmon is displaced when the intracellular calcium increases. At the same time, the myosin light chain get phosphorilated and by mean of that, removed from its inhibitory conformation (Kureishi, Kobayashi et al. 1997). Both the caldesmon displacement and the phosphorilation of the myosin light chain respond to calcium-bound calmodulin. Thus regulation of muscular contraction in smooth fibres involves inhibition/activation cycles of both thin and thick filaments.

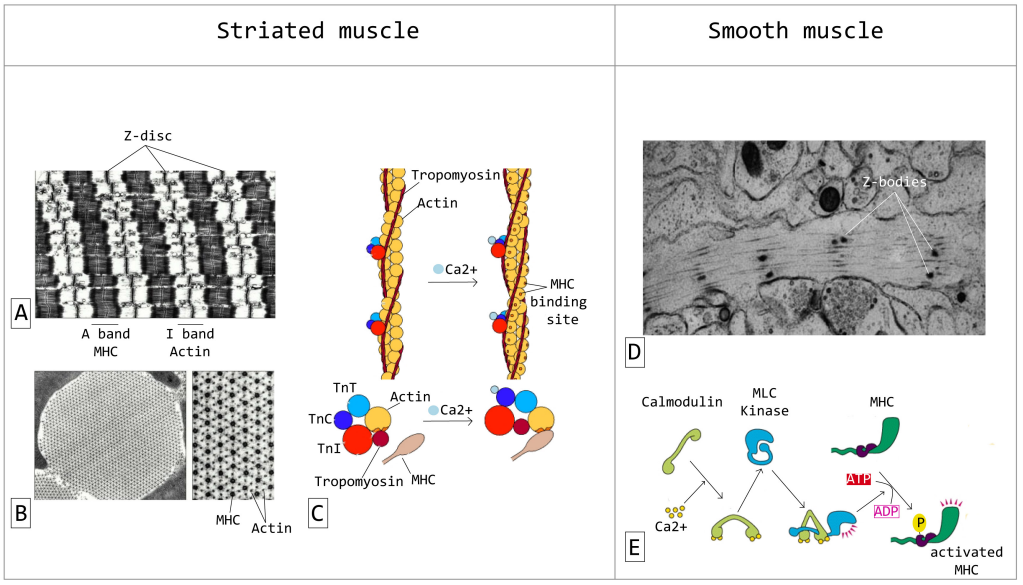


Fig. 8 Ultrastructural and molecular comparison between striated and smooth muscles.
A. Striated muscle in longitudinal section. B. In cross section. C. Molecular regulation of the contraction is carried on by the Troponin complex. All images modified from (Alberts et al. 2008). D. Smooth muscle (from (Salvenmoser, Egger et al. 2010)). E. Molecular regulation of the contraction in the smooth muscle requires the phosphorilation of the Myosin Light Chian (MLC) (from Alberts et al. 2008).

5.4 The mesodermal gene regulatory network and the scopes of this thesis

Recent comprehensive genetic studies on model organisms, *e.g.* flies, nematodes and mice, have revealed that homologous genes have similar tissue specificity and act inside similar gene regulatory networks. These emerging principles are valid for all germ layers, including the mesoderm (Ciglar and Furlong 2009).

Central genes in patterning the bilaterian mesoderm are the orthologues of *Twist*, *Mef2*, several *T-box* as well as *bHLH* factors, among many others (Technau and Scholz 2003). The expression patterns of some of these key

mesoderm factors have been studied also in "non model" organisms (Lartillot, Le Gouar et al. 2002; Boyle and Seaver 2008; Boyle and Seaver 2010; Shimeld, Boyle et al. 2010) showing clearly conserved tissue specific expression. During my project in the laboratory I decided to extend the knowledge of mesodermal gene expression to members of the Acoelomorpha, given that they might hold the key to unravel the evolution of the mesoderm and bilateral symmetry (Baguñà and Riutort 2004; Baguñá, Martinez et al. 2008). However, while I was working on my PhD project a new phylogenomic study has shown that the Acoelomorpha might not represent the earliest bilaterian offshoot (Philippe, Brinkmann et al. 2011). If this turns to be the "final" phylogenetic scenario, the expression of mesodermal genes in acoelomorpha should reveal key aspects of the evolution of new body plans from more complex ones.

Aims of the study

At the time this thesis begun the Acoelomorpha (Acoela+Nemertodermatida) were thought to represent the earliest offshoot of the Bilateria (Ruiz-Trillo, Paps et al. 2002), and as such they were assumed to be pivotal organisms to study and understand the origin and evolution of the bilaterian mesoderm.

During the course of this study however, other phylogenomic approaches have shown that the enigmatic worm *Xenoturbella* is an acoelomorph too, and two alternative phylogenetic positions have been offered for the whole group. In the first study the early branching position of Acoelomorpha within the Bilateria has been confirmed (Hejnol, Obst et al. 2009). In a second study instead, the acoelomorphs have been relocated to the deuterostomes, either as the most basal branching group, or as the sister group of the Ambulacraria (Philippe, Brinkmann et al. 2011).

Independently of their phylogenetic position, acoels remain interesting animals to understand key evolutionary though, at present, they are still poorly characterized.

The aims of my Thesis were:

- to set up acoels as valuable models for experimental studies, with special focus on the species *S. roscoffensis*

- to disentangle the molecular regulatory mechanisms of mesoderm formation in the acoels

- to characterize the principal acoel mesodermal derivatives, *i.e.* the muscles, at the molecular level

- to describe the dynamic patterns of mesodermal gene expression during acoel development and regeneration

INFORME DEL DIRECTOR SOBRE ELS ARTICLES PUBLICATS

Director: Pere Martinez Serra

Els articles inclosos en aquesta tesi doctoral són: dos ja publicats (un com a primer autor i l'altre com a segon), un article de revisió en premsa (que na Marta signa com a segon autor) i un "draft" d' article que ja està enviat a publicar (però del que no tenim resposta encara).

Els articles publicats són, per ordre d'aparició a la Tesi:

R1- Chiodin, M., Achatz, J., Wanninger, A., and Martinez, P. (2011) Molecular architecture of muscles in an acoel and its evolutionary implications. J. Exp. Zool. Mol Dev. Evol. 316(6):427-39.

Factor d' impacte: 2.37

En aquest article es descriu la formació del sistema muscular a Symsagittifera roscoffensis, utilitzant marcadors inmunoquímics (un anticos específic, fet al laboratori, contra la tropomiosina) i hibridació in situ, amb tres gens específic de la formació de muscles. L' anàlisi es va fer utilitzant gens derivats d' una col.lecció d' ESTs que varem fer al laboratori. Totes aquestes eines es van fer servir també per descriure la reconstrucció de la musculatura en animals que regeneraven.
La Marta va fer la major part d'aquest treball, caracteritzant els genes, desenvolupant l' anticos específic de tropomiosina i fent totes les hibridacions in situ.

R2- Chiodin, M., Borve, A., Berezikov, E., Ladurner, P., Martinez, P, and Hejnol, A. (2012) Mesodermal gene expression in the acoel Isodiametra pulchra indicates a low number of mesodermal cell types and the endomesodermal origin of the stem cell system. (acceptat a PLOS One)

Factor d' impacte: 4.35

Aquest article està, fonamentalment, desenvolupat al grup del Dr. Andreas Hejnol (SARS institute for Molecular Marine Biology, Bergen, Noruega). És part d' una col·laboració que el nostres grups tenen i el resultat de l' estada que va fer la Marta en aquell laboratori. La feina, independentment del nombre d' autors (que han contribuint en aspectes com la generació de llibreries d' EST o seqüències genòmiques) ha estat feta, en la major part, per na Marta Chiodin. Ella ha clonat els gens, i ha fet totes les in situs (o quasi totes). El resultat és aquest article que s'acaba d' enviar a publicar.

R3- Semmler, H., **Chiodin, M.,** Bailly, X., Martinez, P., and Wanninger, A. (2010) Steps towards a centralized nervous system in basal bilaterians: Insights from neurogenesis of the acoel Symsagittifera roscoffensis. Develop. Growth Differ. 52 (8) 701-713.

Factor d' impacte: 2.28

En aquest article es descriu, per primera vegada, el sistema nerviós de l'acel Symsagittifera roscoffensis. S'utilitzen marcadors inmunoquimics per descriure els detalls del sistema serotonèrgic, amb una resolució mai aconseguida pels acels. La descripció inclou estadis primerencs (hatchlings) i adults. Per primera vegada varem utilitzar hibridació in situ per detectar l' expressió de gens involucrats en la formació del sistema nerviós, en aquest paper varem incloure l' anàlisi del gen Sox. Més recentment, i gracies al desenvolupament d' aquesta metodologia i l' introducció de genoteques de cDNA i seqüències genòmiques hem pogut esbrinar el paper d' altres gens a la neurogènesi dels acels.
La Marta Chiodin va esser la persona que va fer les hibridacions in situ de Sox i va contribuir definitivament a l' anàlisis inmunoquimic. Mentre l' anàlisi amb anticossos va esser compartit amb l' Henrike Semmler, l'identificació de Sox i les in situs van ser, únicament, tasques portades a terme per na Marta.

R4- The Acoela - on their kind and kinships, especially with xenacoelomorph worms (Bilateria incertae sedis) (2012) Achatz, J. G., **Chiodin, M.,** Salvenmoser, W., Tyler, S., and Martinez, P. Organisms, Diversity and Evolution (en premsa)

Factor d' impacte: 1.65

Aquest és el primer review extens que s' ha publicat sobre aquest grup d' animals. E un Review que s' en ha demanat donada la nostra posició rellevant dintre del camp. Cobreix aspectes morfològics, de sistemàtica i de desenvolupament comparat.
La Marta ha redactat l' apartat dedicat a l' embriologia i la biologia molecular dels acels, una secció especialment rellevant ja que inclou moltes dades recents.
L' article està sent revisat, despres dels comentaris dels referees i no esperem cap problema per la seva publicació, que segurament passarà abans de la lectura d'aquesta Tesi.

És important afegir que una gran part d'aquesta feina s' ha fet en absència de tecnologies adequades. Els acels són un grup molt poc investigat i per tant qualsevol dada ha estat obtinguda després d' un llarg procés que totes les eines i tècniques necessàries s' han hagut de desenvolupar, una a una. Aquest no és un treball de desenvolupament fet amb animals models, amb tècniques establertes i genomes coneguts. Aquesta és una feina fonamental que implica el desenvolupament, pràcticament de cero, d' un nou sistema animal d' investigació.

Vull destacar que tots aquests articles han estat fets amb col·laboració amb altres grups internacionals. Aquesta ha estat una constant en el treball de na Marta Chiodin, una feina realitzada amb contacte permanent amb altres grups del món.

Signat: Pere Martínez Serra

Publications

RESEARCH ARTICLE

Molecular Architecture of Muscles in an Acoel and Its Evolutionary Implications

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART B
MOLECULAR AND DEVELOPMENTAL EVOLUTION

MARTA CHIODIN[1]*, JOHANNES G. ACHATZ[1], ANDREAS WANNINGER[2], AND PEDRO MARTINEZ[1,3]*
[1]Departament de Genètica, Universitat de Barcelona, Barcelona, Spain
[2]Department of Evolutionary Biology, Section of Morphology, University of Vienna, Vienna, Austria
[3]Institució Catalana de Recerca i Estudis Avançats (ICREA), Passeig Lluís Companys, Barcelona, Spain

ABSTRACT

We have characterized the homologs of an actin, a troponin I, and a tropomyosin gene in the acoel *Symsagittifera roscoffensis*. These genes are expressed in muscles and most likely coexpressed in at least a subset of them. In addition, and for the first time for Acoela, we have produced a species-specific muscular marker, an antibody against the tropomyosin protein. We have followed tropomyosin gene and protein expression during postembryonic development and during the posterior regeneration of amputated adults, showing that preexisting muscle fibers contribute to the wound closure. The three genes characterized in this study interact in the striated muscles of vertebrates and invertebrates, where troponin I and tropomyosin are key regulators of the contraction of the sarcomere. *S. roscoffensis* and all other acoels so far described have only smooth muscles, but the molecular architecture of these is the same as that of striated fibers of other bilaterians. Given the proposed basal position of acoels within the Bilateria, we suggest that sarcomeric muscles arose from a smooth muscle type, which had the molecular repertoire of striated musculature already in place. We discuss this model in a broad comparative perspective. *J. Exp. Zool. (Mol. Dev. Evol.) 316:427–439, 2011.* © 2011 Wiley-Liss, Inc.

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Muscles exist exclusively in the Eumetazoa, namely the Cnidaria, the Ctenophora, and the Bilateria, and as such they are pivotal to reconstruct and understand the evolution of animals (Burton, 2008).

In the bilateral animals, the muscles are morphologically distinguished in two basic types: the smooth and the striated muscles. In the smooth muscular cells, the myofilaments (i.e. thin actin and thick myosin filaments, which interaction produces the contraction) are poorly arranged. Conversely, in the striated muscles, the myofilaments are highly organized in units called sarcomeres (Ruppert et al., 2004).

The relation of the two different muscle types, to each other and among different taxa, is still not settled and molecular data has just accumulated enough to allow for the first speculations (Seipel and Schmid, 2005). However, one generally accepted scenario suggests that myocytes, i.e. true muscular fibers lacking any epithelial component, are derived from epitheliomuscular cells, which are the most ancestral type of contractile cells (Rieger and Ladurner, 2003). Myoepithelial cells are abundant within the Cnidaria (sea anemones, corals, jellyfish), which are the sister group of the Bilateria. However, in the swimming life stage of some cnidarians, the medusa, there are myocytes too.

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Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Pedro Martinez and Marta Chiodin, Departament de Genètica, Universitat de Barcelona, Av. Diagonal 645, 08028-Barcelona, Spain. E-mail: pedro.martinez@ub.edu; martachiodin@ub.edu

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These are generally suggested to have evolved convergently to the bilaterian muscles as an adaptation to swimming. If these premises are accepted, the origin of true muscles in the Bilateria dates back to its own origin. Recent molecular phylogenies place acoels as the earliest offshoot of all bilateral animals (Hejnol et al., 2009) and, albeit this position is still controversial (Dunn et al., 2008; Egger et al., 2009; Philippe et al., 2011), a full set of evidences, such as morphological characters and the *Hox* gene complement, support their basal position (Haszprunar, '96; Hejnol and Martindale, 2008, 2009; Moreno et al., 2009). Accordingly, in order to understand the evolutionary origin of muscular cells and the relationship between the cnidarian and bilaterian muscles, data from these simple worms are crucial.

It is generally accepted that the mesoderm has evolved from the endoderm; however, in most of the Bilateria two mesoderm sources exist: the so-called endomesoderm and the ectomesoderm, which usually develops from ectodermal tissues (Martindale and Henry, '99; Technau and Scholz, 2003; Martindale et al., 2004). In acoels, muscles and all other mesodermal tissues develop from endomesoderm because they have no ectomesoderm (Henry et al., 2000). Morphogenesis and embryonic development of the musculature have been investigated in two acoel species: *Isodiametra pulchra* and *Symsagittifera roscoffensis* (Ladurner and Rieger, 2000; Semmler et al., 2008). Accordingly, in both species, the differentiation of muscles proceeds from the anterior to the posterior pole of the embryo, the circular muscles arise before the longitudinal muscles, and the juvenile and adult musculature originate by adding more fibers to an initial grid. Morphological investigations on muscles, either using electron microscopy (Rieger et al., '91) or fluorophore-tagged phalloidin and confocal microscopy (Hooge, 2001; Hooge and Tyler, 2005) are more numerous, cover a much greater number of species, and show that the smooth type is the only type of muscle occurring in these animals.

Investigations on the adult body-wall structure and its development are informative for deciphering the interrelationships of taxa and eventually tracing the evolution of new body plans (Wanninger, 2009), though they don't tell much about the evolution of the muscular tissue itself. Dissecting the molecular fingerprint of muscles in the Acoela could offer important insights into the topic (Arendt, 2008).

We are currently working to establish the acoel *S. roscoffensis* as a model system for molecular developmental biology, and we have characterized, for the first time in any acoel species, the expression pattern of three muscular genes, an actin, a tropomyosin, and an inhibitory subunit of the troponin complex. These three proteins interact in the skeletal muscle of vertebrates and have also been identified in several invertebrates, with two of them, actin and tropomyosin, also existing in the cnidarian muscles (Groger et al., '99; Scholz and Technau, 2003).

Additionally, we have raised a specific antibody against the tropomyosin of *S. roscoffensis*. In order to understand the dynamic expression of some of the muscular markers in a developmental context, we have followed the expression of tropomyosin, gene and protein, during muscle regeneration.

Regeneration can be easily induced and followed in acoels; although, so far, the process has been poorly studied (Gaerber et al., 2007; De Mulder et al., 2009a; Bery and Martinez, 2011). Although the development of muscles in adults has only been studied during the asexual reproduction of four different *Convolutriloba* species (Sikes and Bely, 2008), there is no published data available in animals after experimental excision. For the first time in acoels, we describe the regeneration of muscles using a species-specific muscular marker.

MATERIALS AND METHODS

Animal Collection, Rearing, and Fixation

Adult specimens of *S. roscoffensis* were collected in Carantec (Brittany, France) in 2007 and 2008. The specimens were kept in aquaria with continuous seawater cycling at 15°C. After approximately 1 week, gravid animals released cocoons. These were collected and kept in glass Petri dishes at 15°C as well. Filtered seawater was changed once a day.

Hatchlings were collected and immediately processed for fixation or left to grow for 1–7 days, with filtered seawater replaced once a day. For regeneration studies, adults were sectioned transversally in the mid-body region. The anterior and posterior halves were kept in different dishes. Animals were left to regenerate at 15°C and were subsequently fixed at different time intervals. Within the first 24 hr of regeneration, they were usually fixed at intervals of 4–5 hr.

For immunostaining, specimens were treated with 1% cysteine chloride (pH 7–7.5) in seawater for about 20 min at room temperature, and then rinsed twice in filtered seawater to remove mucous secretions. Subsequently, specimens were relaxed by addition of drops of 7.14% magnesium chloride. Then, the animals were fixed in 4% PFA (dissolved in 0.1 M PBS; pH = 7.5), for either 2 hr at room temperature or overnight at 4°C. The animals were subsequently washed three to five times in PBS and stored at 4°C in PBS+0.1% sodium azide.

For in situ hybridization, the specimens were fixed in a mixture of 0.2% glutaraldehyde+3.7% formaldehyde in PBS for 5 min at room temperature, and then left for 1 hr in 3.7% formaldehyde in PBS at the same temperature. Fixed animals were subsequently washed three to five times in PBS and progressively dehydrated in a methanol series. Specimens were stored in 100% methanol at −20°C.

Gene Isolation and Gene Orthology Analyses

The cDNA clones of this study were identified from an arrayed (and fully sequenced) cDNA library from *S. roscoffensis*

aposymbiotic hatchlings. All inserts were cloned into pBluescript SK- and oriented as to get an antisense riboprobe when using the T7 RNA polymerase (Roche, Hoffman-La Roche Inc., Nutley, NJ), and a sense probe when using the T3 RNA polymerase (Roche).

After regrowing the transformed bacteria from a glycerol stock, the *SrAct*, *SrTnI*, and *SrTrp* were resequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA).

Phylogenetic analyses were performed using protein sequences related to *SrAct*, *SrTnI*, and *SrTrp*, which were downloaded from GenBank. For the actin family we chose muscular and nonmuscular actins, for troponin sequences we used members of all three subunits known to interact in the troponin complex (TnC, TnI, TnT), and for tropomyosin sequences we selected "long-muscular" and "short-nonmuscular" tropomyosin isoforms. Alignments of the protein sequences were done using the MAFFT program, included in the Geneious package (Drummond et al., 2010). Considering the level of sequence identity, the matrices used for generating the alignments were BLOSUM80 for SrAct, BLOSUM30 for SrTnI, and BLOSUM45 for SrTrp. To calculate the trees, a maximum likelihood methodology was performed with RAxML 7.0.3 (Stamatakis et al., 2008) on the Vital-IT server (http://phylobench.vital-it.ch/raxml-bb/), using the model of evolution suggested by ProtTest (Abascal et al., 2005). Accession numbers of the sequences (S1, S2, an S3) and alignments used (S8, S9, and S10) are provided in the supplementary material. The original alignments, in nexus or phylip format, are available on demand.

Antibody Production and Immunostaining

The anti-SrTrp antibody was made against the peptide LDKTNHQLDDANKE of the deduced protein sequence of *SrTrp*. For predicting the antigenicity of the peptide, based on local average hydrophylicity, the Hopp and Woods' method (Hopp and Woods, '81) was applied to the whole aminoacidic sequence of the protein. The highest hydrophilicity is suggested for the peptide containing the residues 64–77.

The peptide was synthesized, purified by semi-preparative reverse-phase HPLC, and its purity was verified by analytical HPLC and amino acid analysis. The corresponding immunogens were prepared by coupling the peptides synthesized to KLH and then injected into rabbits for developing antibodies. The animals were boosted at first after 6 weeks and then every 4 weeks. Blood was collected 2 weeks after each booster injection. The antisera obtained were evaluated for their titer and cross-reactivity using an enzyme-linked immunoabsorbent assay.

Immunostaining of whole mount juveniles or adult specimens was performed following the protocol published by Semmler et al. (2010). The primary antibody working concentration was determined empirically. A 1/50 dilution of anti-SrTrp in 6% Normal Goat Serum (Sigma, St. Louis, MO) in PBS yielded the best signal-to-background ratio. Controls with preimmune serum, at the same working concentration of anti-SrTrp, yielded no signal.

For phalloidin staining, adults' specimens were first permeabilized in PBS+3% triton for 2 hr at room temperature. Subsequently, they were incubated in a solution of Alexa633-phalloidin (Molecular Probes, Eugene, OR) in PBS diluted 1/40, for 3 hr at room temperature. This step was performed in the dark, as all the following washing steps in PBS (6 × 10 min). Finally, the specimens were processed for analysis.

All specimens were mounted in either Vectashield (Vector Laboratories, Burlingame, CA) or FluoromountG (Southern Biotech, Birmingham, AL). The preparations were observed and analyzed on either a Leica TCS SP2 or a TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

In Situ Hybridization

Digoxigenin-labeled sense and antisense riboprobes were synthesized using the Dig-RNA labeling kit from Roche (Hoffman-La Roche Ltd., Basel, Switzerland), following the manufacturer's instructions. The clones recovered in the EST library were used as templates for riboprobe synthesis. All clones contained the complete ORFs and both the 5′ and 3′ untranslated regions. The respective antisense probe lengths for the *SrAct*, *SrTnI*, and *SrTrp* genes were 1,478 bp, 1,480 bp, and 2, 7 kb, respectively.

In situ hybridization on whole mount specimens was performed following the protocol published by Semmler et al. (2010), with the only exception of skipping the proteinase K step plus the following glycine washes and the refixation step. Hybridization was done as follows: *SrAct*, 0,1 ng/μL of sense or antisense probe concentration and 60°C of hybridization temperature; *SrTnI* 1 ng/μL of sense or antisense probe concentration and 60°C of hybridization temperature; *SrTrp* 1 ng/μL of sense or antisense probe concentration and 50°C of hybridization temperature.

All specimens were mounted in 70% glycerol (Sigma) in PBS and analyzed on a Zeiss Axiophot (Carl Zeiss MicroImaging, GmbH) or on a Leica BMLB (Leica Microsystems, Wetzlar Germany) microscope, both equipped with a ProgRES C3 camera (Jenoptik, Germany).

RESULTS

Molecular Characterization of Actin, Tropomyosin, and Troponin I Orthologs From *S. roscoffensis*

In order to understand the phylogenetic affinities of some molecular components of the acoel muscles, we isolated a few clones that represent well-known components of the muscular architecture. These clones are the potential homologs of actin, troponin I, and tropomyosin (*SrAct*, *SrTnI*, and *SrTrp*).

The clone *SrAct* is 1,478 bp long and it contains an open reading frame (ORF) of 1,129 nucleotides, which conceptual translation is a 377 amino acids–long protein. Phylogenetic

analysis was performed using full-length sequences of cytoplasmic and muscular isoforms from several taxa (supplementary material, S8). In agreement with previous results, we have recovered the muscular actins of the vertebrates and those of the insects as independent monophyletic groups (Vandekerckhove and Weber, '84; Mounier et al., '92) (supplementary material, S4). *SrAct* clusters with the deuterostomes' muscular actins; however, the bootstrap values for the nodes in this topology are very low with RAxML (supplementary material, S4).

The clone *SrTnI* is 1,480 bp long and it contains a 529 bp ORF. The deduced translation of the ORF results in a protein of 175 amino acids. Phylogenetic analyses using sequences of all three subunits of the troponin complex from distantly related taxa show that *SrTnI* is a true ortholog of the inhibitory subunit of the troponin complex (Fig. 1A and supplementary material S5).

The clone *SrTrp* is 2.7 Kb long and has an 858 bp ORF. The encoded protein is 285 amino acids long and is a "long-type" tropomyosin (supplementary material, S3 and S10). The long forms of the tropomyosin include 38 amino acids long *N*-terminal motif, which is highly conserved even among distantly related organisms, but is never recovered in nonbilateral animals (green box in S7). Given the complexity of the gene and the lack of information on paralogous genes and different isoforms in acoels, we have included in the analysis both long and short isoforms from as many taxa across the Eumetazoa as possible (supplementary material, S10). The phylogenetic trees show that tropomyosins of the diploblasts (Cnidaria and Ctenophora) cluster separately from the tropomyosins of Bilateria. It is noteworthy that *SrTrp* clusters with the chordates' muscular isoforms in the RAxML analyses (Fig. 1B and supplementary material, S6).

Remarkably, all our phylogenies would support a position of the acoels within the deuterostomes, as recently suggested by Philippe et al. (2011), and contradict the so far accepted basal postion of the acoels within the Bilateria. However, our phylogenies are based on single gene analysis (one single gene per tree) and the support values for the "deteurostome" topologies are low (all below 85).

Expression Pattern of SrAct, SrTnI, SrTrp Genes and the SrTrp Protein

SrAct is broadly expressed in juveniles of *S. roscoffensis*. This pattern is not surprising because the gene is very likely to be expressed in differentiating myoblasts and myocytes, which are expected to be widely distributed along the whole body axis of a growing juvenile (Ladurner and Rieger, 2000; Semmler et al., 2008). However, the expression pattern of the gene is not uniform along the antero–posterior body axis (Fig. 2A). The number of *SrAct* positive cells is lower in the anterior-most part of the juvenile, around the frontal organ, whereas the highest density of positive cells is always found in the region of the mouth, where the U-shaped, accessory, and ring muscles are located (Fig. 2A, asterisk; Semmler et al., 2008). In adult worms, *SrAct* is also widely expressed along the body, but the highest signal is seen around the male genital opening and the region anterior to it (Fig. 2A"). The male genital opening is surrounded by circular and gonopore-specific muscles and is equipped with accessory muscles in the anterior region (Semmler et al., 2008), which is also strongly stained. The highest signal though is found in special muscular cells, the muscle mantles of sagittocysts (Fig. 2A"). These are present only in adults, between the female and male genital openings, at the posterior-most tip of the body, and along the posterior lateral edges (Fig. 2A", B", D, E; Semmler et al., 2008).

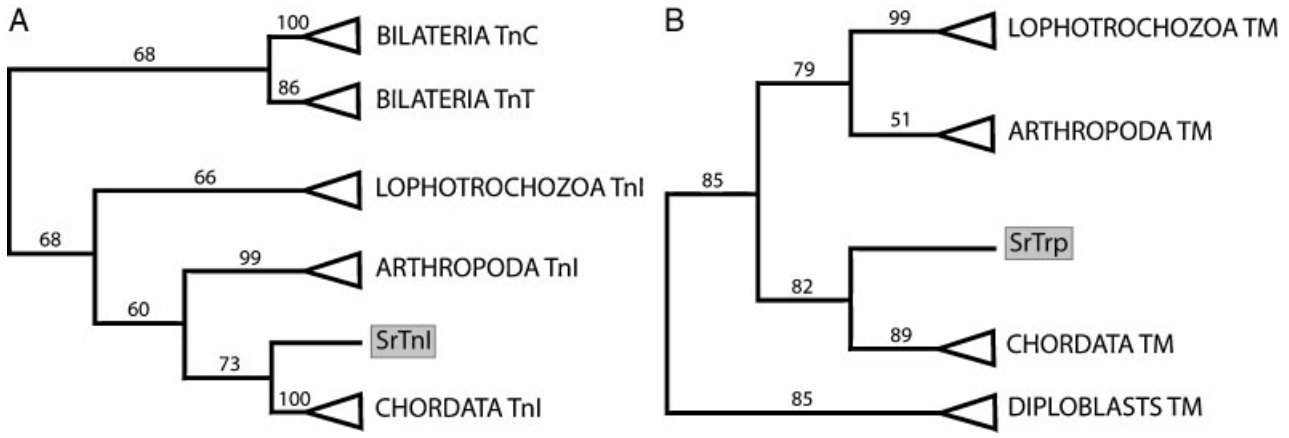

Figure 1. Schematic representation of phylogenetic trees calculated by RAxML. All nodes with support values below 50 have been collapsed. (**A**) Tree calculated using the WAG model. The tree includes all the three subunits of the Troponin complex. TnC, calcium-binding subunit. TnI, inhibitory subunit. TnT, tropomyosin-binding subunit. The grey box highlights the acoel sequence SrTnI. (**B**) Tree calculated using the RETREV model. The tree includes long and short isoforms of the tropomyosin proteins which are not specifically shown for easy representation purposes. The acoel sequence, SrTrp, is grey boxed.

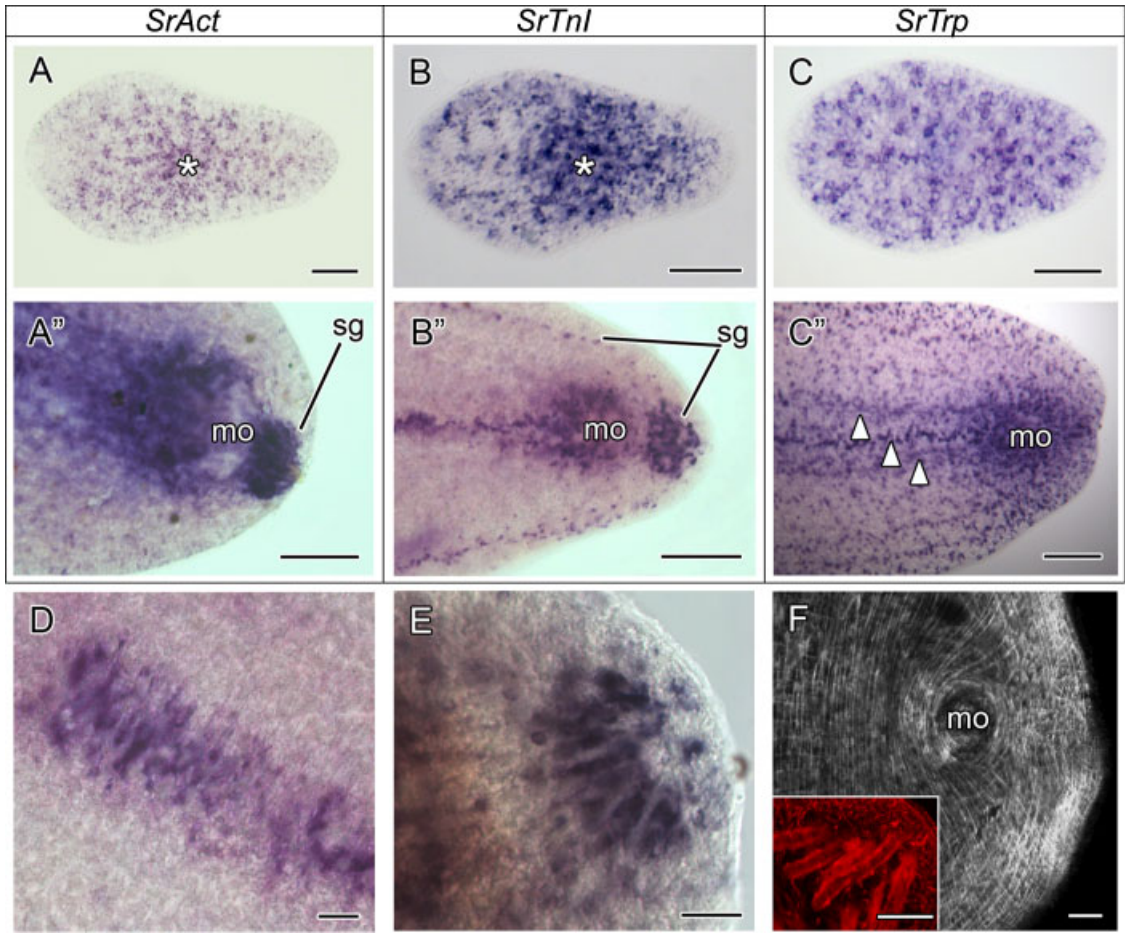

Figure 2. *SrAct*, *SrTnI*, and *SrTrp* gene expression patterns in the acoel *Symsagittifera roscoffensis*. Anterior is toward the left in all aspects. **(A)** *SrAct* expression in a juvenile. The positive cells appear scattered along the AP axis of the juvenile. The highest concentration of actin-expressing cells is around the mouth region (asterisk), where the mouth accessory muscles and U-shaped muscles are present. **(A")** *SrAct* expression in the posterior tip of an adult. The expression is restricted to the male genital opening (mo), the region frontal to it, and the muscle mantles of sagittocysts (sg). **(B)** *SrTnI* expression in a juvenile specimen. The expression of the *troponin I* gene appears scattered with the highest concentration of positive cells in the mouth region (asterisk). **(B")** *SrTnI* expression in the posterior part of an adult. The expression of *SrTnI* is recovered in the region of the male gonopore (mo), in a longitudinal band anterior to it, and in the muscle mantles of sagittocyts at the posterior-most tip and in two lateral longitudinal bands (sg). **(C)** *SrTrp* expression in a juvenile. Contrary to the *SrAct* and the *SrTnI* genes, *SrTrp* is more broadly expressed along the body of the juvenile. The frontal region has a greater number of *SrTrp*-positive cells if compared with *SrAct* and *SrTnI*. **(C")** *SrTrp* expression in the posterior part of an adult. As in the juvenile, the *SrTrp*-positive cells are more scattered than *SrAct* and *SrTnI* ones. High expression levels are seen in the region of the male gonopore and in longitudinal bands anterior to it (arrowheads). **(D)** Detail of the muscle mantles of the sagittocysts in the region between the female and the male genital opening as revealed by the anti-*SrTnI* riboprobe. **(E)** Detail of the muscle mantles of the saggitocysts, revealed by the anti-*SrTnI* riboprobe, at the posterior-most tip of an adult specimen. **(F)** Anti-SrTrp antibody staining in the caudal end of an adult. The antibody detects all types of muscles, except for the muscle mantles that surround the sagittocysts. The latter, labeled by the phalloidin, are shown in the inset. Scale bars: A-C 50 μm, A"–C" 100 μm, D–F and inset 25 μm. mo, male genital opening; Sg, muscle mantle of the sagittocyst.

The gene *SrTnI* is also broadly expressed in the juvenile *S. roscoffensis*. Its expression is reminiscent of that of *SrAct*. A lower number of *SrTnI* positive cells is found in the anterior part of the juvenile, whereas the majority of *SrTnI*-expressing cells congregate around the mouth (Fig. 2B, asterisk). In adults, the male genital opening and the muscle mantles of the sagittocysts are clearly stained by the *SrTnI* probe (Fig. 2B").

The expression patterns of *SrAct* and *SrTnI* are remarkably similar, especially in the adult stage where the highest expression domains correspond to the male gonopore and the muscle mantles of the sagittocysts.

The tropomyosin gene, *SrTrp*, is expressed all along the body axis of juveniles (Fig. 2C). However, compared with *SrAct* and *SrTnI,* its expression is more broad and uniform. There is no lack of *SrTrp*-expressing cells in the frontal part of the animal and the higher density of positive cells in the mouth region is less pronouced (Fig. 2C). In the adult, similar to what we detect in the juvenile, the *SrTrp*-positive cells are more evenly distributed, compared with *SrAct*- and *SrTnI*-positive cells (Fig. 2C''). The highest expression of the gene occurs at the male gonopore. The strongly labeled longitudinal bands found in front of the male genital organ might coincide with known strong muscles present between the female and male gonopore, although they could also be longitudinal nerve cords or longitudinal bands of gland cells (Fig. 2C'', arrowheads). This has to be determined using detailed histological analysis. Surprisingly, no tropomyosin expression is found in the muscle mantles of the sagittocysts.

In all cases, control in situ hybridizations run with sense probes of all the three genes yielded no signal (data not shown).

A better understanding of the tropomyosin pattern is uncovered by the use of a complementary tool, a specific antibody. The specific anti-SrTrp antibody recognizes the peptide LDKTNHQLDDANKE, which is located at the *N*-terminal region of the protein, covering the residues 64–77 of the deduced translation of the *SrTrp* gene. The anti-SrTrp antibody recognizes specifically the muscles of *S. roscoffensis*. When applied to juveniles, it reveals the body-wall musculature, the inner thick longitudinal, outer thin circular, and intermediate diagonal cross-over muscles on the dorsal side (Fig. 3A), plus the longitudinal, diagonal, circular, and U-shaped muscles, as well as specialized ring muscles and accessory fibers associated with the ventral side of the mouth (Fig. 3C). Besides the body-wall musculature anti-SrTrp stains parenchymal muscles, showing that dorsoventral muscles are denser in the posterior half of the juvenile than in the anterior one (Fig. 3B, double arrowhead). This asymmetry correlates with the presence of prominent organs, such as the nervous system, the statocyst, and the frontal organ, which are all located in the anterior half of the body (Bery et al., 2010).

In adult specimens, the overall organization of the body-wall musculature is maintained. Specialized muscles associated with the copulatory organs are detected at the ventral side. The male genital opening at the posterior-most tip has the greatest number of specialized muscles and is the region that shows the strongest signal (Fig. 2F). Contrary to the fluorophore-tagged phalloidin, which has been successfully applied in *S. roscoffensis* to label muscles and other structures, our antibody does not recognize any kind of sensory structure and does not mark the muscle mantles of sagittocysts, neither in juveniles nor in adult specimens (Semmler et al., 2008; Fig. 2F, inset).

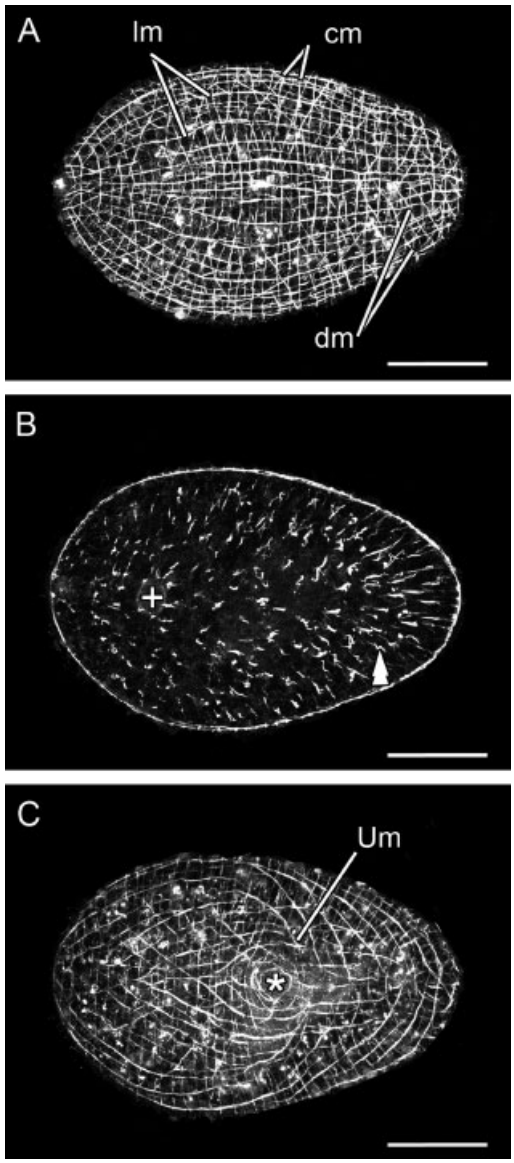

Figure 3. *SrTrp* protein expression detected by a specific anti-SrTrp antibody in a juvenile specimen. Anterior is toward the left in all aspects. **(A)** Confocal projection of dorsal sections. Muscles of the body wall are evident. **(B)** Confocal projection of sections in the mid-body plane. Parenchymal muscles are highlighted by the double arrowhead. They run dorsoventrally and their density is higher in the region posterior to the mouth than anterior to it. The statocyst is highlighted by the symbol+. **(C)** Confocal projection of ventral sections. In addition to the longitudinal, circular, and diagonal muscles, specialized U-shaped muscles are present on the ventral side of the animal. The mouth opening (asterisk) is surrounded by specialized circular muscles. Scale bars: 50 µm in all aspects. hpa, hours post amputation. cm, circular muscles; dm, diagonal muscles; lm, longitudinal muscles; Um, U-shaped muscles.

SrTrp Gene and Protein Expression During Muscle Regeneration in *S. roscoffensis*

Once cut transversally in a median plane, *S. roscoffensis* is able to regenerate both the anterior and posterior halves of its body. Generally, the head fragment regenerates the missing half slightly faster than the tail fragment does (data not shown). The process of muscle regeneration is similar in both cases. For simplicity, we describe only the regeneration of the posterior end of the animal, within the first 24 hr after amputation. During this time frame, the common pattern of the body-wall muscles is completely restored. However, the copulatory organs are not formed until much later (personal observations).

At the site of the wound, a translucent blastema becomes visible 12–16 hr after amputation (hpa) (Fig. 4I). Within 1 hpa, no major rearrangement of the muscles occurs, but a contraction of the circular muscles around the wound rim reduces its exposed surface (Fig. 4A). At 5 hpa, the preexisting body-wall muscles lose their regular arrangement, both at the dorsal and ventral sides of the animal but only in the region proximal to the wound (Fig. 4B and C). Along the rim of the wound, discrete regions of stronger signal are observed. At this time, the longitudinal muscles bend and converge to those regions (Fig. 4B and C, arrowhead). An outgrowth of dorsal longitudinal muscles occurs immediately after this local loss of the regular muscle pattern (about 9 hpa). The longitudinal fibers bend and grow toward the ventral side, thus provoking a ventral shift of the wound (Fig. 4D and E). By 16 hpa, the wound is closed and has completely shifted to the ventral side. In this area, the wound is covered by a faint web of young fibers, which are slender if compared with the old ones (Fig. 4G, double arrowheads). The dorsal side of the body wall has recovered, at this time, a more regular arrangement of the musclature (Fig. 4F). We have not observed any changes in the level of tropomyosin gene expression in connection with muscle rearrangement, growth, or differentiation within the first 16 hpa (Fig. 4H and I). At 16 hpa, a domain of increased tropomyosin expression appears next to the wound border (Fig. 4I). This domain of increased *SrTrp* expression persists until 24 hpa, even though it becomes progressively more restricted (Fig. 4J). At 24 hpa, the body-wall muscles have restored their original arrangement. The increased expression of the tropomyosin is a clear sign that new muscles are still differentiating in the posterior growing tip.

DISCUSSION

Muscular Genes in a Basal Bilaterian

Much of our knowledge on the physiology of muscular contraction comes from studies conducted in vertebrates (Gordon et al., 2000). Nevertheless, the three genes characterized in this study are known to interact in the striated muscles of both invertebrates and vertebrates (Bullard et al., '73; Hooper and Thuma, 2005). The thin filaments of the muscles are double helices of F-actin (filaments of polymerized actin monomeres). In both smooth and striated muscles, the thin filaments interact with thick filaments made of myosin heavy chain, in order to produce the muscular contraction. Although in the striated muscles the thin and thick filaments are highly organized in a structure, sarcomere, giving these muscles their distinct appearance, they are arranged less strictly in the smooth muscles (Clark et al., 2002). Another difference between smooth and striated muscles is how they respond to variations in calcium concentration. When calcium levels increase in reaction to a stimulus, the tropomyosin is displaced from its resting position, allowing the myosin and the actin to interact and lead to the contraction (Lees-Miller and Helfman, '91). Although in the smooth muscles the calcium response is mediated by a calmodulin–caldesmon complex, through the phosphorylation of a myosin light chain (Rasmussen et al., '87; Kureishi et al., '97; Morgan and Gangopadhyay, 2001), in the striated muscles the same mechanism is mediated by the proteins of the troponin complex (Galinska-Rakoczy et al., 2008; Lehman et al., 2009). This complex is formed by three different subunits: the calcium binding subunit (TnC), the tropomyosin-binding subunit (TnT), and the inhibitory subunit (TnI), whose main role is to inhibit the actomyosin ATPase by interacting at the same time with the actin, the tropomyosin, and the other two subunits of the complex (Farah and Reinach, '95).

Actin belongs to a highly conserved multigene family present in all eukaryotes and is highly conserved. Each gene encodes for different isoforms which are classified into two main groups in the Metazoa: the muscular isoforms and the nonmuscular or cytoplasmic isoforms (Mounier and Sparrow, '97). Although the differential usage of the isoforms is well understood in vertebrates and to a lower extent in the arthropods, this is far from being clear in other invertebrates (Mounier and Sparrow, '97). The muscular isoforms arose independently from a cytoplasmic ancestor in the vertebrates (Vandekerckhove and Weber, '84), the insects (most likely in the whole Ecdysozoa) (Mounier et al., '92), and most probably in the lophotrochozoans as well (Carlini et al., 2000). Whether there are muscle- and nonmuscle-specific actins in cnidarians is not clear yet (Aerne et al., '93).

In our phylogenetic analysis, SrAct resembles more the deuterostomes' muscular actins. This result is surprising, because it has been shown that the chordates' muscular actins arose independently from a cytoplasmic ancestor (Vandekerckhove and Weber, '84). However, care should be taken evaluting these results owing to the extreme conservation and the frequent occurrence of adaptive substitutions of amino acids in this protein (Mounier and Sparrow, '97).

In juveniles, *SrAct* is broadly expressed from the anterior to the posterior end of the animal. However, the presence of unstained cells and tissues, such as in the anterior-most region, suggests that *SrAct* is not ubiquitous (as expected for a cytoplasmic actin) and that it might be only specific of myoblasts

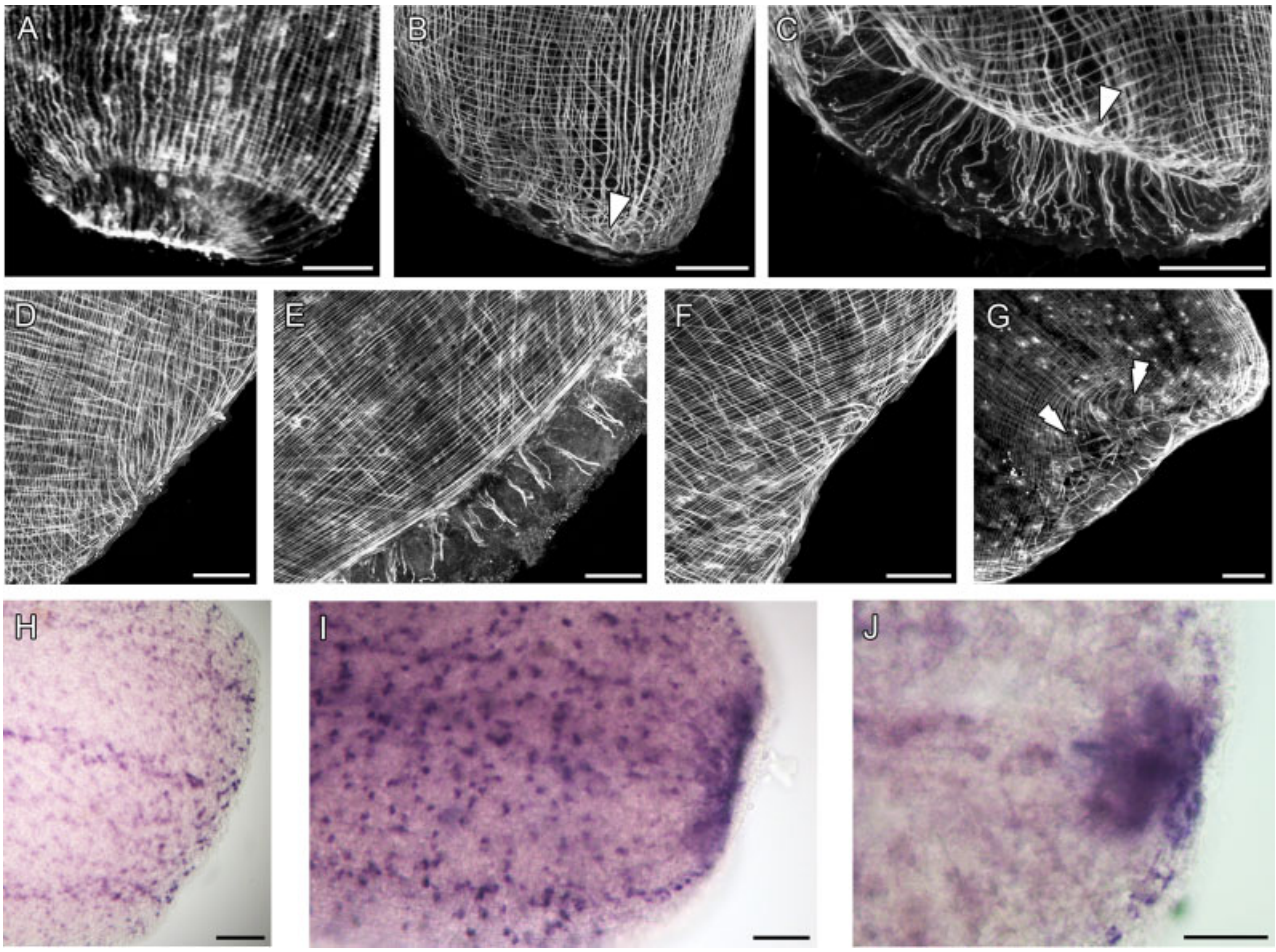

Figure 4. Regeneration of the musculature in an adult *S. roscoffensis* revealed by anti-SrTrp antibody staining and in situ hybridization of the *SrTrp* gene. All specimens are anterior halves in the process of regenerating the posterior part of the animal. **(A)** 1 hpa. An initial contraction is visible. Anterior toward the top. **(B)** 5 hpa, dorsal view. **(C)** 5 hpa. Ventral view of the same specimen as in B. In both cases, disorganized muscles are evident at the wound border, both at the dorsal and ventral side (arrowhead). Anterior is up in both panels. **(D)** 9 hpa, dorsal view. Longitudinal muscles appear still disorganized, bending and growing toward the ventral side. Anterior is toward the top left. **(E)** 9 hpa, ventral view of the same specimen. In this case, no outgrowth of muscles is observed; thus, the wound is progressively shifted toward the ventral side. Anterior is toward the top left. **(F)** 16 hpa, dorsal view. The outgrowing muscles at the dorsal side of the animal seem to be more organized than in the previous stage. Anterior is toward the top left. **(G)** 16 hpa, ventral view of the same specimen as in F. The wound region (double arrowheads) has completely moved to the ventral side. The wound is now covered by a faint web of very thin muscles. Anterior is toward the top left. **(H)** *SrTrp* mRNA expression in a specimen 1 hpa. The levels of *SrTrp* transcript expression at the wound border are comparable to the expression level in the rest of the body. Anterior is toward the left. **(I)** 16 hpa. A translucent blastema has appeared in the region of the wound. Along the wound border, a domain of increased expression of the tropomyosin gene is now observed. Anterior is toward the left. **(J)** 24 hpa. The wound is already covered by the ciliated epidermis. A restricted domain of increased tropomyosin expression is still present in the former wound region, indicating that differentiation of new myocytes is still going on at this stage of regeneration. Anterior is toward the left. Scale bars: 50 μm in all aspects.

and differentiated myocytes. Differentiating myoblasts and myocytes are expected to be widely distributed, especially in the body of a growing juvenile. Additionally, it is known for both invertebrates and vertebrates (Cox et al., '86; Sassoon et al., '88; Kelly et al., 2002) that actin transcripts accumulate in myoblasts before they differentiate into myocytes. Expression analysis of *SrAct* in adult worms further supports its muscular role. The highest signal is recovered around the male gonopore, which is the most muscular structure of adults and in the muscle mantles of sagittocysts (Semmler et al., 2008). The latter are extrusomes

used for defense and copulation, and are produced by a specialized cell, the sagittocyte. The distal tip of the sagittocyte is wrapped by a muscular cell (the muscle mantle), the contraction of which causes the discharge of the sagittocyst (Gschwentner et al., 2002).

Although actin and tropomyosin proteins exist in different isoforms and are present both in muscular and nonmuscular cells (Lees-Miller and Helfman, '91; Pittenger et al., '94; Mounier and Sparrow, '97), troponins are only known from muscles of bilateral animals, whereas no troponin orthologs have been found in the available nonbilaterian genomes (U. Technau, personal communication). Unquestionably, *SrTnI* is a muscular gene, as it is shown to be the homolog of the inhibitory subunit of the troponin complex in our phylogenetic analyses and by its expression in the male genital opening and in the adults' muscle mantles of the sagittocysts. The expression of *SrTnI* is strikingly similar to the expression of *SrAct* in juveniles, thus the coexpression of the two genes is likely. Troponin proteins are key regulators of the muscular contraction in the sarcomeric muscles of both invertebrates and vertebrates, with the only exception known from the ascidian *Ciona intestinalis*, whose body-wall smooth muscles contain troponin and a striated muscle isoform of the tropomyosin (Meedel and Hastings, '93; Endo et al., '96). Interestingly, *S. roscoffensis* has exclusively smooth muscles (Semmler et al., 2008), a condition likely to be ancestral for all acoels, because no striated muscles have ever been described in any acoel species so far investigated (Rieger et al., '91; Hooge, 2001).

Tropomyosin is an elongated protein that assembles in dimers and forms a coiled-coil structure. In the vertebrates' muscles, but very likely in the invertebrates' muscles too (Bullard et al., '73; Lehman et al., 2000), each tropomyosin dimer lies in the groove of the filamentous actin and its role is to inhibit the actin–myosin interaction, thus preventing contraction during resting conditions. Tropomyosin genes exist in multiple copies in the genomes of all metazoans as well as in other eukaryotes so far sequenced (Lees-Miller et al., '90; Lees-Miller and Helfman, '91; Irimia et al., 2010). In the Bilateria, one tropomyosin gene encodes for short (about 250 aa) and long (usually 284 aa) isoforms. Any tropomyosin gene can generate several different isoforms, either by alternative splicing or differential promoter usage (Lees-Miller and Helfman, '91). The two forms (long and short) differ by a 38 amino acid-long peptide at the *N*-terminus of the protein (Greenfield et al., '98), which is highly conserved among distantly related bilaterians, although it is never recovered in nonbilateral animals (green box in supplementary material, S7). This domain is necessary for head-to-tail interactions between two consecutive tropomyosins and for their stability in the F-actin furrow. Mutations in the *N*-terminal domain result in loss of affinity of the tropomyosin for the actin filaments (Greenfield et al., '98). With a few exceptions (Weinberger et al., '93; Pittenger et al., '94; Perry, 2001), the long forms are expressed in muscles, whereas the short isoforms are expressed in other cell types (Lees-Miller and Helfman, '91; Pittenger et al., '94; Irimia et al., 2010). In line with these findings in other organisms, we show that the long tropomyosin *SrTrp* (supplementary material, S7) is muscular, using a specific anti-SrTrp antibody. However, it is possible that the ISH of this gene could be misleading, because the riboprobe generated against the full length of the tropomyosin clone might recognize transcripts of the short tropomyosin as well, thus labeling muscles and, perhaps, also other cell types (Pittenger et al., '94). This would explain why *SrTrp* seems more widely expressed than *SrAct* and *SrTnI*.

Accordingly, one could interpret the longitudinal bands of cells strongly stained in the region frontal to the male genital opening as nerve cords (Fig. 2C''), a real possibility because some long isoforms of the tropomyosin are known to be present in the nervous system (Weinberger et al., '93). However, we have to point out that *S. roscoffensis* has six longitudinal nerve cords (Bery et al., 2010; Semmler et al., 2010), meaning that four of the nerve cords would not express the gene. In our view, there are two ways to explain this domain of expression. First, these cells could be accessory muscles, because the region anterior to the male gonopore is rich with them (Semmler et al., 2008; this study), or alternatevely, they could be special gland cells, which we have found to be distributed in a paired manner that coincides with the observed pattern (unpublished data).

The genes *SrAct*, *SrTnI*, and *SrTrp* show mostly overlapping expression domains in both juvenile and adult *S. roscoffensis*, thus suggesting that in the acoel the three proteins might physically interact, as they do in the striated muscles of other bilaterians. However, this might be restricted only to a subset of muscles because tropomyosin is not transcribed (no ISH signal) or translated (no antibody signal), for instance, in the muscle mantles of sagittocysts (see below).

Tropomyosin Expression During Muscle Regeneration in the Acoel *S. roscoffensis*

In acoels, the dynamic pattern of muscular gene expression can be best studied in regenerating animals.

The first sign of a reorganization of the musculature is a constriction of the circular muscles along the rim of the wound. As seen in *Macrostomum lignano*, the initial constriction might help in reducing the wound surface (Salvenmoser et al., 2001). Subsequently, the muscles of the body wall lose their local regular organization, and some longitudinal fibers grow from the dorsal to the ventral side, causing a ventral shift of the wound. The wound closure by an initial layer of preexisting muscles takes approximately 12 hr. Right afterwards, an undifferentiated blastema becomes visible, the tropomyosin gene expression is upregulated in the region behind the blastema, and most likely new myocytes begin to differentiate. It is very likely (as is the case in *M. lignano*) that in addition to help in closing the wound,

the old fibers also serve as scaffold for the growing blastema (Salvenmoser et al., 2001).

During regeneration of the platyhelminth planarian *Girardia tigrina,* muscles are formed anew in the blastema, without any previous wound closure. However, few of the old fibers are observed to invade the blastema having, probably, the function of guiding differentiating cells into it (Cebrià et al., '97). Similarly, during the regeneration of the nervous system in *Convolutriloba longifissura* and *S. roscoffensis,* some of the existing nerve cords invade the blastema before the differentiation of new neurons starts (Gaerber et al., 2007; Bery and Martinez, 2011). It is, therefore, likely that old muscle fibers provide guidance cues for undifferentiated cells once they migrate into the blastema during the regeneration of *S. roscoffensis*.

The tropomyosin gene is up-regulated in the wound area, simultaneously or immediately after to the wound closure by preexisting muscles. The up-regulation of the gene indicates that differentiation of myocytes might occur in this area. In fact, accumulation of the tropomyosin transcripts has been observed in invertebrates and vertebrates during embryonic muscle development. In the gastropod *Haliotis rufescens,* the tropomyosin gene is expressed before myofibrillogenesis (Degnan et al., '97), whereas in *Xenopus laevis*, tropomyosin transcripts accumulate in the somites and in the embryonic heart long before mature myocytes are formed (Gaillard et al., '98).

The great regenerative capacity of acoels is owing to their unique stem cell system. The stem cells, neoblasts, are located exclusively in the parenchyma and they are able to differentiate in any cell type. A subpopulation of neoblasts expresses the gene *piwi*, which in other bilaterians is a germ line specification factor (De Mulder et al., 2009a,b; Egger et al., 2009). During regeneration of *I. pulchra*, *piwi*-expressing cells appear in the blastema approximately 1 day after amputation. These cells have most likely migrated into the blastema from other regions of the body (De Mulder et al., 2009a). We propose here that in *S. roscoffensis* a similar migration of undifferentiated cells could occur after the wound has been closed by preexisting muscle fibers. These migrating cells would be guided into the newly formed blastema by the same fibers. After proliferating, the neoblasts would initiate their differentiation into various cell types, by expressing tissue-specific genes (e.g. *SrTrp* in differentiating myocytes).

Evolutionary Implications

Our data show that the smooth muscles of *S. roscoffensis* have a molecular architecture similar to the striated muscles of other bilaterians, although only smooth muscles have been reported in Acoela (Rieger et al., '91; Hooge, 2001). In line with this and taking into account most recent animal phylogenies (Egger et al., 2009; Hejnol et al., 2009) that suggest the Acoela as the earliest offshoot of the Bilateria, it is tempting to suggest that the segregation of specialized muscle cells from "ancestral" epithelial muscle cells coincided with the diploblast–triploblast transition. Accordingly, the first bilateral animals possessed only smooth muscles with the molecular repertoire necessary to build a striated muscle.

Even though it has been proposed that the evolution of the long tropomyosin of the Bilateria might be linked to the evolution of the sarcomere (Irimia et al., 2010), and that the same reasoning could be applied to the evolution of the troponin which do not exist in nonbilaterians (U. Technau, personal communication), our data suggests that it is more parsimonious to regard striated muscle cells as a sister cell type to the smooth muscle cells. In this scenario, striated and smooth muscles would have arisen in the stem lineage that led to the Nephrozoa (i.e. all Bilateria exclusive the acoelomorphs) (Hejnol et al., 2009), from an "acoel-like" smooth muscle, by segregation and divergence of functions and through differential recruitment of additional genes (Arendt, 2008).

In myocytes, which express troponin genes, the myofibrils would have assembled eventually into a sarcomere, whereas the smooth muscles of the nephrozoans recruited new components, such as the calmodulin and the caldesmon among others, to regulate their contraction.

However, the case is far from being settled. Myocytes (true muscular cells) are also present in Cnidaria and Ctenophora (Seipel and Schmid, 2005), and cnidarian muscles express genes that are also present in bilaterian muscles (Schuchert et al., '93; Groger et al., '99; Renfer et al., 2010). Striated muscular cells have been described for one species of the ctenophores and in all medusozoan cnidarians (cubozoans, hydrozoans, and scyphozoans). However, the latter are the most derived classes of cnidarians and their striated muscles have a different ultrastructure from the bilaterian ones, making homology very unlikely (Burton, 2008).

In most bilaterians, smooth and striated muscles coexist and in the invertebrates the distinction between striated and smooth muscles, on a molecular basis, is not as clearly defined as in the vertebrates (Hooper and Thuma, 2005). For example, the smooth body-wall muscles of the ascidian *C. intestinalis* are regulated by the troponin (Endo et al., '96), or in the planarian *Dugesia japonica*, striated muscular isoforms of the myosin heavy chain are expressed in its smooth muscles (Kobayashi et al., '98). The latter example would indicate, for instance, that most likely in turbellarian flatworms striated muscles have been reduced (Ruppert et al., 2004). All these cases suggest that a "striated muscle" molecular architecture in smooth muscles is not exceptional to acoels. The independent loss of the sarcomeric organization in the muscles of some lineages, such as the planarians or *Ciona*, would be easily explained as an adaptation to a lifestyle that does not require the presence of fast striated muscles. Admittedly, the same argument could be applied to acoels as well. In this case, the ancestor of all Bilateria could have had striated muscles and they have been lost in the Acoela, among other lineages.

Philippe et al. (2011) have recently proposed that the Acoelomorpha (i.e. acoels+nemertodermatids) and Xenoturbella group together within the deuterostomes (as already suggested in a previous article by Philippe et al., 2007) instead of being basal bilaterians (Hejnol et al., 2009). If this scenario would be true, referring to the Acoelomorpha condition (or Xenoacoelomorpha, *sensu* Philippe et al., 2011) as ancestral to all Bilateria would be unfounded. Now we would have to assume following the most parsimonious reasoning that the protostome–deuterostome ancestor would have had striated muscles that have been lost in some lineages of the protostome (e.g. in turbellarian flatworms) and most probably in the whole Xenoacoelomorpha.

Obviously, to better understand the evolution of muscles, a final settlement within the metazoan tree of pivotal groups, such as Ctenophora, Acoelomorpha, and *Xenoturbella*, would be critical. Additionally, the acquisition of molecular data from *Xenoturbella*, which also exhibits only smooth muscles (Ehlers and Sopott-Ehlers, '97), and from the Nemertodermatida would be essential as well. However, it must be pointed out that we need further analyses of the connection between molecular composition of muscles and their morphology and function, before a final reconstruction of the stepwise evolution of musculature in the Metazoa is possible.

In summary, though we are far from a complete understanding of how the various types of muscles evolved over time, at present, three points may be considered:

1. Myocytes evolved from epithelial muscle cells (Rieger and Ladurner, 2003).
2. The first true myocytes were most likely of the smooth type, as it is hardly possible that such a complex and organized structure as the sarcomere evolved promptly from an epithelial muscle cell type.
3. The striated muscle cell type is not the sister of "the" smooth muscle type, but to one of numerous smooth cell types as there are cryptic subtypes of smooth muscles even in basal cnidarians (Renfer et al., 2010).

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Resúmen del primer artículo, R1

Arquitectura molecular de la musculatura en un acelo y sus implicaciones evolutivas

Hemos caracterizado los homólogos de un gen de una Actina, una Troponina I, y una Tropomiosina en el acelo *Symsagittifera roscoffensis*. Estos genes se expresan en los músculos y probablemente se co-expresan en al menos un subconjunto de ellos. Además, y por primera vez para los acelos, hemos producido un marcador muscular específico para esta especie, un anticuerpo contra la proteína Tropomiosina. Hemos descrito la expresión del gen y de la proteína Tropomiosina durante el desarrollo postembrionario y durante la regeneración posterior de adultos amputados, mostrando que las fibras musculares preexistentes contribuyen al cierre de la herida. Los tres genes caracterizados en este estudio interactúan en la musculatura estriada de vertebrados e invertebrados, donde la Troponina I y la Tropomiosina son reguladores clave de la contracción del sarcómero.

S. *roscoffensis* y todos los demás acelos descritos hasta el momento sólo tienen musculatura lisa pero la arquitectura molecular de éstos es la misma que la de las fibras estriadas de otros Bilateria. Dada la posición basal que se ha propuesto para los acelos dentro de los Bilateria, sugerimos que los músculos sarcoméricos surgieron de un tipo de músculo liso que ya tenía en su lugar el repertorio molecular de la musculatura estriada. Discutimos este modelo desde una perspectiva comparativa amplia.

Mesodermal gene expression in the acoel *Isodiametra pulchra* indicates a low number of mesodermal cell types and the endomesodermal origin of the stem cell system

Marta Chiodin[1], Aina Børve[2], Eugene Berezikov[4], Peter Ladurner[3], Pedro Martinez[1,5] and Andreas Hejnol[2*]

1 Departament de Genètica, Universitat de Barcelona, Barcelona, Spain
2 Sars International Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway
3 Institute of Zoology and Center for Molecular Biosciences, University of Innsbruck, Innsbruck, Austria
4 Hubrecht Institute, Utrecht, Netherlands
5 Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

*Communicating author

Abstract

Acoelomorphs are bilaterally symmetric small marine worms that lack a coelom and possess a digestive system with a single opening. Their phylogenetic position in the animal tree of life is debated as either forming the sister group to all remaining Bilateria and as a morphologically simple stepping stone in bilaterian evolution or as derived and thus morphologically highly reduced deuterostomes. Acoels and their relatives Nemertodermatida and *Xenoturbella* (together forming the Acoelomorpha) possess a very limited number of cell types. To further investigate the diversity and origin of mesodermal cell types we describe the expression pattern of 12 orthologs of bilaterian mesodermal markers including *Six1/2*, *Twist, FoxC*, *GATA4/5/6*, in the acoel *Isodiametra pulchra*. All the genes are expressed at least in part of the acoel musculature and in the stem cells (neoblasts) and gonads. Most genes are expressed in endomesodermal compartments of *I. pulchra* developing embryos similar to what has been described for their cnidarian orthologs. Our results deliver molecular evidence of the presence of a very limited number of mesodermal cell types and suggest an endomesodermal origin of the gonads and the stem cell system. Since the evolutionary interpretation largely depends on the phylogenetic position of the Acoelomorpha we discuss our results in the light of both phylogenetic hypotheses.

Introduction

The mesoderm is the embryonic germ layer that develops between the endoderm and the ectoderm. It is regarded as a key innovation that led to the diversification of organ systems and cell types present in bilaterally symmetrical animals (Bilateria) [1,2,3,4,5]. In the Bilateria the mesoderm gives rise to structures such as body wall musculature, supporting skeletons and secondary body cavities (coeloms). In some lineages these body cavities evolved into new organ systems such as the excretory and circulatory system that in turn allowed the evolution of larger body sizes [6,7,8]. Thus, the origin and evolution of the mesoderm have been central tenets in formulating hypotheses of the transition from a relatively simple radially symmetric ancestor to a complex bilaterian. A crucial topic in the different scenarios is the homology of coelomic cavities and how often they originated in animals [3,9,10,11]. According to the 'archicoelomate hypothesis' or 'enterocoely hypothesis' [3,12] the coelomic cavities of bilaterians

evolved from evaginations of the gastric epithelium of a cnidarian polyp-like ancestor. This mode of coelom development (enterocoely) is observed in extant deuterostomes such as echinoderms and hemichordates, in which it gives rise to a tripartite organization of coelomic cavities. According to Remane [12], such tripartite organization of body cavities is the ancestral bilaterian state and the acoelomate and "pseudocoelomate" conditions would have arisen by independent reductions of the coeloms in multiple animal lineages [13].

The mesoderm of extant coelomate animals consists of defined muscular layers and coeloms. Coeloms can be lined by a simple epithelium (pleura) or by an epithelio-muscular lining (myo-epithelium), and often both linings are present in the same taxon. A myo-epithelium consists of alternating epithelial cells and epithelio-muscular cells, which are epithelial cells with accumulated contractile filaments (mainly actin and myosin) at their basal portion, and are supposed to represent the ancestral contractile cells types [8,14]. According to some authors, a separation of the contractile myoepithelial cells and the epithelial cells would have occurred in the myo-epithelial lined coelom of the bilaterian last common ancestor (archicoelomate) [15].

A different scenario for the origin of the mesoderm is suggested by the acoeloid-planuloid hypothesis [4,16,17]. Here, the separation between the muscular contractile basal portion and epithelial apical portion would have occurred in the endoderm of a planula-like ancestor. In this scenario, individual myocytes, most likely arranged in an orthogonal grid of circular and longitudinal muscles, would be the first type of mesodermal tissue. According to this theory, the last common bilaterian ancestor was an organism that was similarly organized to extant acoelomorphs [18], which possess this type of muscular arrangement. In cnidarians, the sister group of the Bilateria, bilaterian 'mesodermal' genes are expressed in the endoderm [2] suggesting that the mesoderm evolved from the endoderm. However, it is an open question as to how and when this transition occurred.

In this study we present the expression patterns of 12 bilaterian mesodermal markers (Fig. S1) in *Isodiametra pulchra* (Acoela, Acoelomorpha). Acoelomorphs are unsegmented, acoelomate worms, sometimes referred as the proxy of the ancestral bilaterian in planuloid-acoeloid theory [4,16,19]. Recent molecular phylogenies and most modern phylogenomic approaches have supported this proposition by showing that acoelomorphs branched off the bilaterian tree before the protostome-deuterostome split [20,21]. However, a different phylogenomic study that applied a site-

heterogeneous model shows acoelomorphs as the sister group of the Ambulacraria [22], thereby implying that the morphological simplicity of the acoelomorphs is due to a loss of many characters, *e.g.* the anus, coelomic cavities and excretory system, possibly by neoteny (paedomorphosis) [23]. Since the phylogenetic position is still in debate [24], we discuss our results in the light of both hypotheses.

The musculature is the most prominent mesodermal derivative in *I. pulchra* and its ontogeny and architecture have been thoroughly studied [18,25]. Furthermore a mesenchymal tissue, called the parenchyma, fills the body space between the digestive syncytium and the body wall. The parenchyma develops from endomesodermal precursors and it is declared as mesodermal tissue only on the base of its location in adult worm [26,27]. Gonads and neoblasts are also located in the parenchyma too, but their embryonic origins not yet known [28,29].

In this study, we compare the expression patterns of mesodermal genes in *I. pulchra* with the expression of the orthologs in the Bilateria and Cnidaria and try to infer the ancestral condition of bilaterian mesoderm.

Results

Anatomy of *I. pulchra*

I. pulchra is an acoel that lives abundantly in the mud of the northeast Atlantic Ocean [30,31]. As in all other acoel species, a single anterior statocyst can be recognized at low magnification (Fig. 1A). A dense net of muscular parenchymal fibers (Fig. 1B) is in sunk with respect to the body wall, *i.e.* the epidermis and the body wall outer-circular and inner-longitudinal muscles (Fig. 1B). Additional diagonal muscular fibers are intermingled between the circular and longitudinal layer in *I. pulchra* [18,25]. The mouth is ventral and is surrounded by specialized muscles, which form a ring around the buccal opening (Fig. 1C). There is no pharynx, instead the mouth opens directly into the digestive syncytium. A pair of thick parenchymal muscles crosses each other dorsal to the mouth, between its posterior rim and the anterior wall of the female genital organ (Fig. 1C).

Immediately posterior to the mouth there is the female genital organ, which consists of a muscular vagina and the sausage-shaped bursal nozzle (Fig. 1D), a sclerotized structure necessary for sperm storage [25]. Posteriot to the female genital organ is the

male genital organ with its prominent muscular structure, the seminal vesicle that is complemented by a muscular tubular penis. Gonads are paired, consisting of ventral ovaries and dorso-lateral testes [29]. The gonads are not lined by any tissue and lie in the parenchyma. In close proximity, the neoblasts, the acoel somatic stem cells are also located in the parenchyma [28]. Hatchlings and juvenile worms of *I. pulchra* have a very similar body plan, although they lack the reproductive organs.

Gene selection and orthology

All of the genes characterized in this study are orthologous to bilaterian mesoderm markers, in addition to being expressed in the endoderm of cnidarians (see Fig. S1 for detailed references list). Although some of the genes characterized here are not specifically restricted to the bilaterian mesoderm, their broad usage in bilaterian mesoderm patterning justifies their interest for this study. These genes specifically are the orthologs of: *Mef2*, which can trigger either myogenesis or neurogenesis depending on splice variants in cnidarians and bilaterians [32,33]; *Six1/2,* also used in neurogenic and myogenic circuits in Cnidaria and Bilateria [34,35,36,37]; *Pitx*, whose expression seems not to be germ layer specific in Bilateria, nevertheless it is consistently expressed in the coelomic mesoderm of the deuterostomes [38,39,40]; *Tbr*, whose expression varies from neural to endomesodermal in different taxa [41,42,43,44,45].
Finally, *FoxA* orthologs are central nodes of the endoderm gene regulatory network across the Bilateria [46]. Consistently, in the acoel *Convolutriloba longifissura*, *FoxA* is expressed in the endoderm during embryonic development and in freshly hatched worms [47]. However, *FoxA* is also necessary for the development of the muscular apparatus associated to the digestive system, *e.g.* the muscular pharynx [48,49,50].
The orthology assignments for all genes are given in the supplementary material (Fig. S2-S9). In the case of the tropomyosin gene *IpTrp*, no phylogenetic analysis was conducted given the high amino-acid sequence similarity across all Eukaryota. *IpTrp* shares 90% of identical positions to a tropomyosin of another acoel species (*SrTrp*, Fig. S10) [51].

Gene expression

Genes that are broadly expressed in *I. pulchra* mesoderm (muscles, parenchyma, gonads and neoblasts): *IpmuscleLIM, IpPitx IpFoxA1 and IpFoxC*

MuscleLIM genes are expressed in muscles in a wide range of bilaterians [52,53,54] and cnidarians [55]. In *I. pulchra* juveniles *IpmuscleLIM* is expressed subepidermally along the whole anterior-posterior axis (Fig. 2A), with a higher concentration of transcripts in the anterior region where the muscular net is denser (Fig. 1B). In adult worms, the gene is strongly expressed in the anterior region and in two bilaterally symmetrical domains, approximately where the gonads and the majority of neoblasts are located. Additionally, the gene is expressed in the cross muscles (Fig. 2B, asterisk).

The anterior region is densely packed with myocytes and neurons, and a few scattered cell bodies of secretory cells and the insunk cell bodies of epidermal cells [56]. The high intensity and homogenous distribution of *IpmuscleLIM* positive cells in the anterior region does not correlate with neural or gland and epidermal cells expression. Furthermore, the high expression of *IpmuscleLIM* in the muscular copulatory organs, as well as the consistent muscular expression of *muscleLIM* orthologs across the eumetazoans, indicate that, in the anterior domain, *IpmuscleLIM* is expressed in the myocytes of the head. By fluorescent *in situ* hybridization (FISH) we achieved a better resolution of the cell types that express the gene. We clearly could detect expression in the gonads (testes and ovaries) of adult worms. The expression in cells surrounding the digestive syncytium indicates the *IpmuscleLIM* is expressed in parenchymal cells (Fig. 2B and C). In order to understand if these genes are expressed in the neoblasts of *I. pulchra*, we combined EdU labeling of S-phase cells (neoblasts) with FISH, and indeed we identified *IpmuscleLIM* expression in the neoblasts (Fig. 2C1-C3).

IpPitx expression in juvenile worms mirrors *IpmuscleLIM* expression (Fig. 2D). *IpPitx* is expressed subepidermally, similar to the expression pattern observed for *IpmuscleLIM*. Since in freshly hatched worms no peripheral parenchyma can be detected (Hejnol, Seaver and Martindale, unpublished data) [56], we feel confident in assigning *IpPitx* expression to the juvenile myocytes. Likewise, *IpmuscleLIM* and *IpPitx* are similarly expressed in adult worms (Fig. 2E). Clear muscular expression was detected in the genital organs and the mouth (Fig. 2F). Additionally, *IpPitx* is expressed in the parenchyma, gonads and in a subset of the neoblasts (Fig. 2F and F1-F3, open white arrowheads).

One of the two *FoxA* orthologs, *IpFoxA1*, is expressed in the juvenile digestive syncytium (Fig. 2G) whereas in adults its expression gets extended to the anterior

mesoderm as well as to the peripheral parenchyma, the ring muscles encircling the mouth and to a pair of accessory muscles connected to the male copulatory organ (Fig. 2H). By FISH, we detected expression in the gonads (Fig. 2I) as well as in the neoblasts, as confirmed by the co-localized EdU and FISH signals. It can be concluded that *IpFoxA1* is primarily endodermal, since we have also observed its expression in the vegetally invaginated endomesoderm of the embryo (Fig. S11 A) and it gets recruited to mesodermal components only in later postembryonic development. The embryonic expression of *IpFoxA1* at the vegetal pole differs from that of *IpmuscleLIM* and *IpPitx*, which are expressed at the animal pole after gastrulation (Fig. S11 E and F), where myogenesis is initiated [18].

The ortholog of *FoxC* is expressed subepidermally along the anterior-posterior axis of the juvenile, but contrastingly from *IpmuscleLIM* and *IpPitx,* its expression intensity does not decrease towards the posterior end of the animal (Fig. 2L). In adults, *IpFoxC* expression is restricted to more specific domains in a similar fashion to *IpmuscleLIM* and *IpPitx* (Fig. 2M). Again, we detected anterior expression, most likely in the "head-myocytes", in the cross muscles and in the lateral domains encompassing both gonads and neoblasts (Fig. 2M, 2N and 2N1-N3). In embryos, *IpFoxC* is detected at the anterior animal pole after gastrulation, as *IpmuscleLIM* and *IpPitx* are, suggesting its likely myogenic activity (Fig. S11 B).

In conclusion, all the genes described in this section have a broad expression in *I. pulchra* juvenile and adult specimens. They all are expressed in muscles, or at least in a subset of them, and in the peripheral parenchyma. Moreover those genes are expressed in the gonads and in a subset of neoblasts, whose embryonic origins are not yet understood.

Mesodermal genes expressed in muscles, gonads and neoblasts of *I. pulchra*: *IpFoxA2, IpGATA456, IpMef2, IpSix1/2*

The second *FoxA* ortholog, *IpFoxA2*, is expressed subepidermally along the whole anterior-posterior axis of the hatchling, and has a broader expression domain than its paralog (Fig. 3A). Yet, the strongest expression of *IpFoxA2* is in the region of the digestive system, suggesting that the expression of the two *IpFoxA* paralogs overlap in the digestive system during juvenile development (Fig. 3A). In adults, *IpFoxA2* expression becomes restricted to more discrete domains. Here, *IpFoxA2* is expressed in the head myocytes, in the cross muscles (Fig. 3B) and in gonads and neoblasts (Fig. 3C

and 3C1-C3). The weak signal detected by FISH in the cells surrounding the digestive syncytium (Fig. 3C) is most likely background signal, given that we observed no parenchymal expression in the more sensitive enzymatic reactions (Fig. 3B).
IpGata456 is expressed in scattered cells in the anterior region of juveniles, approximately around and posterior to the statocyst. The most posterior positive cells are arranged along the midline (Fig. 3D). This expression domain persists in older worms. Furthermore, we found that at this stage, *IpGata456* is expressed in the head-myocytes, in the cross muscles (Fig. 3E) and in the gonads (Fig. 3F) and neoblasts (Fig. 3F1-F3).
IpMef2 transcripts are concentrated in the head region and in two longitudinal bands of cells in juveniles (Fig. 3G). The high level of *IpMef2* expression in the region where the copulatory organs develop corroborates the expression of this gene in differentiating myocytes (Fig. 3G). In sexually mature worms, we were able to detect expression of *IpMef2* in the head region. In this region the signal persists higher in what we interpret to be the anterior and posterior commissures of the brain (Fig. 3H, small white arrowheads), and weaker between the two commissures, therefore in the head myocytes (Fig. 3H). These results are consistent with dual neural and myoblasts expression of *Mef2* orthologs as observed in other eumetazoans [32,33]. In adults, the gene is additionally expressed in close proximity to the male genital organ (Fig. 3H) as well as in the gonads (Fig. 3I) and in the neoblasts (Fig. 3I1-I3).
In juveniles, *IpSix1*/2 is expressed anterior and in two longitudinal bands in cells that are flanking the digestive syncytium (Fig. 3O). We infer that the anterior domain might correspond to neural expression, given that the strongest labeled spots coincide with the location of the two anterior and two posterior neurite loops of the brain, as well as in a transversal stripe which likely is the posterior brain commissure (Fig. 3O, small white arrowheads). The posterior connection of the two lateral expression domains of *IpSix1*/2 corresponds to the developing female genital organ (Fig. 3O, inset). In adult worms *IpSix1/2* expression has considerably decreased with the exception of the gonads (Fig. 3M and N). Weak expression persists in the anterior region in cells that we infer to be myocytes, due to their distribution; even weaker expression is detected in the cross muscles (Fig. 3M and 3N). By double EdU and FISH labeling, we detected expression in few neoblasts (Fig. 3N1-N3).
To summarize, the genes *IpFoxA2, IpGATA456*, *IpMef2*, *IpSix1*/2 are expressed in myocytes, gonads and neoblasts, but they are not expressed in cells of the peripheral

parenchyma. Therefore the genes are confined to fewer cell types than those described in the previous section.

Genes expressed in a limited amount of cell types: *IpTwist1 and IpTwist2*, *IpTbr* and *IpTrp* (*tropomyosin*)

We have cloned two *Twist* orthologs (*IpTwist1* and *IpTwist2*). We did not detect early juvenile or embryonic expression of both orthologs. In the adult, *IpTwist1* is mainly expressed in the gonads and in the male copulatory organ (Fig. 4A and B). The expression in ovaries was only clear after fluorescent *in situ* hybridization. However, by mean of FISH, we could not observe expression in the male genital organ (Fig. 4B). Double labeling with EdU and *IpTwist1* antisense probes, revealed weak expression of the gene in only few neoblasts (Fig. B1-B3). The second *Twist* ortholog, *IpTwist2*, has an overlapping expression with *IpTwist1* in the gonads and weakly in the copulatory organs (Fig. 4C). Several neoblasts are *IpTwist2* positive (Fig. 4D and D1-D3). Although the discrepancies between FISH and standard *in situ* hybridization patterns are difficult to explain, it is relevant for this study that expression of both *Twist* orthologs is restricted to a few cells and few cell types, namely a subset of myocytes, neoblasts and part of the gonads, whereas neither are expressed in the parenchyma. *IpTbr* is not expressed in hatchlings whereas it is detected at later stages in the developing oocytes (Fig. 5A), when the juveniles start reproductive development. In mature adults *IpTbr* is expressed at all stages of oocyte development, *i.e.* from oogonia to mature oocyte (Fig. 5B-C). Thus, *IpTbr* is maternally expressed and it may be necessary for the endomesodermal patterning, as we detected expression in the endomesoderm during embryonic development (Fig. S11 G). *IpTbr* transcript was not colocalized with EdU labeling of neoblasts (Fig. 5C1 and C3).

Finally we have found the general muscle marker *IpTrp* (tropomyosin) to be broadly, but exclusively, expressed in the musculature of *I. pulchra*. In juveniles the gene is widely expressed subepidermaly along the antero-posterior and dorso-ventral axes (Fig. 5D). In comparison to the juvenile, the adult *I. pulchra* has developed female and male genital organs, which express *IpTrp* (Fig. 5E-F). The strong expression is located in muscular ring surrounding the male genital opening (Fig. 5E-F, arrowhead), which is the most muscular structure in adult *I. pulchra* and also in other acoels [18,51,57,58,59]. Strong *IpTrp* expression is also found in the sphincter of the female genital organ (Fig. 5E and F, arrow). *IpTrp* transcript levels are very high at all stages of development,

from embryo to adult. The uniform distribution of *IpTrp* positive cells indicates that this gene is a pan-muscular marker. The head region, with high condensation of myocytes, did not stain stronger as *e.g.* in the case of *IpmuscleLIM*, because we shortened the time of the staining reaction.

Overall, we did not observe *IpTrp* expressed in neoblasts, although some *IpTrp* positive cells exhibited faint EdU labelling (Fig. 5F1-F3, see below), suggesting they could be neoblasts that have undergone differentiation (see discussion). To summarize, the two *Twist* orthologs characterized in this study are expressed in neoblasts, gonads and a subset of myocytes, but their expression is restricted to few cells, especially when compared with *IpFoxA2*, *IpGATA456*, *IpMef2* and *IpSix1*/2 that are expressed in the same cell types. *IpTbr* and *IpTrp* instead are expressed in single cell type, the oocyte and the myocytes, respectively.

Considerations on neoblasts expression

In the EdU assay, the fluorescent signal is detected by a modified uridine, which is incorporated in the nuclei of the proliferating cells. We observed two different patterns of incorporation into *I. pulchra* neoblasts. One type, called type I (after Gschwentner and colleagues, [60]) incorporate the uridine homogeneously at the periphery of the nucleolus (Fig. 2F3). The others, type II neoblasts, incorporate the uridine in a less uniform fashion, so that the glowing nucleus has a granular aspect (Fig. 2F3). We have observed that the genes *IpFoxC*, *IpTwist1* and *IpTwist2* are preferentially expressed in type II, granular neoblasts, whereas all other genes do not exhibit any preference (Table 1). The genes *IpmuscleLIM* and *IpPitx* were expressed in all EdU-labeled neoblasts (data not shown) that we examined, whereas other investigated genes seemed to be expressed only in a subset of labeled neoblasts (Table 1). Finally the genes *IpFoxA1*, *IpFoxC* (Fig. 2I1 and I3; Fig. 2N1 and N3), *IpFoxA2* (Fig. 3C1 and C3) and *IpTrp* (Fig. 5F1 and F3) were generally expressed in very few neoblasts, with a very low level of EdU incorporation (Table 1).

Discussion

Acoel mesoderm and the differential expression of mesodermal genes in *I. pulchra* musculature

Acoelomorphs have a unique early cleavage pattern that is called 'duet cleavage' because the two animal micromeres are already formed at the second cleavage by asymmetric cell division [26,61,62]. The fate map of the acoel species *Neochildia fusca* shows that the digestive system, the muscles and the peripheral parenchyma derive uniquely from the third pair of vegetal macromeres, the endomesodermal precursors [26].

Muscles in acoelomorphs are fibrous, mononucleated and of the smooth type, which are arranged in an orthogonal grid of inner-longitudinal and outer-circular muscles plus some diagonal muscles, interposed between the two other layers and crossing each other at the body midline [27]. In *I. pulchra*, specialized parenchymal muscles cross the body dorso-ventrally while specialized muscles are also found around the mouth opening and the copulatory organs (Fig. 1D). Acoels do not possess body cavities; instead parenchymal tissue fills the space between the epidermis and the digestive syncytium. This tissue bears the parenchymal cells [27,63], the neoblasts (*i.e.* the acoel somatic stem cells) [28,60] and the germ cells, plus all stages of gamete maturation (gonads). Gonads are not lined by any tissue (asacular) in any acoelomorph taxa [29,64,65]. The somata of epidermal, glandular and muscular cells are usually sunken below the body wall, making it difficult to recognize them from the other parenchymal cells. The anterior region of acoels is densely packed with myocytes, neurons, and scattered epidermal and gland cell bodies, but neoblasts and parenchymal cells are usually absent from this area of the body [28,56]. The posterior tip of the animal also has no peripheral parenchyma but it is occupied by the myocytes of the copulatory organs, glands, and a spacious posterior chordoid vacuole (Fig. 1D).

In this study we have shown that all genes that we have characterized and which are orthologs of bilaterian mesodermal markers are expressed in *I. pulchra* muscles, with the only exception being *Eomes/Tbrain/Tbx21* ortholog *IpTbr* (Fig. 6).

Here we have also shown that the different *I. pulchra* muscles express different genes (summarized in Table 2), whereas only the gene *IpTrp* (tropomyosin) is, as expected, a pan-muscular marker. Different muscle types were previously described based on their ultrastructure, *e.g.* the pseudostratification of the anterior "head"-myocytes (Fig. 1B) [25], and we assume that more acoel muscle cell types can be identified on the level of their molecular fingerprint [14].

We show here that the female and male genital organs express different sets of genes and therefore might have independent origins (Table 2). However, the same genes that

are expressed in the female genital organ are also expressed in the crossed muscles, e.g. *IpSix1/2* is expressed in the anlagen of the female genital organ during late juvenile development and in the cross muscles in adults. The two muscular structures have likely a common origin in a single muscle type. Indeed the cross muscles are physically connected to the female genital organ, and cross each other exactly at the intersection between the mouth and the genital organ (Fig. 1C). Given that *I. pulchra* deposits the fertilized eggs through the mouth [27], we suggest that the cross muscles might be used in reproductive and feeding behaviors. Indeed the *I. pulchra* cross muscles express *FoxA* and *GATA* 456 orthologs which are expressed in the muscular pharynx of several other eumetazoans [2,48,49,66,67]. Because *I. pulchra* lacks a pharynx, which is present in less derived acoel groups [30,68,69], we propose that the complex ventral musculature [25] as well as the cross muscles stand in for the absent pharynx during feeding behaviors. Notably, silencing of the gene *IpPostHox*, which is expressed in the same area of *IpFoxA* and *IpGATA456*, produces worms incapable of normal feeding and with disrupted posterior musculature [70]. Likewise, we predict those worms would not be able to lay the eggs. The myogenic specification factor *IpMef2* is expressed in the anlagen of the copulatory organs and is not detected in the adult structure, which is consistent with a role in early myocyte specification as seen in other bilaterians [37]. In *Drosophila*, the gene *Twist* acts as an early myogenic factor [71], whereas in the mouse it is a myogeneic inhibitor [72], thus its function in the mature copulatory organs of *I. pulchra* is difficult to envision. Besides being expressed in the developing musculature, we also found that *IpSix1/2* and *IpMef2* are expressed in cells of the nervous system (Fig. 3E and 3G). The expression of both genes in muscles and neurons is well documented for bilaterians and cnidarians [32,36,73,74,75]. For example, different *Mef2* splice variants regulates myogenesis and neurogenesis in *N. vectensis* [32]. Since our antisense probe encompasses the MADS and Mef2 domains that are invariably present, it is possible that we detected all the transcript variants.

Mesodermal gene expression in *I. pulchra*: neoblasts, gonads and parenchyma

Neoblasts and gonads lie in close proximity to the longitudinal bands that run parallel to the digestive syncytium, and they do not overpass the posterior border defined by the copulatory organs. The neoblasts are the only dividing cells in the body of *I. pulchra* and they can differentiate into several cell types, presumably all, including germ cells [28]. It is generally believed that the metazoans germ cells evolved from totipotent

somatic stem cells, similar to the acoelomorph neoblasts or the cnidarian interstitial cells [76]. Indeed, key regulators of metazoan germ cells development, *e.g. piwi*, are also expressed in acoels and platyhelminth neoblasts as well as cnidarian interstitial cells [28,77,78,79]. In this study we extended considerably the list of factors that are commonly expressed in the germ line and in the neoblasts of the acoel *I. pulchra*. With exception of the gene *IpTbr*, all other mesodermal genes characterized here are expressed in the neoblasts and in the gonads of *I. pulchra* (Fig. 6). Remarkably, no ortholog of these genes is expressed in the stem cell system and/or in the germ line of platyhelminthes species that have been investigated by large scale expression profiling [80,81,82,83,84].

A 'genome-wide totipotency' is proposed to be necessary for the maintenance of the stem-cell and germ-cell pluripotency [80,82,83,84,85]. Such feature could be maintained by post-transcriptional regulative mechanisms that rely on the presence of numerous RNA binding proteins that act as translation inhibitors [80,83]. We speculate that a similar mechanism might be at the base of the differentiation potential of *I. pulchra* neoblasts. We suggest that the genes studied here are targets of RNA binding proteins that prevent translation and thus allow a later prompt activation for differentiating pathways. Alternatively, the genes that we have characterized might have a regulative role of stem cell and germ cell biology in *I. pulchra*, suggesting that this might stand on completely different regulative mechanisms than in other metazoans. The expression of the genes *IpFoxA1*, *IpFoxC* (Fig. 2I1-I3 and N1-N3), *IpFoxA2* (Fig. 3C1-C3) and *IpTrp* (Fig. 5F1-F3) in cells with low EdU signal might also indicate that these cells already entered the post-mitotic phase (and therefore have reduced by half the uridine incorporation) and undertaken a differentiation pathway. We however consider this hypothesis less likely since the time frame between EdU incubation and fixation was very short.

Neoblasts and germ cells are suspected to segregate during embryonic development, because neoblasts and primordial germ cells are already present in freshly hatched worms [28]. In light of our results showing the expression of mesodermal genes in these neoblasts, it follows that neoblasts most likely arise from endomesodermal tissue during embryogenesis (Fig. S11). However, under this scenario, the neoblast capacity to differentiate into ectodermally derived cells, such as epidermal cells and neurons, remains unclear. Since we also do not know whether all neoblasts express all

endomesodermal genes or not, we cannot exclude their developmental origin from all the three germ layers.

Finally, only few of the mesodermal genes characterized here (*IpmuscleLIM*, *IpPitx*, *IpFoxA1*, and *IpFoxC*) are expressed in the acoel peripheral parenchyma. *MuscleLIM* orthologs are exclusively expressed in the muscles of bilaterians [53,54] whereas *Pitx*, *FoxA* and *FoxC* orthologs are expressed in the mesoderm as well as in the endoderm of several bilaterians and they are all expressed in the cnidarian endoderm [47,66,86,87,88,89,90,91]. From the acoel embryology it is known that the peripheral parenchyma differentiate from the same precursors of the acoel endoderm (digestive syncytium). Likewise, the evolutionary origins of the acoel peripheral parenchyma from endoderm was proposed by Smith and Tyler [63] after they observed that this tissue is absent in less derived acoelomorph taxa such as the Nemertodermatida - the acoel sister group - and *Xenoturbella,* whose position within the Acoelomorpha receives support [21,22,92]. Both nemertodermatids and *Xenoturbella* have an epithelial digestive system and lack the peripheral parenchyma [10,93]. Previous researchers convincingly connected the origin of the peripheral parenchyma with the evolution of the syncytial digestive system, and thus both characters are specializations of the acoel lineage. This process exemplifies how mesodermal tissue can originate anew from the endoderm. Interestingly, the acoel *Paratomella rubra*, a distant lineage to the more commonly studied Acoela species [30,58], has a digestive system that consists of a lumen surrounded by digestive cells (digestive parenchyma) but lacks a proper peripheral parenchyma. *Paratomella* might thus represent the link between the more ancestral acoelomorphs and the derived acoels, e.g. *I. pulchra* [10,63]. In this scenario one should therefore expect the orthologs of *muscleLIM*, *Pitx*, *FoxA* and *FoxC* to be expressed in the epithelial digestive system of nemertodermatids and *Xenoturbella,* as well as in the digestive parenchyma of *Paratomella rubra*.

Muscles, neoblasts and gonads express the majority of the mesoderm-specific genes characterized in this study, which indicates an endomesodermal origin for these tissues. Since all the acoel parenchymal genes are also expressed in the endoderm of other bilaterians and cnidarians, our findings support the statement that this tissue developed and evolved in the acoel lineage from digestive precursors. It is thus questionable to define the acoel parenchyma as “mesodermal”. According to the position between digestive syncytium and ectoderm and without any homology statement it can be called

"mesoderm" (see Ruppert [6]). However, in case a common evolutionary origin is implied it should be better called "endodermal parenchyma".

Acoelomorphs as derived deuterostomes: does the acoel parenchyma represent the extant vestige of an ancestral coelomic cavity?

Albeit the monophyly of the Acoelomorpha (*Xenoturbella* + (Nemertodermatida+Acoela)) initially proposed on morphological observations [92] is recovered in the most recent phylogenomic studies [21], their placement inside the Bilateria remains one of the major debated topic in animal phylogeny [24]. Both molecular and morphological phylogenies agree that the Acoela bear many derived characters *e.g.* digestive syncytium and parenchyma (see above), whereas *Xenoturbella* and Nemertodermatida retain most ancestral traits such as an epithelial digestive system. However, one phylogenomic study that used massive taxon and gene sampling, places the clade as sister to all remaining bilaterians [21], while a different phylogenomic study that uses a site-heterogenous model of protein evolution but much less molecular sequence data, places the clade as sister to the Ambulacraria (Hemichordata + Echinodermata) [22]. Alternatively, after microRNA and mitochondrial genome analysis, the same study proposes that Acoelomorpha is nested within the Deuterostomia as a sister lineage to the Ambulacraria (echinoderms and hemichordates) [22]. The phylogeny of Philippe and colleagues [22], implies the loss of several deuterostome diagnostic characters such as gill slits, enterocoelic formation of the mesoderm, and possibly a tripartite coelomic organization of the adult body plan [22].

The origin of an acoelomate body plan from a coelomate ancestor is of course possible, given that it is observed in extant animal species, such as *e.g.* interstitial annelids [7,8,94] (Fig. 7). It is generally assumed that the acoelomate condition is achieved through progenesis [13,94] and an attempt of deriving the acoelomorph body plan from neotenic juvenile hemichordates in which the coelom has not yet been formed, has been previously suggested [23]. The observation that in extant echinoderm species some body-wall muscles develop from the myo-epithelial coelomic lining where all progressive stages are present in a single specimen [95], has led some authors to generalize this model as the bilaterian model of muscle evolution [10,15,94]. However, there are no embryonic or adult traces of an anlage or degenerated coelom present in

acoelomorphs. Thus, it remains unclear how the musculature might have separated from the former myo-epithelium of the coelomate ancestor (Fig. 8).

On first glance, an obvious conclusion would be that the acoel parenchyma represents the extant vestige of an ancestral coelom, given that the parenchymal cells also express the gene *Pitx* which is also expressed in the right coelomic pouch of echinoderms and in the left enterocoelic mesoderm of the cephalochordates [38,39,40]. Likewise, *FoxC* is expressed in the parenchyma of *I. pulchra*, is expressed in the coelomic pouches of sea urchin [87], and in the segmental mesoderm of cephalochordates [89] that initially forms by enetrocoely from the dorsal roof of the archenteron [96].

The assumption that the acoel parenchyma represents the vestige of the ancestral coelomic cavity would nevertheless lead to the least parsimonious implication that a peripheral parenchyma was present in the acoelomorph ancestor and must have been lost twice, in the lineage to *Xenoturbella* and in the nemertodermatid lineage (see discussion above and Fig. 7). We can exclude the enterocoelic formation of the peripheral parenchyma [56] since it is nearly absent in hatchlings (Hejnol, Seaver and Martindale, unpublished data) and the endoderm is syncytial early in development and transient coelomic pouches are absent. The series of transitions from an epithelial digestive tract to the syncytial digestive system demonstrated by Smith and Tyler [63] offers a more plausible explanation of parenchyma evolution, *i.e.* as an acoel apomorphy (Fig. 7), that differentiates from neoblasts in late development. Therefore, even if acoelomorphs are deutorostomes, the parenchyma is unlikely to represent the remnant of a collapsed coelom.

Acoelomorph as derived deuterostomes: are the gonads the vestige of the ancestral coelomic cavity?

If the acoel peripheral parenchyma does not represent the extant vestige of a coelomic cavity, does the mesodermal gene expression in the acoel gonads support a coelomate acoelomorph ancestor? The assumption is plausible as the majority of bilaterians form their gonads from coelomic cavities that are connected to the exterior through special ducts called gonocoels. Even though acoels do not have any of these structures, they still have genital openings, *i.e.* the female and male genital organs, and even though the fertilized eggs are released through the mouth instead of the female genital organ, the acoel genital opening could be the reduced gonopores of an ancestral gonocoele. In this study we show that the genes *IpFoxC*, *IpGATA456*, *IpPitx*, *IpSix1/2* and *IpTbr* are

expressed in neoblasts and/or gonads of *I. pulchra* (Fig. 6), whereas the echinoderm orthologs are expressed in the coelomic mesoderm [39,87,97,98,99]. Likewise the *Branchiostoma* orthologs of *Mef2, Pitx*, *Six1/2*, *Tbr* and *Twist* - all expressed in *I. pulchra* neoblasts and/or gonads - are expressed in the Hatscheck's diverticulum [38,40,44,100,101,102] that forms by evagination from the anterior tip of the archenteron and is traditionally homologized to the protocoelic cavity of hemichordates [103] (but see Stach [104] for a different opinion). In addition, the lancelet's orthologs of the genes *FoxC*, *Mef2*, *Six1*/2 and *Twist* are expressed in the larvae segmented mesoderm [89,100,101,102], which develops through enterocoely [96]. Many of the genes for which we show expression in the gonads in *I. pulchra* (germ cells and differentiating gametes) are expressed in the coelomic lining of deuterostomes, making it plausible to recognize the acoel gonads as the remnant of the ancestral coelomic cavity of the deuterostomes.

Furthermore, the acoel neoblasts and gonads express the same mesodermal gene set indicating a possible common origin of the two cell types. Remarkably, bilaterians that have coelomic gonads develop their germ cells from a specialized region of the coelomic epithelium, the germinative region, by de-differentiation of the epithelial cells into somatic stem cells which subsequently develop (and evolved, see [76]) into germ cells [8]. We therefore might conclude that the acoel neoblasts and gonads can represent the vestige of the germinative epithelium of an ancestral gonocoele.

This hypothesis must be enriched by further data such as *e.g.* gene expression in other acoelomorph taxa. Especially relevant would be the investigation of orthologous gene expression in *Xenoturbella*, which has endodermal gonads instead of separate "mesodermal" gonads of the acoels and the nemertodermatids [27,29,64], which can deliver evidence that the gonads did not separate from the endoderm in this lineage. Accordingly we expect to find the orthologs of genes that are expressed in the *I. pulchra* gonadal tissue (Fig. 6) to be expressed in the *Xenoturbella* endoderm.

Lastly, it must be noticed that the genes characterized in this study are not coelomic "markers", but these genes are more generally used for mesoderm patterning across the Bilateria. Indeed, a *Tbr* ortholog is used to pattern the mesenchymal mesoderm in sea urchin, whereas it is expressed in the coelomic mesoderm of starfishes [99]. Thus co-option of the genes for patterning different tissues is common even among closely related species, and is even more likely to happen in more distantly related taxa such as acoelomorphs and echinoderms or cephalochordates.

To summarize, we cannot detect remnants of a former coelomic cavity in acoels. The coeloms of the coelomate ancestor must have disappeared without leaving embryonic and adult traces. This would be the first case of a complete coelomic reduction demonstrated in animals. Miniaturization per se does not necessarily imply that coeloms are lost, *e.g.* in the example of interstitial priapulids [105] or hemichordates [106]. All clearly secondary acoelomate conditions show at least a coelom-anlage (interstitial polychaetes, [107,108]) or the reduction of the coelom can be traced during embryogenesis as *e.g.* in the anterior somitomeres in *Branchiostoma* [6] or in the dwarf male of the echiuran *Bonellia* [109]. Alternatively, the complete absence of the coelomic remnants could indicate the independent origin of the coelomic cavities in hemichordates and echinoderms, a possibility which has been suggested previously (Fig. 8) [8].

Acoelomorphs as sister group to all remaining bilaterians: the original state of mesoderm and how a parenchyma evolved from the endoderm

In contrast to the recently proposed deuterostomic affiliation of the Acoelomorpha, previous phylogenomic studies have placed the group as the sister to all remaining Bilateria [20,21], thereby implying that some of their fundamental morphological and developmental traits might be ancestral to the Bilateria. The cnidarians, the sister group to Bilateria, have ectoderm and endoderm as the only embryonic and adult tissue layers, although some polyps and most medusa stages have evolved individual muscle cells between the ectoderm and the endoderm [1,2,55,110,111]. In general, however, contractile cells of cnidarians are epithelio-muscular cells, named according to their epithelial organization in their apical part with contractile filament extensions at the basal portion [7,8]. Possible scenarios about how true myocytes arose are either by a detachment of the contractile basal portion of the cell from the apical-epithelial portion or the emigration of the contractile cells into the space between endoderm and ectoderm [8,14]. Given that cnidarian polyps have epithelio-muscular cells in both the ectoderm and the endoderm it is obvious that individual muscle cells can arise from both layers. They develop from the ectoderm in hydrozoan cnidarians [110,111], from the endoderm in ctenophores and acoels [26,112] and finally from both germ layers in spiralians [113] as well as some ecdysozoans [114]

One convincing answer to the question of whether the bilaterian mesoderm originated from the endoderm or the ectoderm or from both tissue layers is offered by the

expression of bilaterian mesoderm orthologs in anthozoan cnidarians [2,32,115,116,117,118].

In the anthozoan *Nematostella* mesodermal orthologs are expressed in the endoderm, but the *Nematostella* orthologs to *FoxA, GATA* and *Mef2* are also expressed in the ectoderm [2,32,115]. Cnidarian orthologs of the *Tbr/eomes/Tbx21* T-box subfamily have not yet been characterized and thus might be a bilaterian novelty [119,120]. An expression study of the *Nematostella* tropomyosin genes [121] has not yet been published. However we can anticipate that some of those genes might be expressed in the endoderm given that several different isoforms of the protein have been detected in the endoderm of polyps of the closely related species *Anthopleura japonica* [122].

The evolution of muscles from the cnidarian endoderm (Fig. 8) easily accommodate to a phylogenetic frame where the acoelomorphs are the sister group to the Nephrozoa, especially considering that all genes expressed in the anthozoan endoderm are also expressed in most of the acoel muscles (Fig. 6 Table 2) [26] (this study).

Under this scenario, our data also indicate that the myocytes, which are the only confirmed mesodermal cell types in the most basal acoelomorph taxa, represent the ancestral mesodermal cell type whereas different structures, *e.g.* coeloms, and their enterocoelic development, must have evolved later (Fig. 8). Thorough comparative molecular developmental investigations on the protostome groups e.g. Brachiopoda, and Chaetognatha, would further clarify if coeloms evolved once or multiple times in the Nephrozoa (see literature in Nielsen [103]).

The developmental origins of neoblasts in the Acoelomorpha still remains unsolved, since fate mapping studies do not show a high enough resolution and need to be combined with early EdU labeling. At present we can only predict that the nemertodermatids' neoblasts would express a similar set of genes to the acoels. Whether or not those neoblasts represent a subpopulation committed to endomesodermal fates whereas a second population segregate from the ectoderm and becomes committed to epidermal and neural differentiation, is an open question.

Conclusions

In this study we show that most of the acoel homologs of bilaterian mesodermal transcription factors are also expressed in mesodermal compartments of the acoel, which only consist of muscles, gonads and neoblasts [26]. Our gene expression study suggests that some neoblasts and germ cells might derive from endomesodermal

precursors and are thus true mesoderm. If the acoelomorphs are nested inside the deuterostomes [22], it is likely the acoelomate condition in acoels arose from a coelomate ancestor. However, we find no traces or anlage of mesodermal tissue that indicates the former presence of a coelom in a coelomate ancestor. Only the gonads could represent the 'vestige' of a secondary coelomic cavity. In case the Acoelomorpha are the sister group to the remaining Bilateria [20,21], mesoderm evolution by 'enterocoely' is less parsimonious. In this scenario, myocytes that form an orthogon of circular and longitudinal musculature are likely the first mesodermal cell type that evolved in Bilateria. Other mesodermal tissues such as coeloms or connective tissue must have evolved independently as secondary separations from the endoderm - similar to the secondary separation of the parenchyma in the acoel lineage. However, a solid phylogenetic framework of animals is needed to trace the path of mesoderm evolution and differentiation.

Materials and Methods

Gene cloning and orthology assignment

Putative orthologs of genes of interest were identified by BLAST search against *I. pulchra* transcriptome (Berezikov et al., manuscript in preparation) using known sequences. Gene orthology of *I. pulchra* sequences were tested by reciprocal blast against NCBI Genbank. For all the sequences supported by reasonable e-values, we designed pairs of gene specific primers or RACE primers, and we performed PCR on cDNA from *I. pulchra* juveniles, amplified with the SMARTer RACE cDNA Amplification kit (Clontech). PCRs were performed using the manufacturer instructions. Primer sequences are available on request. Amino acid alignments were made with MAFFT and corrected by hand for obvious alignment errors (NEXUS files are available upon request). MrBayes3.2 [123] was used to conduct a Bayesian phylogenetic analysis. The models used for each analysis were JTT+I+G. The results are a consensus of two converged runs of 2,000,000 (*fox* genes 50,000) generations sampled every 1000 generations and four chains. Gene excession numbers: *IpFoxA2:* JX853975, *IpFoxA1:* JX853976, *IpFoxC:* JX853977, *IpGata456:* JX853978, *IpmuscleLIM:* JX853979, *IpMef2:* JX853980, *IpPitx:* JX853981, *IpSix1/2*: JX853982, *IpTbr:* JX853983, *IpTrp:* JX853984, *IpTwist1:* JX853985, *IpTwist2:* JX853986.

Animal rearing and labeling

Adult specimens of *Isodiametra pulchra* (Smith & Bush 1991) (formerly *Convoluta pulchra*) were reared as described by De Mulder et al. 2009 [28]. Ripe adults filled with oocytes were selected from culture plates and transferred to Petri dishes with filtered seawater and starved overnight. Deposited eggs were collected daily and fixed and processed for *in situ* labeling as described by Hejnol and Martindale [47]. To penetrate the eggshell, the fertilized eggs were treated with 0.01% Pronase (Sigma) and 0.1% thioglycolate (Sigma) in seawater, before fixation. Juveniles and adults were collected periodically and fixed for enzymatic *in situ* hybridization. Fluorescent *in situ* labeling was conducted using the TSA Plus Cy3 or Cy5 Kit (Perkin Elmer). Phalloidin stainings were conducted after a published protocol [59]. EdU-ClickIT labeling (Invitrogen) was performed following the manufacturer's instructions after incubating starved worms for 2h at room temperature in filtered artificial seawater containing EdU at a concentration of 100 µM.

Documentation

Digital images of *in situ* hybridized specimens were taken with a microscope equipped with Nomarski optics and processed through Aperture 3.0 software (Apple inc.). Fluorescent-labeled specimens were analyzed with a SP5 confocal laser microscope (Leica, Germany) and processed by the ImageJ software 1.43u (Wayne Rasband, NIH). Final figure plates and phylogenetic trees images were arranged with Photoshop CS3 and Illustrator CS3 (Adobe).

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Figure legends

Figure 1: Anatomy of *Isodiametra pulchra.* The adult and confocal projections of the main muscular structures of the adults.

A. A sexually mature adult living specimen imaged with a Differential Interference Contrast. Few structures are easily recognized: the anterior statocyst (st), the digestive syncytium (ds) occupies the larger part of the body volume. The brown color of the syncytium is due to the ingested algae (diatoms). The mouth (m) is indicated, although not easily recognized. It is located ventrally and slightly posterior from the mid body region. The paired gonads (go) flank the digestive system of *I. pulchra*. Together with the posterior-ventral female and male genital organ (fo and mo, respectively) they form the reproductive system of the acoel. **B.** Magnification of the anterior part of an *I. pulchra* specimen whose muscular fibers have been labeled with fluorescently labeled phalloidin. The parenchymal muscular net, highlighted by the dashed line, is especially dense around the statocyst. **C.** Phalloidin labeled ventral and dorsal side of the *I. pulchra* mouth region. On the left, the ventral side with the specialized ring muscles encircling the mouth at the level of the mouth opening is shown. On the right image, the dorsal side of the same worm is pictured. Note the thick parenchymal muscles crossing each other and delimiting the anterior digestive syncytium (ds) and the lateral flanking gonads (go). The bright spots in the digestive syncytium are the undigested diatoms. **D.** Posterior most ventral tip of a phalloidin labeled *I. pulchra* adult specimen. A bursal nozzle (bn), the female copulatory organ (fo) and the posterior most male genital organ (mo) are strongly labeled by phalloidin indicating the strong musculature. Note the circular shape of both organs. Scale bars are 50 μm in all aspects**.**

Figure 2: Expression of orthologs of bilaterian mesodermal genes that are broadly expressed in *I. pulchra*

In the left panel whole-mount *in situ* hybridization of juvenile (left column) and adult (central and right columns) specimens are shown. Expression in the specimens in the right column is detected with fluorescent signal (purple). Anterior to the left, scale bar

50 µm in all aspects**.** In the right panel the neoblasts localization of the transcript is shown. Left columns all show double stained worms with EdU, which labels S-phase cells, and with the fluorescent antisense probe of the corresponding gene. The center and right columns show RNA transcripts and neoblasts staining alone. All aspects show a single confocal plane.

A-C. *IpmuscleLIM* expression. **A.** A 2-3 days old juvenile. *IpmuscleLIM* expression is uniform along the AP axis. hm: head myocytes. **B.** *IpmuscleLIM* in adult specimen is stronger in the anterior "head-myocytes" (hm) and in the region of the gonads and neoblats (nb/go). Additionaly *IpmuscleLIM* is expressed in the parenchymal cells (pa) closely located to the digestive syncytium (ds). The asterisk highlights the intersection of the cross parenchymal muscles between the mouth and the female genital organ (arrow). **F.** Same than in B, but detected with fluorescently labeled probes. **C1.** Double labeled worms with antisense *IpmuscleLIM* and EdU. The open white arrowheads indicate co-localization of the signal in some of the cells. **C2.** Same than C1 but only *IpmuscleLIM* transcript signal is shown. **C3.** Same than C1 and C2, where only the EdU signal is shown. **D-F.** *IpPitx* expression. **D.** In a 2-3 days old juvenile, the expression is stronger in the anterior head mesoderm (myocytes). **E.** *IpPitx* in strongly expressed in all mesoderm derivatives of adult *I. pulchra*. Hm, head myocytes, pa, parenchymal cells m, mouth and nb/go neoblast and gonads. The black arrowheads point the male copulatory organ. **F.** Same than in E, but detected by fluorescent signal which clearer shows the gonadal cell types: t, testes; oo, oocytes. **F1.** Neoblast localization of the signal in double labeled specimens. **F2-F3.** Same as in F1, single RNA and EdU, respectively, signals are shown. **G.** In a 2-3 days old juvenile *IpFoxA1* is expressed in the digestive syncytium (ds) and in muscles connected to the developing copulatory organs (open black arrowhead). **H.** In adult specimens *IpFoxA1* expression extends to cells of the periphereal parenchyma,to the ring muscles encircling the mouth and muscles connected to the copulatory organs. **I.** Same as in H, but fluorescent. **I1.** *IpFoxA1* transcripts are localized in some of the neoblasts. **I2** and **I3**, same as in I1 but only RNA transcripts and EdU, respectively, signals are shown.

L. 2-3 days old juvenile. *IpFoxC* is expressed along the whole antero-posterior axis. **M.** In adults, *IpFoxC* is expressed in myocytes, neoblasts and gonads. **N.** Same as in M, but transcripts are detected by fluorescently labeled probes. We additionally observed *IpFoxC* expression in parenchymal cells. **N1.** Some of the neoblasts express *IpFoxC*.

N2-N3 same as in N1 but only RNA transcripts and EdU, respectively, signals are shown.

Figure 3. Expression of orthologs of bilaterian mesodermal genes that are expressed in subsets of endomesodermal tissues in *I. pulchra*. The whole figure is structured as in Figure 2. In the left panel, whole mount *in situ* hybridization are shown. Anterior is to the left in all aspects and the scale bar is 50 µm**.** The right panel shows the localization of the corresponding transcripts in the neoblasts, labeled by EdU. **A.** *IpFoxA2* expression is ubiquitous in the endo-mesoderm of 1-day old juvenile. **B.** In adult specimens the expression is restricted to the anterior head myocytes (hm), in the neoblasts and gonads (nb/go), the crossed parenchymal muscles (asterisk) and the sphincter of the female copulatory organ (arrow). No expression is detected in parenchymal cells. **C.** Same as in B. Very weak expression is also detected in the parenchymal cells (pa). nb: neoblasts, oo: oocytes, t: testes. **C1.** Double labeled specimens by fluorescent anti sense probe and EdU (for neoblast detection). The gene is expressed in some of the neoblasts. **C2.** RNA transcripts signal of *IpFoxA2* alone. **C3.** EdU labeled neoblasts cells alone. **D.** In juveniles *IpGATA456* is restricted to few anterior cells. **E.** In adult specimens the expression is extended to neoblasts and gonads and the crossed parenchymal muscles. **F.** Same as in E, but detected with fluorescent labeled antisense probes. **F1.** *IpGATA456* is expressed in several neoblasts. **F2-F3.** Same as in F1, where transcript and EdU signals, respectively are shown alone. **G.** In juveniles *IpMef2* is expressed in the anterior two thirds of the worm, and its expression is stronger in the region where the copulatory organs will develop. hm: head myocytes. **H.** In adult specimens, *IpMef2* expression is down-regulated, with the exception of the anterior and posterior brain commissures (small white arrowheads) and in the neoblasts/ gonads regions. Weak expression surrounds the male genital organ (black arrowheads). **I.** *IpMef2* fluorescent *in situ* labeled adult specimen. **I1.** Several neoblasts express *IpMef2*. **I2-I3**. Same as in I1, where only the transcript and EdU signals, respectively, are shown. **L.** 5-6 days old juvenile. *IpSix1/2* is expressed in the anterior and posterior neurite loops of the brain (small white arrowheads) and strongly in the developing female copulatory organ (arrow, magnified in the inset). **M.** In adult specimens the expression of *IpSix1/2* is stronger in the oocytes (oo) and weak in the testes and neoblasts (nb/t). **N.** Fluorescent *IpSix1/2* transcripts labeled specimen. The expression is

strong in the oocytes. **N1.** *IpSix1/2* is expressed in a subset of neoblasts. **N2-N3**. Same as in N1, where only transcripts and EdU signals, respectively, are shown.

Figure 4. Expression of two *Twist* orthologs in adult specimens of *I. pulchra*. No expression was detected in juvenile specimens and therefore they are not shown. Whole mount specimens are shown in the left panel whereas in the right panel specimen double labeled with antisense probe and EdU (S-phase cells) are shown. **A.** *IpTwist1* is expressed in testes (t) and in the male copulatory organ (black arrowhead). **B.** By fluorescent *in situ* hybridization we also detected *IpTwist1* expression in the oocytes (oo). **B1.** *IpTwist1* is additionally expressed in few neoblasts (open white arrowhead). **B2.** *IpTwist1* RNA transcripts signal shown alone. **B3.** EdU (S-phase cells) signal shown alone.

Figure 5. Expression of *IpTbr* and *IpTrp* (*tropomyosin*) in juvenile and adult *I. pulchra* specimens. The left panel shows whole mount *in situ* hybridization whereas in the right panel the localization of the gene transcripts in neoblasts (EdU labeled cells) is shown. Anterior is to the left and scale bar 50 µm in all whole-mount aspects. **A.** *IpTbr* expression in about one week old juvenile. The gene is expressed in the maturing oocytes (oo). **B-C.** Close-up of *IpTbr* expression in all stages of oocytes (oo) maturation in adult worms. **C1-C3**. Expression of *IpTbr* is not detected in the neoblasts. **D.** *IpTrp* expression in a juvenile specimen. **E-F.** *IpTrp* expression in an adult specimens. Arrow points to the muscular sphincter of the female genital organ and arrowhead to the male genital organ. m: mouth. **F1-F3.** *IpTrp* is expressed in cells with low EdU incorporation.

Figure 6. Summary of mesodermal gene expression in adult *I. pulchra*.
Columns represent tissue types, rows the gene identity. Question marks represent detection ambiguities between standard and fluorescent *in-situ* hybridization protocols (see text for details). On the right side is a schematic representation of an adult worm. The tissue is color-coded according to gene expression on the left. Body wall and parenchymal muscles are in blue. Not all muscles are represented for clarity purposes. Peripheral parenchyma is in sandy-brown. Ovaries are in dark red and testes in light orange. Only single ovary and testes are represented not reflecting the real bilateral symmetric status of *I. pulchra* gonads. The same asymmetric representation is given for

the neoblasts (dark purple, with big grey nucleus), which are distributed in two symmetric rows in living animals. Examples of gene expression for each structure are given in the insets. bm: bodywall muscles; pm: parenchymal muscles; bn: bursal nozzle; fo: female copulatory organ; mc: male copulatory organ.

Figure 7. Tissue conditions in the digestive tract of different acoelomorph taxa. Schematic cross sections of the digestive tract of different acoel taxa (after Tyler & Smith [63]). **A.** Reconstructed ancestral condition of the acoelomorph stem species based on outgroup comparison (Cnidaria and/or Bilateria respectively). The epithelial digestive endoderm with lumen borders directly to the muscular grid. No parenchyma is present. **B.** Nemertodermatida and *Xenoturbella* posses an epithelial endoderm with gland cells, but lack a lumen. **C.** *Paratomella* (Acoela) possesses a digestive parenchyma in which no epithelial connections are present. Not all parenchymal cells are in contact with the digestive lumen. **D.** *Diopisthoporus* (Acoela) possesses a thick parenchymal layer that is forming a sheet around the digestive syncytium. **E**. Derived condition found in most acoel taxa as *e.g.* also in *Isodiametra*. Parenchymal cells surround the large syncytium but are only forming a relative thin sheet of cells with extensions into the digestive syncytium.

Figure 8: Different scenarios about mesoderm evolution depending on the phylogenetic position of Acoelomorpha. Two possible phylogenetic positions of Acoelomorpha either as sister to the remaining Bilateria or as sister group to Ambulacraria (discussed in the text). Musculature in red. Four possible scenarios are numbered. Scenario 1: A cnidarian-like ancestor with epithelial-muscle cells that form ring and longitudinal musculature form the orthogonal musculature of acoels. The musculature would be the first cell type of mesoderm [63]. Scenario 2: A similar cnidarian-like ancestor is forming myoepithelial coelomic cavities as outpouchings from the gastric cavity (according to enterocoely hypothesis [124]). In the lineage to the Acoelomorpha the orthogonal muscle grid of acoels is formed from the coeloms. After the formation of the muscle grid coeloms got reduced. This scenario includes several losses and gains and is thus not parsimonious and can be rejected. Scenario 3: In case the last common ancestor of Deuterostomia had coeloms, the coeloms got reduced in the lineage to the Acoelomorpha without any traces [12]. Scenario 4: Coelomic cavities of Ambulacraria are not homologous with those in other animal lineages [22] and are

formed independently from the endoderm of a acoelomorph-like ancestor *e.g.* by enterocoely.

Table1. Expression of mesodermal genes in *I. pulchra* neoblasts

	Type II neoblasts	Low EdU signal	Type I and II neoblasts	Expressed in all examined neoblasts
IpTwist1	Yes			
IpTwist2	Yes			
IpFoxC	Yes	Yes		
IpFoxA2		Yes		
IpFoxA1		Yes		
IpTrp			Yes	
IpGATA456			Yes	
IpMef2			Yes	
IpSix1/2			Yes	
IpmuscleLIM			Yes	Yes
IpPitx			Yes	Yes

Table 2. Differential mesodermal gene expression in *I. pulchra* adult musculature.

Head muscles	Mouth ring muscles	Cross muscles	Female copulatory organ	Male copulatory organ
IpTrp	*IpTrp*	*IpTrp*	*IpTrp*	*IpTrp*
IpmusleLIM		*IpmusleLIM*		*IpmuscleLIM*
IpPitx	*IpPitx*		*IpPitx*	*IpPitx*
IpFoxA1	*IpFoxA1*			
IpFoxA2		*IpFoxA2*	*IpFoxA2*	
IpFoxC		*IpFoxC*	*IpFoxC*	
IpGATA456		*IpGATA456*	*IpGATA456*	
IpMef2				*IpMef2*
IpSix1/2		*IpSix1/2*	*IpSix1/2*	
				IpTwist1
				IpTwist2

Table 3. Acoel gonadal expression of mesodermal genes, compared to gene expression in the coelomic mesoderm of deuterostomes.

Acoel's gonadal orthologs	Echinoderm coelomic mesoderm	Cephalochordate Hatscheck's diverticulum	Early cepahalochordate segmented mesoderm
FoxC	Yes	No	Yes
GATA456	Yes	Unknown	Unknown
Mef2	Unknown	Yes	Yes
Pitx	Yes	Yes	No
Six1/2	Yes	Yes	Yes
Tbr	Yes	Yes	No
Twist	No	Yes	Yes

Figure 1
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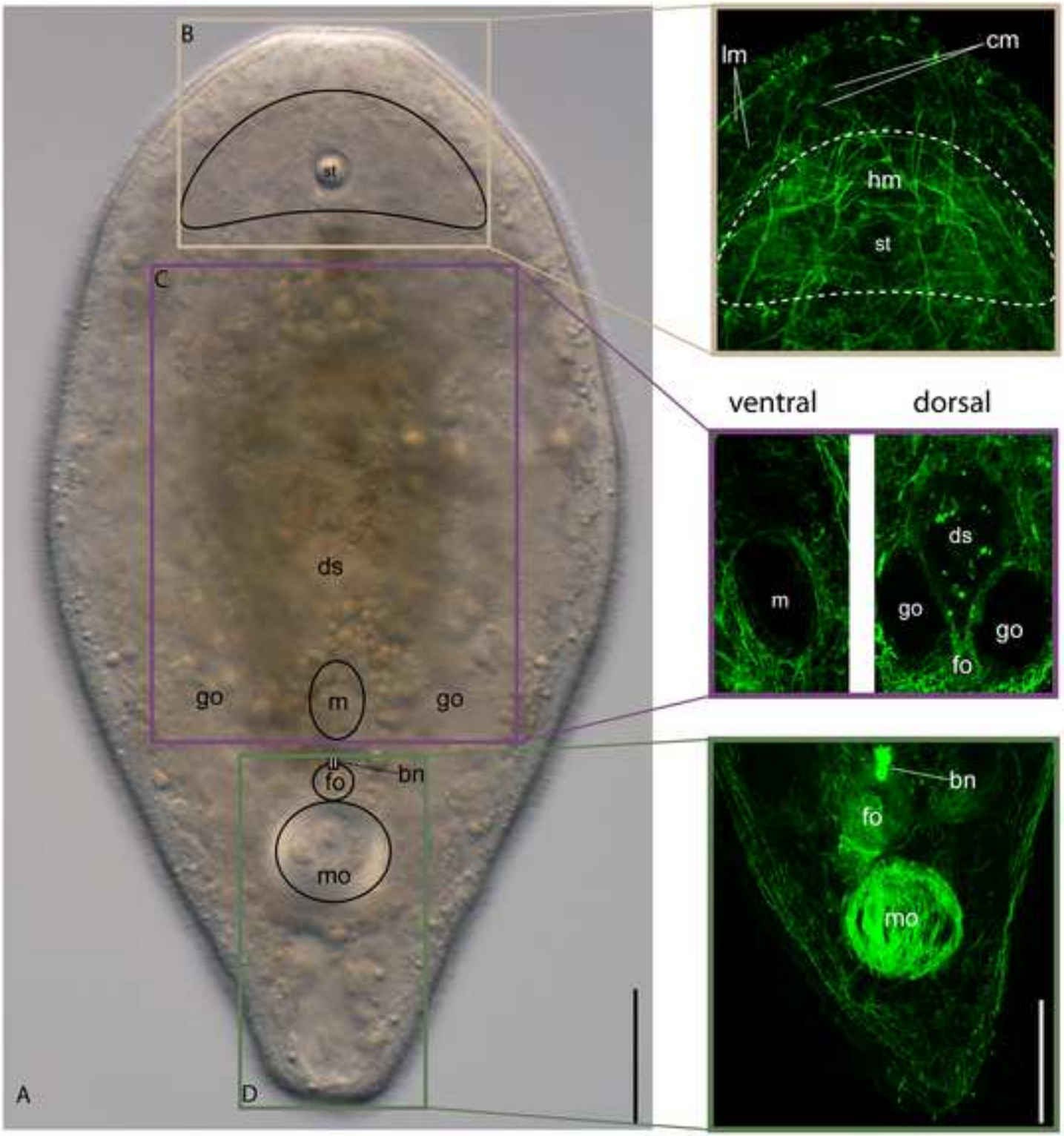

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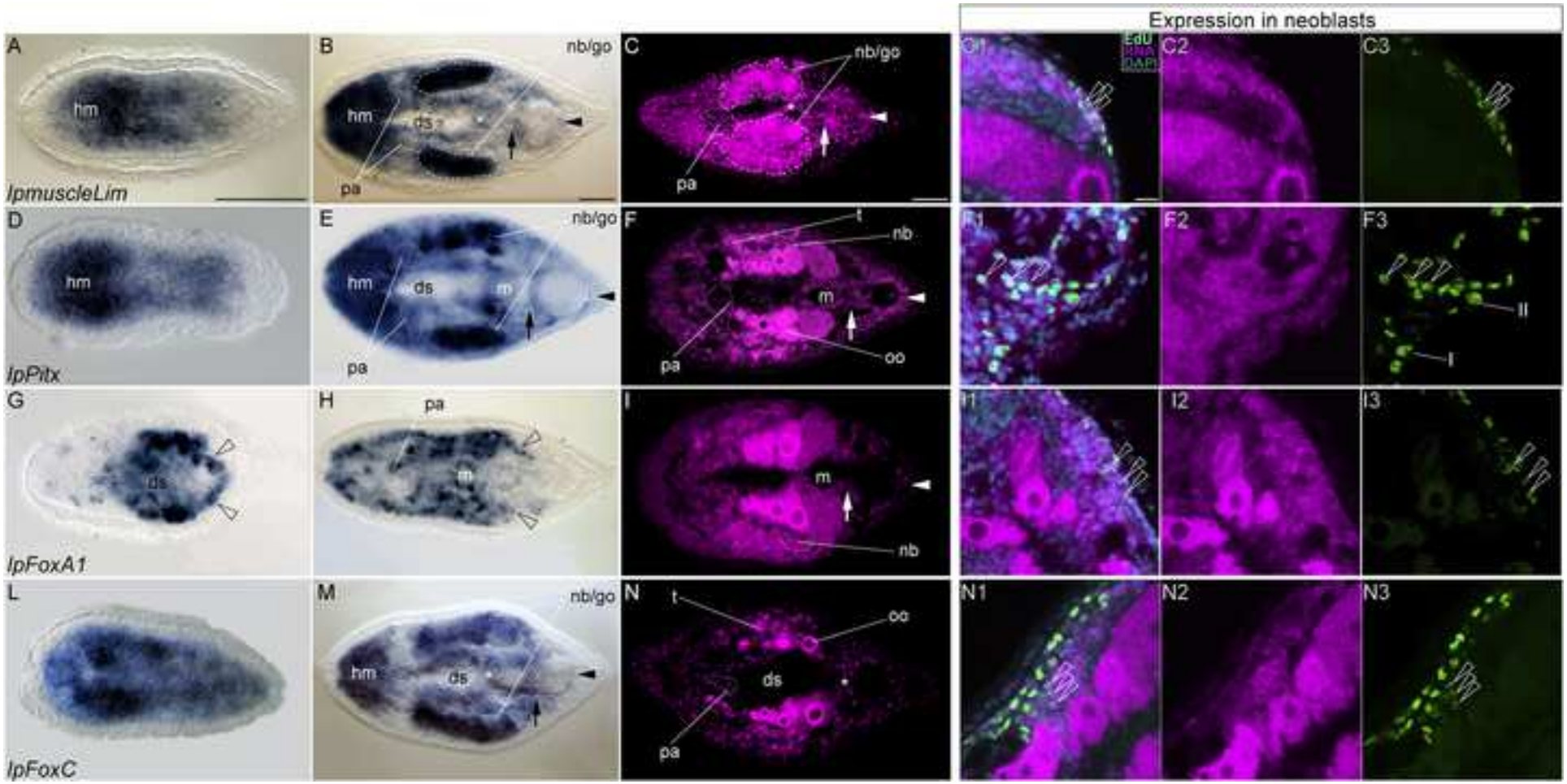

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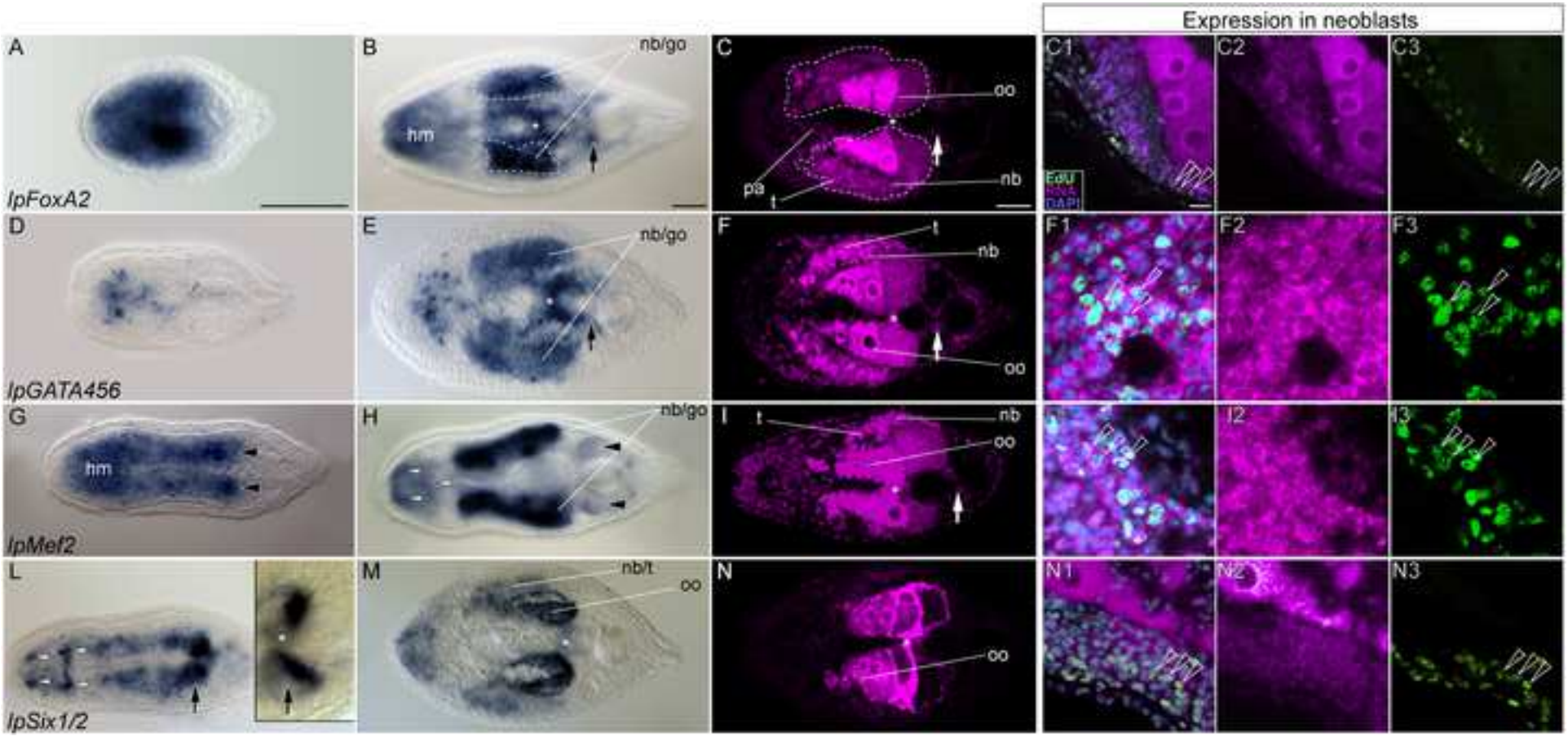

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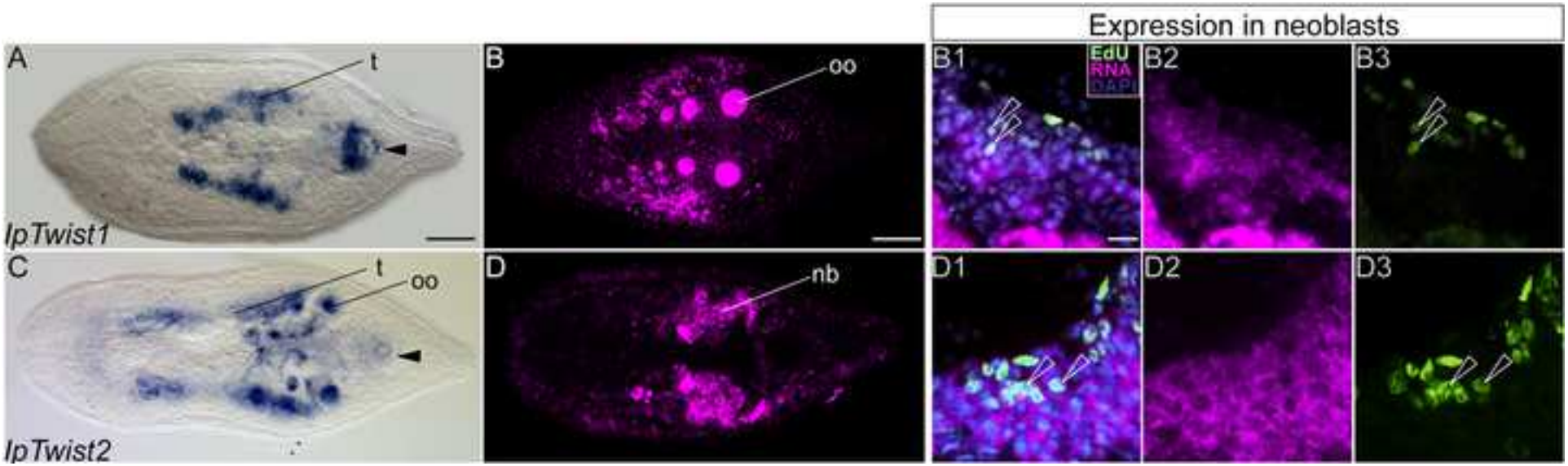

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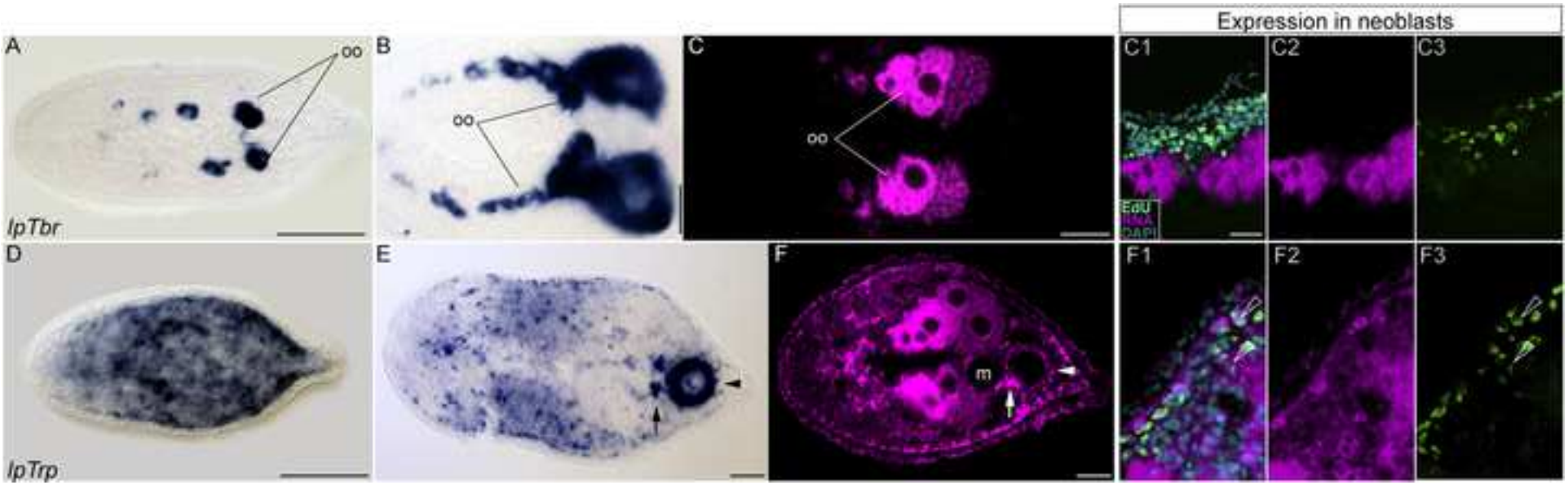

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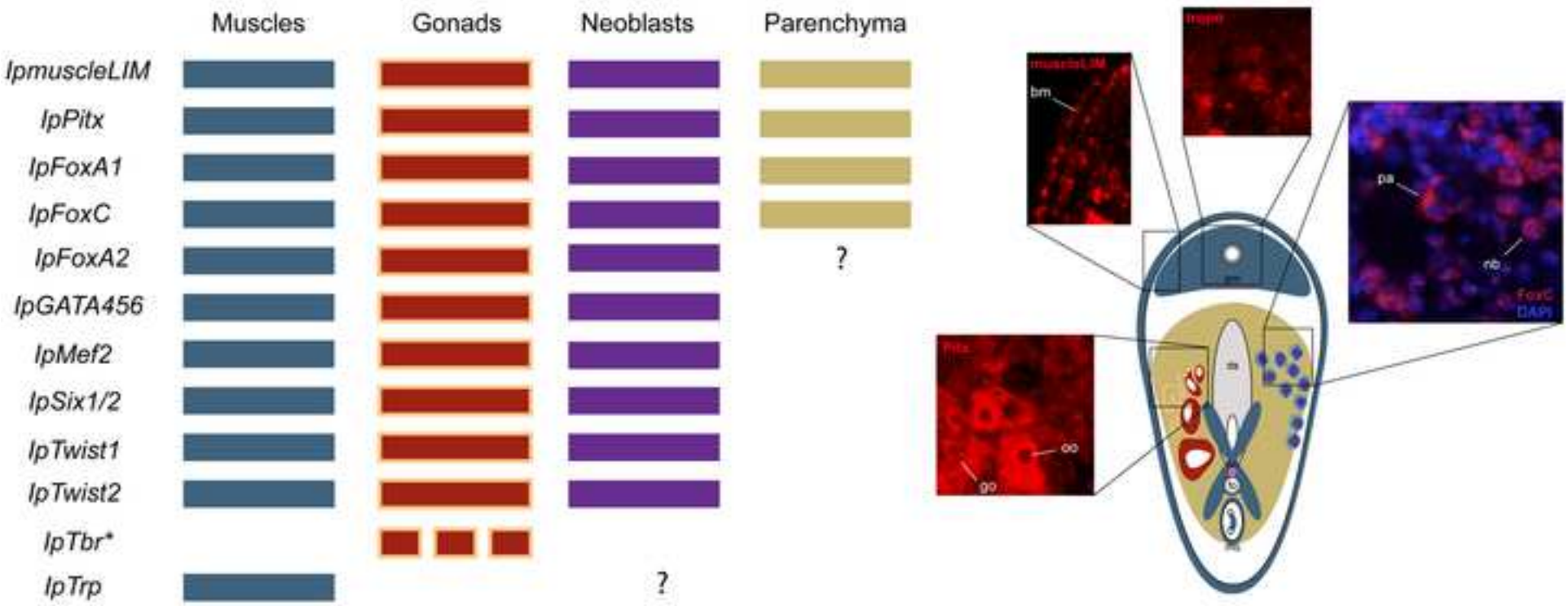

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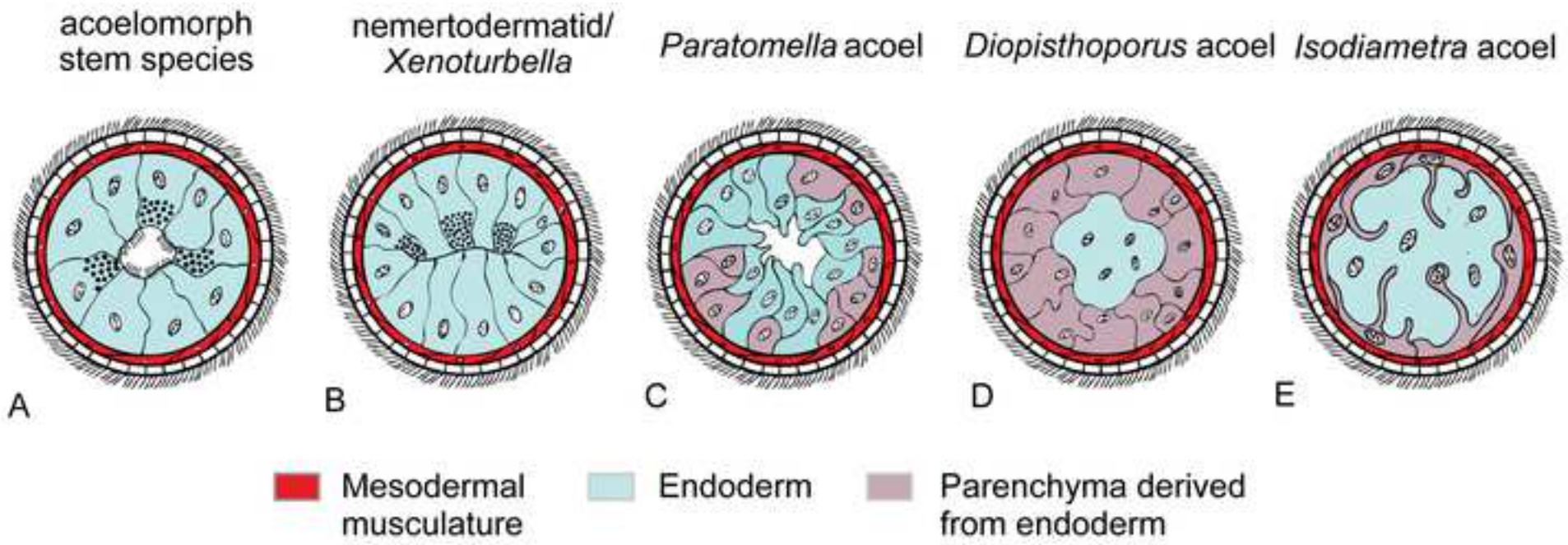

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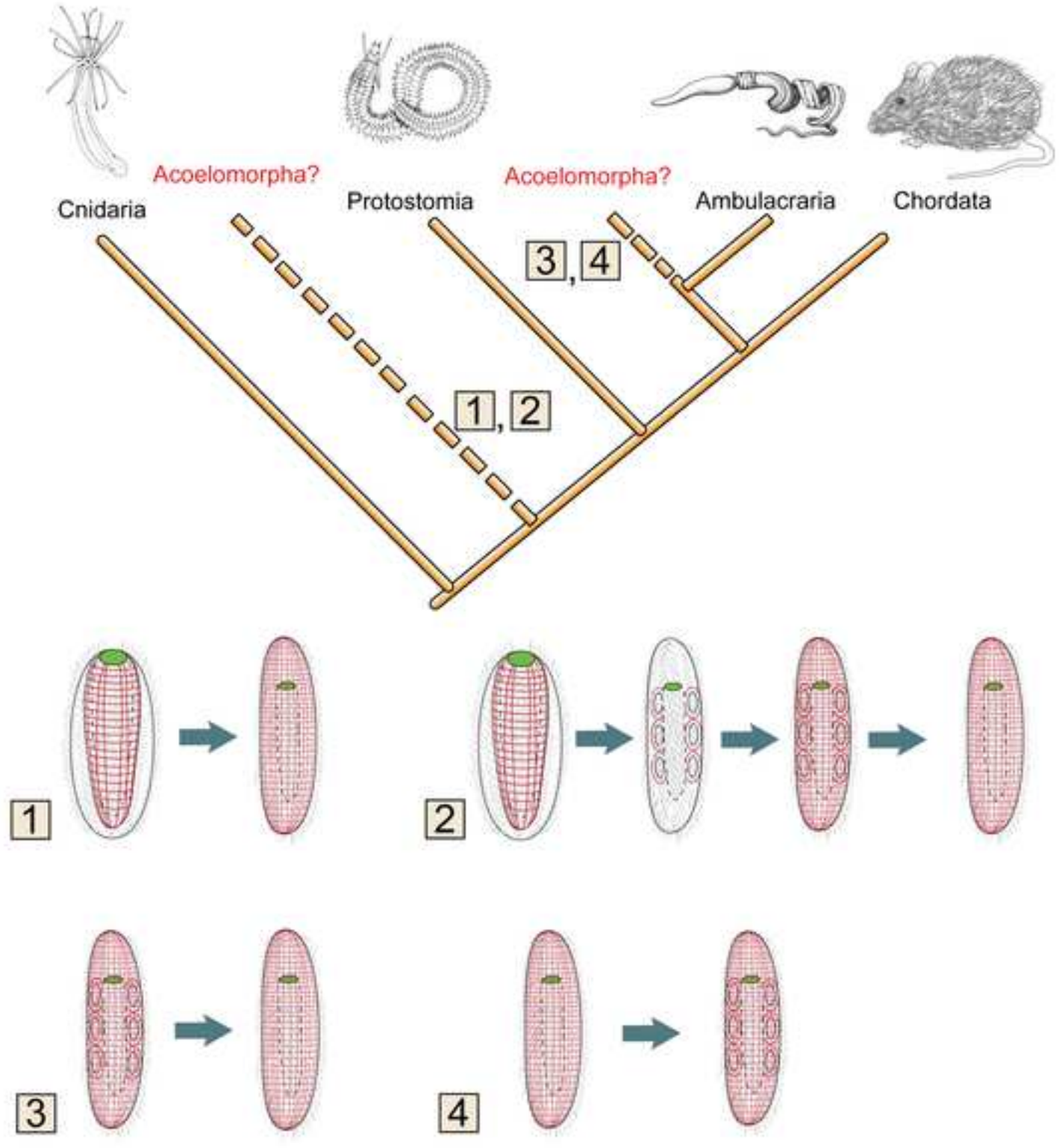

Supporting Information

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Resúmen del segundo artículo, R2

La expresión de genes mesodermales en el acelo *Isodiametra pulchra* indica una baja complejidad de células mesodermales y el origen endo-mesodermal del las células madres.

Los acelomorfos son pequeños gusanos marinos de simetría bilateral que carecen de celoma y poseen un sistema digestivo ciego, es decir que únicamente tienen el orificio oral. Su posición filogenética en el árbol de la vida animal es todavía tema de debate al no ser claro si representan una simple y primera etapa en el desarrollo de la evolución de los bilaterales o si descienden directamente de los deuteróstomos, y consecuentemente su simplicidad morfológica es secundaria. Los acelos y sus parientes más relacionados, los nemertodermátidos y xenoturbellidos (que juntos forman los acelomorfos) poseen un número limitado de tipos celulares. Con el fin de investigar el origen y la diversidad del mesodermo y sus derivados, hemos descrito el patrón de expresión de 12 ortólogos de marcadores de mesodermo en bilaterales incluyendo los ortólogos de *Six1/2*, *Twist*, *FoxC*, *GATA4/5/6*, en el acelo *Isodiametra pulchra*. Todos los genes están expresado por lo menos en partes de la musculatura, en parte de las células madres (neoblastos) y en las gónadas. La mayoría de los genes están expresados en el compartimento endo-mesodermal de los embriones de *I. pulchra* de una forma parecida a la que fue descrita para los genes ortólogos en cnidarios. Nuestros resultados proporcionan la evidencia molecular de la presencia de un numero muy limitado de células mesodermales y sugieren una

origen endomesodermal de las gónadas y de al menos un subconjunto de las células madres.

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Original Article

Steps towards a centralized nervous system in basal bilaterians: Insights from neurogenesis of the acoel *Symsagittifera roscoffensis*

Henrike Semmler,[1] Marta Chiodin,[2] Xavier Bailly,[3] Pedro Martinez[2,4] and Andreas Wanninger[1]*

[1]*Research Group for Comparative Zoology, Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark;* [2]*Departament de Genetica, Universitat de Barcelona, E-08028 Barcelona, Spain;* [3]*Station Biologique, F-29682 Roscoff Cedex, France; and* [4]*Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain*

Due to its proposed basal position in the bilaterian Tree of Life, Acoela may hold the key to our understanding of the evolution of a number of bodyplan features including the central nervous system. In order to contribute novel data to this discussion we investigated the distribution of α-tubulin and the neurotransmitters serotonin and RFamide in juveniles and adults of the sagittiferid *Symsagittifera roscoffensis*. In addition, we present the expression pattern of the neuropatterning gene *SoxB1*. Adults and juveniles exhibit six serotonergic longitudinal neurite bundles and an anterior concentration of serotonergic sensory cells. While juveniles show an ''orthogon-like'' arrangement of longitudinal neurite bundles along the anterior-posterior axis, it appears more diffuse in the posterior region of adults. Commissures between the six neurite bundles are present only in the anterior body region of adults, while irregularly distributed individual neurites, often interconnected by serotonergic nerve cells, are found in the posterior region. Anti-RFamide staining shows numerous individual neurites around the statocyst. The orthogon-like nervous system of *S. roscoffensis* is confirmed by α-tubulin immunoreactivity. In the region of highest neurotransmitter density (i.e., anterior), the HMG-box gene *SrSoxB1*, a transcription factor known to be involved in neurogenesis in other bilaterians, is expressed in juvenile specimens. Accordingly, *SoxB1* expression in *S. roscoffensis* follows the typical pattern of higher bilaterians that have a brain. Thus, our data support the notion that Urbilateria already had the genetic toolkit required to form brain-like neural structures, but that its morphological degree of neural concentration was still low.

Key words: Acoela, Bilateria, evolution, nervous system, Sox.

Introduction

The evolution of the bilaterian nervous system has been a matter of debate for more than a century. In this context, acoelomorph flatworms (i.e., Acoela and Nemertodermatida) occupy a central position because they are often proposed to constitute the earliest extant offshoot of Bilateria (Ruiz-Trillo *et al.* 1999, 2002; Hejnol *et al.* 2009). Acoelomorphs express a high plasticity in their neural organization with only a low degree of centralization in their anterior body region (Raikova *et al.* 2004a; Hejnol & Martindale 2008a; Kotikova & Raikova 2008; Raikova 2008). Traditionally, the acoelomorph nervous system has been diagnosed as an ''orthogon'', with variable numbers of nerve cords (i.e., longitudinal neurite bundles) (Bullock & Horridge 1965; Reuter *et al.* 1998). Thereby, the term ''orthogon'', as introduced by Reisinger (1925, 1972), describes a rectangular network consisting of longitudinal neurite bundles which are interconnected by commissures. However, a regular orthogon *sensu* Reisinger seems to be absent in a number of acoelomorph taxa (Haszprunar 1996), whereby the nervous system may comprise one to five pairs of neurite bundles that are only irregularly interconnected by commissures (Kotikova 1991; Raikova *et al.* 2004a). The anatomical deviation of the acoelomorph nervous

*Author to whom all correspondence should be addressed.
Email: awanninger@bio.ku.dk
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system from the classical definition of an orthogon has more recently resulted in the term ''cordal nervous system'' for these early bilaterians (see Kotikova 1991; Raikova *et al.* 1998).

The high plasticity of the acoelomorph nervous system raises questions about the putative anatomy of the ancient acoelomorph neural architecture (Rieger *et al.* 1991; Raikova *et al.* 2000a, 2004b). In order to shed more light on neurogenesis in Acoelomorpha, we investigated the distribution of the neurotransmitters serotonin in early juvenile and adult stages and RFamide in adults of the acoel *Symsagittifera roscoffensis* (von Graff, 1891). We complement our data with expression data of the transcription factor *SoxB1 (Sox = Sry related HMG box),* which is known to play an important role in germ layer specification and nervous system patterning in cnidarians and bilaterians (Crémazy *et al.* 2000; Sasai 2001; Overton *et al.* 2002; Shinzato 2007), and specifically address the question as to what extent a centralized nervous system (''brain'') might have been part of the acoelomorph – and urbilaterian – groundpattern.

Materials and methods

Collection and fixation

Adults were collected in the intertidal zone, off the Bretonic Coast on the Ile Calot close to Carantec, France and off the Spanish northwest coast around Gijón. Adults were kept in aquaria with natural sea water. Cocoons containing embryos were isolated and cultured in Petri dishes at room temperature (RT). Prior to fixation, 7% $MgCl_2$ was applied to avoid muscle contraction. Specimens were fixed in 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PB) for 0.5–2.0 h at RT, washed thrice in PB and stored at 4°C in 0.1 mol/L PB with 0.1% sodium azide (NaN_3) added to prevent bacterial and fungal growth.

For *in situ* hybridization, specimens were fixed for 5 min in 0.2% glutaraldehyde + 3.7% formaldehyde, followed by 1 h in 3.7% formaldehyde at RT. This was followed by several washes in PB containing 0.1% Tween 20 (PTw). After dehydration through a graded methanol series for 20 min per step the samples were stored in 100% methanol at –20°C.

Scanning electron microscopy

Fixed and stored adults and juveniles were postfixed in 1% osmium tetroxide in distilled water for 1 h, followed by two washes in distilled water. Specimens were dehydrated in a graded ethanol series. Subsequently, they were transferred into a 1:3, then 1:1 and 3:1 acetone–ethanol solution, which was followed by two washes in 100% acetone. The specimens were critical point dried with a Baltec CPD 030 critical point dryer (BAL-TEC AG) and sputter-coated with platinum–palladium for 100 s in a JEOL JFC 2300HR sputter coater (Jeol Ltd.). Specimens were analyzed with a JEOL JSM-6335F scanning electron microscope (Jeol Ltd.).

Immunocytochemistry

After fixation and storage the specimens were incubated in 6% normal goat serum (Sigma-Aldrich) in PB with 5% Triton-X 100 (blockPBT) at 4°C overnight. This was followed by incubation in the first antibody for 24–48 h at 4°C. Anti-serotonin (5-HT) (Calbiochem and Molecular Probes) was applied in a 1:100 dilution, anti-tyrosinated tubulin (Sigma-Aldrich) in a 1:100 dilution, and anti-RF-NH_2 (courtesy of Thomas Leitz, Kaiserslautern, Germany) in a 1:800 dilution in blockPBT. The samples were washed for 6–12 h (four changes) in blockPBT. All following steps were carried out in the dark. As secondary antibodies, goat anti-rabbit TRITC (Jackson ImmunoResearch) and goat anti-rabbit Alexa 594 (Molecular Probes) were applied in a 1:100 dilution in blockPBT. Goat anti-mouse Alexa488 (Molecular Probes) was used in a 1:200 dilution in blockPBT. Specimens were incubated in the secondary antibody for 24–48 h at 4°C. The samples were washed four times in PB for 15 min each, dehydrated in a graded ethanol series, and embedded in benzyl benzoate:benzyl alcohol (2:1 concentration). To exclude signal from autofluorescence, negative controls were performed by omitting the primary or the secondary antibody, respectively, and yielded no signal.

The samples were analyzed with a Leica DM RXE 6 TL fluorescence microscope equipped with a TCS SP2 AOBS confocal unit (Leica Microsystems). Maximum projection images and light micrographs were recorded for each sample. The images were edited with Leica confocal software, Photoshop CS2, and Illustrator CS2 (Adobe Systems).

In situ *hybridization*

One 1121 bp clone recovered from the expressed sequence tag (EST) library, containing the entire open reading frame of the Sox gene plus the 5′ and 3′ untranslated regions (UTRs), was used as a template for riboprobe synthesis. Both, sense and antisense probes were generated using the digoxygenin (DIG) RNA labeling kit (Roche). After precipitation, the riboprobe was diluted in hybridization buffer to a final working concentration of 1 ng/μL.

Specimens were rehydrated through 60% methanol/40% PB with 0.1% Tween-20 detergent (PTw), followed by a 30% methanol/70% PTw wash for another 10 min. Four washes in PTw followed. After 15 min 1 μg/mL protease K (Sigma-Aldrich) in PTw digestion at RT, samples were washed in PTw containing 2 mg/mL glycine (Sigma-Aldrich), followed by a wash step in 1 mL of 1% triethanolamine (Sigma-Aldrich) in PTw, to which 1.5 μL acetic anhydride was added twice. After several washes in PTw, specimens were refixed in 3.7% formaldehyde in PTw for 1 h at RT and rinsed several times in PTw. The hybridization buffer (HB) consisted of 50% deionized formamide, 5× sodium chloride-sodium citrate buffer (SSC), 50 μg/mL Heparin, 0.1% Tween-20, 1% sodium dodecyl sulfate (SDS) and 100 μg/mL salmon testes single stranded DNA (Sigma-Aldrich). Two washes in HB at RT were done before the overnight incubation at 50°C in HB. The denatured RNA probe was incubated in HB for at least 3 days at 50°C. Washes with gradient HB/2× SSC at hybridization temperature and 0.05 × SSC/PTw at RT, followed by several PTw washes, were required before blocking in 1% blocking buffer (Boehringer–Mannheim), which was diluted in maleic acid buffer for 1 h at RT. Incubation with a 1:5000 dilution of the anti-Digoxigenin-AP Fab fragments (Roche) was carried out overnight at 4°C. Several washing steps in PB + 0.2% Triton X-100 and 1% bovine serum albumin (PBT) and three washes in alkaline phosphatase (AP) buffer followed. The color reaction with 1% 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche) in AP buffer was carried out in the dark at RT. The reaction was stopped by rinsing the specimens several times in PTw, after optimal signal-background ratio had been reached. The specimens were mounted in 70% glycerol and analyzed with a Zeiss Axiophot microscope (Carl Zeiss MicroImaging GmbH) equipped with a Leica DFC 300FX camera.

Sequence alignment and phylogenetic analysis

The EST library prepared from aposymbiotic hatchlings of *Symsagittifera roscoffensis* contained 14 overlapping Sox cDNA clones, which were re-sequenced using dye terminator chemistry (BigDye Terminator v3.1 Cycle sequencing kit; Applied Biosystems). Sequence similarities were determined using the BLAST package from the National Center for Biotechnology Information (NCBI). Several high mobility group (HMG)-box genes from nine metazoan taxa were selected and their HMG domains aligned with ClustalW, via Bioedit (Tom Hall, Ibis Biosciences) with manual correction. The data comprised 43 sequences and 79 characters.

Only the HMG domain (amino acid) sequences were included in the analysis. The evolutionary tree was calculated using the neighbor-joining method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to calculate the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 79 positions in the final dataset. Phylogenetic analyses were conducted by Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA4).

Results

Gross morphology of juvenile and adult Symsagittifera roscoffensis

Juvenile and adult specimens are entirely covered by cilia (Fig. 1A,B). The mouth is situated in the center of the body axis in the juvenile and in the anterior third of the body axis in the adult. Anterior to the mouth a funnel groove is formed by the lateral edges, which bend ventrally in this area (Fig. 1A–C,E). The male gonopore is situated almost at the posterior tip of the animal and shows a slit-like opening (Fig. 1D). The female gonopore is situated at the end of the anterior third of the body axis (not shown). Juveniles and adults exhibit pores of 1 μm diameter distributed over the entire body surface, which are presumably gland openings (Fig. 1F–H; see Oschman 1967). In the juvenile, these pores are especially numerous and randomly distributed on the ventral side and in two rows along the lateral edges (Fig. 1F,G).

Neural structures in the juvenile

After hatching, the white juvenile measures approximately 220 μm in length and possesses a serotonergic nervous system, which consists of six longitudinal neurite bundles that are regularly interconnected by commissures (Fig. 2A). The commissures between the median neurite bundles and the lateral neurite bundles are not continuous. In the anterior region, the neurite bundles are interconnected by two serotonergic commissures (Fig. 2A, double arrows). The median neurite bundles are connected to each other by commissures anterior and posterior to the statocyst and at least once again in a more posterior region. At least four commissures are present between the median and the lateral and between the two lateral neurite bundles,

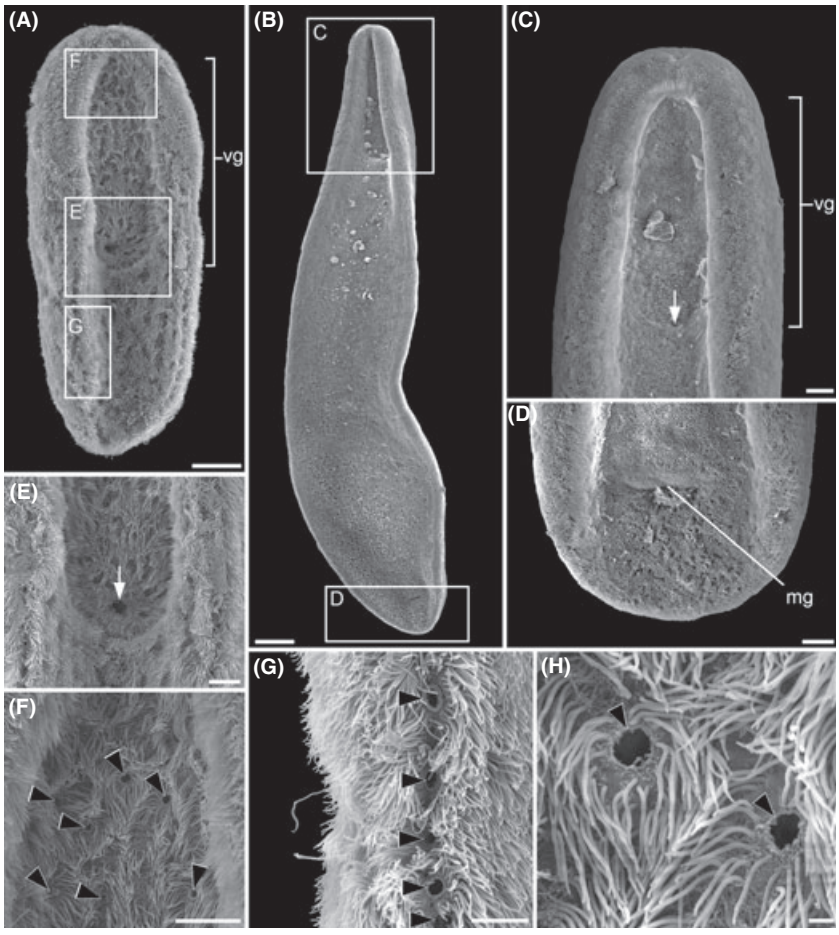

Fig. 1. Scanning electron micrographs of juvenile and adult *Syms-agittifera roscoffensis*. Anterior faces upwards. Juveniles (A) and adults (B) are entirely covered with cilia. A ventral groove (vg) is found anterior to the mouth (arrow). (C) Close-up of (B), showing the ventral groove. (D) The male gonopore (mg) is present in the posterior tip of the adult. (E) Close-up of the mouth opening of the specimen shown in (A). (F–H) Over the entire surface pores (arrowheads), which might constitute gland openings, are present. While irregularly distributed in the ventral groove (F), these pores line up along the lateral edges (G). (H) Close-up of two pores. Scale bars: A, C, D: 25 μm; B: 100 μm; E, F: 10 μm; G: 5 μm; H: 1 μm.

respectively. The highest density of serotonergic perikarya is present in the anterior region of the animal. Juveniles, shortly after hatching, possess about 120 such serotonergic perikarya.

In 1-week-old juveniles, which are about 235 μm long, the gradient of the serotonin expression from anterior to posterior is even more obvious than in younger juveniles (Fig. 2B). The immunofluorescent signal is stronger in the six longitudinal neurite bundles, but fewer commissures are observable and only a few serotonergic perikarya are present. Staining using the pan-neural marker tyrosinated α-tubulin revealed a similar overall neural architecture in the juvenile, which consisted of three pairs of longitudinal neurite bundles, which are interconnected in the anterior part by several commissures (Fig. 3).

Adult serotonin- and RFamide-positive structures

Some serotonergic perikarya are found peripheral to the bodywall musculature in the anterior tip of adult animals. Six neurite bundles run along the anterior-posterior body axis. The two median neurite bundles lie in rather dorsal position, whereas the other four are in a more ventral position (Fig. 4). The first, i.e., anteriormost commissure is located in a more dorsal position than the second commissure (Fig. 4C, double arrows). Dorsal to the statocyst, an anterior and a posterior commissure are present (Fig. 4C). Posterior to the statocyst two weakly stained neurites, which extend median-anteriorly into the direction of the statocyst, are found (Fig. 4C, arrows). We did not find any neurites directly connected to the statocyst. In the anterior-median region of the animal the commissures between the neurite bundles are still serially arranged (Fig. 4D), whereas more posteriorly, there is no such regular pattern and the neural structures resemble more a nerve net (plexus) (Fig. 4E,F, empty arrowheads). In the ventral anterior tip of the animal serotonergic perikarya are particularly numerous (Fig. 4C, arrowheads).

Similar to the anti-serotonin staining, six neurite bundles are visible by anti-RFamide staining (Fig. 5A). In general, RFamide-immunoreactivity is less intense compared to the immunoreactivity of serotonin (Fig. 5A,B). There are several RFamidergic cell bodies present in the anterior tip (Fig. 5C–F). The anterior

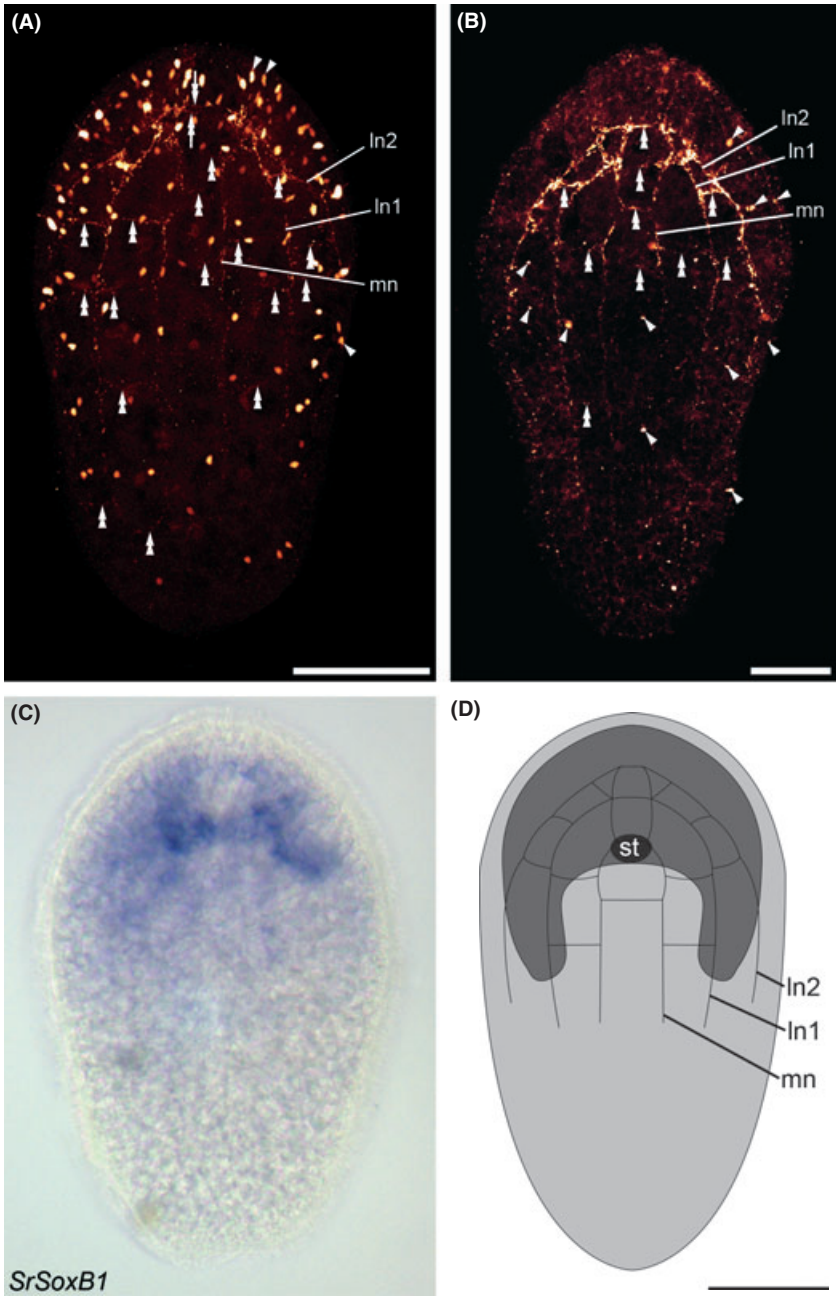

Fig. 2. Serotonergic nervous system and *SoxB1* expression in *Symsagittifera roscoffensis* juveniles. (A) Confocal laser scanning micrographs showing the serotonergic nervous system in a specimen a few hours after hatching. The six longitudinal neurite bundles, comprising two median (mn) and four lateral (ln1 and ln2) ones, extend along the anterior-posterior body axis and are interconnected by several commissures (double arrowheads). Two commissures (double arrows) are found at the anterior pole of the animal. Several serotonergic perikarya are marked by an arrowhead. (B) Serotonergic nervous system of a 1-week-old juvenile. (C) *SrSoxB1* is expressed in the anterior region. (D) Schematic drawing in which *SrSoxB1* expression (dark grey area) and the serotonergic nervous system of a 1-week-old juvenile have been plotted onto each other. The position of the statocyst (st) is indicated. Scale bars, 50 μm.

concentration around the statocyst is more elaborate than in the anti-serotonin staining (Fig. 5A,C–F). In the area of the statocyst several neurites run in a dorso-ventral direction but lack a bilateral symmetrical arrangement (Fig. 5C–F, encircled).

Sox *expression in the juvenile*

The gene isolated from the EST library encodes for a protein with an HMG-domain, typical for proteins such as Sox, T-cell factor (TCF), Mating yeast transcription-factor 2 (MAT2), and High Mobility Group-box-containing protein/Upstream binding factor (HMG/UBF). This isolated sequence clearly aligns with genes from this big protein superfamily (Fig. 6). The Sox group shows a characteristic box of 79 amino acids and it can be further divided into differentiated subfamilies (A–H). The *Symsagittifera roscoffensis* sequence shows the characteristic amino acid strings typical for the Sox-subfamily B (Fig. 6; Bowles *et al.* 2000; Koopman *et al.* 2004; Jager *et al.* 2006). The phylogenetic analysis of our sequence also reveals that the gene is a member of the subfamily SoxB, clustering within the SoxB1 group (Fig. 7; Koopman *et al.* 2004).

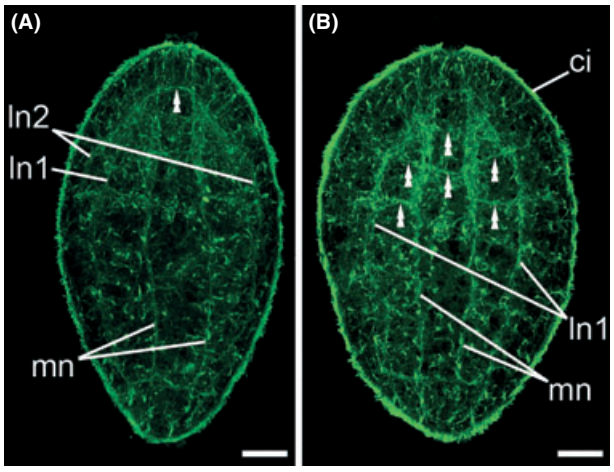

Fig. 3. Nervous system of a juvenile *Symsagittifera roscoffensis* as visualized by anti-tyrosinated α-tubulin staining. (A) The dorsal region shows the two pairs of lateral neural bundles (In1, In2) and the paired median longitudinal neurite bundle (mn). The anterior commissure is marked with a double arrowhead. (B) In a more ventral region one pair of lateral neurite bundles, the paired median longitudinal neurite bundle, and numerous commissures (double arrowheads) are present. Scale bars, 25 μm.

This transcription factor that we call *SrSoxB1*, with the initials Sr denoting the species name, is most strongly expressed in a horseshoe-shaped pattern that includes the region around the statocyst (Fig. 2C,D). This region is also the one of the highest intensity of serotonin signal in the juvenile (Fig. 2B,D). Its scattered distribution may be due to the fact that the RNA of this neural marker gene is present in neuronal precursor cells that do not (yet) express the respective neurotransmitter.

Discussion

Comparative adult neuroanatomy of the three flatworm-shaped taxa Acoelomorpha, Platyhelminthes, and Xenoturbella

Recent phylogenetic analyses indicate that the three flatworm-like taxa, Acoelomorpha, Platyhelminthes, and *Xenoturbella* constitute distinct phyla. While Platyhelminthes clearly nests within the protostomian Lophotrochozoa, Acoelomorpha is considered the earliest extant bilaterian offshoot, and *Xenoturbella* has recently been reconsidered to cluster with the ambulacrarian deuterostomes (Haszprunar *et al.* 1991; Ruiz-Trillo *et al.* 2002; Halanych 2004), although alternative positions close to the acoels have also been suggested (Hejnol *et al.* 2009). Despite this, the representatives of all three phyla exhibit several similarities concerning their nervous system, together with other morphological features such as a frontal glandular system and the lack of accessory centrioles in the locomotory cilia complex. These characters have previously been interpreted as synapomorphies and have been used to unite these three groups in the Platyhelminthes, although most of these characters are only expressed in one of the three groups (Smith *et al.* 1986; Haszprunar 1996).

Platyhelminthes and Acoelomorpha exhibit a nervous system consisting of an anterior concentration with an anterior commissure and one or several pairs of longitudinal neurite bundles, which are interconnected by rectangular commissures. However, Platyhelminthes have a nerve ring around the foregut, while Acoelomorpha only have a few neurites in the anterior body region (Reuter *et al.* 2001a; Kotikova *et al.* 2002; Morris *et al.* 2007). Although earlier recordings mentioned a dorsal ''ganglion'' in the acoelomorph *Childia crassum*, a postcerebral organ in *Childia groenlandica*, or a so-called ganglionic ''endonal brain'' mass above the statocyst and suggest a more extensive nervous system around the statocyst (Westblad 1948; Ivanov & Mamkaev 1973; Ehlers 1985; Bedini & Lanfranchi 1991; Raikova *et al.* 1998, 2004a), no ganglionic cell mass has ever been confirmed for Acoelomorpha using immunocytochemical methods (Raikova *et al.* 2001). Only commissural fibers associated with few cell bodies and several neurites in the dorsal and lateral vicinity of the statocyst have so far been described (Reuter & Gustafsson 1995; Raikova *et al.* 2001; Reuter *et al.* 2001a; Raikova *et al.* 2004a; present study). Remarkably, for the juveniles of the acoel *Neochildia fusca*, a three to four cell diameter-thick layer of neurons that form a cortex surrounding a neuropil was described, thus fitting the histology-based definition of a ''ganglion'' (Ramachandra *et al.* 2002).

Instead of being regularly distributed along the longitudinal neurite bundles as in most Platyhelminthes, the commissures in Acoela are irregularly arranged along the anterior-posterior body axis, while the proposed acoel sistergroup Nemertodermatida only shows a few very thin serotonergic commissures in the anterior part of the longitudinal neurite bundles (Raikova *et al.* 2000a, 2004b). Moreover, many acoelomorphs have equally pronounced neurite bundles (Raikova *et al.* 1998, 2000a; Reuter *et al.* 1998), thus contrasting the situation found in Platyhelminthes, where the ventral or lateral neurite bundles are often the most prominent ones and are therefore commonly referred to as ''main cords'' (Reuter & Gustafsson 1995). One case in Acoela is known, in which two neurite bundles appear more prominent than others, whereby the dorsal (median) neurite bundles are more pronounced, thus

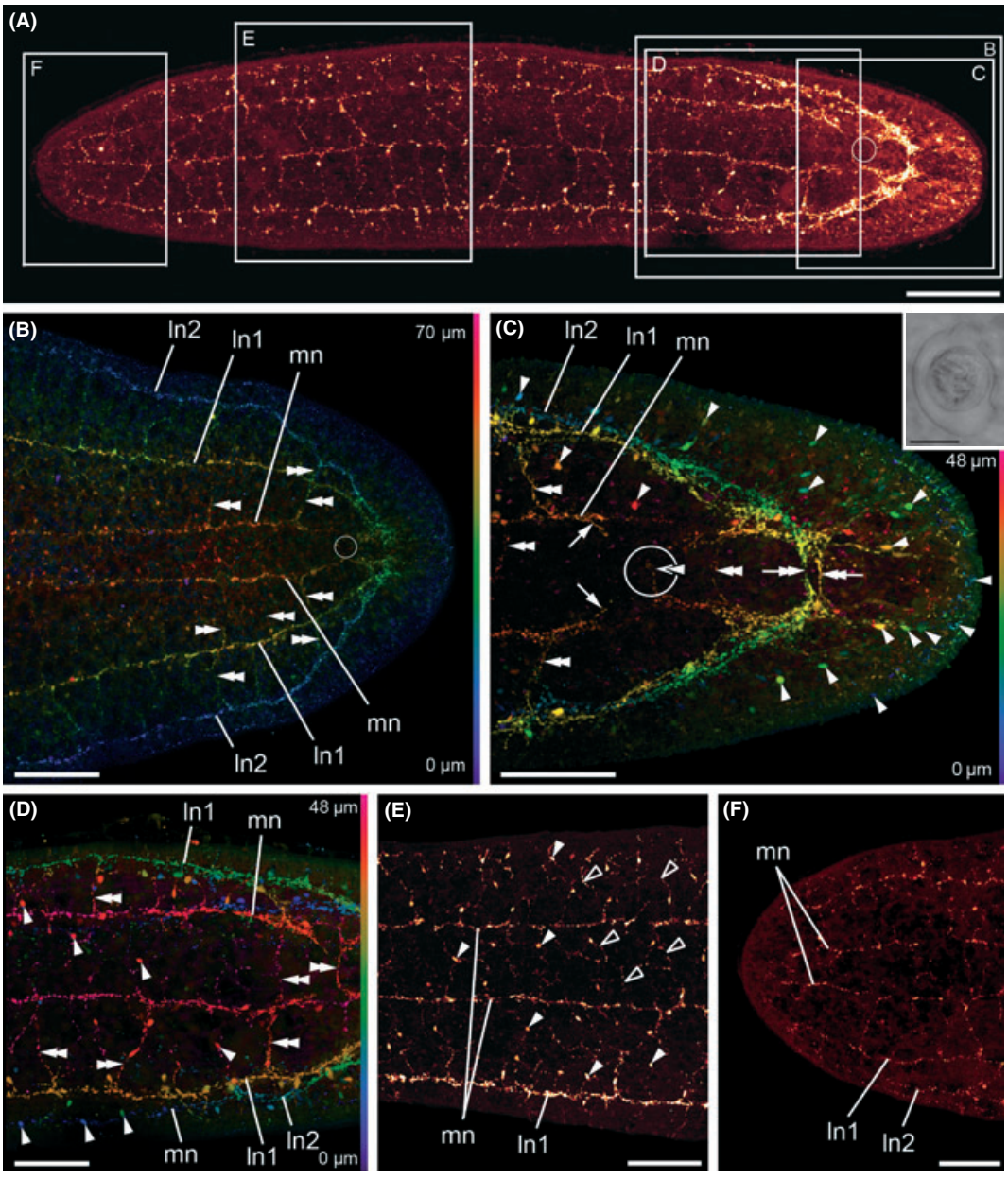

Fig. 4. Adult serotonergic nervous system of *Symsagittifera roscoffensis*. (A) The plexus-like nervous system shows weak centralization in the anterior region of the animal. The circle marks the position of the statocyst. (B) The median (mn) and the two lateral (In1, In2) neurite bundles, which are interconnected by commissures (double arrowheads), run along the anterior-posterior body axis. (C) In the vicinity of the statocyst two neurites (arrow) are present, which extend towards the statocyst. The two most anterior commissures are visible (double arrows). Serotonergic perikarya are more frequent in the anterior part of the animal (arrowheads). The boxed area in (C) shows the detail of the statocyst. (D) The nervous system is most regularly structured in the anterior part of the animal. (E) The median region of the animal exhibits a plexus-like serotonergic nervous system. Empty arrowheads mark irregularly distributed neurites. (F) Plexus-like nervous system in the posterior region of the animal. Scale bars: A, B: 100 µm; C–F: 50 µm; box in C: 10 µm.

likewise contrasting the situation found in Platyhelminthes (Reuter *et al.* 2001b).

Symsagittifera roscoffensis exhibits six equally prominent neurite bundles, whereas other acoelomorphs exhibit between two and 10. Since recent data on the presumably most basal acoel clades Paratomellidae, Solenofilomorphidae and Hofsteniidae are still missing, deductions concerning the basal acoelomorph condition remain speculative (Hooge *et al.* 2002, 2007). Childiidae possess eight to 10 neurite bundles, while

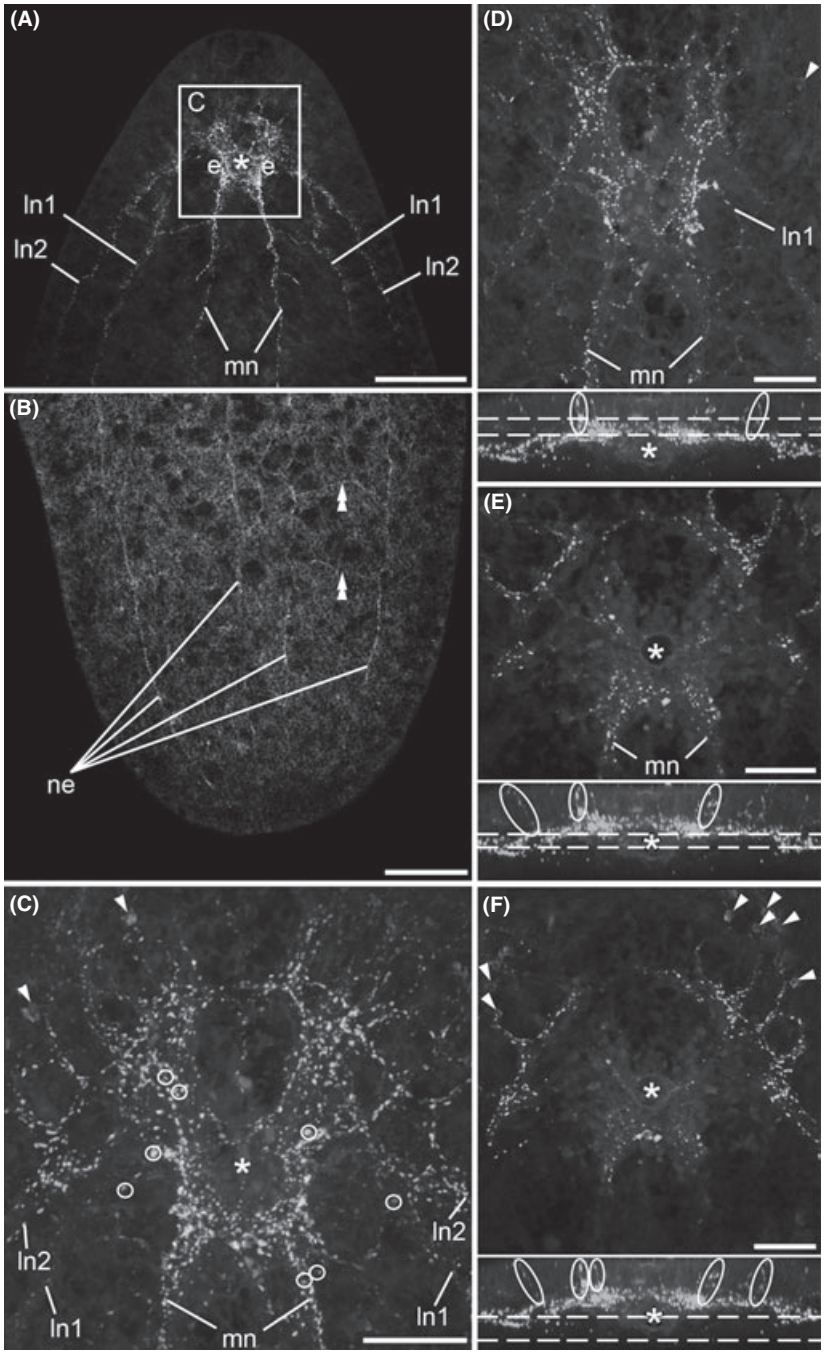

Fig. 5. Adult RFamidergic nervous system of *Symsagittifera roscoffensis*. Anterior faces upwards. (A) The RFamideric nervous system shows two median (mn) and two pairs of lateral (In1, In2) neurite bundles, which arise from an anterior commissure-like neurite (boxed area). The eyes (e) are situated lateral to the statocyst (asterisk). (B) Four weakly stained neurite bundles (ne) are present in the posterior part. Several commissures (double arrowheads) are visible. (C) Details of the anterior concentration of RFamidergic structures are shown. Several neurites run in dorsoventral direction (circles). Nerve cells are present in the periphery of the animals (arrowheads). (D–F) The dorsoventral projection of the part of the confocal stack that is marked by dashed lines in the image below shows a dorsal (D), median (E), and ventral (F) region of C. Scale bars: A, B: 100 μm; C–F: 30 μm.

their sistergroup Mecynostomidae has six to eight (Hooge *et al.* 2002; Raikova *et al.* 2004a). The closely related Sagittiferidae and Convolutidae have six neurite bundles, while the Anaperidae exhibit 10 (Raikova *et al.* 1998; Hooge *et al.* 2002; Gaerber *et al.* 2007; present study). Six neurite bundles are also present in

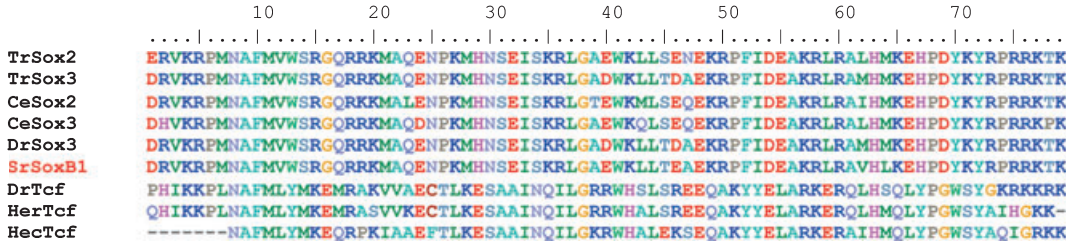

Fig. 6. The *SoxB1* gene of *Symsagittifera roscoffensis* aligns with *Sox* genes from the following taxa. In parenthesis the National Centre for Biotechnology Information (NCBI) gene numbers of the genes included in the analysis are stated. Ce, *Caenorhabditis elegans* (gil193210553, gil17567586); Dr, *Danio rerio* (gil3769679, gil85060503); Hec, *Hydractinia echinata* (gil109238636); Her, *Heliocidaris erythrogramma* (gil42521331); Sr, *Symsagittifera roscoffensis*; Tr, *Takifugu rubripes* (gil33415915l, gil33415917). The amino acids are colored as follows: basic amino acids in blue (K, R) and pink (H); non-polar aliphatic amino acids in green (A, I, L, M, V), in brown (P, G), and in red (C); non-polar amino acids (N, Q, S, T) in grey blue; acidic amino acids in red (D, E); non-polar aromatic amino acids in mint green (Y, W, F).

Haploposthiidae and Actinoposthiidae, while Isodiametridae either possess four or eight (O. I. Raikova, unpubl. data, 2004). In contrast to the Acoela, the two nematodermatid species studied to date show either two or four neurite bundles (Raikova *et al.* 2000a, 2004b). This variety in the overall neural architecture hampers solid inferences concerning the number of longitudinal neurite bundles in the acoelomorph (and therefore also bilaterian) groundplan. While in Acoelomorpha the anterior concentration and the neurite bundles are formed by only one solely submuscular plexus (Rieger *et al.* 1991), Platyhelminthes possesses a pharyngeal or stomatogastric nervous system (Ehlers 1985; Reuter 1994) and additional epidermal, intraepithelial and genital plexi (Reuter & Halton 2001), again illustrating the differences in the neural organization of both phyla.

The proposed deuterostome *Xenoturbella* probably has the least concentrated nervous system among Bilateria. It solely consists of an intraepidermal neural plexus (Raikova *et al.* 2000b; Bourlat *et al.* 2003, 2006). The simplicity of the nervous system of *Xenoturbella* has traditionally been considered as an indication for a basal position of the Xenoturbellida within the Bilateria. Recent molecular data, however, have argued for inclusion of *Xenoturbella* within the deuterostomes (although alternative views do exist; see, e.g., Hejnol *et al.* 2009), thus confirming independent morphological investigations on the nervous system, epidermis, spermatozoa, and statocyst (Reisinger 1960; Ehlers 1991; Bourlat *et al.* 2003). Taking into account the low degree of nervous system concentration in the acoelomorphs, it appears tempting to speculate that *Xenoturbella* might have retained the simple, plexus-like nervous system, which might have been present in the last common bilaterian ancestor. This scenario is supported by expression data of neuropatterning genes in the enteropneust *Saccoglossus,* which point to the direction of a nerve net as being basal for this clade (Lowe *et al.* 2003). A final statement, however, can only be made once more comparative data on the evolution of the nervous system of basal deuterostomes become available and once the phylogenetic position of *Xenoturbella* has eventually settled.

Traces of cephalization during acoel neurogenesis?

In juvenile *S. roscoffensis* the serotonergic nervous system appears very regular, consisting of six longitudinal neurite bundles with regular commissures and an anterior concentration of sensory cells. The commissures between the median longitudinal neurite bundles is not observable in the adult anymore. A similar condition is found in the anterior body region of the adults, while more posteriorly no commissures between the neurite bundles are found. Instead, a net of irregular neurites is present (Fig. 8). In addition to the serotonergic perikarya, which are mostly restricted to the anterior region of the juvenile, the adult exhibits perikarya over the entire length of the body. The gradual decrease of serotonergic structures from anterior to posterior is not observable any more in the adult. By contrast, serotonergic structures are distributed uniformly along the anterior-posterior body axis and are generally more numerous in the adult than in the juvenile. The cordal nervous system pattern of the juvenile is only recognizable in the anterior-most region of the adult. This might indicate that the anterior part of the adult retains the juvenile neural arrangement, while novel neural components are added in the adult by posterior growth (Jacobs *et al.* 2005; Egger *et al.* 2009). This notion is corroborated by expression data of the anterior patterning gene *ClEmx*, which is expressed along the entire anterior-posterior body axis and not only in the anterior region in juveniles of the sagittiferid *Convolutriloba longifissura* (Hejnol &

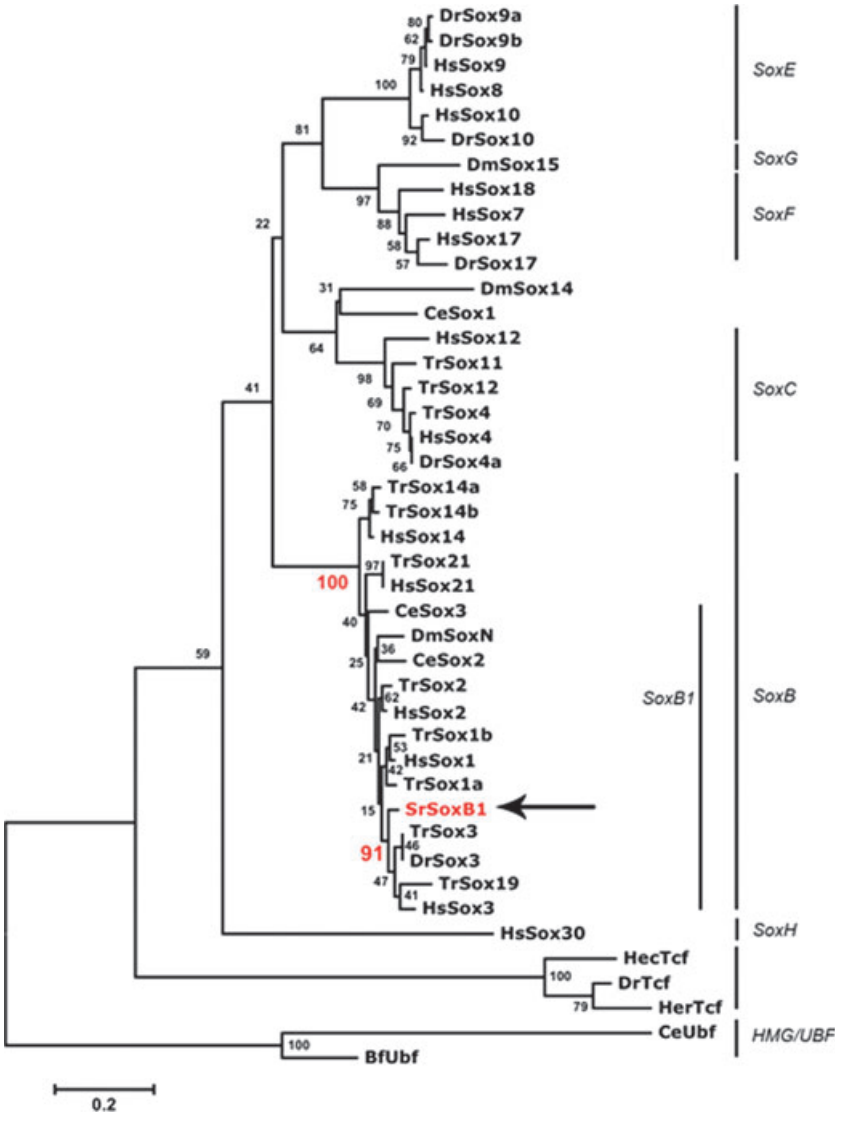

Fig. 7. Evolutionary relationships of high mobility group (HMG)-box genes of nine taxa. *Sox* genes comprise the subfamilies A–J. The gene *SrSoxB1* (arrow) from *Symsagittifera roscoffensis* groups highly supported together with several *Sox1*, *Sox2*, *Sox3* genes, which are all classified to the *SoxB1* family (Koopman *et al.* 2004). The evolutionary tree was calculated using the neighbor-joining method. The optimal tree with the sum of branch length = 6.35648486 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to calculate the phylogenetic tree. The taxa with the National Centre for Biotechnology Information (NCBI) gene numbers of the genes included in the analysis in parenthesis are as follows: Br, *Branchiostoma floridae* (gil219418451); Ce, *Caenorhabditis elegans* (gil71986275, gil193210553, gil71981100, gil17567586); Dm, *Drosophila melanogaster* (gil19549767, gil24653573, gil24582930); Dr, *Danio rerio* (gil3769679, gil56711293, gil18859408, gil18859396, gil85060503, gil18859410, gil124430741); Hec, *Hydractinia echinata* (gil109238636); Her, *Heliocidaris erythrogramma* (gil42521331); Hs, *Homo sapiens* (gil21264338, gil36552, gil8894592, gil16943719, gil145275218, gil2909359, gil30179902, gil182765453, gil30581116, gil31563384, gil46094055, gil30061555, gil29826338, gil30179899); Sr, *Symsagittifera roscoffensis*; Tr, *Takifugu rubripes* (gil33415911, gil33415913, gil33415915l, gil33415917, gil33415919, gil33415921l, gil33415923, gil33415925, gil33415927, gil33415929, gil33415931).

Martindale 2008b). Genes, such as *Nk2.1* and *Otx*, which are expressed over the entire body axis in adult cnidarians, are only expressed in the anterior region in most adult bilaterians (Grens *et al.* 1996; Smith *et al.* 1999; Meinhardt 2002). Comparison of these expression profiles supports the hypothesis that the bilaterian

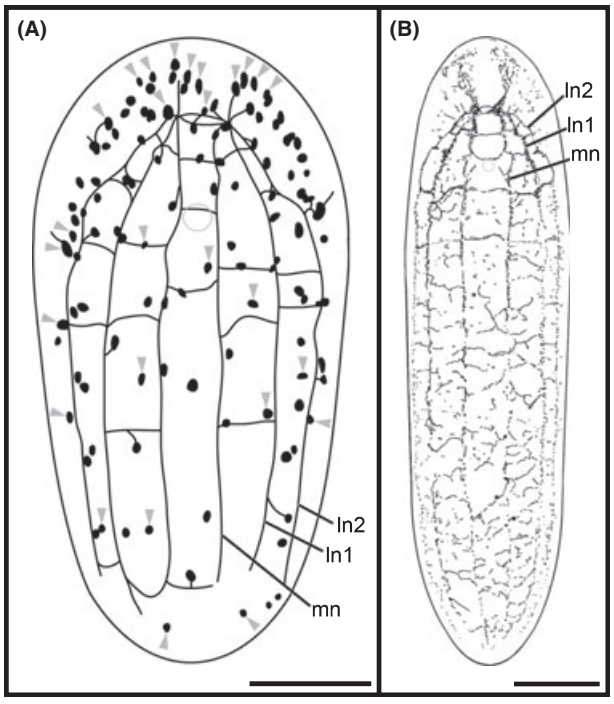

Fig. 8. Semi-schematic sketch drawing of the juvenile (A) and the adult (B) serotonergic nervous system of *Symsagittifera roscoffensis*. Six longitudinal neurite bundles extend along the anterior–posterior body axis: two median (mn) and four lateral (In1 and In2) ones. The position of the statocyst is encircled and serotonergic perikarya are marked with arrowheads. Scale bars: A: 50 μm; B: 100 μm.

body plan evolved by growth from a posterior terminus (Meinhardt 2002; Jacobs *et al.* 2005). By contrast, *SoxB1*, a regulator of neuronal development, only shows expression in the anterior pole of larvae of the cnidarian *Nematostella vectensis* (Sasai 2001; Magie *et al.* 2005) and in juveniles of *Symsagittifera roscoffensis*. In the late embryo of the hemichordate *Saccoglossus kowalevskii*, *Sox1/2/3* is strongly expressed in the prosome (i.e., in the anterior part of the animal), and to a much lesser extent in the metasome, thus corresponding to the decreasing neuronal density in the posterior region of this species (Lowe *et al.* 2003). Accordingly, expression of *SoxB* genes is commonly associated with the developing central nervous system in protostomes as well as in deuterostomes (Uchikawa *et al.* 1999; Crémazy *et al.* 2000; Holland *et al.* 2000; Le Gouar & Guillou 2004). This anterior concentration of expression of the *SoxB1* gene in the aboral part of *Nematostella vectensis* planula larvae as well as in *S. roscoffensis* juveniles is in accordance with an anterior concentration of sensory cells and a decreasing anterior-posterior gradient of the RFamide peptide in *Hydractina carnea* larvae and in *S. roscoffensis* (Seipp *et al.* 2007). In addition, anterior-posterior orientated RFamidergic and tyrosine-
tubulinergic neurites show a gradual development from anterior to posterior in *H. carnea* planula larvae. In competent larvae, these neurites disappear and a nerve net is gradually formed (Gröger & Schmid 2001). In contrast to the regular neural arrangement present in planula larvae, adults show an epithelial nerve net of interconnected neurons. Some medusoid adults have unequally distributed ring-shaped or longitudinal neurites and regions with condensed neuronal cell bodies along the body axis, but until now it remains unclear whether these have been carried over from the larval stage or form *de novo* in adults (Watanabe *et al.* 2009).

In summary, recent data on neurogenesis and gene expression patterns suggest that cnidarians and acoels both develop their nervous system with an anterior-posterior gradient and this could be interpreted as a first evolutionary step towards nervous system centralization, which is not yet fully expressed in these two basal metazoan clades. Nevertheless, neurogenesis data on more basal Acoela are necessary to further assess this notion. Increasing evidence seems to emerge that the genetic toolkit needed for the formation and patterning of a centralized nervous system (''brain'') had already been in place before the emergence of Bilateria from their radial symmetrical ancestor (Lemons *et al.*, 2010), without being fully expressed on the morphological level. Following this line of reasoning, the plexus-like morphological arrangement of the nervous system with little or no anterior concentration has been retained in the acoelomorphs and in basal deuterostomes (*Xenoturbella* and partly in Echinodermata and Hemichordata), with independent concentration events at the base of Protostomia (i.e., Ecdysozoa + Lophotrochozoa) and in various deuterostome lineages.

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Resúmen del tercer artículo, R3

Pasos hacia un sistema nervioso centralizado en bilaterales basales: comprensión desde la neurogénesis del *Symsagittifera roscoffensis*

Debido a la posición basal en el árbol de la vida de los bilaterales que se ha propuesto para los acelos, estos podrían tener la clave para nuestra comprensión de la evolución de un importante número de rasgos corporales, incluido el sistema nervioso central.

Para contribuir con nuevos datos a esta discusión hemos investigado la distribución de tubulina y de los neurotransmisores serotonina y RFamida en ejemplares juveniles y adultos de *Symsagittifera roscoffensis*.

Adicionalmente presentamos la expresión del gen neurogénico *SoxB1*.

Los ejemplares adultos y juveniles exhiben seis cuerdas nerviosas longitudinales y una concentración anterior de células sensoriales marcadas por el anticuerpo contra la serotonina.

Mientras los juveniles muestran una disposición ortogonal de cuerdas nerviosas y comisuras transversales a lo largo del eje anterior-posterior, el sistema nervioso de adultos parece más irregular en la región posterior. Las comisuras transversales están presentes sólo en la región anterior de los adultos, mientras que en la región posterior se encuentran neuronas distribuidas individualmente de manera irregular, a menudo interconectadas, por las células serotoninérgicas. El anticuerpo contra el neurotransmisor RFamida marca numerosas neuronas individuales alrededor del estatocisto. La immunoreacción frente a la α–

tubulina confirma la presencia de un sistema nervioso ortogonal en *S. roscoffensis*.

Los genes de clase *SoxB1* pertenecen a la familia de factores HMG-box, y se conocen por su función neurogénica en bilaterales. El gen *SrSoxB1* se expresa en la región de mayor densidad del neurotransmisor serotonina, es decir en la parte anterior, de forma consistente con el patrón de expresión de sus ortólogos en los demás bilaterales dotados de cerebro.

Así, nuestros datos sostienen la tesis de que el ancestro de los bilaterales ya tenía el compendio instrumental necesario para formar estructuras neuronales proto-cerebrales, siendo el grado de densidad neuronal de las mismas todavía bajo.

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REVIEW

ORGANISMS DIVERSITY & EVOLUTION

The Acoela: on their kind and kinships, especially with nemertodermatids and xenoturbellids (Bilateria incertae sedis)

Johannes G. Achatz · Marta Chiodin · Willi Salvenmoser · Seth Tyler · Pedro Martinez

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Abstract Acoels are among the simplest worms and therefore have often been pivotal in discussions of the origin of the Bilateria. Initially thought primitive because of their "planula-like" morphology, including their lumenless digestive system, they were subsequently dismissed by many morphologists as a specialized clade of the Platyhelminthes. However, since molecular phylogenies placed them outside the Platyhelminthes and outside all other phyla at the base of the Bilateria, they became the focus of renewed debate and research. We review what is currently known of acoels, including information regarding their morphology, development, systematics, and phylogenetic relationships, and put some of these topics in a historical perspective to show how the application of new methods contributed to the progress in understanding these animals. Taking all available data into consideration, clear-cut conclusions cannot be made; however, in our view it becomes successively clearer that acoelomorphs are a "basal" but "divergent" branch of the Bilateria.

Keywords Acoelomorpha · *Xenoturbella* · Morphology · Development · Systematics · Phylogeny

J. G. Achatz · M. Chiodin · P. Martinez
Department of Genetics, University of Barcelona,
Av. Diagonal, edifici annex, planta 2a,
08028 Barcelona, Spain

J. G. Achatz (✉) · W. Salvenmoser
Department of Evolutionary Developmental Biology,
University of Innsbruck,
Technikerstrasse 25,
6020 Innsbruck, Austria
e-mail: Johannes.Achatz@gmx.at

S. Tyler
School of Biology and Ecology, University of Maine,
5751 Murray Hall,
Orono, ME 04469, USA

P. Martinez (✉)
Institució Catalana de Recerca i Estudis Avançats (ICREA),
Passeig Lluís Companys, 23,
08010 Barcelona, Spain
e-mail: pedro.martinez@ub.edu

Introduction

Acoels are bilaterally symmetric, microscopic worms, typically in the millimeter-size range, that are found predominantly in benthic marine habitats. They can easily be recognized by the presence of a characteristic statocyst at the anterior end (see sensory organs; Figs. 1, 2a, b, d). Most are translucent or somewhat milky, but some are colored by pigmentation, by algal symbionts, or by glandular secretions called rhabdoids (Figs. 1, 2a, b, 5a). Their body shapes correlate with their habitat: species living in sand are long and slender, those moving on or in mud are compact and droplet-shaped, those moving on or beneath stones and corals are broad and flat, epiphytic species have ventrally enrolled sides, and pelagic species have a disc-shaped body or enrolled sides (Figs. 1, 3, 5a).

Acoels are acoelomate, the space between gut and body wall being filled with parenchymal cells that occasionally contain chordoid vacuoles and the insunk bodies of epidermal and gland cells. The name 'acoel' comes from their lack of a cavity in the gut, which is typically a solid syncytium.

Of the nearly 400 described species (Tyler et al. 2012, The Turbellarian Taxonomic Database, http://turbellaria.umaine.edu; Wallberg 2012, The Stylet–Diversity and Systematics of Acoela and Nemertodermatida, http://acoela.myspecies.

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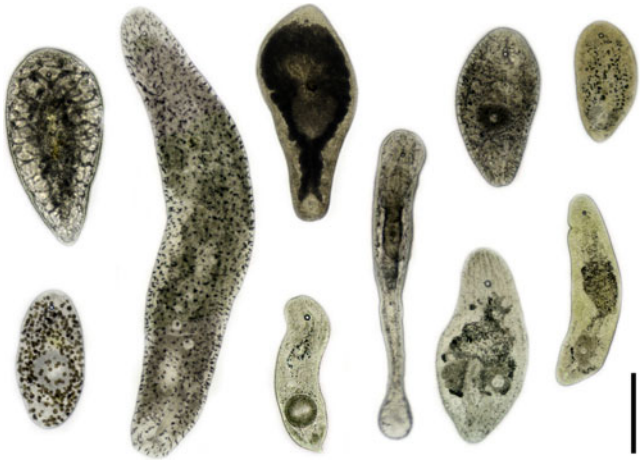

Fig. 1 Images of various live acoels found in a beaker of sublittoral sand from the Indian Ocean. Animals are oriented with the anterior end to the top. Note the statocyst in all and mature oocytes in some animals. Scale bar: 200 μm

info), by far the majority are free-living, but seven are parasites or endosymbionts in the digestive system of echinoderms (Jennings 1971), and two are found in fresh water (Ax and Dörjes 1966; Faubel and Kolasa 1978). Their diet varies as much as their habitat, ranging from bacteria and unicellular algae to crustaceans, small bivalves, and worms (including other acoels); some are known for cannibalism (e.g., *Conaperta flavibacillum*).

Morphology

Epidermis Like most microscopic worms, acoels move predominantly by ciliary gliding. The epidermis is multiciliated, and the cilia have the common configuration of nine peripheral microtubule doublets and two central microtubules (9×2 +2). The shape of the cilia is distinctive, having a marked shelf at the tip where the doublets 4–7 terminate (Tyler 1979; Ehlers 1985; Smith and Tyler 1985a; Smith et al. 1986; Rieger et al. 1991). Even more distinctive of the cilia is their rootlet system, which interconnects them: from the major, rostrally directed rootlet on each cilium, two lateral rootlets project and connect to the tips of the adjacent cilia, and from a caudal rootlet two bundles of fibers project to join the knee-like bend of those same adjacent rootlets (Hendelberg and Hedlund 1974; see Fig. 1 F in Rieger et al. 1991).

Glands Unicellular glands that typically richly populate the epidermis include the above-mentioned rhabdoid glands (Smith et al. 1982), which may be colored, and mucous glands. Glands occurring at special positions include sagittocytes that produce needle-shaped extrusomes (sagittocysts, Fig. 4c) predominantly near the reproductive organs; prominent mucous glands of the frontal organ that discharge together through a pore at the anterior terminal end of the body (Smith and Tyler 1985b, 1986; Klauser et al. 1986; Smith et al. 1986; Rieger et al. 1991; Figs. 2b, 3); and frontal glands of a variety of types that discharge near the anterior tip. The nuclei of all these gland cells with the exception of most pigment cells are usually positioned below the body-wall musculature.

Sensory organs Specifically distinctive of acoels, the statocyst comprises a lithocyte bearing one statolith encompassed in a capsule formed by two lining parietal cells (Ehlers 1985; Figs. 2d, 3, 4a). Occasionally, animals that have been reproduced asexually may lack the statocyst (Hanson 1960, Hendelberg and Åkesson 1988; Åkesson et al. 2001; see Fig. 5a), whereas panther worms (Hofsteniidae) have been reported to occasionally possess more statoliths after regeneration of the anterior body region (Steinböck 1966).

In a small percentage of species paired eyespots, which are probably photoreceptive, occur at the anterior end

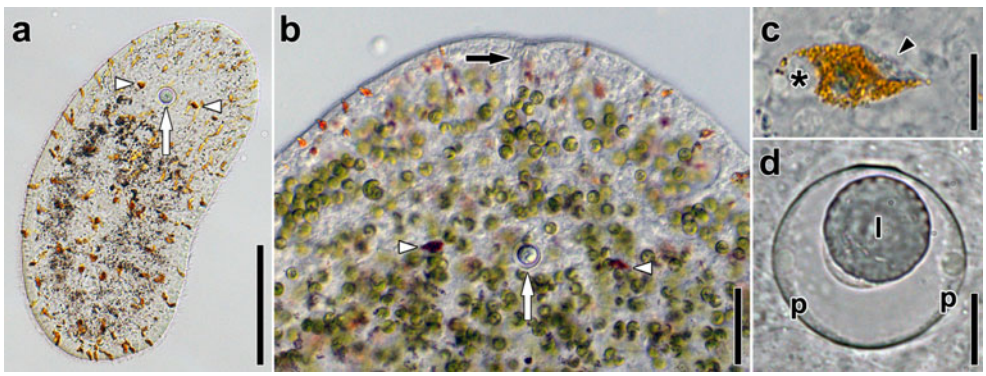

Fig. 2 Images of sensory structures of live *Symsagittifera roscoffensis*. **a** Hatchling. *Arrowheads* point to eyes, *arrow* to statocyst. Note absence of symbionts and presence of orange rhabdoids. **b** Anterior end of adult with symbionts and rhabdoids. *White arrowheads* point to eyes, *white arrow* to statocyst, *black arrow* to frontal organ. **c** Eye of an adult. *Asterisk* marks nucleus, *arrowhead* points to concrements. **d** Statocyst of an adult. Abbreviations: *l* lithocyte; *p* parietal cells. Scale bars: **a** 100 μm; **b** 50 μm; **c** 10 μm; **d** 10 μm

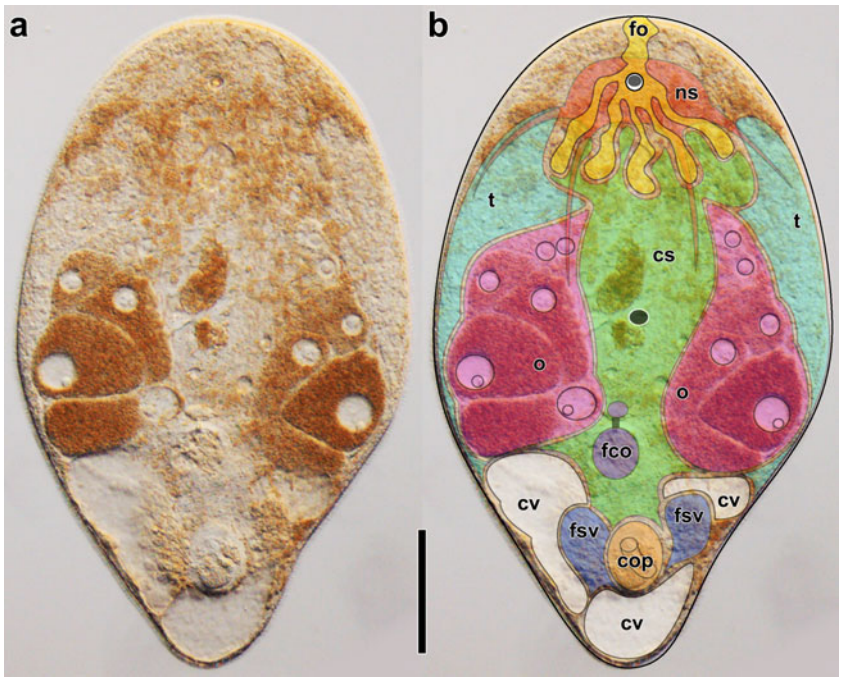

Fig. 3 Image of a mature and live specimen of *Isodiametra pulchra* without (*left*) and with superimposed colors (*right*) to illustrate the general morphology of acoels. From top to bottom: *yellow*: frontal organ (*fo*); *red*: nervous system (*ns*); *green*: central syncytium (*cs*); *cyan*: testes (*t*); *pink*: ovaries (*o*); *gray*: mouth; *purple*: female copulatory organs (*fco*) composed of seminal bursa, bursal nozzle, and vestibulum (from posterior to anterior); *white*: chordoid vacuoles (*cv*); *blue*: false seminal vesicles and prostatoid glands (*fsv*); *orange*: male copulatory organ (*cop*) composed of muscular seminal vesicle and invaginated penis. Scale bar: 100 μm

(Figs. 2a, b, c). Lanfranchi (1990) described the eyespots of *Otocelis rubropunctata* as specialized epidermal cells with typical 9×2+2 cilia and with pigment granules and many synapses and axonemal outgrowths on the basal surface, but he was unable to prove photoreceptive function. Yamasu (1991) suggested the photoreceptive capacity of the eyes of *Praesagittifera naikaiensis* by relating experimental ablation with behavioral assays. In this and some other species, the eyespots don't have ciliary or rhabdomeric elements but consist of a pigment cell containing a vacuole with refractive inclusions called concrements and up to three nerve cells to relay the stimulus. The same configuration of cells has also long been known in *Convoluta convoluta* (Popova and Mamkaev 1985), and such eyespots have subsequently been recognized to be characteristic for a derived group within the Acoela, the Convolutida (Hooge and Tyler 2005; Achatz et al. 2010). In all likelihood, many species of the Acoela can detect light (and behave accordingly) through photoreceptive sensory cells of the epidermis—cells that are difficult to identify because they are not accompanied by pigment cells.

Other known sensory organs in acoels are single-celled receptors, which are mostly monociliary. These can be classified into several types on a morphological basis (Todt and Tyler 2007 and references therein), each type occurring in a specific region of the body that is species-specific (Todt and Tyler 2007; Bery et al. 2010).

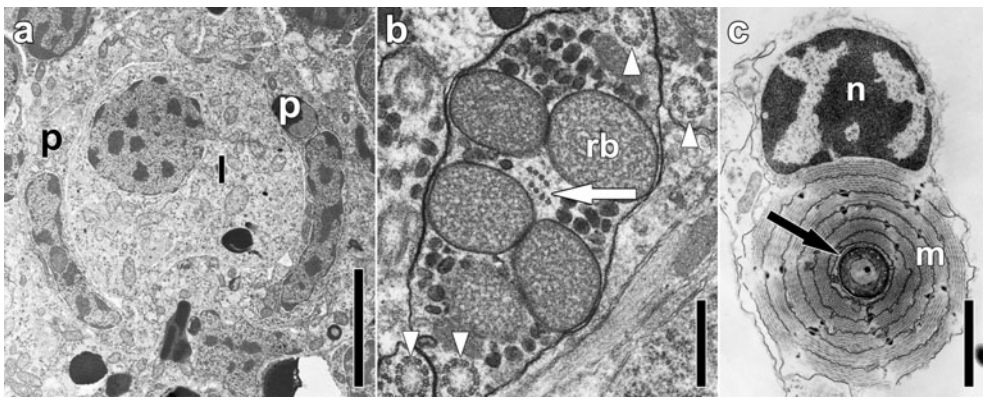

Fig. 4 Electron micrographs of structures with phylogenetic significance. **a** Statocyst of a hatchling of *Isodiametra pulchra* with two parietal cells (*p*) and a lithocyte (*l*). **b** Sperm of *Convoluta niphoni* (Convolutidae) with axial microtubules (*white arrow*) and axonemes without central microtubules (*white arrowheads*). **c** Extrusion apparatus of *Convolutriloba hastifera* consisting of a sagittocyst (*black arrowhead)* and a wrapping muscle mantle. Abbreviations: *m* muscle mantle; *n* nucleus of muscle mantle; *rb* refractive body; *p* parietal cell. Scale bars: **a** 4 μm; **b** 0.5 μm; **c** 2 μm

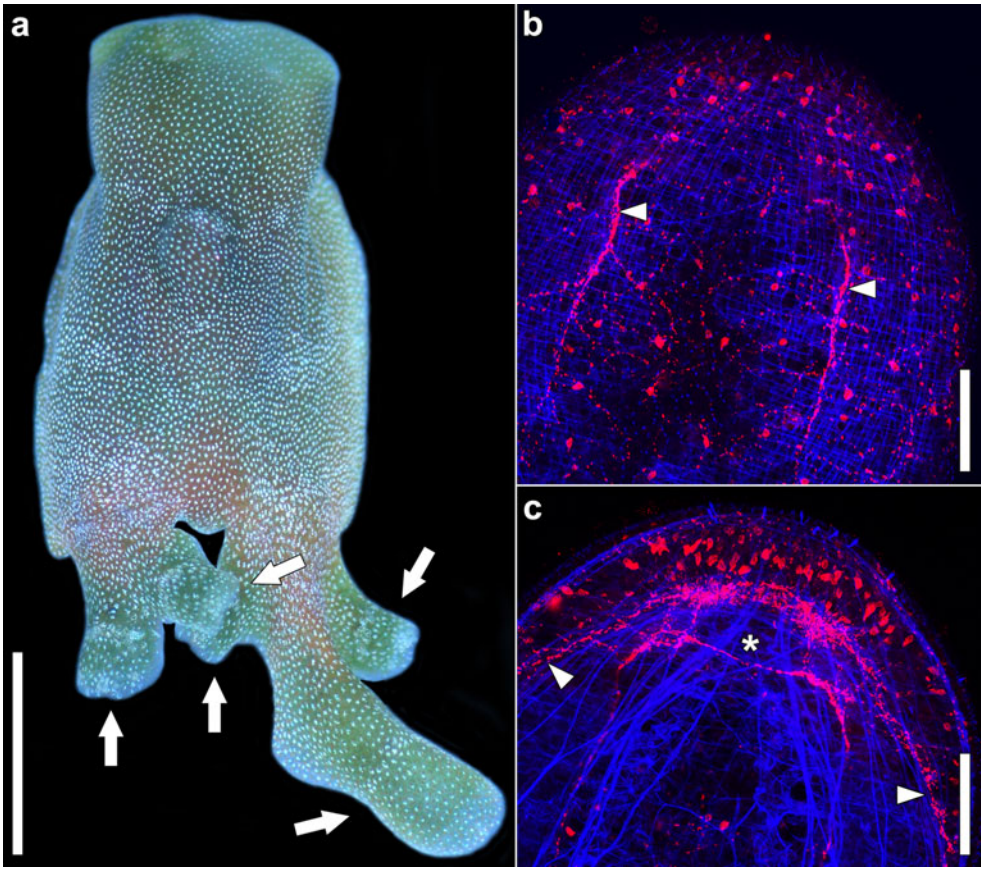

Fig. 5 **a** Image of a live specimen of *Convolutriloba retrogemma* reproducing asexually by budding. *White arrowheads* point to buds. Note the reversed polarity. **b**, **c** CLSM projections showing muscles (*blue*) and serotonin-like immunoreactive nervous system (*red*) in dorsal (**b**) and central (**c**) planes of a mature *Isodiametra pulchra*. *White arrowheads* point to neurite bundles, *asterisk* marks the position of the statocyst. Scale bars: **a** 1 mm; **b** and **c** 50 μm

Nervous system The nervous system itself consists of a supramuscular plexus, a submuscular plexus, 3–5 pairs of longitudinal neurite bundles (terminology after Richter et al. 2010), and a brain, which is shaped like a ring, a barrel, or a bilobed mass, with a complex connectivity of various fibers forming connectives and commissures (Raikova et al. 1998; Reuter et al. 2001a, b; Gaerber et al. 2007; Achatz et al. 2010; Bery et al. 2010; Semmler et al. 2010; Figs. 3, 5b, c). Serotonin-like immunoreactive (Raikova et al. 1998, 2004a; Reuter et al. 2001a, b; Gaerber et al. 2007; Semmler et al. 2010; Figs. 5b, c), RFamide-like immunoreactive (Raikova et al. 2004a; Reuter et al. 1998), and cholinergic (Gaerber et al. 2007; Bery and Martinez 2011 and references therein) parts of the nervous system have been revealed by immunohistochemistry and conventional histochemistry. The neurite bundles are generally distributed evenly around the anteroposterior axis and are similar in diameter; however, the dorsal or ventrolateral neurite bundles may be more pronounced (Rieger et al. 1991; Raikova et al. 1998, 2001).

Muscles Besides ciliary gliding, acoels use muscles to move. Abundant dorsoventral muscles serve to flatten the body, and the musculature of the body wall and parenchymal muscles generate bending, shortening, and lengthening movements. The body-wall musculature comprises circular-, diagonal-, longitudinal-, longitudinal crossover-, spiral-, U-shaped-, reversed U-shaped, and pore muscles (Hooge 2001; Tekle et al. 2005; Semmler et al. 2008; Achatz et al. 2010; Figs. 5b, c). The arrangement and complexity of the ventral body-wall musculature led Tyler and Rieger (1999) to hypothesize that it serves in ingesting food and so functionally makes up for the lack of a true pharynx.

Pharynx Pharynges are present in the acoel families Diopisthoporidae, Hallangidae, Hofsteniidae, and Solenofilomorphidae, and the genera *Oligochoerus* (Convolutidae) and *Proporus* (Proporidae). Detailed morphological analyses of these pharynges show that they are very diverse with respect to musculature, the nature of the lining cells, and the types of receptors present (Karling 1974; Crezée 1975; Doe 1981; Rieger et al. 1991; Todt and Tyler 2007; Todt 2009). Nowhere else in the animal kingdom is the position of the mouth as variable as it is in acoels. Even though it is most commonly situated mid-ventrally, the mouth can be anywhere from subterminally at the anterior (*Proporus*, *Hallangia*, *Hofstenia,* and some species in the Isodiametridae) to terminally on the posterior end (*Diopisthoporus*) and anywhere in between along the ventral midline.

Gut The gut is syncytial and lacks a lumen in most investigated species and is therefore commonly termed a central syncytium, but central parenchyma is a common term as

well (Fig. 3); however some species, notably representatives of the Paratomellidae, have a lumen without an epithelial lining (its cells are parenchymal, packed in a jumble, and lack the aligned polarity and cell junctions characteristic of epithelia—Smith and Tyler 1985a; Ehlers 1992a). All acoels hitherto studied, covering a wide range of sizes and phylogenetic distribution (compare species studied in Smith and Tyler 1985a and phylogeny of Jondelius et al. 2011), lack glandular cells, as would be typical of the gut of most animals (including the sister group, the Nemertodermatida —see below) in the digestive tissue.

Excretory organs No typical excretory organs have been found in acoels. Cells that resemble the cyrtocytes of protonephridia (so-called "pulsatile bodies" with waving cilia found below the epidermis) have been shown to be degenerating epidermal cells that are in the process of being resorbed (Mamkaev 1967; Tyler et al. 1989; Ehlers 1992b; Lundin 2001). Cells lacking cilia and resembling the canal cells of protonephridia (with a branching system of lacunae and tubules connecting to the outside) have been proposed to be excretory cells in *Paratomella rubra* (Ehlers 1992c).

Symbionts Symbiotic algae are found in many acoels living in sun-exposed habitats (Figs. 2b, 5a) and are essential for the survival of the host (Shannon and Achatz 2007). These can be either zoochlorellae or zooxanthellae, or both together in some species (see Achatz et al. 2010 for more detail). Transfer is commonly horizontal, meaning that the symbionts are acquired anew by each generation. Vertical transmission, whereby the symbionts are passed to the next generation in the egg, is known for *Amphiscolops carvalhoi* (Marcus 1952) and *Waminoa brickneri* (Barneah et al. 2007). The establishment of symbioses with algae happened at least twice within the Acoela (Achatz et al. 2010).

Gonads Acoels are simultaneous or slightly protandric hermaphrodites. The gonads are always asaccate (asacular in Rieger et al. 1991), meaning that the germ cells are not lined and separated from the surrounding parenchyma by specialized tissue called tunica (Fig. 3; for exceptions see also below—What is primitive in the Acoelomorpha?; Rieger et al. 1991, pp 88 and 93; the notion of Boone et al. 2011 that testes in acoels can be saccate must be a misinterpretation of the literature). The position of the ovaries and testes is highly variable even with regard to each other; they can be paired or unpaired, and in a few species (e.g., *Antigonaria*) their germinative zone is mixed, producing both sperm and ova (Rieger et al. 1991). The oocytes are entolecithal and in many cases accompanied by accessory cells, but contrary to occasional claims (Mark 1892; Dörjes 1968; Winsor 1988), the ovary is never differentiated into germarium and vitellarium (Achatz et al. 2010). Sperm are described in more depth as they provide important characters for the internal phylogeny of acoels. During the early development of sperm—spermatogenesis—spermatids grow two free flagella at the distal end, which are subsequently incorporated into the body of the sperm in a proximal direction. They run its entire length or close to the distal end of the nucleus, which is positioned at the proximal end of the sperm (Hendelberg 1969, 1977). The flagella lose their membrane after fusion, but the axonemes remain. In most cases these axonemes show the typical configuration of nine peripheral microtubule doublets and two central microtubules (as in locomotory cilia); however, in some species there is only one central microtubule (9×2+1) or none (9×2+0—see Fig. 4B). There are additional microtubules in the cytoplasm of the sperm, most likely to provide some rigidity to the cell. These cytoplasmic microtubules are positioned either under the plasma membrane, forming a kind of cytoskeletal sheath (so-called cortical microtubules) or run through the central axis of the sperm in between the two axonemes (axial microtubules) (Figs. 4b, 7).

Canal system Sperm usually aggregate within spaces in the parenchyma close to the male copulatory organ. If these spaces are encompassed by specialized tissue (including muscles that provide pressure to eject the sperm and secretions), they are called seminal vesicles; if the parenchyma has no obvious differentiation, they are called false seminal vesicles; however, both types can be present in the same individual (Fig. 3). The male copulatory organs are highly diverse and range in general anatomy from being absent or simple invaginations of the body wall (antrum) to complicated arrangements comprising muscular or sclerotized parts that are combined with glandular parts and muscular bulbs that provide pressure for the ejection of sperm (Westblad 1948; Dörjes 1968). The male gonopore can be situated anteroventrally along the ventral midline up to the posterior end, its position, as well as that of the copulatory organ, depending on the position of the testes and the direction of maturation of the sperm.

The female copulatory organ consists of gonopore(s), vagina(e), seminal bursa(e), and one or many bursal nozzles (Figs. 3, 6a, b, c), but some or all of these parts can be missing, leaving the animal with a kind of inconspicuous bursal tissue or no obvious adaptation at all. A seminal bursa is a distinct "pocket" made up of parenchymal cells that serves to store and digest sperm received from a mating partner (Brüggemann 1985a; Petrov et al. 2006; Achatz et al. 2010; Fig. 6a). Bursal nozzles are structures, stiffened by F-actin-rich cells, that accompany or are part of the seminal bursa; they appear to select and modify sperm (Brüggemann 1985a; Petrov et al. 2006; Achatz et al. 2010; Figs. 6a, b, c).

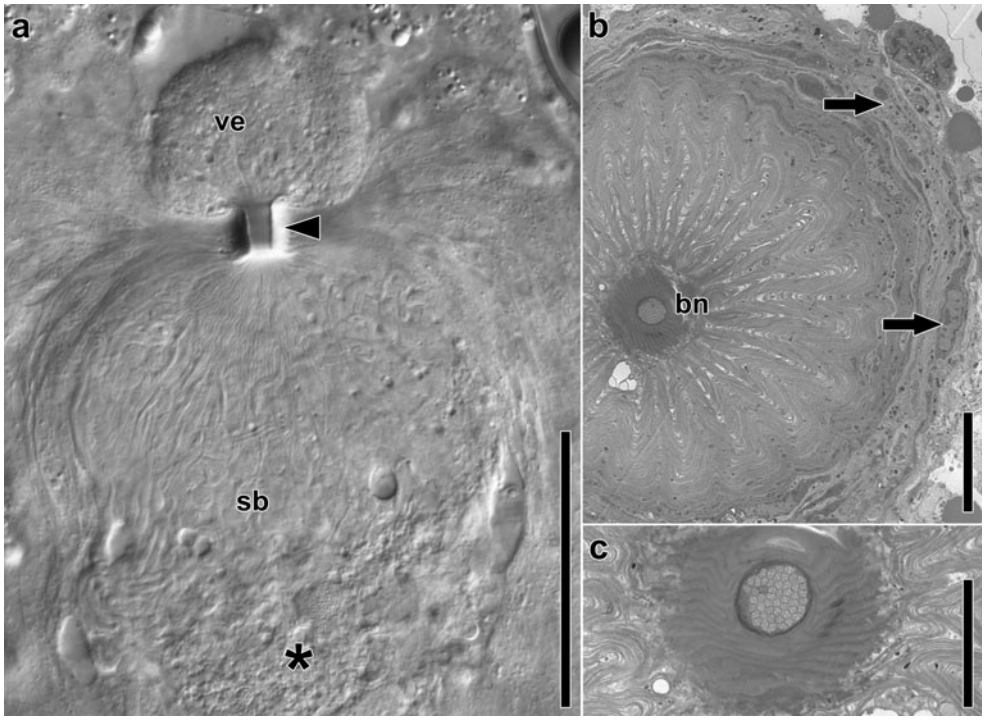

Fig. 6 Female copulatory organs in *Isodiametra pulchra*. **a** Image of female copulatory organs in a live and squeezed specimen. Note the mass of elongated and convoluted sperm in the seminal bursa (*sb*) that merge towards the bursal nozzle (*arrowhead*) and a few "heads" extending into the vestibulum (*ve*). *Asterisk* marks bursal stalk connecting the bursa with the digestive parenchyma, *arrowhead* points to bursal nozzle. **b** Electron micrograph showing cross section through the bursal nozzle (*bn*). *Arrows* point to nuclei of cells of the bursal wall. **c** Counterclockwise rotated detail of **b**. Note the density of sperm in the duct of the bursal nozzle. Abbreviations: *bn* bursal nozzle; *sb* seminal bursa; *ve* vestibulum. Scale bars: **a** 50 μm; **b** 10 μm; **c** 5 μm

Reproduction and development

Sexual reproduction Fertilization is always internal; the mode of copulation varies considerably and seems to be related to the environment (Apelt 1969). Among the modes of sperm transfer are mutual exchange (Hyman 1937; Costello and Costello 1938; Westblad 1946; Apelt 1969), hyperdermal transmission (Bush 1975), and hypodermal injection (Apelt 1969). In general, in the first two cases, a simple opening in the epidermis, an antrum, or a soft, muscular penis serves to transfer sperm; in the last case the epidermis of the partner is commonly punctured with sclerotized accessory structures like needles or a stylet.

Eggs are laid individually or in clusters through the mouth, the female gonopore, or through rupture of the body wall (Costello and Costello 1939; Apelt 1969; see Rieger et al. 1991).

Development Embryonic development is direct and follows a distinct spiral duet cleavage pattern that likely originated independently from the common quartet spiral cleavage of the lophotrochozoan phyla (Bresslau 1909; Apelt 1969; Boyer et al. 1996; Henry et al. 2000). The cleavage pattern is only known for a few species of acoels, all belonging to the Crucimusculata, with the exception of *Diopisthoporus*, which is viviparous and in which embryonic development is difficult to follow. Nevertheless, it is clear that cleavages are spiral and that the second, asymmetric and horizontal cleavage leads to the production of micromeres (Apelt 1969). As in quartet spiral cleavage, the first horizontal cleavage is unequal and so produces micromeres, but it occurs at the two-cell stage instead of the four-cell stage, so the micromeres appear as duets instead of quartets. The micromeres arise in a leiotropic direction with respect to the animal-vegetal axis, as do all subsequent micromeres, unlike the micromeres in spiral quartet cleavage, which are alternately leio- and dexiotropic. Also distinct from spiralian cleavage is its more bilateral nature: the sagittal plane (and so the antero-posterior axis) of the adult is defined by the first cleavage, whereas this plane and axis lie oblique to the quadrants in quartet spiral cleavage (Henry et al. 2000). The first, second, and third micromere duets give rise to all ectodermal structures, while endodermal (parenchyma) and mesodermal (muscles) structures are derived from the third duet of macromeres. Gastrulation occurs by growth of the micromeres upon the macromeres, and the mouth is formed at a site other than the blastopore (Boyer et al. 1996; Henry et al. 2000).

Unlike the canonical spiralian development, acoel duet spiral development shows no ecto-mesoderm source. Internal tissues arise either by delamination—that is, mitoses are oriented so as to produce digestive parenchyma, musculature,

and nervous tissue toward the interior of the embryo (of *Neochildia fusca*: Ramachandra et al. 2002) or by immigration of cells that form the endoderm and mesoderm (in *Convolutriloba longifissura*: Hejnol and Martindale 2008a). By the time gastrulation is complete, the embryo looks layered: the outermost layer is the epidermal primordium, a middle layer contains progenitors of muscles and neurons, and the innermost cells are those that will develop into the digestive syncytium. The segregation of organs starts afterwards, when the ciliated epithelium plus sub-epithelial muscle fibers form and when the nervous system begins to differentiate at the anterior end of the embryo.

While knowledge of the development of the nervous system remains incomplete, the development of the musculature of the body wall has been studied in two species, *Isodiametra pulchra* and *Symsagittifera roscoffensis*. By means of labeling of F-actin filaments, Ladurner and Rieger (2000) and Semmler et al. (2008) found that primary myocytes appear in the anterior half of the embryo of both species about halfway through development. Complete circular fibers form before longitudinal fibers, in an anteroposterior progression. In *I. pulchra* the first myocytes appear as single cells separated from each other in latitudinal positions; by elongating and connecting to each other with fine endings, these fibers completely encircle the embryo (Ladurner and Rieger 2000). Longitudinal fibers appear in a bilateral pattern and follow a similar developmental course. In contrast, in *S. roscoffensis*, the circular, longitudinal and diagonal primary myocytes seem to form simultaneously (Semmler et al. 2008). In both species, the primary muscle fibers serve as a template for the formation of secondary and further muscle fibers, a mechanism that is also used during muscle regeneration (see below). Accessory muscles, such as the sphincter muscles of the mouth, develop shortly before hatching.

Asexual reproduction While all acoels reproduce by sexual reproduction, many can also reproduce asexually through a variety of mechanisms. Paratomy—the preformation of organs before separation—occurs in the Paratomellidae (Dörjes 1966) and results in a chain of zooids; architomy, by which the organs form after the separation of mother and daughter, is common in the family Convolutidae, namely among the genera *Adenopea* (du Bois-Reymond Marcus 1955), *Amphiscolops* (Hanson 1960), and *Symsagittifera* (Marcus and Macnae 1954), and in species of *Convolutriloba* (Bartolomaeus and Balzer 1997); and budding occurs in other species of *Convolutriloba*, whereby the daughter individual develops with its anteroposterior axis perpendicular to or reversed in relation to that of the mother (Hendelberg and Åkesson 1988; Åkesson et al. 2001; Shannon and Achatz 2007; Sikes and Bely 2008, 2010; see Fig. 5a).

Regeneration Acoels exhibit great regenerative capacity after fission or after experimental amputation (see Egger et al. 2007). In all species studied to date, the process involves an initial muscle contraction that helps to close the wound. Muscle fibers that develop in the wound area are largely randomly oriented initially and only gradually achieve their orthogonal arrangement. Pre-existing muscle fibers and longitudinal neurite bundles invade the newly formed blastema and serve as a template for the differentiation of new myocytes and neurons (Gschwentner et al. 2001; Gaerber et al. 2007; Sikes and Bely 2008; Bery and Martinez 2011; Chiodin et al. 2011). Development, regenerative processes, and tissue homeostasis are controlled by somatic stem cells called neoblasts (De Mulder et al. 2009). These neoblasts usually show a high nucleus/cytoplasm ratio with little cytoplasmic differentiation and are referred to as totipotent, meaning that they can differentiate into all cell types. Somatic neoblasts are localized exclusively within the parenchyma, in contrast to the epidermal positions of stem cells in other metazoans, with the exception of rhabditophoran flatworms (for more detail, see De Mulder et al. 2009 and Egger et al. 2009). The germ cells and a subpopulation of somatic neoblasts in *I. pulchra* express a homolog of the gene *piwi*, the silencing of which does not affect cell proliferation in adult worms but does affect their ability to produce offspring; silencing also eventually kills juveniles treated during development (De Mulder et al. 2009). In most bilateral animals, *piwi* is a germline marker (and is found in the germline of *I. pulchra* as well), whereas it is found only in somatic stem cells of sponges, cnidarians, and rhabditophoran flatworms; thus its function in stem-cell specification must be primal (De Mulder et al. 2009).

Phylogenetic relationships within the Acoela

As acoels only show a paucity of variable organs, and only rarely bear consistently sclerotized structures, they offer few characters on which to base classification. Additionally, their microscopic size makes them difficult to investigate. The first acoel described, *Convoluta convoluta*, was classified as a planaria simply by its overall similarity to better-known triclad turbellarians (Abildgaard 1806), and subsequent descriptions of acoel species variously reported acoels to have no nervous system (Uljanin 1870; Graff 1882) and confused the terminal pore of the frontal organ with the mouth opening (Graff 1891). Growing knowledge of acoel diversity (Graff 1905; Luther 1912; Westblad 1940, 1942, 1945, 1946, 1948; Marcus 1947, 1948, 1949, 1950, 1951, 1952, 1954) finally led to the construction of a stable family-level system by Dörjes (1968) that was based primarily on light microscopic traits of the male copulatory organ. However, Dörjes did not develop a phylogenetic hypothesis for the Acoela because, with the characters at hand, there was no striking transformation series between families. It was the progress in investigative tools that paved

the way to clearer concepts of relationships. Electron microscopy made it possible to see details down to cellular substructures and provided more characters on which to establish similarities and differences, and by means of confocal laser-scanning microscopy, in combination with immunocytochemistry and fluorophore-tagged phalloidin (Figs. 5b, c), parts of the nervous system (Raikova et al. 1998, 2004a), the muscles of the body wall (Hooge 2001; Tekle et al. 2005), and the ducts and musculature of copulatory organs (Hooge and Tyler 2005) could be revealed with ease. By applying these techniques, sperm ultrastructure (Hendelberg 1977; Raikova et al. 2001; see Figs. 4b, 7) and body-wall musculature (Hooge 2001) could be discerned and provided a basis for the first substantial hypotheses of family interrelationships. Hooge et al. (2002) and Jondelius et al. (2011) confirmed and further expanded our understanding of these relationships through molecular sequence studies.

The most recent and most data-rich hypothesis of relationships is that of Jondelius et al. (2011); it covers rDNA and COI sequences from about a third of all described species, only missing data from the Anthroposthiidae, and the monotypic Antigonariidae, Nadinidae, and Tauriididae (see Fig. 7 for a simplified phylogenetic scheme). In summary, the analysis shows that the Diopisthoporidae is the most basal family of the Acoela, followed by the Paratomellidae and a clade Jondelius et al. (2011) call Prosopharyngida, comprising the Hallangidae, Hofsteniidae, and Solenofilomorphidae. The basal position of these families is consistent with earlier claims based on morphology, especially for the Paratomellidae (Smith and Tyler 1985a; Ehlers 1992a; Raikova et al. 1997, 2001) and the Hofsteniidae and Solenofilomorphidae, the relationship of which was implied by their possession of a specific type of receptor with an enlarged main rootlet and a smaller posterior rootlet (Todt and Tyler 2007). However, as mentioned by Jondelius et al. (2011), *Hallangia proporides* does not easily fit in the Prosopharyngida, showing characters that are reflective of isodiametrids.

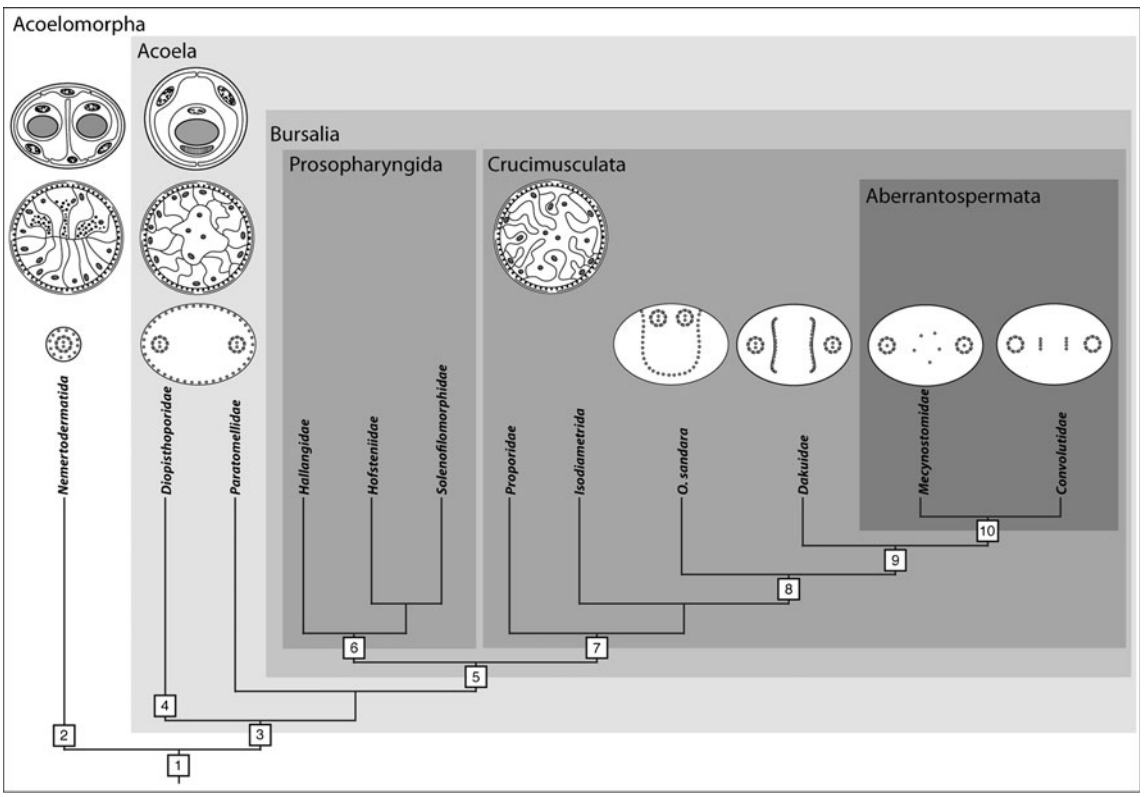

Fig. 7 Cladogram of the Acoelomorpha with partial family-level systematics of the Acoela. *1*. Multiciliated epidermis, ciliary rootlet system, frontal organ, basiepidermal nervous system with ring-shaped brain. *2*. Statocyst with two lithocytes (statoliths) and many parietal cells, sperm with cork screw-like morphology. *3*. Statocyst with one lithocyte (statolith) and two parietal cells, brain sunk below body wall, lateral fibers at knee of rostral rootlet, biflagellated sperm; digestive system becomes depolarized. *4*. Position of mouth at the posterior end. *5*. Specialized parenchymal tissue for reception, storage, and digestion of sperm (seminal bursa). *6*. Subterminal pharynx at anterior end. *7*. Ventral crossover muscles and highly branched wrapping cells. *8*. Cytoplasmic microtubules of sperm partially lose contact with membrane and change position toward the center of the cell. *9*. Cytoplasmic microtubules of sperm change position toward the center of the cell, stacked bursal nozzles with matrix and gland cells. *10*. Central microtubules in axonemes of sperm reduced to allow movement in more than one plane. General scheme after Achatz et al. (2010); schemes of cross sections through statocysts from Ehlers (1985), through bodies after Rieger and Ladurner (2003); systematics and branching after Jondelius et al. (2011)

The five "basal" families are clearly set apart from the "higher acoels," or Crucimusculata (as named by Jondelius et al. 2011), which are identified by the possession of ventral crossover muscles (Jondelius et al. 2011) but also wrapping cells (Smith and Tyler 1985a; see Fig. 7). Because many families within the Crucimusculata were recovered as paraphyletic, Jondelius et al. (2011) synonymized several of them: the Haploposthiidae and Polycanthiidae with Proporidae, Childiidae with Mecynostomidae, and Anaperidae and Sagittiferidae with Convolutidae; they also transferred species of the Otocelididae with copulatory needles and the genus *Philactinoposthia* to the Dakuidae.

Jondelius et al. (2011) also reconstructed the ancestral state via simultaneous analysis of gene sequence data and 37 morphological characters under parsimony and Bayesian optimality criteria. Characters such as the presence or absence of a vagina and seminal vesicle were shown to be uninformative to the phylogenetic relationships, whereas those of the copulatory organs were quite significant at the family level and those of the body-wall musculature at deeper backbone nodes (except in the Mecynostomidae and Proporidae, for which the genitals were reconstructed with a slightly stronger signal than the muscles). By means of these analyses even the characteristics of the common ancestor to all acoels could be determined with some accuracy. However, the results should be taken with a pinch of salt as the character analysis (how morphological characters are selected, how states are defined, delimited, coded, and ordered; Wiens 2001), which is as crucial for the analysis of morphological characters as is the alignment for the analysis of a molecular data set (Pleijel 1995; Freudenstein 2005), lacks accuracy. The presence of a stylet, for instance, was reconstructed in all deep nodes under the model based on Bayesian character reconstructions, with BPPs ranging between 0.95 and 0.97 (see Table 4 in Jondelius et al. 2011), and therefore the presence of a stylet is considered part of the ground pattern in acoels (see Fig. 9 in Jondelius et al. 2011), having been lost repeatedly within the clade. Yet, stylets in the Mecynostomidae are composed of tubulin (Tekle et al. 2007), those in the Dakuidae are composed of actin (Brüggemann 1985b; Hooge and Rocha 2006), and the stylet of *Paratomella rubra* is composed of neither one of those molecules (own unpublished observation). Consequently, following Remane's second homology criterion (similarity in substructure of character), the stylet as such is a homoplasious character. Notably, Xiang and Thomas (2008) showed that reconstruction signals of homolog characters are robust with regard to the analysis method used, whereas those of homoplasious characters are highly dependent on the method used, and not surprisingly, the parsimony reconstruction of the stylet is not consistent with the Bayesian reconstruction. This incongruity further applies to the pharynx. Todt (2009), who was aware of the "basal" phylogenetic position of pharynx-bearing acoels (see her Fig. 10), was unable to find any clear signs or remnants of common ancestry (other than the pharynges of Hofsteniidae and the Solenofilomorphidae). She did not provide an analysis of the characters that she thought indicative of an independent origin of pharynges; however, the same applies to Jondelius et al. (2011), who only used the presence/absence of interconnecting cells to code the diversity of the pharynges, ignoring the known variation in pharynx tube muscle layers and associated tissues, as well as in receptors. To sum up with an example that might be more current to the reader: we think that assessing the homology of eyes in the Bilateria by taking a sequence data set and running an ancestral state reconstruction by coding the eyes as present/absent, not taking the diversity of morphology into account, does not fully represent the complexity of the challenge.

Fortunately, there are robust characters by which the inner phylogeny of the Acoela can be retraced unequivocally, and these include characters of the body-wall musculature, the female copulatory organ (bursa and bursal nozzle) and sperm (Fig. 7). Sperm with cortical microtubules are found in "basal" families; the most divergent families have, instead, axial microtubules; interestingly, taxa that are phylogenetically positioned in between these two groups have an intermediate pattern of cytoplasmic microtubules, revealing an evolutionary transformation series (Petrov et al. 2004). Within the clade possessing axial microtubules, three groups can be distinguished on the basis of the pattern of microtubules in the axonemes: the Dakuidae have two singlet microtubules in the center of the axoneme, as is typical of most cilia (9+2); the Mecynostomidae have, instead, only a single microtubule in this central position (9+1); the Convolutidae typically lack central microtubules (9+0) altogether (Hendelberg 1977; Raikova et al. 2001; see Fig. 4b). Achatz et al. (2010) suggest that changes in the number and position of cytoplasmic microtubules are adaptations of the sperm to accommodate passage through a bursal nozzle. Nozzles are likely bottlenecks for the sperm on their way to fertilize ova and, therefore, should lead to sperm competition. Consequently, sperm and copulatory organs, especially the bursal nozzles, are shaped according to the antagonistic co-evolution between female and male function (sexual conflict), a situation also found in other microturbellarians of the rhabditophoran genus *Macrostomum* (Schärer et al. 2011). It appears that the central microtubules of the axonemes are also subject to this pressure and have become reduced in the Mecynostomidae and Convolutidae, probably to allow bending of the sperm in more than one plane.

Relationships with Nemertodermatida and Xenoturbellida

The first precladistic ideas placing acoels in the tree of life and interpreting their nature can be subsumed to the concept of the "acoeloid-planuloid hyothesis," which was proposed

by Graff (1904) and elaborated upon by Hyman (1951). This hypothesis proposed that a cnidarian-planula-like ancestor would have given rise to an acoel-like stem bilaterian that acquired bilaterality either through decompression of the body followed by a shift of the mouth from terminal to ventral (Graff) or through flattening along the oral-aboral axis and displacement of the nervous center toward one end, which became the new anterior end (Hyman). In this scenario, acoels are viewed as direct descendants of such a simple Urbilateria (Fig. 8a).

Subsequently, the theory and methodology of phylogenetic systematics (Hennig 1950, 1965) were established, and the archicoelomate theory, which postulated an ancestor with features of coelomate bilaterians (Remane 1963; Jägersten 1972), became widely accepted in Europe, whereas most US authors followed Hyman. Consequently, acoels were regarded as secondarily reduced and were classified within the Platyhelminthes, together with the Nemertodermatida forming the Acoelomorpha (Fig. 8b).

Nemertodermatids resemble acoels in general body form and the possession of a statocyst, but the statocyst bears two statoliths as opposed to the one in acoels (Ehlers 1985; Sterrer 1998). They live in mud or the interstices of sand, or are commensal (*Meara stichopus* lives in the foregut of a sea cucumber). Like acoels, they lack excretory organs and special ducts for the germ line. Despite these and other

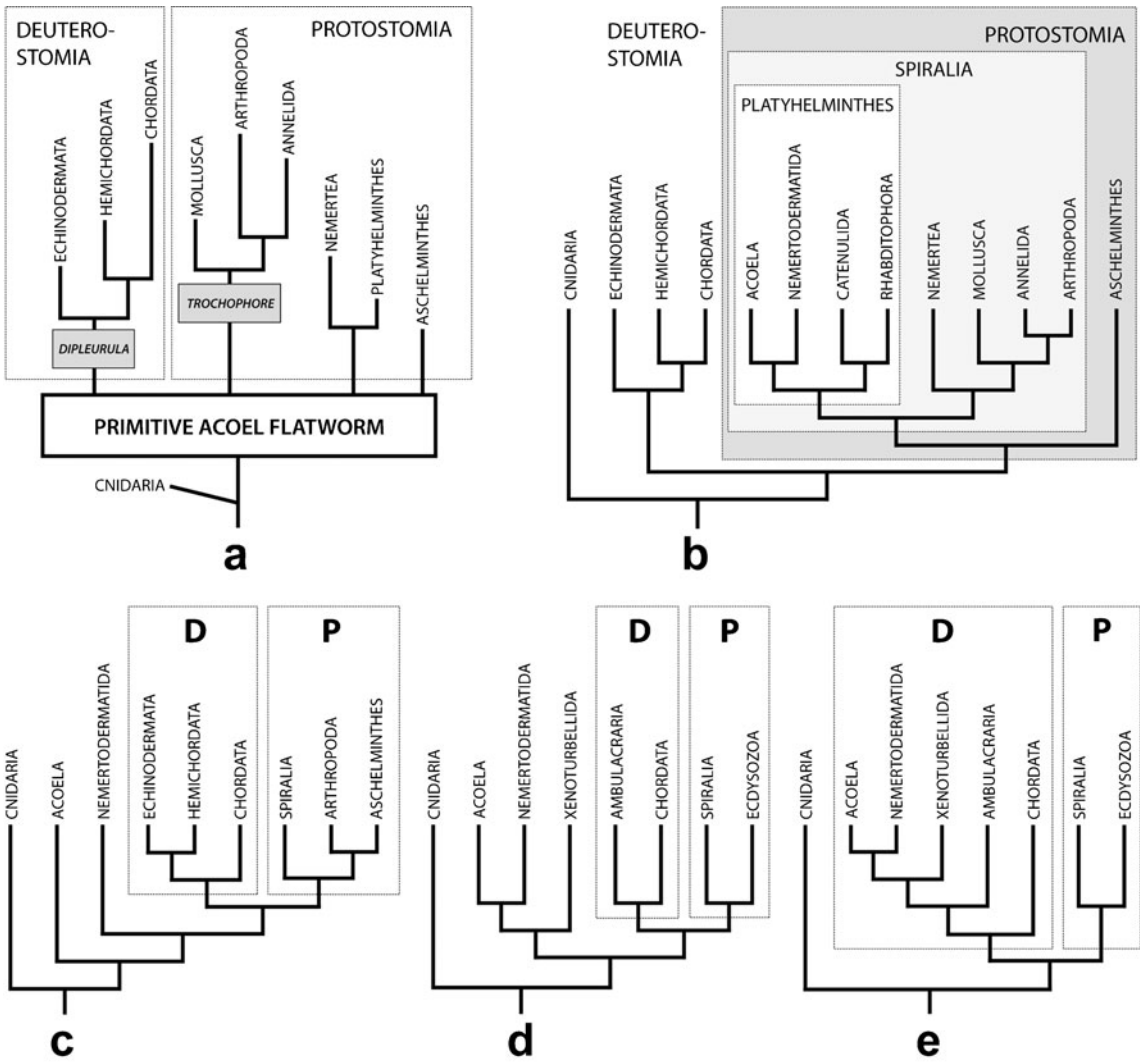

Fig. 8 The Acoela/Acoelomorpha in different schemes of eumetazoan relationships. **a** Precladistic version assuming a small planula-like worm as the ancestor of all bilaterians and with acoels as its direct descendants (after Hyman 1951). **b** Scheme based on morphological characters; the Acoela is part of the Acoelomorpha, which is placed within the Platyhelminthes (after Westheide and Rieger 2007). **c** Phylogeny according to rDNA (Wallberg et al. 2007); the Acoelomorpha forms a paraphylum at the base of the Bilateria. **d** Phylogeny according to phylogenomics I (Hejnol et al. 2009); Acoelomorpha together with the Xenoturbellida forming a clade that is a sister group to all other Bilateria. **e** Phylogeny according to phylogenomics II (Philippe et al. 2011); the Acoelomorpha is placed within the Deuterostomia and derived by progenesis from a coelomate ancestor. Abbreviations: *d* deuterostomia; *p* protostomia

similarities between acoels and nemertodermatids, only two solid characters unite them: the ciliary rootlet system (Tyler and Rieger 1977) and the horizontal orientation of the second, asymmetric cleavage plane (Jondelius et al. 2004).

Nevertheless, knowledge of nemertodermatids is crucial in interpreting various characteristics of acoels. Extracellular matrix (ECM) is present in abundance in virtually all metazoans, but is missing under the epithelia (as basal lamina) in acoels and is also relatively scant to varying degrees in nemertodermatids (Smith and Tyler 1985a). An explanation may be that acoels substitute the mechanical properties of the basal lamina with the network of rootlets and a terminal web, which are both well developed in nemertodermatids as well (Rieger et al. 1991).

The nervous system of "basal" nemertodermatids (see Fig. 2 in Wallberg et al. 2007) consists of ring-like connectives, longitudinal neurite bundles, and a basiepithelial plexus, all positioned in the epidermis (Riser 1987; Raikova et al. 2000a, 2004b); as the nervous systems of many "basal" bilaterians are such "skin brains" (Holland 2003; see box 1), acoels likely have a more derived condition in that the connectives and neurite bundles are sunk below the body wall. The ring-shaped brain in "basal" nemertodermatids and "basal" acoels may represent the ground pattern in acoelomorphs even if most have paired ganglia complete with neuropile and rind as in other bilaterians.

As in the epithelia, ECM is missing in the parenchyma in acoels and is also relatively scant to varying degrees in nemertodermatids (Rieger et al. 1991). Considering that fixed parenchymal cells and chordoid cells are present in acoels but absent in nemertodermatids (Rieger et al. 1991) and that the differences of true parenchymal cells found between various acoelomate taxa suggests convergent evolution of such (see Rieger 1985), again, the character state found in nemertodermatids should be considered the plesiomorphic state for acoelomorphs.

In addition, the syncytial digestive system of acoels may be an extreme of conditions seen in nemertodermatids, which, while having a true epithelium and gland cells in their gut, have a small, relatively occluded lumen (Karling 1974; Smith and Tyler 1985a; see Fig. 7). A remnant of a gut lumen is evident in the acoel *Paratomella rubra* (Smith and Tyler 1985a), and various acoel species only temporarily develop a digestive syncytium after ingestion (Smith 1981).

The specialized form of the sperm in acoels (with two flagella whose axonemes are incorporated into the sperm cell) may be an adaptation to internal fertilization (Fig. 7); the sperm of the Nemertodermatida are moderately modified, presumably also for internal fertilization, but are monoflagellate like most metazoan sperm (Tyler and Rieger 1974, 1977; Hendelberg 1977; Fig. 7).

The embryonic cleavage pattern in Nemertodermatida bears resemblance to patterns in acoels, spiralian phyla (annelids, molluscs), and deuterostomes. Like that of acoels, cleavage in *Nemertoderma westbladi* takes place in a duet pattern, but starts out radial (like the cleavage patterns of deuterostomes); the micromeres later shift clockwise to produce a spiral-like pattern (Jondelius et al. 2004). Whether these differences signify an intermediate position of nemertodermatids between acoels and other animals (either spiralian or radially cleaving phyla) remains to be seen.

All of these features point to the Acoela being rather derived in comparison to the Nemertodermatida, which seem to have retained more characters in states more like those of other basal bilaterians (Tyler and Rieger 1977; Smith and Tyler 1985a; Tyler 2001). Some of these differences—for example, the digestive syncytium, the possession of a pharynx, or the position of the central nervous system below the body wall—may have facilitated diversification in ways not available to nemertodermatids. That diversification is now reflected in the approximately 400 described species compared to only 8 in the Nemertodermatida.

Even though a separate placement of the Acoelomorpha from the Platyhelminthes has been suggested based on morphological characters (Smith et al. 1986) and cladistic analyses of such (Haszprunar 1996), it was the comparison of sequence data on rDNA of the acoel *Paratomella rubra* (Ruiz-Trillo et al. 1999) and some other acoel species with that of other metazoan phyla that paved the way for the acceptance of such a split and the position of acoels at the very base of the Bilateria (Carranza et al. 1997; Ruiz-Trillo et al. 1999; Jondelius et al. 2002; Telford et al. 2003; Wallberg et al. 2007; Jondelius et al. 2011; Fig. 8c). Surprisingly, in these analyses acoels and nemertodermatids were split (Fig. 8c); however, data from amino acid sequences of mitochondrial genomes (Ruiz-Trillo et al. 2004; Mwinyi et al. 2010) and ESTs (Hejnol et al. 2009; Philippe et al. 2011) did re-establish the high probability of a sister group relationship between Acoela and Nemertodermatida and the validity of the Acoelomorpha (Figs. 8d, e). To place the Acoelomorpha, rDNA genes seem unsuitable because of their high A + T content and rather truncated and modified nature (Mallatt et al. 2010). Additionally, even though base composition bias or long branch attraction could be excluded to affect the placement of the Acoela in Wallberg et al. (2007), the limited number of genes likely makes us follow the evolution of these genes more than the organisms from which they have been sequenced.

Unfortunately, the content and order of mitochondrial genomes are unsuitable to infer the phylogenetic position of acoelomorphs because the existing data are either too scarce or, in the case of the complete mitochondrial genome of the highly derived acoel *Symsagittifera roscoffensis*, too divergent (Mwinyi et al. 2010). Consequently, the most reliable hypotheses based on molecular data come from analyses of amino acid sequences in either mitochondrial

genomes or EST collections, and these suggest that the Acoelomorpha is either (1) the earliest offshoot of the Bilateria (Hejnol et al. 2009, Mwinyi et al. 2010) or (2) in a sister group relationship with *Xenoturbella bocki* as the earliest offshoot of the Bilateria (Hejnol et al. 2009; Fig. 8d); or 3) together with *Xenoturbella bocki* among the deuterostomes (Philippe et al. 2011; Fig. 8e).

But what is *Xenoturbella bocki*? It constitutes, together with *Xenoturbella westbladi* (Israelsson 1999), the enigmatic Xenoturbellida (Bourlat et al. 2006) and is a remarkably simple worm, lacking organs other than an anterior statocyst. It is found on deep marine muds off the coasts of Scandinavia and Scotland. While considerably larger than acoels (measuring up to 4 cm in length), it has been linked to them through its simple morphology (worm shape, acoelomate structure, single opening to the gut), similarity in its nervous system, and lack of excretory organs and tissue enclosing the germ cells (Westblad 1949; Hyman 1959). More similarities are discernible through electron microscopy, especially in the shape of the cilia, their axonemal termination patterns, and their rootlets (Pedersen and Pedersen 1986, 1988; Franzén and Afzelius 1987; Lundin 1998). *Xenoturbella* has pulsatile bodies (degenerating epidermal cells) that appear much like those of acoelomorphs (Lundin and Hendelberg 1996; Lundin 2001); the cellular but unciliated nature of its gut is reminiscent of nemertodermatids, and its lack of a somatogastric nervous system (Raikova et al. 2000b) is similarly reminiscent. *Xenoturbella* also shows even stronger affinity with hemichordates and echinoderms through molecular sequence similarity (Bourlat et al. 2003, 2006, 2009) and morphological similarity of its epidermis and statocyst (Reisinger 1960; Pedersen and Pedersen 1986; Stach et al. 2005). However, the occurrence of monociliated parietal cells in the statocysts of apodous sea cucumbers and *Xenoturbella* most likely originated independently (Ehlers 1997).

Interestingly, Philippe et al. (2011) linked *Xenoturbella* to both Acoelomorpha and Ambulacraria (i.e., echinoderms + hemichordates) with sequence data of amino acids in a genomic set and mitochondrial genes. Further support for a close relationship among acoelomorphs, *Xenoturbella*, and deuterostomes comes from shared specific microRNAs, a shared sperm protein (Philippe et al. 2011), and a shared GNE kinase (De Mendoza and Ruiz-Trillo 2011), all of which are present only in these groups. As a cautionary note, however, we stress that the nature of microRNAs is rather problematic inasmuch as losses constantly occur and in the groups in question the data have not been backed up by a genome; the RSB66 sperm protein and epimerase could also have been lost specifically in the protostomes (De Mendoza and Ruiz-Trillo 2011). Additionally, the bootstrap supports for the Xenacoelomorpha are low in the analyses of Hejnol et al. (2009) as well as in Philippe et al. (2011), and one needs to explain the difference in mitochondrial gene order and different codons for serine in the acoelomorphs in comparison to deuterostomes (Bourlat et al. 2003). Furthermore, the morphological features that are similar between acoelomorphs and xenoturbellids could all be shared plesiomorphies or convergent adaptations to the benthic life of small worms (see Pardos 1988 for ciliary tips and rootlets). Taking all these facts into consideration, we are not opposed to but reluctant to accept the validity of a clade Xenacoelomorpha.

What is primitive in Acoelomorpha?

Whether the ancestor to all living bilaterians was a simple acoelomate worm or a more complex coelomate is a long-standing and ongoing debate (Rieger 1986; Holland 2003; De Robertis 2008). Proponents of the former hypothesis commonly refer to the "acoeloid-planuloid hyothesis" (e.g., Salvini-Plawen 1978; Baguñà and Riutort 2004; Wallberg et al. 2007; Hejnol and Martindale 2008a) and interpret acoelomorphs as "conserved" descendants of a simple urbilaterian and the basic acoelomorph body plan—simple basiepidermal nervous system and lack of anus, lining tissue over germ cells, and excretory organs as well as direct development—as primitive in the line leading to the rest of the Bilateria. Proponents of the archicoelomate theory (complex coelomate ancestor) usually suggest that acoelomorphs have acquired their recent organization through secondary loss of many features. The recently recovered position as sister group to the Ambulacraria within the Deuterostomia would support this idea because it is easier to loose characters such as through-gut, nephridia, deuterostomy, and gill slits once opposed to evolve them independently twice within the Deuterostomia. Comparable scenarios have been shown to occur in protostomes through either reduction of the coeloms or progenesis in a coelomate animal with acoelomate or pseudocoelomate larvae or juveniles (Rieger 1980, 1986; Schuchert and Rieger 1990; Fransen 1980a, b; Tyler 2001). One might oppose the latter proposition that the larvae of deuterostomes are coelomate and that the assumption of progenesis does not work in this case. However, the key point is that in acorn worms, pterobranchs, and echinoderms, mesoderm and coelomic cavities do not just appear through enterocoely from the archenteron but also through schizocoely and delamination (Peterson et al. 1999; Ruppert et al. 2004). Consequently, by suppressing the mesenchymal-epithelial transition or forestalling maturity to a developmental stage earlier than the mesenchymal-epithelial transition, the acoelomate condition could also be accomplished in a "deuterostome-like" coelomate.

Unfortunately, no morphological feature helps us to unequivocally decide between the two scenarios outlined

above and the same applies to results from Evo-Devo studies.

The central nervous system with basiepidermal ring commissures and major neurite bundles could just as easily reflect features of the urbilaterian as descent from a basal deuterostome. The homology of its subunits with structures of other bilaterians remains a matter of debate (Rieger et al. 1991; Raikova et al. 1998, 2000a; Bery et al. 2010). Semmler et al. (2010) found *SoxB1* to be widely expressed in the developing brain of *S. roscoffensis*, a finding that is consistent with its expression in developing neural structures throughout cnidarians and bilaterians. However, *SoxB1* is not, strictly speaking, a "brain marker" in that it is also expressed in the apical organ of the larvae of an acorn worm (Taguchi et al. 2002). Finally, the anterior-to-posterior development of the nervous system of acoels and its similarity with the oral-aboral gradient of the nervous system of cnidarians has led some to speculate that it reflects the first steps in centralization of the nervous systems of the Bilateria (Marlow et al. 2009; Semmler et al. 2010).

The proposed ancestral role of the ParaHox genes is the anteroposterior patterning of the digestive system; in particular, *cdx* shows conserved expression in a posterior ectodermal domain that is associated with the formation of the hindgut, and this was taken to mean that the anus of all Bilateria was homologous (Hejnol and Martindale 2008b). In the acoel *C. longifissura*, which like all acoels lacks an anus, *cdx*, together with other homologs of bilaterian hindgut markers such as *brachyury* (*bra*), *orthopedia* (*otp*), and the homeobox gene *nk2.1*, is expressed in a posterior ectodermal domain of juveniles in tissue that later forms the male gonopore (Hejnol and Martindale 2008b).

These findings have profound implications for the evolution of a through-gut. While the expression of genes such as *goosecoid* and *brachyury* in the mouth region of not only acoels and the rest of the Bilateria but also cnidarians indicates homology of the anterior gut opening throughout the Metazoa, the presence of hindgut genes in the region of the future male gonopore in acoels may be interpreted as showing independent, multiple origins of the anus in the bilaterians or of secondary reduction of the hindgut in acoels and its cooption for the gonopore (cf. Gnathostomulida, which have secondarily lost the anus—Knauss 1979). Hejnol and Martindale (2008b) followed Reisinger (1961) in suggesting that the anus evolved as a common opening of the gut and gonoducts (cloaca). If, however, these genes have more general morphogenic functions (if, for instance, *brachyury* simply organizes infolding of epithelia), then these speculations may be premature.

Asaccate gonads can be interpreted as a primitive character of the Acoelomorpha. However, this feature is also found in catenulid platyhelmiths (Rieger et al. 1991) and in the ovaries of several subgroups of Gnathifera, namely the Gnathostomulida (Mainitz 1983) and the Micrognathozoa (Kristensen and Funch 2000). Noteworthily, stromal cells can be found in gonads of the "basal" acoels *Diopisthoporus* ssp. (Westblad 1945, 1948; Smith and Tyler 1985a) and *Nemertoderma* sp. (Tyler and Rieger 1977), perhaps being vestiges of a more primitive condition.

Acoelomorphs appear to fundamentally lack excretory organs, and this is routinely taken to be a primitive feature (Jondelius et al. 2002). If acoelomorphs are progenetic or reduced descendants of a coelomate ancestor that would have relied on a coelomic cavity to produce primary urine, then loss of the cavity in progenesis would have left acoelomorphs without any obvious excretory organ. Deuterostomes do not have protonephridia, and their absence from acoelomorphs could be taken as further evidence in favor of their proper placement outside the protostomes, as the basal-most Bilateria or in the Deuterostomia.

Surveys of the homeodomain via degenerate PCR have identified three bona fide Hox genes in acoels—one anterior, one central, and one posterior—and only the homolog of the posterior ParaHox gene *caudal* (*cdx*—Hejnol and Martindale 2009; Moreno et al. 2009; for discussion see above). As in all Bilateria, the acoel Hox genes are expressed in staggered spatial domains along the anteroposterior axis; however, they are all expressed at approximately the same developmental stage, i.e., after gastrulation during embryonic development and at bud initiation during asexual reproduction (in this latter case with the exception of the central Hox gene, the expression of which is slightly delayed with respect to the anterior and posterior Hox genes). The lack of temporal colinearity in Hox gene expression is best explained by the lack (or disruption) of the Hox gene cluster in the Acoela (Moreno et al. 2009).

The anterior and central Hox genes are expressed in the neuroectoderm of the developing embryo of *Convolutriloba longifissura,* and in the cerebral ganglion and developing neurite bundles of the related species *Convolutriloba retrogemma* and *Symsagittifera roscoffensis* (Hejnol and Martindale 2009; Moreno et al. 2009; Sikes and Bely 2010). Evidence of the neural patterning nature of the anterior and central Hox is reinforced by the overlapping expression of the neural gene *SoxB1* in *C. longifissura* and *S. roscoffensis* (Hejnol et al. 2009; Semmler et al. 2010; our personal observations). The posterior Hox gene is expressed in the three germ layers in *C. longifissura* and in the posterior peripheral parenchyma in *S. roscoffensis* and *I. pulchra*. Its function has been tested in the latter species by RNA interferrence, during adult homeostasis, regeneration, and juvenile development. The gene is necessary for egg maturation and the correct development and maintenance of the posterior musculature, while its function is less clear in the posterior nervous system (Moreno et al. 2010).

Though the most parsimonious interpretation of the data is that acoels bear the primitively minimal set of Hox genes and are themselves a basal clade within the Bilateria, it is also possible that the low number of Hox genes is concordant with a secondary simplification of the body plan. The fact that the left complement includes one Hox gene of each class (anterior, central, and posterior) could be attributed to a reduction that leaves only a minimal set compatible with bilateral organization (Moreno et al. 2011).

The paucity of microRNAs in *S. roscoffensis* and *Childia* and especially the lack of key microRNAs necessary for organogenesis such as *miR-1* (heart) or *miR-9* (brain) correlate with a basal position of acoels and support the aceloid-planuloid hypothesis (Sempere et al. 2006, 2007). However, Philippe et al. (2011) found four additional microRNAs in the more basal acoel *Hofstenia miamia* and thus showed intraphylum variability and that microRNAs may have been lost in most acoels.

Conclusion

Certainly the Acoelomorpha does not belong in the Platyhelminthes, and Acoela + Nemertodermatida is a monophylum. If it were a paraphylum at the base of the Bilateria as suggested by some studies either their similarities in development, ciliary structure, and rootlet system must have originated independently twice, which is very unlikely, or these traits would have to be plesiomorphic for bilaterians, which is even more unlikely. The Acoelomorpha are, furthermore, not members of the protostomes, as they have never been recovered within this clade in molecular sequence analyses; the absence of protonephridia and the endomesodermal origin of muscles further corroborate this assumption.

To us it is clear that the ancestor common to acoelomorphs and other bilaterians was quite different from a present-day acoel or nemertodermatid. In analyses of ribosomal genes and phylogenomic approaches, acoels and nemertodermatids have very long branches (see figure 2 in Wallberg et al. 2007 and figure 3 in Philippe et al. 2011), and while a long branch does not necessarily mean a variation in complexity, it by definition means that the molecules analyzed are quite different from the inferred ancestral state. As an organism and its molecules evolve as an entity, it is difficult to comprehend how an organism could evolve slowly while its molecules are evolving fast. Not a single so-called "living fossil" has shown an extraordinary branch length yet in any analysis (e.g., Webster et al. 2006 for priapulids), and animals that are quite different from the inferred ancestral state show relatively long branches compared to the former (e.g., Struck et al. 2011 for myzostomids).

Animals with a branch length comparable to those of the Acoelomorpha analyzed under the same conditions by Philippe et al. (2011) are suggestively "simple"—platyhelminths, rotifers, and cycliophorans, all believed to be small and simplified by regressive evolution. The descent of the Acoelomorpha from a more "complex" or better quite different ancestor would elegantly account for the long branches in all molecular investigations and their peculiar morphology.

Whether one accepts Acoelomorpha as the sister group to the remaining Bilateria or prefers their placement in the Deuterostomia, together with the placement of the Chaetognatha either basal to ecdysozoans and spiralians (Marlétaz et al. 2008) or nested within one of those clades (see Harzsch and Wanninger 2010 for review), it throws the value of the terms "Deuterostomia" and "Protostomia" into question. Reflecting on the nervous system and development of the Acoelomorpha and Chaetognatha, it might well be anticipated that the term "Protostomia" should be replaced with the term "Gastroneuralia" (Schimkewitsch 1891; Ulrich 1951) and that a new term should be introduced for the clade comprising Ambulacraria and Chordata (and probably Xenacoelomorpha).

Future perspectives

We need more information before the Acoelomorpha can be placed definitely in bilaterian phylogenies and before we can reconstruct the appearance of the ancestor common to the Acoelomorpha and other bilaterians. Information now available from EST collections of acoels (*C. longifissura*, *I. pulchra*, *N. fusca*, *S. roscoffensis*), nemertodermatids (*Meara stichopi*, *N. westbladi*), and *Xenoturbella bocki*, as well as from microRNA libraries (*Hofstenia miamia*, *N. fusca*, *S. roscoffensis*) and BAC (genomic) libraries (*S. roscoffensis*), has yet to be fully tapped. Whole-genome projects on various acoelomorphs and *X. bocki* are pending. Among newer techniques from which we can expect novel phylogenetically relevant information are gene knock-down protocols with double-stranded RNA (as has been applied to *I. pulchra*: De Mulder et al. 2009; Moreno et al. 2010 and *H. miamia* (personal communication Mansri Srivastava)), cryoelectron microscopy (Salvenmoser et al. 2010), immunocytochemistry, staining for mitotic cells (Gschwentner et al. 2001; De Mulder et al. 2009), and in situ hybridization. For *in situ* probes a significant "breakthrough" has been made that provides access to the embryo through the eggshell (Hejnol and Martindale 2008a; Hrouda 2007; De Mulder 2009). However, a method with which the embryo can be made accessible for double-stranded RNA without damaging or alternating the development of the embryo is still required.

The production of transgenic animals would also be a significant development. The creation of stable transgenic lines would allow us to link gene expression and function to morphogenetic events underlying the development of defined structures. A major challenge in transgenesis is the

production of germ line-transgenic specimens able to transmit the transgene to the offspring, avoiding the problems associated with mosaicism. The availability of technologies for functional analysis in these worms is essential to decipher whole gene regulatory networks (GRN) and infer putative ancestral regulatory states controlling cell type and tissue differentiation as well as the developmental origins of defined body plan features (Davidson 2011).

The number of immunocytochemical markers specific to acoelomorphs remains relatively limited—the production of a library of monoclonal antibodies, as has been achieved for other flatworms (Bueno et al. 1997; Ladurner et al. 2005), from carefully selected species would be indispensable. In addition, having a good embryo microinjection technology would help when it comes to lineage tracing and knockdown in specific lineages; 4D microscopy would be beneficial in analyzing such lineages.

All the tools mentioned above need to be applied with an eye to testing the proposed positions of the Acoelomorpha and evaluating the apomorphic or plesiomorphic state of morphological and molecular characters under investigation. Pinpointing this is critical to understanding one of the most important stages in animal evolution. However, regardless of their precise phylogenetic position, they are highly valuable for comparative analyses of genomes and gene features, to unravel how genome and morphology are linked, and as a source of comparison to understand bilaterian features such as the multiciliated epidermis, acoelomate body plan, spiral cleavage, the "centralization" of the nervous system, and its immersion below the body wall.

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Resúmen del cuarto artículo, R4

Los acelos: en su clase y parentescos, especialmente con los nemertodermatidos y xenoturbellidos (Bilateria incertae sedis)

Los acelos están entre los organismos más simples y, por lo tanto, a menudo han sido fundamentales en las discusiones sobre el origen de los animales bilaterales.

Inicialmente, declarados primitivos por su morfología similar a las de las larvas "planulas" de cnidarios, incluyendo un sistema digestivo ciego, fueron posteriormente declarados ser una rama especializada de los plathelminthos.

Sin embargo, desde que la filogénesis molecular les excluyó de los plathelminthos y de cualquier otro phylum, poniéndolos en la base de los Bilateria, se convirtieron en el centro del renovado debate e investigación. Hemos revisado lo que se conoce actualmente sobre las acelos, incluyendo información con respecto a su morfología, desarrollo, sistemática y relaciones filogenéticas; y hemos puesto algunos de estos tópicos en perspectiva histórica para mostrar cómo la aplicación de nuevos métodos investigativos han contribuido al progreso del entendimiento de estos animales.

Teniendo en cuenta todos los datos disponibles, no podemos hacer conclusiones claras. Sin embargo, según nuestra opinión cada vez se hace más claro que los acelomorfos son una rama "basal" y también "divergente" de los Bilateria.

Discussion

1. Convenience of the model species

It has been only for a relative short time that acoels have been the subject of studies in developmental, regenerative and stem cells biology (Hejnol and Martindale 2008; Hejnol and Martindale 2008; De Mulder, Kuales et al. 2009; Hejnol and Martindale 2009; Moreno, Nadal et al. 2009; Bely and Sikes 2010; Moreno, De Mulder et al. 2010; Sikes and Bely 2010; Chiodin, Achatz et al. 2011), whereas abundant (older) literature exist on acoel morphology and systematics (reviewed in (Achatz, Chiodin et al. 2012). At present there is only a handful of species for which molecular and genomic tools are available and all of them belong to the most advanced acoel families. Reasonable criticisms have been addressed about the improper use of derived acoel species as models to infer the ground pattern of the Acoelomorpha ancestor and perhaps of the whole Bilateria as well (Jondelius, Wallberg et al. 2011), which can be hardly contradicted. Nevertheless I would like to underline the several practical advantageous aspects that the two species of this study, namely *Symsagittifera roscoffensis* and *Isodiametra pulchra*, present over other basal acoelomorph taxa, especially nemertodermatids and xenoturbellids.

Specimens of *Symsagittifera roscoffensis* are especially abundant along the Atlantic coast in the north of France. The worms, which live in the interstitial spaces of the sand, have to emerge daily, during low tide, in order to accomplish the photosynthetic needs of the algae that they bear as symbionts. During that time, millions of specimens can be easily collected. More often the samples are monotypic, *i.e.* the only species present is *S. roscoffensis,* and they do not have to be extracted or cleaned from the sand in which they live, thereby laborious

and time consuming processing after sampling can be avoided. *S. roscoffensis* lays the fertilized eggs in cocoons, which is quite convenient at the time of collecting big amount of material. Although the permeabilization of the cocoon membrane and the eggshell has been a hard nut to break, this problem has been finally overcome in our laboratory (see *e.g.* Fig.11A). Still a pending task in setting up *S. roscoffensis* as model system is the establishment of stable laboratory cultures. Although the worms can be kept alive under laboratory conditions, they stop reproducing. This leads to the need of frequent and expensive travelling to the site of collection and, therefore, to deal with the difficulties of handling huge amount of live material, *e.g.* for embryos staging.

Although the process of obtaining *Isodiametra pulchra* is much more laborious than *S. roscoffensis*, the availability of stable laboratory cultures makes it now a suitable model for developmental studies.

Acoels have a high rate of single nucleotide polymorphism that complicates the labor of assembling the genome when the sequences derive from multiple individuals However, we have been able to assemble, already, a big fraction of the *S. roscoffenis* genome, a genome that we and other research groups are currently annotating with detail (unpublished). This problem is a lot reduced in the case of *I. pulchra* given that all working laboratory cultures at present derive from a unique in-bred population. *I. pulchra* is the first acoel species that could be cultured, and as expected most of available protocols for functional developmental biology, such as RNAi gene knockdown have been tested uniquely in *I. pulchra* (De Mulder, Kuales et al. 2009; Moreno, De Mulder et al. 2010). Drawbacks with this species are the susceptibility of the cultures of getting contaminated, mostly from parasites coming with the algae, and the quite time consuming method for embryo collection and cleaning.

2. On the status of the acoels within the Acoelomorpha (Xenoturbellida+ (Nemertodermatida+Acoela)): the significance of the nervous and the reproductive systems.

In the introduction we have discussed already what characters are or can be considered advanced in the acoels, with respect to their relatives, the nemertodermatids and *Xenoturbella* (Fig.3).

Especially the latter seems to have retained the more ancestral metazoan traits, most evident in its epithelial digestive system, the presence of an intra-epidermal nervous system (Ehlers and Sopott-Ehlers 1997; Raikova, Reuter et al. 2000; Bourlat, Nielsen et al. 2003; Nielsen 2010) and, in my opinion, the organization of the reproductive system. To my knowledge, this latter character, so far, has been quite neglected in the analysis of the evolutionary relationships within the acoelomorphs. The gametes in *Xenoturbella* develop in the endoderm (Obst, Nakano et al. 2011). This fact is most reminiscent of what happens in the anthozoan cnidarians, whose 'gonads' are located in the mesenenteries, which are infoldings of the endoderm (Extavour, Pang et al. 2005; Saina and Technau 2009). Furthermore, the lack of copulatory organs in *Xenoturbella* would suggest that the fertilization is external and that actually the gametes are released through the mouth.

It has been recently suggested that the evolution of internal fertilization might have driven the evolution of a more complex centralized nervous system, due to the necessity of a fine control in the reproductive behavior. This condition is nicely exemplified in the Acoela, which must have evolved a submuscular

nervous system in parallel with the more complex reproductive organs (including mesodermally located gonads, article R2) (Achatz, Hooge et al. 2010; Achatz and Martinez In press).

Concerning the complexity of the acoel nervous system, some considerations about the expression of the orthologueous gene *SoxB1* in the species *S. roscoffensis* would be of interest. As shown in the article R3 (Semmler, Chiodin et al. 2010), I have found that the acoel orthologue of the pro-neural gene *SoxB1* is expressed in the anterior region of the hatchling, most likely in the developing brain. The gene however, in older juveniles, is also expressed in two parallel stripes of cells, continuous with the brain and that extend towards the posterior end of the juvenile, without reaching it (data not shown). The expression of the gene is similar to that of the *SrHox1*, the *S. roscoffensis* orthologueue to the anterior class *Hox* genes, for which a neural patterning function has been postulated (Moreno, Nadal et al. 2009). In Bilateria *SoxB1* orthologues are expressed in the anterior brain region, whereas the anterior *Hox* genes pattern the frontal most part of the hindbrain (or its equivalent), even in those organisms that have diffuse nerve net instead of a centralized nervous system (Lowe, Wu et al. 2003). Thus, the expression of both *SrSoxB1* and *SrHox1* along the whole AP axis of *S. roscoffensis* (but see also (Hejnol and Martindale 2009) for non-regionalized animal-vegetal expression of *SoxB1* in *C. longifissura* embryos) is not consistent with the regionalization of the nervous system of the *e.g.* basal deuterostomes *S. kowalevsky* (Lowe, Wu et al. 2003), but is reminiscent of the expression observed in the anthozoan *N. vectensis* (Magie, Pang et al. 2005). Keeping in mind that the non-regionalized *SrSoxB1* expression could be the result of a modified role for the gene, or specific to the acoel lineage – the anterior neural marker *ClSix3*/6 is restricted to

the anterior nervous system of the developing *C. longifissura* (Hejnol and Martindale 2008; Hejnol and Martindale 2009)- it would be interesting to learn how the orthologueous genes are expressed in *Xenoturbella*. This should provide us with insights into the origin of the nervous system patterning in the deuterostomes, and probably in the whole Bilateria.

3. Mesodermal genes in acoels

Understanding the nature of the mesoderm in the ancestor of Bilateria is central in deciphering its evolution. In my opinion the results showed in this study are compelling evidence that myocytes were the first mesodermal cell types.

In the article R2, I have analyzed the expression of twelve bilaterian mesodermal genes. These included from genes encoding for transcription factors, often involved in early specification of mesoderm (*e.g.* the gene *Twist* in *Drosophila* (Castanon and Baylies 2002)) to genes for terminal differentiation proteins (*e.g.* the *Tropomyosin*). All genes but one (the orthologue of the bilaterian *T-brain* genes) are expressed in the muscles of the acoel *I. pulchra*, some of them being expressed in all muscles whereas others are expressed only in a small subset of them, *e.g.* the genes *IpSix1*/2 and *IpTwis1* and *IpTwist2*.

Interestingly all anthozoan orthologues of the genes characterized in *I. pulchra* are also expressed in the endoderm of anthozoan cnidarians (Fritzenwanker, Saina et al. 2004; Martindale, Pang et al. 2004; Marlow 2010; Genikhovich and Technau 2011), suggesting an obvious evolutionary connection between the cnidarian epithelio-muscular endoderm and the acoel musculature. The fact that in the acoel more basal relatives, namely *Xenoturbella* (Ehlers and Sopott-

Ehlers 1997) and the nemertodermatids, the only mesodermal structures are the muscles -and probably also the gonads and neoblasts in the nemertodermatids (Rieger, Tyler et al. 1991; Rieger and Ladurner 2003)- enhances the possibility that the mesoderm arose first as musculature. This scenario, obviously, contemplates the acoelomorphs as basal bilaterians.

Under the second scenario, *i.e.* acoelomorphs are secondarily simplified deuterostomes, one should also expect conserved expression patterns between the cnidarian endoderm and the acoelomorphs' musculature. In this case, we should assume that the pseudo stratified ancestral coelom (Fig.6) would have first inherited the genes expression from the cnidarian endoderm (Fig.5) (Remane 1963). The same genes expression would have been preserved in the bilaterian muscles in the following evolutionary steps, the one leading to the separation of muscular and epithelial layers from the ancestral pseudo-stratified coelomic lining (Fig.6) (Rieger and Lombardi 1987). Then the coelomic cavity would have disappeared in the lineage leading to the acoelomorphs.

In my opinion the major problem with the 'bulging model' of muscles' evolution (Fig.6) (Schmidt-Rhaesa 2007) is that it does not explain how two cells, the myocyte and the epithelial cell, each one with its own nucleus, would be born from an already differentiated cell, *i.e.* the epithelio-muscular cell. The evolution of myocytes from the myoepithelial lining of a coelom is more "credible" than the 'bulging model' in the sense that it only implies the separation of two different cell types from an already pseudostratified layer (Fig.6).

Obviously, the bulging model can only be understood when the epithelio-muscular cell precursor undergoes a cell division that originate two daughter cells of which one will take the fate of the myocyte and the other will

differentiate into the epithelial cell. Hence, one should expect that the two daughter cells would take different cell fates by inheriting complementary regulatory states from that of the ancestral epithelio-muscular cell. I think that this condition is indeed realized during acoel development. In fact, none of the muscular genes studied in the article R2 is simultaneously expressed in the digestive syncytium (endoderm) and in the musculature of *I. pulchra,* except the two *FoxA* orthologues. By cell-lineage experiments we know that the acoel musculature derive from the same embryonic precursors from which the digestive system develops, namely the third duet of vegetal macromeres (Henry, Martindale et al. 2000). Following the fate of the progeny of the third macromeres duet is technically impossible and therefore it is also not possible to determine the exact moment of the seggregation of the endodermal and the mesodermal lineages. Yet the first myocytes are known to appear at the anterior animal pole, where all *I. pulchra* mesodermal genes, but *IpFoxA1* (*IpFoxA2* embryonic expression is unknown), are activated (Fig.9).

In conclusion, the expression of cnidarians endodermal orthologues in the musculature of *I. pulchra* cannot be used to dismiss any of the two models of bilaterian evolution (planuloid-acoeloid versus archycoelomate hypotheses), in the absence of a better-known phylogenetic position of the Acoelomorpha. Nevertheless, the lack of any developmental trace of enterocoelic development during acoel development, or of coelomogenesis, points to some difficulties for accepting the archycoelomate hypothesis.

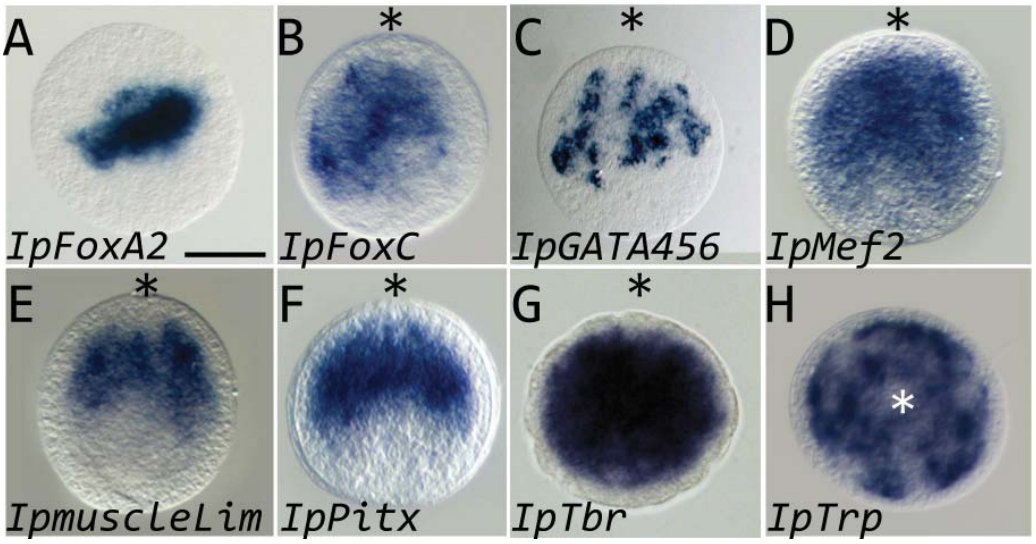

Fig.9 Embryonic expression of I. *pulchra* mesodermal genes.

The asterisk indicates the animal pole. All embryos are post-gastrulae stages. Scale bar 50 μm.

4. The molecular architecture of the acoel musculature.

Acoels, and acoelomorphs in general, have only smooth mono-nucleated muscles (Rieger, Tyler et al. 1991; Ehlers and Sopott-Ehlers 1997) although some species can exhibit a pseudo striated pattern (Tyler and Rieger 1999; Todt and Tyler 2006). In the article R1 (Chiodin, Achatz et al. 2011), I have analyzed the expression of key regulators of the muscular contraction in Bilateria, namely *Actin*, *Tropomyosin* and the inhibitory subunit of the *Troponin* complex. Actin and Tropomyosin have several different functions and are present in all eukaryotes, however they are also consistently expressed in the musculature of cnidarians and bilaterians (Steinmetz, Kraus et al. 2012 and references therein).

The proteins of the Troponin complex are a bilaterian innovation (Steinmetz, Kraus et al. 2012), and they are responsible of regulating the muscular contraction in response to an increased intracellular calcium concentration (Fig. 8C). Surprisingly I have found that one *Troponin* gene is expressed in the musculature of *S. roscoffensis*, thereby suggesting that the contraction of the acoel smooth muscles is similarly regulated than the bilaterian striated muscles.

There are two possible explanations for this. In the case that the acoelomorphs are direct descendents of the bilaterian ancestor, the most parsimonious explanation would be that the acoel molecular architecture of the muscles represents a first evolutionary step towards the evolution of the eubilaterian striated muscle. If instead the acoelomorphs are derived deuterostomes, it is then most plausible that the muscles lost the striation pattern while keeping still the striated-muscle molecular architecture. This is not an impossible evolutionary process since it has happened already in the ascidians (Meedel and Hastings 1993; Endo, Matsumoto et al. 1996) and the planarians (Kobayashi, Kobayashi et al. 1998) body wall musculature.

In a recent comparative investigation of the molecular architecture of non-bilaterian and bilaterian muscles, Steinmetz and colleagues (Steinmetz, Kraus et al. 2012) found that some proteins involved in the regulation of the bilaterians smooth musculature, such as the Myosin light chain Kinase and Phosphatase, have evolved in the metazoan ancestor and thus plausibly they argue that the ancestral regulative mechanism of acto-myosin sliding might have relied on these proteins. I have found the orthologues of a *Myosin light chain* (*MLC*) and a *Calmodulin*, in the transcriptome of *S. roscoffensis* (not published) and I have next checked the expression of both orthologues in this species. In bilaterians, both genes are expressed in smooth and striated musculutare whereas, quite

unexpectedly, I did not found them to be expressed in *S. roscoffensis* musculature (Fig.10A). Although I did not demonstrate the nature of the *SrMLC* and *SrCalmodulin* positive cells, I have good reasons to think that these are primordial germ cells.

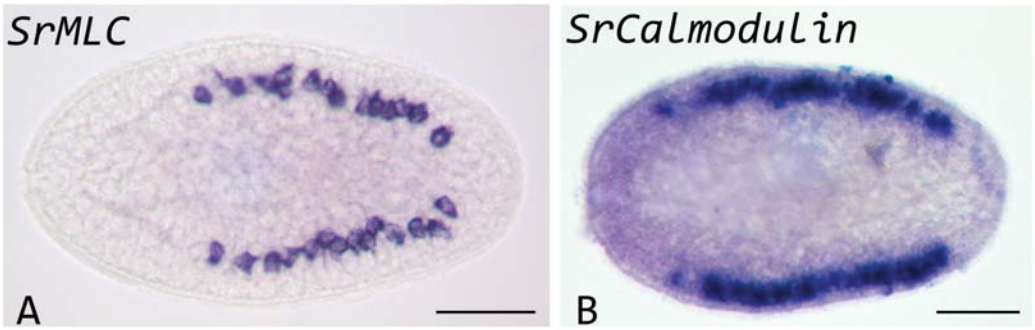

Fig.10 Expression of *S. roscoffensis Myosin light chain* (*MLC*) and *Calmodulin* orthologues. Anterior is to the left in both aspects. Scale bar 50 µm. A. *SrMLC* in a juvenile. B. *SrCalmodulin* in a juvenile.

To this point it is interesting to notice that the *S. roscoffensis* orthologueue of the gene *Twist (SrTwist)* is expressed with a similar pattern to that of *SrMLC* and *SrCalmodulin* orthologues (though I could not demonstrate co-expression of the genes) (Fig.11B and Fig.10A-B). Because in adult worms *SrTwist* is expressed in the testis (Fig.11C), as it is the gene *IpTwist1*, the *I. pulchra* orthologue (article R2, Fig.4), the juvenile (primordial germ cells) and adult (spermatogonia) expression domains can be easily related.

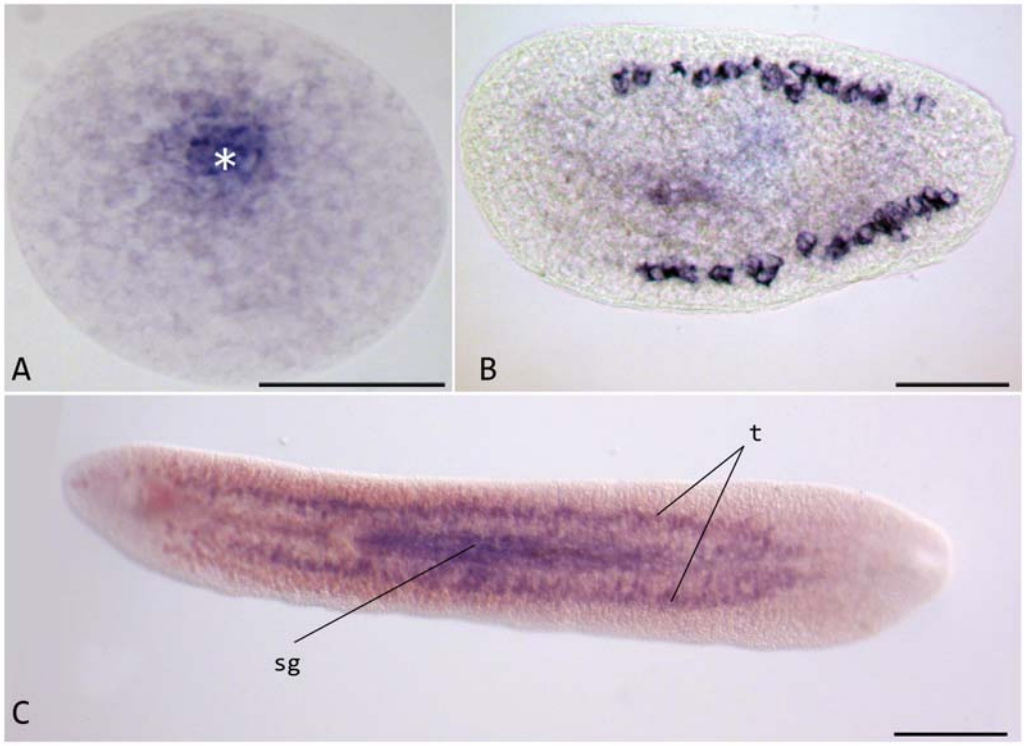

Fig. 11 Expression of a *Twist* orthologue in *S. roscoffensis*.

A. During embryogenesis *SrTwist* is expressed at the animal (future anterior) pole, most likely in the myocytes of the animal spiral muscle (see Semmler et al. 2008). B. In juvenile *SrTwist* is expressed in, most likely, primordial germ cells. C. In adult worms *SrTwist* is expressed in the testis (t), and in the muscle mantle of the saggitocysts (sg)

If the ancestral regulation of acto-mysoin contraction was in fact based on the phosphorilation of a MLC (Steinmetz, Kraus et al. 2012), it might be reasonable to assume that acoels have lost this mechanism (Fig.12, scenarios 1 and 2). To my knowledge, there is at present no clear evidence that *MLC* or the *MLC-Kinase* and *MLC-Phosphatase* are expressed in non-bilaterian muscles and hence the possibility that this mechanism evolved independently in the Bilateria should be considered. Thus, under this other scenario,b it is more likely that

either MLC regulation could have evolved in the Eubilateria (Fig.12, scenario3), or be lost in the Acoelomorpha when they are deuterostomes (or their sister group) (Fig.12 scenario 4).

Though a preliminary search of the drafted genomes of *Xenoturbella bocki* and *S. roscoffensis (data not published yet)* I have found *bona fidae* orthologues of the four Z-disc bilaterian proteins: titin, α-actinin, lbd3 (LIM binding protein) and muscleLIM (data not shown) (Steinmetz, Kraus et al. 2012). I have characterized the expression of a *muscleLIM* orthologue in the acoel *I. pulchra* , which is, then, restricted to the musculature of the worm (article R2, Fig.2A-C). It is therefore reasonable to expect that the orthologues of *titin*, *α-actinin* and *lbd3* will be also expressed in the acoel musculature.

In summary, although the acoel musculature has the ultrastructural appearance of smooth muscles, its molecular architecture is closer to that of bilaterians striated muscles. Furthermore, the acoel musculature appears to be quite unique among animals, having no obvious expression of the *MLC* orthologues (Craig, Smith et al. 1983; Steinmetz, Kraus et al. 2012), although it remains possible that a muscle specific paralog has not yet been identified in our EST collections or genome assemblies.

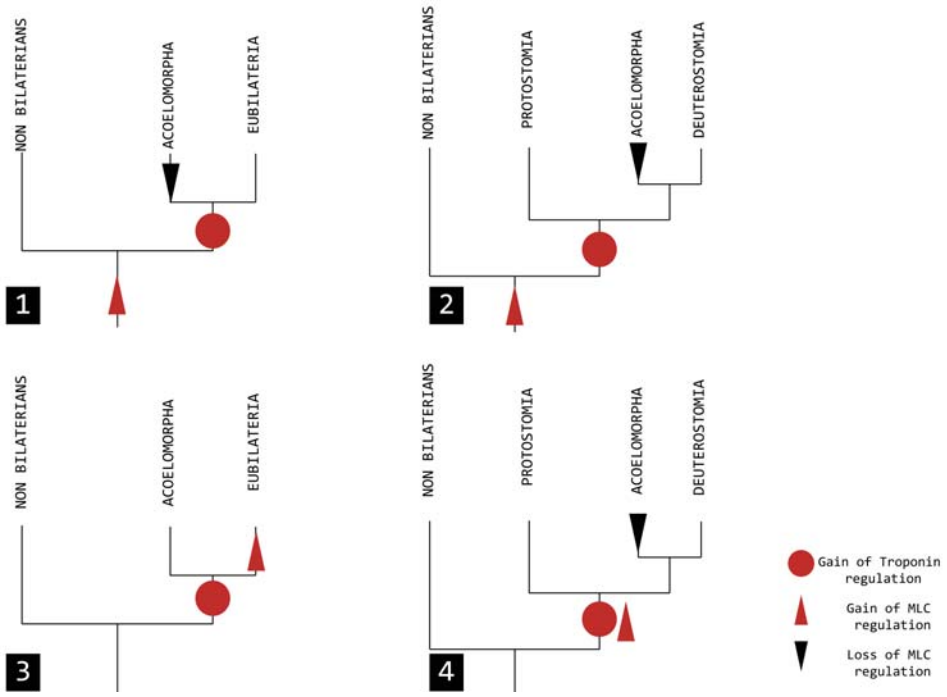

Fig.12 Possible scenarios of evolution of MLC regulation in eumetazoans muscles. See the text for details.

5. Do acoels have a conserved bilaterian mesoderm gene regulatory network?

In the course of this thesis I have looked at the expression of several bilaterian mesodermal genes in acoels. Some of the genes I have looked for, e.g. *FoxC*, are expressed during early mesodermal specification in a wide range of bilaterians (Wilm, James et al. 2004; Mazet, Amemiya et al. 2006; Tu, Brown et al. 2006; Wotton, Mazet et al. 2008; Shimeld, Boyle et al. 2010; Janssen, Budd et al. 2011) whereas other factors, *e.g. Mef2*, are expressed after mesoderm

specification and are necessary to initiate mesoderm differentiation programs such as myogenesis (Sandmann, Jensen et al. 2006).

In *Drosophila*, *Twist* is expressed in the ventral region of the early blastula and, if suppressed, the embryo fails to gastrulate (Leptin 1999). Once gastrulation is accomplished, *Twist* is expressed in the mesoderm and becomes an essential myogenic factor (Baylies and Bate 1996; Sandmann, Girardot et al. 2007). However, such a central role of the gene *Twist* in mesoderm development is only known from *Drosophila*. In all other bilaterians, albeit being expressed in the mesoderm, *Twist* is never activated before gastrulation (Yasui, Zhang et al. 1998; Nederbragt, Lespinet et al. 2002; Dill, Thamm et al. 2007; Price and Patel 2008), except in sea urchin (Wu, Yang et al. 2008). Arguably, in these organisms *Twist* is needed for mesoderm patterning and not for its early specification. No *Twist* functional study has been carried out outside the model organisms, however it is sufficient to mention that in the mouse, for instance, *Twist* is a myogenic suppressor. In acoels, as well as in the other bilaterians, *Twist* expression is quite enigmatic and variable even within related species. At first, I did not observe *Twist* embryonic expression during the development of *I. pulchra* whereas in *S. roscoffensis*, I recovered *Twist* expression during the late embryogenesis, most likely in the anterior spiral muscle (Semmler, Bailly et al. 2008) (Fig.9A, asterisk). In adults, *Twist* is expressed in the muscles of the copulatory organs of *I. pulchra* (article R2, Fig.4A-D) and in the ventral sagittocysts of *S. roscoffensis*, which have a muscular mantle (Gschwentner, Baric et al. 2002; Semmler, Bailly et al. 2008; Chiodin, Achatz et al. 2011). In summary, in both acoel species *Twist* is expressed in the muscles, but only in a subset of them and when they are already differentiated. To me, the better

explanation for this expression pattern is that this gene is mainly used for myocytes' maintenance rather than used for their differentiation.

I propose here that the acoel *Twist* might be a "master regulator" of the acoel male reproductive system, since in both species the gene is consistently expressed in the testis and in the saggitocysts and penis, which both are structures used for sperm transfer in *S. roscoffensis* (Gschwentner, Baric et al. 2002; Semmler, Bailly et al. 2008) and *I. pulchra* (Hooge and Tyler 2005).

In *Drosophila* Twist activates the myogenic transcription factor Mef2 (Sandmann, Girardot et al. 2007), that in turn switches on muscle specific genes such as *tropomyosin* or *muscleLIM* (Stronach, Renfranz et al. 1999; Sandmann, Jensen et al. 2006). Most likely, this is not the case in *I. pulchra*. *IpMef2* and *IpmuscleLIM* are activated at the anterior animal pole in post gastrulae embryo, exactly at the time and place where myogenesis begins (article R2, Fig.10D-E) (Ladurner and Rieger 2000). Neither one of the two *Twist* orthologues were observed to be similarly expressed during *I. pulchra* embryonic development, nor their expression domains seem to overlap those of *IpMef2* and *IpLIM*, in juveniles or adults. Consistently with a probable myogenic role, *IpMef2* (Sandmann, Jensen et al. 2006; Potthoff and Olson 2007) is broadly expressed in juveniles and downregulated in adult (article R2, Fig.3G-I). *IpmuscleLIM* is similarly expressed to *IpMef2* in juveniles, but in a different pattern in the adults (article R2, Fig.2A-C). There the gene *IpmuscleLIM* is still broadly expressed, especially in the musculature, in a fashion consistent with having a dual role in other bilaterians (Arber, Halder et al. 1994; Kong, Flick et al. 1997; Stronach, Renfranz et al. 1999; Broday, Kolotuev et al. 2004): as a myogenic promoter (in embryos and juveniles) and as essential component of Z-disc (adults).

To summarize, in acoels *Twist* is expressed only in a small subset of mesodermal derivatives, whereas in most of bilaterians the gene is broadly expressed in the mesoderm. The two other key myogenic factors, *Mef2* and *muscleLIM,* have a more conserved expression pattern between acoels and the rest of Bilateria, therefore supporting the ancestral myogenic activity of these genes (Martindale, Pang et al. 2004; Genikhovich and Technau 2011). Additionaly, other genes such as the orthologues of *FoxC, Pitx* and *GATA* factors, which are usually quite upstream in the mesoderm differentiation pathways (Boorman and Shimeld 2002; Carlsson and Mahlapuu 2002; Duboc, Röttinger et al. 2005; Rojas, De Val et al. 2005; Gillis, Bowerman et al. 2007; Boyle and Seaver 2010) are instead expressed in both early and already differentiated mesoderm derivatives in the acoels, consistently with a possible role in the development and maintenance of the acoel mesodermal structures.

6. Did the acoelomorph have a coelomate ancestor? Evidences from mesodermal gene expression patterns

The functional plasticity of body cavities has been central to the bilaterian body plan divergence, as it boosted the growth of larger body size, the change in feeding and locomotory behaviors and the occupation of new ecological niches. Given the key role played by coeloms in the evolution of new body plans, it is reasonable to ask if the acoelomate condition has been achieved as secondary reduction (archycoelomate hypothesis) or if this has been the ground pattern from which coelomate bilaterian body plans have evolved (planuloid-acoeloid hypothesis) (Fig.5).

Some acoelomate bilaterians are clearly derived from coelomate ancestors. This is very well exemplified in *e.g.* interstitial annelids or in miniaturized acorn worms (Rieger, Purschke et al. 2005; Worsaae, Sterrer et al. 2012). Most often these organisms fail to develop a coelom, whose anlage nevertheless appears during embryonic development. Progenesis, *i.e.* the achievement of sexual maturity before the full development of the adult body plan in the acoelomate larvae, is the developmental pathway that leads to the evolution of acoelomate bodyplans (Schuchert and Rieger 1990; Rieger 1994; Tyler 2001; Rieger, Purschke et al. 2005; Worsaae, Sterrer et al. 2012).

In this context, supporters of the archycoelomate hypothesis, or more generally of a coelomate bilaterian ancestor, usually explain the evolution of acoelomate body plans by heterochrony, in a similar fashion to that observed in extant acoelomate annelids or hemichordates, more often assuming an ancestor with a biphasic life cycle (Rieger 1985; Rieger 1994).

According to the archycoelomate hypothesis, the ancestral mode of coelomogenesis is enterocoely though this developmental process can be obviously modified as it happened in, *e.g.* schizocoelus spiralians or ecdysozoans (Boyer and Jonathan 1998; Hejnol and Schnabel 2006) and references therein). In this scenario, the acoelomate body plan of the plathelminthes would have been evolved by the loss of schizocoelus development, having left an unsegmented mesenchymal parenchymal tissue as the only extant trace of the ancestral body cavity (summarized in (Willmer 1990)).

Acoels have a parenchymal tissue too, but its development is rather enigmatic since neither there is a sign of enterocoely or schizocoely during their embryonic development (Henry, Martindale et al. 2000) nor has this tissue

already formed in freshly hatched worms (Smith and Bush 1991) and (Hejnol, Seaver and Martindale unpublished data). Furthermore the parenchyma is not part of the acoelomorph ground pattern (Smith and Tyler 1985; Ehlers and Sopott-Ehlers 1997; Rieger and Ladurner 2003), and as such it can hardly be considered as the remnant of the ancestral coelomic cavity.

So far a genetic approach has not been taken to decipher possible signs of ancestral coelomic cavities in acoelomate taxa. If coeloms were part of the bilaterian ground pattern then we should expect that its development and patterning are controlled by a conserved gene toolkit, as for example it is realized during antero-porterior patterning of Bilateria by staggered expression of *Hox* genes (reviewed in, *e.g.* (Martindale 2005)). To my knowledge there is at present not even a proposal for the nature of such genomic toolkit. By carefully searching into the literature, I have identified a core gene set consistently expressed in the developing coelomic cavities of basal deuterostomes (which have enterocoelic development). This core genes set include the orthologues of the genes *Six1/2, FoxC, Pitx, GATA456* and *T-brain* (Fig.13) (Boorman and Shimeld 2002; Lee and Davidson 2004; Duboc, Röttinger et al. 2005; Mazet, Amemiya et al. 2006; Tu, Brown et al. 2006; Kozmik, Holland et al. 2007; Yankura, Martik et al. 2010).

In agreement with the morphological and developmental data, in my opinion, the gene expression data does not support any putative homology between the acoel parenchyma and the coelomic mesoderm of other basal deuterostomes. Only the orthologues of *Pitx* and *FoxC* are expressed in the acoel parenchymal cells when one would expect a more extensive conservation of gene expression, which is the case in ambulacrarians and basal chordates (Fig.13).

The complete set of orthologue 'coelomic' genes is instead expressed in the acoel gonads, where additionally I found expression of the orthologues of the genes *Mef2* and *Twist*, which are also expressed in the cephalochordates coelomic mesoderm (Yasui, Zhang et al. 1998; Zhang, Wang et al. 2007) (Fig. 13). Given that in the nemertodermatids the gonads are mesodermally located (Rieger, Tyler et al. 1991) and in *Xenoturbella* the gametes develop in the endoderm (Obst, Nakano et al. 2011) -which is nevertheless the coelom precursor according to enterocoely model- these structures appear to be stronger candidates for representing the vestiges of the ancestral coelomic cavity.

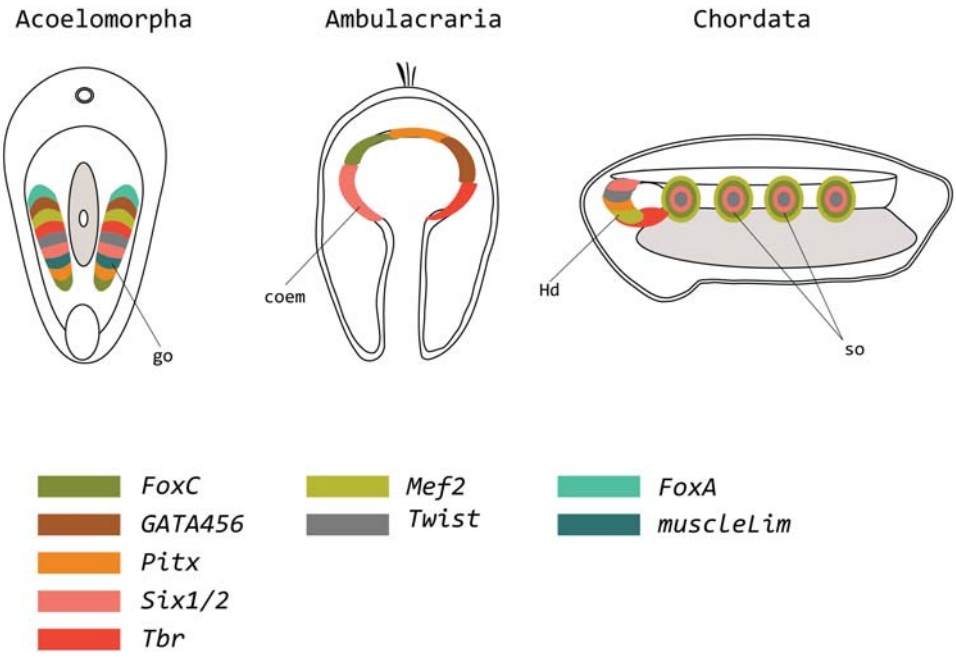

Fig.13. Comparison of mesodermal gene expression between the acoel I. pulchra and other basal deuterostomes. All genes expressed in the developing coelomic cavities of ambulacrarians and cephalochordates are expressed in the acoel's gonads. go, gonad; coem, coelomic mesoderm; Hd, Hatschek's diverticulum; so, somites.

7. The acoelomorph gonads: a connection to the coelom or the link between cnidarian and eubilaterian gonads?

I will refer to the acoelomorph gonads as mesodermally located gonads, meaning that, except in *Xenoturbella*, they are located in the space between the digestive system and the bodywall (Rieger, Tyler et al. 1991; Boone, Willems et al. 2010; Boone, Bert et al. 2011). In the acoels therefore the gonads are surrounded by parenchymal cells, which is not the case in the nemertodermatids, a group lacking parenchyma (Smith and Tyler 1985; Rieger and Ladurner 2003). This fact also implies that in the nemertodermatids the gonads, and the neoblasts, are the only cells filling the body space, and as such I prefer to use the term mesodermally instead of parenchymally located, which might be appliable specifically only to the acoels (De Mulder, Kuales et al. 2009; Egger, Steinke et al. 2009). Of course the term mesodermally implies mesodermal developmental origins, which is not incorrect here, because the gene expression of mesodermal genes in the gonads of *I. pulchra* actually suggests their mesodermal origins (article R2).

Differently from the majority of eubilaterians, which have epithelial sac-like gonads, the acoelomorphs gonads are not lined by any tissue (Rieger, Tyler et al. 1991; Boone, Willems et al. 2010; Boone, Bert et al. 2011). In the sac-like eubilaterian gonads the epithelium is usually a germinative epithelium which encloses the maturing gametes (Schmidt-Rhaesa 2007). In the acoelomorphs the gonads are compact and regionalized, *i.e.* it is possible to distinguish germinative and growing regions for the gametes, and therefore the term gonad

is justified albeit not corresponding to the standard type, as they do not form a separate compartment of the acoelomorphs' body.

Gonoducts, generally present in the eubilaterians gonads, are lacking in the acoelomorphs, albeit, at least in the acoels, female and male gonopores are usually present (there is just a single male gonopore in the nemertodermatids).

In my view, the male gonopore can be indistinctly called gonopore or copulatory organ, given that its function is to transfer the sperm. On the other hand, the function of the female gonopore is only that of receiving the sperm but is not involved in the release of gametes, and thus it should preferentially be called female copulatory organ.

As already pointed out the *Xenoturbella* gonads are quite different from those of the other acoelomorphs since there the gametes develop and mature in the endoderm (Obst, Nakano et al. 2011). If the gametes develop in the endoderm, it is also reasonable to assume that they are released through the mouth and that the fertilization is external (so far there has been no copulation observed) (Hiroaki Nakano, personal communication). Often, in nemertodermatids and acoels, the fertilized eggs are released through the mouth too, thus this might reflect an ancestral condition. It is also clear that in the acoels the male and female copulatory organs evolved independently. The female copulatory organ expresses the same regulatory genes than the 'mouth cross muscles' (see article R2, Table 2), which suggests a common developmental origins for the two structures. The male gonopore expresses different genes which, with the exception of *IpPitx* (also expressed in the female copulatory organ), are specific to this structure (article R2, table 2).

The developmental mode of germ cell segregation and gonad development is quite variable in eubilaterians and it is difficult to define a plesimorphic

condition (Extavour and Akam 2003; Extavour 2007; Schmidt-Rhaesa 2007). In some eubilaterians, the gametes develop inside a coelomic cavity (*e.g.* in annelids) or, as in the case of some echinoderm species, the epithelial lining of the gonad separates from the coelomotoel (reviewed in (Schmidt-Rhaesa 2007)). If the acoelomorph ancestor is assumed to be a coelomate organism (scenario favored in (Philippe, Brinkmann et al. 2011)), it is more plausible to assume that the gonads represent the only extant vestiges of a former 'germinative' coelomic cavity (or coelomic gonad), because the genes expressed in the gonad of *I. pulchra* are also expressed during coelom formation in basal deuterostomes (Fig.13).

The acoel germ cells differentiate from the neoblasts (Gschwentner, Ladurner et al. 2001; De Mulder, Kuales et al. 2009), and consistently with this notion I have recovered almost identical expression profiles between *I. pulchra* neoblasts (at least a subpopulation of neoblasts) and gonads (article R2, Fig. 6). In this context and under the hypothesis that the acoel gonad is the vestige of a former 'germinative' coelomic cavity, it can be speculated that the neoblasts derive from the disrupted germinative epithelium of the putative ancestral germinative coelom. This assumption consequently implies the endomesodermal origins of both the subpopulation of those neoblasts producing the germcells and the gonads (in the fllowing sections I will be using the term neoblast-gonad system).

8. On the common origins of the stem and germ cells system and the affinity with the cnidarians.

One problem with homologizing the neoblast-gonad system of acoels to an ancestral germinative coelom is related to the necessary implication that this assumption has for the endo-mesodermal origins of the neoblasts-gonadal system. I have no problem in accepting the endomesodermal origin of the acoelomorph gonads as this is a well conserved character across the Bilateria (Extavour 2007) but I find problematic to assume the endomesodermal segregation of the acoel neoblasts, notwithstanding their parenchymal location and the expression of mesodermal genes (article R2, Fig.6). Indeed, if the neoblasts segregate exclusively from the endomesoderm, *i.e.* undertake an endomesodermal cell fate, they supposedly would only differentiate into endomesodermal derivatives (unless they reset their genetic program) and as such, their capability of differentiating into ectodermally derived epidermal cells (Egger, Steinke et al. 2009), would remain unexplained.

To me, a more parsimonious explanation is that the mesodermal genes are expressed only in a subset of the acoel neoblasts whereas the others, most likely segregated from the ectoderm, would not, obviously, be detected by mesodermal gene expression.

Remarkably, I found that all genes expressed in the gonads are also expressed in the neoblasts (or better said in the endomesodermal neoblast population) (article R2, Fig.6), except the pan-muscular marker *tropomyosin* (Chiodin, Achatz et al. 2011; Steinmetz, Kraus et al. 2012) and a *T-brain* orthologue, which is likely to

be a maternally expressed factor necessary for the proper development of the oocyte (Croce, Lhomond et al. 2001; Horton and Gibson-Brown 2002).

Overall, this coincident gene expression between gonads and neoblasts suggests to me that I might actually have been detecting exclusively those neoblasts that will differentiate into the acoel germ cells (Gschwentner, Ladurner et al. 2001; De Mulder, Kuales et al. 2009).

My data, additionally, supports the notion that germ cells have evolved from totipotent stem cells, similar to what we see in the acoelomorph neoblasts or the cnidarian interstitial cells (Extavour and Akam 2003). In fact, while in most bilaterians there is a clear morphological and molecular separation between stem and germ cell lines, this separation is lacking in basal metazoans lineages such as sponges and cnidarians; whereas amongst Bilateria, such separation is only missing in acoels (most likely in the whole acoelomorphs) and rabditophoran flatworms. In the aforementioned taxa, the lack of molecular differentiation between stem and germ cells is exemplified by their shared expression of the bilaterian germ line marker *Piwi* (Seipel, Yanze et al. 2004; Reddien, Oviedo et al. 2005; Bosch and Funayama 2008; De Mulder, Kuales et al. 2009; De Mulder, Pfister et al. 2009; Egger, Steinke et al. 2009).

Notably, the data exposed in the article R2 considerably extends the list of genes simultaneously expressed in stem and germ cells (article R2, Fig.6), further supporting the evidence of common developmental (and evolutionary) origins.

It is worth noting the remarkable similarity between the acoel and hydrozoan gonads. This similarity does not only imply morphological similarity, the hydrozoan gonads consist of compact germ cells and developing gametes

condensed in specific regions of the mesoglea (the space between mesoderm and endoderm), but it is extensive when considering the molecular patterning.

As it happens with the gene *Cniwi* (the cnidarian orthologue of *Piwi*), the orthologues of the genes *Twist, Mef2* and *Six1*/2 are expressed in the high proliferative region of the jellyfish gonads, encompassing both stem and germ cells (Spring, Yanze et al. 2000; Spring, Yanze et al. 2002; Seipel, Yanze et al. 2004; Stierwald, Yanze et al. 2004; Hroudova, Vojta et al. 2012).

Although the homology of the hydrozoan and acoelomorph gonads cannot be assumed, because the former originate in the ectoderm (Seipel, Yanze et al. 2004), it might be that these genes are common to an ancestral genetic network leading to the specification of germ cells, and that this network has been coopted by the hydrozoan jellyfishes to specify their gonads. As such, I would also expect to find other, not yet studied, orthologues of acoelomorphs 'gonad' genes being expressed in the hydrozoan gonad.

In conclusion, there is no reason in my opinion to homologize the acoel neoblast-gonad system to a putative ancestral coelomic cavity given that there is not even a developmental hint of its formation. Moreover, the conserved gene expression cannot be used as a strong criterion, as there it could be extensive co-option of these same genes to pattern gonadal tissues, as it seems to be the case in cndarians.

9. Final remarks on the ancestry of coeloms and the plasticity of the mesoderm

To summarize what has been discussed above, it is difficult to speculate about the ancestral condition of the mesodermal germ layer in the absence of a more solid phylogenetic frame.

Certainly, the placement of phoronids, brachiopods and chateognats within the protostomes (Edgecombe, Giribet et al. 2011) calls for a re-evalution of the earlier proposed archycoelomate hypothesis. These three taxa have been traditionally affiliated with the deuterostomes, because of their tripartite organization of coelomic cavities (Nielsen 2012). According to the supporters of the archycoelomate hypothesis this would be the plesiomorphic bilaterian condition.

While in older phylogenetic schemes (Willmer 1990), this condition was uniquely realized in the deuterostomes, making it difficult to reconcile it with general ‘Urbilateria’ models, in the new phylogenies, an archycoelomate body plan architecture is assumed for protostome taxa, enhancing the arguments to support the archycoelomate body architecture as an ancestral bilaterian trait. In order to make any final statement it will be crucial to resolve the acoelomorphs’ phylogenetic position. In the case that the Acoelomorpha are placed as the sister group of the Bilateria, the archycoelomate hypothesis can almost be totally and confidently discarded.

In case the Acoelomorpha are confirmed to be derived deuterostomes, the discussion about the archycoelomate bilaterian ancestor will remain open. Yet, the opinion of most

authorities in zoology is that coeloms might have evolved multiple times within the Bilateria. This is based on the appreciation of the different modes of coelom development and the plasticity of this structure when it comes to its differentiation into new organ systems (Clark 1964; Willmer 1990; Schmidt-Rhaesa 2007; Nielsen 2012).

My opinion is that the whole mesoderm, regardless of his status, is a rather plastic germ layer. The advantage of evolving a third germ layer between the endoderm and the ectoderm is obvious, given that, during the evolution of Metazoa, this happened already twice (Steinmetz, Kraus et al. 2012) and possibly three times (Dunn, Hejnol et al. 2008; Hejnol, Obst et al. 2009), depending on the phylogenetic position of the Ctenophores. Even within the Bilateria, while it can be taken for granted that the endomesoderm evolved once (Technau and Scholz 2003), the ectomesoderm instead probably evolved more than once.

It is remarkable that orthologueous endo-mesodermal genes are expressed in the ectomesoderm of spiralians (Lartillot, Le Gouar et al. 2002; Nederbragt, Lespinet et al. 2002) and in the entocodon of hydrozoan cnidarians (Spring, Yanze et al. 2000; Spring, Yanze et al. 2002; Seipel, Yanze et al. 2004; Stierwald, Yanze et al. 2004), indicating that co-option is really extensive across the Eumetazoa when it comes to pattern structures analogous to the endomesoderm.

In conclusion, of the three bilaterians germ layers, the mesoderm is most likely the more plastic one in terms of originating new cell types and structures and in terms of its developmental and evolutionary origins. Therefore, as it has been already pointed out (Nielsen 2012) it might not be so relevant to put too much attention into the mesoderm in order to depict possible scenarios for bilaterian

evolution. It is, nevertheless, essential to increase our knowledge of comparative mesoderm development in order to understand how new organ systems can evolve and to what extent the effect of convergent evolution must be taken into account when drawing different models of animal evolution.

Conclusions

- The musculature of *S. roscoffensis* has a molecular architecture similar to that known for the bilaterian striated musculature although they appear as smooth muscles in histological preparations. This condition could represent a first step in the evolution of the bilaterian striated musculature, or, alternatively, a secondary reduced condition.

- The muscular mantle of the sagittocysts in *S. roscoffensis* does not express the *Tropomyosin* gene although they do express one *Troponin* gene. This apparent oddity might be linked to their unique function.

- Myocytes differentiation during the posterior regeneration of *S. roscoffensis* starts only after the wound has been closed. The wound closure is accomplished through the use of the already existing body-wall musculature and it precedes the formation of the wound epithelium.

- A large set of bilaterian mesodermal markers is expressed in the musculature of *I. pulchra*. Because the same genes are expressed in the epithelio-muscular endoderm of cnidarians there is a strong possibility that the muscles were, in fact, the first mesodermal cell types to evolve.

- Different subsets of *I. pulchra* muscles express different mesodermal genes, and therefore they are more likely differentially regulated.

- Instead of being a broad marker for the whole acoel mesoderm, *Twist* is most likely involved in patterning the male reproductive system, i.e. the testis and the male copulatory organs of the adult worms.

- The male and female genital organs in *I. pulchra* evolved independently.

- The germ cells and gonads of the acoel *I. pulchra* originate in the endo-mesoderm, most likely from a subpopulation of neoblasts that segregates from the endo-mesoderm.

Resumen

Los acelos son unos gusanos mayoritariamente marinos, de tamaño reducido caracterizados por la falta de cavidades corporales (calidad de las que toman su nombre, a-celo quiere decir sin celoma) y por tener un sistema digestivo con una sola apertura: la boca.

Hasta el día de hoy se han descrito aproximadamente unas 400 especies y aunque muchas de ellas son parte de la fauna intersticial marina, todas presentan adaptaciones y estilos de vida diversos. En esta tesis se ha trabajado con dos especies diferentes, con *Symsagittifera roscoffensis* (von Graff 1891), que vive en la costa atlántica europea e *Isodiametra pulchra* (Smith and Bush 1991), que vive la costa atlántica de América del Norte, fundamentalmente en el estado de Maine. *Symsagittifera roscoffensis* se localiza especialmente en el norte de Francia. La mayor ventaja en trabajar con esta especie es la facilidad de muestreo de animales adultos, que además son fértiles durante todo el año. Especialmente durante la temporada de Abril y Junio se pueden recoger ingentes cantidades de embriones

Isodiametra pulchra es también una especie atlántica, pero se puede encontrar en las costas del norte de América. Esta especie es particularmente valiosa en cuanto que disponemos de cultivos permanentes en el laboratorio. Aunque no sea tan fecunda como *S. roscoffensis*, de media un gusano adulto pone un huevo al día. La posibilidad de disponer de material embrionario y adulto en el mismo laboratorio nos ha permitido el desarrollo de los protocolos habituales de biología molecular, incluyendo un protocolo de RNAi (ARN de interferencia) para bloquear especificadamente la actividad de los genes escogidos (De Mulder, Kuales et al. 2009; Moreno, De Mulder et al. 2010)

Los acelos se caracterizan por una clara simetría bilateral que se reconoce gracias a la presencia de una concentración anterior del sistema nervioso y de los órganos sensoriales (normalmente un estatocisto) además de la presencia de un órgano frontal de carácter glandular. El sistema nervioso consta de un cerebro (aunque el uso de la palabra cerebro contestadazo es aceptable para algún autor (Raikova, Reuter et al. 1998)) concentrado en la región alrededor del estatocisto, y cordones nerviosos longitudinales que no están preferentemente desplazados ni hacia la parte dorsal ni hacía la ventral (Bery, Cardona et al. 2010; Achatz, Chiodin et al. 2012; Achatz and Martinez In press). El eje dorso-ventral está caracterizado por la presencia en la parte ventral de la boca y de los órganos copuladotes. El sistema digestivo de los acelos es ciego, le falta el orificio anal, y consiste en un sincitio, dando lugar a una digestión intracelular, en vez que extracelular, como ocurre en la mayoría de metazoos que tienen un tubo digestivo delimitado por un epitelio.

No solo falta un cavidad digestiva en los acelos sino que también no disponen de cavidades corporales secundarias (celomas). El espacio entre el sistema digestivo y la epidermis está ocupado por tejido parenquimático, las gónadas, que suelen ser pares (dos ovarios y dos testículos) y los neoblastos , es decir el conjunto de células madres pluripotentes (y presentes también de otros gusanos planos) (De Mulder, Kuales et al. 2009; Egger, Steinke et al. 2009).

En los animales bilaterales una de las principales funciones de las cavidades corporales es dar soporte interno (rigidez) al organismo. En los acelos esta función la mantiene la musculatura, que es especialmente densa. En estos animales los músculos forman una estructura ortogonal de músculos longitudinales y circulares; los últimos orientados perpendicularmente y externamente a los primeros, que rodean circunferencialmente el cuerpo del

acelo. Entro las dos capas de músculos longitudinales y circulares, suelen encontrarse fibras orientadas con cierto ángulo respecto al eje antero-posterior (las fibras musculares diagonales). Las fibras que atraviesan el cuerpo en dirección dorso-ventral se suelen definir como fibras parenquimáticas por su localización en el parénquima. Varios músculos accesorios existen en las diversas especies y su posición y naturaleza son variables entre ellas. Estos suelen ser principalmente músculos asociados a los órganos genitales (los órganos copuladores), y por tanto se les suelen considerar parte del sistema reproductor (Ladurner and Rieger 2000; Semmler, Bailly et al. 2008). La fertilización es interna y recíproca (todos los acelos son animales hermafroditas). Las gónadas difieren bastante de las gónadas de los demás animales bilaterales, ya que no están encapsuladas por ningún tejido y no tienen conductos que las comuniquen con el exterior (Rieger, Tyler et al. 1991). La región germinativa de las gónadas se puede distinguir (en el espacio) de la región donde maduran los gametos, y, de forma muy interesante se ha observado que las células germinales se diferencian a partir de los neoblastos (o células madre), una situación que raramente se da entre los animales bilaterales, un caso similar se da en los platelmintos (Reddien, Oviedo et al. 2005; De Mulder, Pfister et al. 2009; Egger, Steinke et al. 2009), aunque parece ser que esta debiera ser la condición plesiomórfica ya que también se da en los cnidarios, el grupo hermano de los animales bilaterales (Seipel, Yanze et al. 2004). El desarrollo de los acelos es único entre el de los animales bilaterales. Un aspecto clave que conviene resaltar para la comprensión de estas tesis es que el mesodermo se origina exclusivamente a partir de precursores procedentes del endodermo (Henry, Martindale et al. 2000). Se asume que tanto los músculos como las células del parénquima periférico son los únicos tejidos que se

diferencian a partir del mesodermo, aunque las gónadas y neoblastos ocupen también en el adulto una localización mesodérmica, es decir entre el sistema digestivo y la epidermis.

Los acelos pertenecen al filo de los Acelomorfos (*sensu* (Haszprunar 1996), es decir (Xenoturbellida+(Nemertodermatida+Acoela)). A día de hoy parece establecido el estado monofilético de este grupo, aunque su posición filogenética dentro de los bilaterales es un tema todavía muy controvertido (Hejnol, Obst et al. 2009; Philippe, Brinkmann et al. 2011). Xenoturbella y nemertodermátidos presentan caracteres morfológicos mas ancestrales, con respecto a los observados en los acelos. Estos son, por ejemplo, un sistema nervioso intra o sub-epidérmico y un sistema digestivo epitelial, características éstas afines o compartidas con los cnidarios.

La limitada complejidad corporal de los acelomorfos, por ejemplo la carencia del orificio anal y la presencia de un sistema nervioso no centralizado (*Xenoturbella* tiene solo una red nerviosa difusa), podría haberse heredado directamente de los cnidarios, y por eso justificaría, en cierto modo, la colocación de los acelomorfos como grupo hermano de los demás animales bilaterales (Hejnol, Obst et al. 2009).

Alternativamente, se ha propuesto que los acelomorfos puedan ocupar una posición filogenética derivada dentro de los deuteróstomos, como grupo hermano de todos los demás deuteróstomos, o bien como grupo hermano de los Ambulacraria (equinodermos+hemicordados) (Philippe, Brinkmann et al. 2011). Con esta hipótesis habría que asumir que muchos de los caracteres que son diagnósticos de los deuteróstomos, se habrían perdido en los acelomorfos. Entre éstos habría que incluir la pérdida de un tubo digestivo completo, con

boca y ano y la pérdida también de cavidades celómicas y la forma en como éstas se desarrollan, o sea por la separación de divertículos del tubo digestivo embrionario, o arquenterón. Este último proceso se denomina enterocélia, y su ancestralidad es un tema central en la formulación de hipótesis sobre la evolución de los animales bilaterales.

El 99% de las especies animales poseen una simetría bilateral, es decir son simétricas con respecto a su eje antero-posterior. A los orígenes de la simetría bilateral se suman (en el tiempo) el origen de un sistema nervioso centralizado, con una concentración anterior de los órganos sensoriales, y un sistema digestivo completo (con boca y ano) además del origen de una tercera capa embrionaria, el mesodermo.

Estas innovaciones fueron críticas par la amplia y rápida diversificación de estos animales bilaterales, y por tanto entender como aparecieron dichas innovaciones es clave para entender lo que al fin y a la cabo son también nuestros orígenes biológicos.

Mas de un modelo se ha propuesto sobre la evolución de los animales bilaterales a partir de cnidarios, sus parientes mas cercanos. Entre todos, dos siguen siendo los modelos mas debatidos, y sobre los cuales, hay que admitirlo, se han construido todos los demás.

Según la 'Teoría planuloide/aceloide' (von Graff 1891) un organismo de complejidad morfológica parecida a la de una larva plánula de cnidarios dio origen a los primeros animales bilaterales que debieran parecerse, hasta un cierto punto, a los modernos acelomorfos. Como ocurre en todas las plánulas existentes, la plánula que dio origen a los bilaterales debía de tener una sola apertura digestiva y posterior en el embrión, el blastoporo, además de un

sistema nervioso difuso. La adaptación a un estilo de vida béntico causó, probablemente, el desplazamiento del orificio del blastoporo hacia la parte ventral y también a una cierta concentración anterior de las neuronas y de los órganos sensoriales. En pasos evolutivos sucesivos, un cerebro evolucionó a partir de esa concentración anterior de neuronas, y el sistema nervioso central se centralizó desplazándose en una de dos posibles direcciones, o bien hacía el lado ventral, como en los actuales protóstomos, o bien hacía el lado dorsal, como en los actuales deuteróstomos. En esta propuesta no se menciona como debiera haberse originado el mesodermo, aunque parece mas obvio, que en estas condiciones, el primer tejido mesodérmico haya dado lugar a los músculos, dado que las plánulas tienen células del tipo epitelio-muscular, a partir de las cuales se supone que han evolucionado los 'verdaderos' miocitos, es decir células con contráctiles que carecen de esa parte apical de tipo epitelial. Según la teoría planuloide/aceloide la actual complejidad morfológica se habría alcanzado mediante incrementos graduales.

El segundo gran modelo de evolución de los animales bilaterales se denomina la 'Hipótesis del arquiacelomado' (Remane 1963), que propone los primeros organismos bilaterales serían mas parecidos a los actuales pólipos adultos de cnidarios antozoos. Según este modelo, el eje oral-aboral de los cnidarios rotó 90 grados para dar lugar al eje antero-posterior de los animales bilaterales. La rotación del eje corporal principal fue también acompañada de un alargamiento del orificio oral de los cnidarios a lo largo del nuevo eje antero-posterior. La posterior oclusión mediana del orificio dio lugar a la formación sincrónica de boca y ano. Esta misma teoría propone también que las cavidades celómicas se formaron en el ancestro de todos los bilaterales a partir de la separación de los divertículos gástricos que son parte integral de la arquitectura corporal de los

actuales pólipos de los cnidarios. Además el proceso de evolución de las cavidades celómicas estaría recapitulado en los bilaterales actuales, por ejemplo en los hemicordados que desarrollan su mesodermo por enterocélia. Las conclusiones a las que se llega utilizando este modelo evolutivo son casi totalmente opuestas a las anteriores. Siguiendo la hipótesis de los arquiacelomados, el ancestro de los bilaterales debería haber tenido una arquitectura corporal algo elaborada, incluyendo un intestino completo (con boca y ano) más cavidades celómicas. Esta teoría no menciona el sistema nervioso, pero dada la complejidad morfológica asumida para el ancestro común de los bilaterales, se podría asumir que el sistema nervioso también tenía cierta complejidad. Esta complejidad estaría ahora reflejada en la conservación de múltiples dominios de expresión de genes ortólogos en los sistema nerviosos de animales bilaterales relacionados de forma muy distante (Denes, Jékely et al. 2007).

Evidentemente el favorecer una hipótesis u otra depende fundamentalmente de la posición filogenética de muchos grupos calve, entre ellos la que se asignan a los acelomorfos. En el caso que se determine que representan el grupo hermano de los demás bilaterales, la teoría planuloide/aceloide tendrá un fuerte soporte, mientras que si se estableciera que los acelomorfos son deuteróstomos cuya simplificación ha sido secundaria la teoría del arquiacelomado quedará como una posibilidad a tener en cuenta.

Durante el periodo de mi tesis he decidido investigar, fundamentalmente, la arquitectura molecular del mesodermo y sus derivados en acelos, ya que la invención de este tejido embrionario ha sido una de las grandes innovaciones de los organismos bilaterales.

En el primer artículo R1 he empezado a investigar la composición molecular de un importante derivado de dicho tejido: los músculos.

La presencia de tejido muscular no es exclusiva de los animales bilaterales, ya que se conocen especies de cnidarios y ctenóforos (diploblásticos) que también tienen miocitos entre el endodermo y el ectodermo. De todas formas ahora sabemos, con certidumbre, que el tejido muscular evolucionó de forma independiente en los animales diploblásticos y en los bilaterales (Steinmetz, Kraus et al. 2012). En los animales bilaterales se reconocen dos tipos fundamentales de músculos, por su aspecto en las preparaciones histológicas: el músculo estriado y el músculo liso. Estos dos tipos difieren también en su arquitectura molecular y mas precisamente por la presencia de diferentes proteínas que regulan el mecanismo de la contracción muscular.

La contracción se basa un mecanismo de deslizamiento de filamentos ligeros de actina sobre filamentos pesados hechos de miosina. Un proteína clave en la contracción muscular, la tropomiosina, inhibe esta interacción , y en consecuencia el deslizamiento los filamentos en las condiciones de reposo.

En el músculo estriado, en respuesta a un estimulo de contracción, las troponinas se encargan de desplazar la tropomiosina y así facilitar el deslizamiento de filamentos ligeros y pesados.

Por otra parte, en el músculo liso, aunque haya tropomiosina, el mecanismo regulador de la contracción se basa en la inhibición de la miosina por parte de la cadena ligera de la miosina, cuya fosforilación posterior al estímulo libera la miosina permitiendo así que ésta interactúe con los filamentos de actina (una revisión exhaustiva en: (Alberts, Johnson et al. 2008)).

Este último mecanismo parece ser el ancestral ya que todas las proteínas necesarias para ello existen desde el origen de los animales, mientras que las troponinas se han originado mas recientemente, con los organismos bilaterales (Steinmetz, Kraus et al. 2012).

Curiosamente, *S. roscoffensis* que como todos los acelos que tienen exclusivamente musculatura de tipo liso, expresa un gen ortólogo a la troponina, además de, y como es esperable, de expresar los ortólogos de actina y tropomiosina. Esta peculiaridad se añade a otra, la de que la musculatura de acelos no expresa el ortólogo de la cadena ligera de la miosina, una proteína que está en la musculatura lisa y estriada de todos los bilaterales, y probablemente también de los cnidarios (aunque en estos últimos nos faltan datos de expresión).

Probablemente la explicación a la segunda condición sea que en acelos hemos detectado una isoforma no muscular de la cadena ligera de miosina. La primera condición, es decir la expresión de troponina en musculatura lisa, tiene importantes consecuencias , según sea la posición filogenética que asumamos para los acelos.

Bajo la hipótesis de que los acelomorfos representen la primera rama de diversificación de los bilaterales, la explicación mas parsimoniosa sería que en los acelos se han implantado ya las bases moleculares para la evolución de los músculos estriados, aunque la aparición de este tipo muscular necesite de otros pasos adicionales en la evolución de los bilaterales.

Si, por el contrario, aceptamos la hipótesis de que los acelomorfos son deuteróstomos derivados, entonces la explicación mas simple es que en los acelos se haya perdido el característico aspecto estriado de la musculatura, aunque se haya mantenido su arquitectura molecular. Este segundo caso no

puede ser descartado ya que conocemos otros organismos bilaterales que perdieron la musculatura estriada, aún teniendo predecesores que la tenían, por ejemplo las ascidias (Endo, Matsumoto et al. 1996).

En el artículo R2 he analizado la expresión de genes mesodérmicos durante el desarrollo embrionario y post-embrionario del acelo *I. pulchra*, para inferir a que nivel el mesodermo de acelos está relacionado con el mesodermo de otros bilaterales mas complejos.

A este fin, es interesante puntualizar que el mesodermo se originó muy probablemente a partir del endodermo, ya que ortólogos de genes que se identifican como marcadores de mesodermo en bilaterales se expresan en el endodermo de cnidarios; y que además mientras todos los bilaterales tienen una fuente de mesodermo que se origina a partir de precursores endodérmicos (endomesodermo), solo unos pocos también tiene una fuente derivada del ectomesodermo. Los acelos, no tienen esta última fuente. Desde el endomesodermo de los acelos se diferencian los músculos y el tejido parenquimático periférico (Henry, Martindale et al. 2000). Además de estos dos tejidos, las gónadas y los neoblastos ocupan también una posición mesodérmica aunque sus orígenes embrionarios son, hoy por hoy, desconocidos. El origen de las gónadas es desconocido dado que en los experimentos de marcaje celular de blastómeros embrionarios no se ha podido seguir el destino de estas células hasta el estadio de adulto, debido a la dilución del marcador y el debilitamiento de su señal fluorescente. En el caso de los neoblastos sus orígenes también permanecen enigmáticos, ya que con este tipo de marcajes los neoblastos son difíciles de distinguir de otros tipos celulares.

He analizado la expresión de 12 marcadores diferentes de mesodermo y he encontrado que todo estos genes, con la excepción de el ortólogo de *T-brain*, se expresan en la musculatura de *I. pulchra.* Además, todos estos genes, esta vez con la excepción del ortólogo de la *tropomiosina*, se expresan en las gónadas y una subpoblación de neoblastos; mientras solo una pequeño grupo de ellos se expresan en el tejido parenquimático. Mis datos apuntan a un posible origen mesodérmico de las gónadas, condición que se aprecia en la mayoría de bilaterales (Extavour 2007).

La expresión coincidente de genes ortólogos en el endodermo de cnidarios y en la musculatura de acelos encaja muy bien con el conocido origen endodérmico del mesodermo, además de que crea una conexión directa entre las células epitelio-musculares de cnidarios y los músculos de bilaterales. Sorprendentemente, las gónadas de las medusas de hidrozoos, una clase muy derivada dentro de los cnidarios, expresan varios de los mismos genes que aparecen activados en las gónadas de acelos. Los dos tipos de gónadas no pueden ser homólogas, pues en los cnidarios se diferencian del ectodermo. Da la impresión de que el fenómeno que observamos aquí es el de una amplia co-opción de genes ortólogos para modelar tejidos análogos.

Aún si los acelos se confirmarán como deuteróstomos, en su mesodermo debiera encontrarse la huella de las cavidad celómicas que tenían sus ancestros. Dado que no hay huella alguna de la formación de cavidades celómicas durante el desarrollo de acelos, he investigado si esta huella pudiera residir en el patrón de expresión de genes mesodérmicos. Gracias a una búsqueda intensiva en la literatura he identificado un grupo central de genes que se expresan consistentemente durante la formación de las cavidades celómicas de los

deuteróstomos menos derivados y he encontrado que todos sus ortólogos se expresan en las gónadas de *I. pulchra.*

Como en varios organismos bilaterales las gónadas se desarrollan a partir de celomas, es plausible suponer que las gónadas de acelos son los restos de la antigua y colapsada cavidades celómicas. De todas formas, esta conclusión, en mi opinión se ha de tomar con cuidado ya que, como he comentado más arriba, estos mismos genes han sido co-optados en cnidarios para moldear un tejido muy parecido. Estudios posteriores, incluyendo la expresión de otros genes así como el efecto de supresión de la actividad de genes ya estudiados puede darnos la información clave para resolver este problema evolutivo.

Finalmente, en el último articulo, R3, he presentado los resultados de una colaboración con el grupo de morfología comparada de la Universidad de Copenhague, en un proyecto de descripción de el sistema nervioso del acelo *S. roscoffensis*. El sistema serotonérgico y FMRFamidérgico se compone de una agrupación anterior de neuronas, alrededor del estatocisto y tres pares de cuerdas nerviosas, interconectadas a través de varias comisuras transversales. Este patrón se mantiene de forma similar en los animales juveniles y en los adultos, fundamentalmente en la parte mas anterior. La estructura ortogonal regular se pierde en la parte mas posterior del organismo. Estos resultados sugieren que el adulto se desarrolla, probablemente, a partir de una zona de crecimiento posterior, y este proceso podría recapitular el modo de evolución del sistema nervioso. De acuerdo con esta hipótesis, hemos encontrado que el gen *SoxB1* se expresa solamente en la parte anterior de los juveniles de *S. roscoffensis,* mientras que en la especie relacionada *Convolutriloba longifissura,* el ortólogo de *SoxB1* se expresa a lo largo del eje animal-vegetal

que es también el futuro eje antero-posterior de los juveniles (Hejnol and Martindale 2009).

Estas hipótesis deberían ser contrastadas en el acelomorfo *Xenoturbella*, que a diferencia de los acelos tiene un sistema nervioso intra-epitelial, mas similar al de los cnidarios que al de los bilaterales, y que, por tanto, pudiera representar el estado ancestral del sistema nervioso de todos los animales bilaterales. En *Nematostella vectensis*, el ortólogo de *SoxB1* no está regionalizado (Magie, Pang et al. 2005), mientras si lo está en el hemicordado *Saccoglossus kowalewsky,* que también tiene un sistema nervioso intraepitelial difuso, pero con patrones moleculares complejos y similares a los de los cordados (Lowe, Wu et al. 2003). Aparentemente nuestros resultados de expresión de *SoxB1* en *S. roscoffensis* son mas parecidos a los que se obtienen en los hemicoordados, pero dado que los acelos han evolucionado su sistema nervioso de forma independiente, a partir de un condición similar a la que se encuentra en *Xenoturbella,* los datos de expresión en *Xenoturbella* del gen *SoxB1* y de otros genes neuronales son ahora indispensables para acabar de entender como evolucionó el sistema nervioso central.

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