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Sipuncula in Evolutionary Developmental Biology

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ABSTRACT. Adult sipunculans are currently placed within Annelida, mainly on the basis of molecular phylogenetic analyses. Here, we review recent advances in morphogenetic studies that have revealed numerous shared features between sipunculans and other annelids, including a metamerically formed nervous system, supporting the notion of a sipunculan/annelid clade. Similar to annelids, sipunculan myogenesis starts with the formation of four separate longitudinal muscle strands that develop from anterior to posterior, suggesting that this mechanism of myogenesis was present in the last common ancestor of both taxa. A dense arrangement of longitudinal body wall muscles in the vicinity of the retractor muscles suggests that the latter evolved from fused longitudinal body wall muscles. Although circular body wall muscles do not develop in a segmental manner during sipunculan ontogeny, traits of segmentation during neurogenesis strongly support recent molecular analyses and argue for a segmented last common ancestor of sipunculans and annelids. The establishment of a detailed morphogenetic sipunculan framework enables a careful interpretation of gene expression patterns that might shed further light on the evolution and partial loss of segmentation in Sipuncula and Annelida.

INTRODUCTION

Adult sipunculan worms uniformly exhibit an unsegmented body that is subdivided into a posterior trunk and a retractable anterior introvert. Internally, a U-shaped gut leading to a dorsally placed anus, a pair of nephridia (in some genera only a single nephridium), an unpaired ventromedian nerve cord, one to four introvert retractor muscles, and an undivided trunk coelom are present (Rice, 1993; Jaeckle and Rice, 2002; Kristof and Maiorova, 2016). Although morphological characters and molecular data strongly support the monophyly of Sipuncula, their internal relationships are still debated (Maxmen et al., 2003; Schulze et al., 2005, 2007; Kawauchi et al., 2012). The majority of sipunculan species for which development has been examined have planktotrophic larvae with either one (trochophore) or two (trochophore and pelagosphera) larval stages, but direct development has been described as well, whereby the embryo develops inside the egg envelope into the crawling juvenile worm (Rice, 1967, 1975a, 1975b, 1976). The spiral cleavage pattern, a trochophore larva with an apical tuft, a circumferential ring of prototroch cells, and other shared developmental traits (e.g., a "molluscan cross") place Sipuncula morphologically close to spiralian taxa such as Annelida and Mollusca (Rice, 1985; Scheltema, 1993, 1996; Cutler, 1994; Westheide and Rieger, 2007; Schulze and Rice, 2009a). Recent molecular studies place Sipuncula as the sister group to Annelida (Mwinyi et al., 2009; Sperling et al., 2009) or even inside Annelida (Boore and Staton, 2002; Bleidorn et al., 2006; Struck et al., 2007, 2011, 2015; Dunn et al., 2008; Hejnol et al., 2009; Shen et al., 2009; Zrzavy et al., 2009; Dordel et al., 2010; Lemer et al., 2015; Weigert and Bleidorn, 2016). In congruence with the latter data, neurogenesis and the distribution of

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proliferating cells show transitional stages of segmentation during development, thus supporting a sipunculan-annelid affiliation (Wanninger et al., 2005, 2009; Kristof et al., 2008, 2011; Kristof and Maiorova, 2016). Herein, we review published data on neuromuscular development in Sipuncula and discuss the significance of Sipuncula in deducing the ancestral conditions and developmental processes of the last common sipunculan-annelid ancestor.

SIPUNCULAN DEVELOPMENT AND ANCESTRY

Immunocytochemistry and F-actin labeling in conjunction with confocal microscopy have proven to be useful for reconstructing possible ancestral neuromuscular features and thus may provide important insights into body plan evolution (Hessling and Westheide, 2002; Raikova et al., 2004; de Rosa et al., 2005; Müller, 2006; Denes et al., 2007; Wanninger, 2009; Boyle and Seaver, 2010; Kristof and Klussmann-Kolb, 2010; Nielsen and Worsaae, 2010; Kristof et al., 2016). So far, eight sipunculan species representing two families and three different developmental modes have been investigated using the abovementioned methods (Table 1; Wanninger et al., 2005; Kristof et al., 2008; Schulze and Rice, 2009b; Kristof, 2011; Kristof et al., 2011; Kristof and Maiorova, 2016).

MYOGENESIS

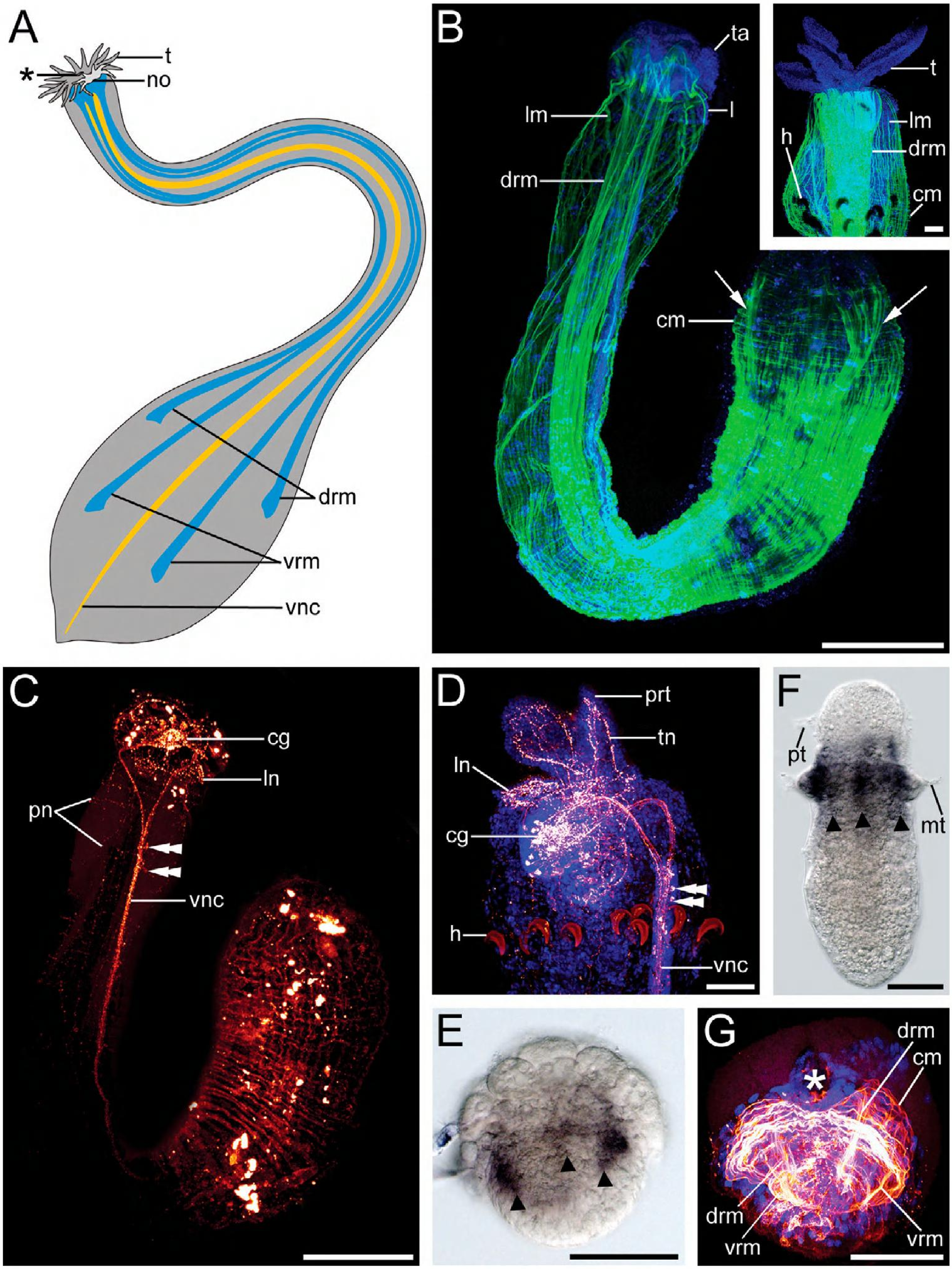
Adult sipunculans may exhibit one (e.g., *Phascolion cryptum*; Schulze and Rice, 2009b) to four (e.g., *Sipunculus nudus*; Gibbs, 1977; Figure 1A) longitudinal introvert retractor muscles, but their myogenesis commonly starts with the simultaneous formation of four introvert retractor muscles that develop from

TABLE 1. List of species investigated by the fluorescense markers serotonin and FMRFamide for neurotransmitters and peptides, phalloidine for F-actin of the musculature, and EdU (5-ethynyl-2′-deoxyuridine) for proliferating cells. Sipunculan family classification is *sensu* Kawauchi et al. (2012). Developmental modes are I, direct development; II, indirect development with a single pelagic lecithotrophic stage; III, indirect lecithotrophic stage; and IV, indirect planktotrophic stage. A dash (—) indicates not investigated.

Species (family) and developmental mode	Neurogenesis	Myogenesis	Cell proliferation
Phascolion strombus[a] (Golfingiidae), III	Serotonin, FMRFamide	F-actin	—
Phascolion psammophilus[b] (Golfingiidae), III	—	F-actin	—
Phascolion cryptum[b] (Golfingiidae), I	—	F-actin	—
Nephasoma pellucidum[b] (Golfingiidae), IV	—	F-actin	—
Themiste lageniformis[b] (Golfingiidae), III	—	F-actin	—
Themiste pyroides[c,d,e] (Golfingiidae), III	Serotonin, FMRFamide	F-actin	EdU
Thysanocardia nigra[c,d,e] (Golfingiidae), III	Serotonin, FMRFamide	F-actin	EdU
Phascolosoma agassizii[d,e,f] (Phascolosomatidae), IV	Serotonin, FMRFamide	F-actin	—

[a] Wanninger et al. (2005).
[b] Schulze and Rice (2009b).
[c] Kristof et al. (2011).
[d] Kristof (2011).
[e] Kristof and Maiorova (2016).
[f] Kristof et al. (2008).

FIGURE 1. (*Opposite page*) Sipunculans in evolutionary developmental biology. Anterior faces upward, and scale bars represent 150 µm in (B) and (C) and 50 µm in the inset and in (D)–(G). Dorsoventral views are given in all aspects, except in (D) and (F), where ventral is to the right. (A) Schematic drawing of an adult sipunculan (*Golfingia* spp.) with tentacles (t) around the mouth opening (asterisk) and a lobed nuchal organ (no) on the dorsal side. Internally, one ventral pair (vrm) and one dorsal pair (drm) of retractors are shown, along with the nonmetameric ventral nerve cord (vnc; redrawn from Strand and Sundberg, 2010). (B) *Phascolion psammophilum* juvenile with tentacles anlagen (ta) and lip (l) showing cell nuclei (blue) and F-actin (green; musculature) labeling. The fusion process of the dorsal retractor muscles (drm) has begun in the anterior region, whereas posteriorly, they are still separated (arrows). Larvae have one dorsal and one ventral pair of retractors initially, whereas adults exhibit a single large dorsal and one small ventral retractor muscle (Schulze and Rice, 2009b); lm marks the longitudinal body wall muscles, and cm marks the circular body wall muscles. The inset shows a slightly older juvenile with four tentacles, hooks (h) on the anterior part of the introvert, and a prominent fused dorsal retractor muscle. (C) Same juvenile as in (B), showing the serotonergic nervous system with the prominent cerebral ganglion (cg) and ventral nerve cord (vnc) with few associated perikarya (double arrowheads), lip neurites (ln), and peripheral neurites (pn). (D) Slightly older juvenile with developed hooks and developing primary tentacles (prt), which are innervated by serotonergic neurites (tn). (E) *Themiste pyroides*, early trochophore larva (2 days after fertilization) showing expression of *Tp-mhc* (myosin heavy chain) in the developing retractor muscles (arrowheads). (F) *Themiste pyroides*, pelagosphera larva (3 days after fertilization) with *Tp-mhc* expression in the retractor muscles; pt marks the ciliated prototroch, and mt marks the metatroch. (G) Same stage as in (E), showing the rudiments of the paired ventral and dorsal longitudinal retractor muscles, as well as numerous circular body wall muscles. Musculature is shown in red, and cell nuclei are illustrated in blue.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anterior to posterior (Åkesson, 1958; Hall and Scheltema, 1975; Wanninger et al., 2005; Schulze and Rice, 2009b; Kristof et al., 2011). Hence, the reduced number of adult retractor muscles is a secondary condition due to loss and/or fusion processes during later juvenile stages (Åkesson, 1958; Figure 1B and inset), suggesting that the last common sipunculan ancestor had four separate longitudinal retractor muscles that developed from anterior to posterior. At the same time as the formation of the four retractor muscles, a considerable number of outer circular body wall muscles develop. The circular muscles develop simultaneously along the anterior-posterior axis and always earlier than the inner longitudinal retractor muscles (Wanninger et al., 2005; Schulze and Rice, 2009b; Kristof et al., 2011). Interestingly, longitudinal body wall muscle fibers increase in number throughout sipunculan ontogeny and form a pattern of dense arrangement in the area of the retractor muscles, whereas they are loosely arranged toward the mid-body region (Kristof et al., 2011). This pattern might suggest that the longitudinal retractor muscles have evolved from fused longitudinal body wall muscles. Myogenesis follows a similar pattern in all investigated sipunculan species, although minor differences do occur. Directly or indirectly developing lecithotrophic species (e.g., *Themiste pyroides* and *Thysanocardia nigra*; Kristof et al., 2011), for instance, lack a terminal organ (this structure enables pelagosphera larvae to attach to substrates) and develop the buccal organ (a vertable pharyngeal pouch used for feeding) considerably later than the species with planktotrophic development (e.g., *Phascolosoma agassizii* and *Nephasoma pellucidum*; Schulze and Rice, 2009a, 2009b; Kristof et al., 2011).

NEUROGENESIS

Regardless of the mode of development (indirect lecithotrophic or indirect planktotrophic versus direct), neurogenesis is remarkably similar in all investigated sipunculans and always gives rise to the adult with a nonmetameric ventral nerve cord and an anteriorly positioned dorsal brain (Figure 1A; Wanninger et al., 2005; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). Early neuronal development in all investigated sipunculans is restricted to the apical organ, which is immunoreactive against the neurotransmitters serotonin and FMRFamide and exhibits two flask-shaped cells in *Themiste pyroides*, *Thysanocardia nigra*, and *Phascolion strombus* (only FMRFamide) and up to four flask-shaped cells in *Phascolosoma agassizii* (Wanninger et al., 2005; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). During subsequent development two neurites grow posteriorly and form a scaffold for the future ventral nervous system, while formation of the adult cerebral ganglion starts at the base of the apical organ (Wanninger et al., 2005; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). In addition, all but one (*P. strombus*) investigated species have a neurite that underlies the metatrochal ciliary bands and that is immunoreactive against serotonin and FMRFamide. *Phascolion strombus* lacks a metatrochal neurite, probably because of its short-lived pelagosphera stage (12–24 hours at 12°C–16°C), which is considerably shorter than in *T. pyroides, T. nigra* (10–14 days at 17°C–19°C), and *P. agassizii* (several months in the open ocean; Wanninger et al., 2005; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). However, during subsequent development, interconnecting commissures and pairs of perikarya appear in an anterior to posterior progression along the paired ventral nerve cord, resulting in a rope-ladder-like ventral nervous system, thus indicating the presence of a posterior growth zone (Wanninger et al., 2005, 2009; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). A median neurite appears in the FMRFamidergic ventral nervous system toward metamorphosis, whereas the serotonergic longitudinal neurites gradually fuse and the metameric arrangement of the associated perikarya disappears. At the same time, the adult cerebral ganglion elaborates, whereas the serotonergic and FMRFamidergic cells in the larval apical organ slowly disappear (Wanninger et al., 2005, 2009; Kristof et al., 2008; Kristof, 2011; Kristof and Maiorova, 2016). Moreover, the fusion and cell migration processes seem to continue also into the first juvenile stages before the adult condition of the ventral nervous system is achieved (Figure 1C,D). Taken together, the currently available data strongly suggest a serotonergic neurite that innervates a ciliated locomotory organ (e.g., prototroch, metatroch), a serotonergic (and maybe also FMRFamidergic) apical organ comprising approximately four flask-shaped cells, a paired ventral neurite bundle with metamerically formed pairs of perikarya, and a median neurite as part of the ancestral sipunculan body plan. Interestingly, the sipunculan metameric mode of neurogenesis is coherent with findings of a transient, metameric distribution pattern of mitotic cells. These originate from the ventral posterior trunk area, thus indicating a posterior growth zone and thereby further supporting a segmented ancestry of Sipuncula (Kristof et al., 2008, 2011; Wanninger et al., 2009).

GENE EXPRESSION

The first, and so far only, gene expression study on a sipunculan, *Themiste lageniformis*, was published by Boyle and Seaver (2010). This study found a similar expression pattern of the genes *FoxA* and *GATA456* between the polychaete *Chaetopterus* and the sipunculan *T. lageniformis*. The genes *FoxA* and *GATA456* are known to be involved in gut development throughout Metazoa (Roberts, 2000; Stainier, 2002). In both species, *FoxA* appears to define the anterior and posterior regions of the digestive system since it is expressed in the area of the foregut and hindgut before the definite gut tube is formed. *GATA456*, by contrast, is largely expressed in the developing midgut and the associated mesoderm as well as along the entire hindgut region (Boyle and Seaver, 2010). It has to be noted, however, that there are species-specific differences such as the *FoxA* expression in a patch of ectodermal cells outside the gut that persist after metamorphosis in *T. lageniformis* and *GATA456* expressing cells in the anterior ectoderm of *Chaetopterus*. *FoxA* and *GATA454* are expressed in distinct regions that correspond to the three digestive system compartments (e.g., foregut, midgut, and hindgut) of both worms, resembling the patterns reported for mouse, fly, nematode, and mollusk embryos and larvae (Boyle and Seaver, 2008, 2010, and references therein). Hence, this study suggests a core role of *FoxA* and *GATA454* in gut development of annelids including sipunculans and provides further support for this pattern being a shared feature throughout the Bilateria.

FUTURE PERSPECTIVES

The ontogenetic establishment and loss of a metamerically arranged organ system has never been described for any animal before, thus rendering Sipuncula and its body plan formation interesting for developmental studies. Since modern high-throughput sequencing technologies (e.g., 454 FLX Genome Sequencer, Illumina genome analyzer, PacBio) are becoming less expensive, they provide exciting opportunities to investigate nonmodel organisms such as sipunculans from a molecular perspective. The abovementioned morphogenetic data enable detailed interpretations of gene expression patterns in larvae and juveniles for ongoing, initiated, and future studies that aim to unravel molecular mechanisms in sipunculan body plan formation (see Boyle and Seaver, 2010; Figure 1E–G). In this context the putative sipunculan "segmentation" process can be assessed by analyzing the role of developmental genes involved in body plan patterning, which are known from model system animals (e.g., *Drosophila* [fly], *Tribolium* [beetle], *Mus* [mouse], and *Danio* [fish]), and such studies may also reveal possible new functions of some of these genes. With such studies, the visibility of Sipuncula in evolutionary developmental biology should increase significantly by contributing to our understanding of developmental patterns and mechanisms in metazoan animals—a key question in the field of "evodevo."

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