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The *Macrostomum lignano* EST database as a molecular resource for studying platyhelminth development and phylogeny

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Abstract We report the development of an Expressed Sequence Tag (EST) resource for the flatworm *Macrostomum lignano*. This taxon is of interest due to its basal placement within the flatworms. As such, it provides a useful comparative model for understanding the development of neural and sensory organization. It was anticipated on the basis of previous studies [e.g., Sánchez-Alvarado et al., Development, 129:5659–5665, (2002)] that a wide range of developmental markers would be expressed in later-stage macrostomids, and this proved to be the case, permitting recovery of a range of gene sequences important in development. To this end, an adult *Macrostomum* cDNA library was generated and 7,680 *Macrostomum* ESTs were sequenced from the 5' end. In addition, 1,536 of these aforementioned sequences were sequenced from the 3' end. Of the roughly 5,416 non-redundant sequences identified, 68% are similar to previously reported genes of known function. In addition, nearly 100 specific clones were

obtained with potential neural and sensory function. From these data, an annotated searchable database of the *Macrostomum* EST collection has been made available on the web. A major objective was to obtain genes that would allow reconstruction of embryogenesis, and in particular neurogenesis, in a basal platyhelminth. The sequences recovered will serve as probes with which the origin and morphogenesis of lineages and tissues can be followed. To this end, we demonstrate a protocol for combined immunohistochemistry and in situ hybridization labeling in juvenile *Macrostomum*, employing homologs of *lin11/lim1* and *six3/optix*. Expression of these genes is shown in the context of the neuropile/muscle system.

Keywords Platyhelminth · Embryo · Morphogenesis · Gene expression · EST

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Introduction

Fundamental aspects of the genetic control of nervous system development are conserved across the bilateria as is documented by a comparable expression and implied function of a large number of regulatory genes. Examples include genes required for the development of photoreceptors and other sensory organs, such as the *six/sine oculis* genes, which occur already in neural structures basal to the Bilateria (Bebenek et al. 2004). Notable examples in case are also provided by the *LIM* genes associated with dorso-ventral neural differentiation (e.g., Thor and Thomas 2002), the homeobox genes *ind*, *vnd*, and *msh* required for medio-lateral organization (e.g., Arendt and Nübler-Jung 1999; Jacobs et al. 1998), and *otd/ems* and the *Pax* genes that play a role in the subdivisions of the tripartite brain (e.g., Lichtneckert and Reichert 2005). Such findings suggest the hypothesis that the bilaterian ancestor might have possessed a nervous system in which olfacto- and photoreceptors, various brain structures, and neuroendocrine cells were laid out in a way similar to the one found in present-day taxa, and were specified by the molecular mechanisms that already included the majority of the

elements still encountered today in derived bilaterians, like vertebrates and flies (Wada et al. 1998; Chang et al. 2001; Reichert 2002; Hirth et al. 2003).

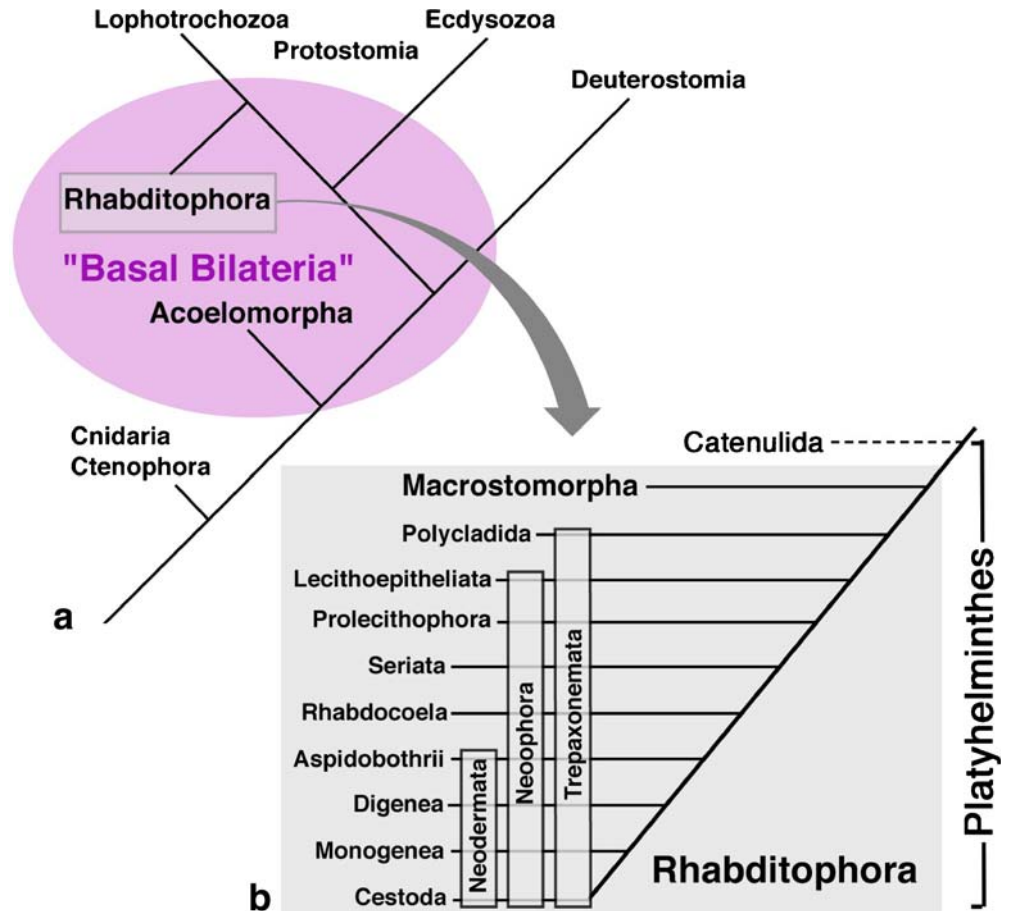
However, as numerous authors have pointed out, many fundamental aspects of neural development are different in arthropods and vertebrates; the fact that the organization of the CNS is basiepithelial in vertebrates (and deuterostomes in general), and is subepidermal in arthropods may serve as one example (reviewed in Holland 2003). It is often difficult to discern whether unique features of an organism's neural development and morphology are ancestral or derived. In many cases such as the eye of vertebrates and arthropods, both lineages may have derived attributes relative to a common ancestor. To better reconstruct the ancestral condition of bilaterian neural developmental systems, additional systems need to be studied in sufficient detail for effective comparison of the derived arthropod and vertebrate systems.

Flatworms (platyhelminths) are of particular interest for the comparative study of neural development. Current phylogenetic analyses, based on structural and molecular characters, separate the Acoelomorpha from the Platyhelminthes *s. str.* (Catenulida and Rhabditophora). Many workers now consider acoelomorph flatworms to be the taxonomic sister of Bilateria, while catenulids and rhabditophorans are basal taxa within the bilaterian taxon Lophotrochozoa (see Ruiz-Trillo et al. 1999; Baganà and Riutort 2004; Telford et al. 2005), the large superphylum

that contains annelids, mollusks, lophophorates, and related protostome phyla (Fig. 1a). In regard to neural structure and development, acoelomorphs and rhabditophorans share many essential characters with each other, as well as with outgroups of bilaterians (coelenterates); these characters are likely to be primitive (Raikova et al. 2004; Reuter et al. 2001a,b; Reuter and Halton 2001).

Molecular studies in Platyhelminthes initially focused on planarians, members of the Tricladida, after their traditional use in studies of regeneration (Saló and Baganà 2002; Agata 2003; Reddien and Sánchez-Alvarado 2004; Cardona et al. 2005a,b; Orii et al. 2005). Triclad EST collections are now available to the scientific community (Sánchez-Alvarado et al. 2002). However, triclads are not a basal branch and are not thought to be primitive within Platyhelminthes (Doe 1981; Ehlers 1985; Baganà et al. 2001; Rieger 2001; Tyler 2001). The study of triclads should, therefore, be complemented by analyses of more basal flatworm taxa. Cladistic analysis of morphological and molecular data yields a pectinate phylogeny of groups within the Platyhelminthes *s. str.*, with more derived forms in the higher branches (Ehlers 1985; Ax 1996). Criteria that are thought to be differentiable into ancestral and derived conditions include: 1) the mode of cleavage [quartet-spiral being primitive among the Catenulida and Rhabditophora, duet spiral being typical for acoels, but see recent discovery of a duet and radial cleavage pattern among the

Fig. 1 a Cladogram showing basal branches of metazoa. Acoelomorphs are shown as sister taxon of all other bilateria; rhabditophoran flatworms appear as a basal branch of the lophotrochozoa (after Telford et al. 2005). **b** Cladogram of rhabditophoran flatworms (after Westheide and Rieger 1996; for details, see text)



Nemertodermatida (Jondelius et al. 2004)]; 2) the presence or absence of compact glandular secretions called rhabdites (Rieger et al. 1991); 3) the delivery of yolk (yolk within the oocyte being primitive, and yolk supplied by specialized yolk cells being derived); 4) increasing complexity of the pharynx; and, 5) the formation of the epidermis (Doe 1981; Joffe 1987; Ehlers 1992). Based on these characters, macrostomids are the most primitive taxon within the Platyhelminthes *s. str.* for which embryos can be obtained; only catenulids, for which sexual reproduction has rarely been observed, are considered more primitive than macrostomids.

In this paper, we introduce an EST database for the macrostomid flatworm *Macrostomum lignano* (Ladurner et al. 2005b). Not only has the development of this worm been described in detail (Morris et al. 2004), but a detailed map of the brain is currently being produced (Morris et al. 2006, submitted). We present in this paper the isolation and sequence characterization of 5,416 non-redundant expressed sequence tags (ESTs) in *Macrostomum*. A reliable in situ hybridization/immunohistochemistry protocol has been developed, permitting integration of expression patterns of genes recovered via EST with a detailed brain map. These tools in combination will help to provide insight into developmental processes underlying neurogenesis in this basal flatworm. Comparative use of these data will further our understanding of the evolution of bilaterian neural organization.

Materials and methods

Animal culture and collection

Macrostomum lignano was cultured in the lab according to the protocol set forth in Morris et al. (2004). Briefly, diatom plates containing *Nitzschia curvilineata* were grown to confluence in modified F/2 media for roughly 10 to 14 days. One hundred fifty *Macrostomum* adults were collected and transferred to a confluent algae plate on a monthly basis. Eggs were subsequently laid and collected on a daily basis and were allowed to develop for 5 days until hatching.

Library preparation

Significant numbers (approximately 10^5) of *Macrostomum* adults and juveniles were collected and were rinsed 5–10 times in sterile seawater. Samples were flash-frozen in liquid nitrogen, and were stored at -80°C until significant quantities were reached for library preparation. Using the custom services of Invitrogen, three separate cDNA libraries were produced. For the libraries, total RNA was extracted using the Trizol reagent. Standard procedures were used for Poly-A RNA selection, and to generate and size-select oligo-dT primed cDNAs (Gubler and Hoffman 1983). cDNAs were then directionally inserted into pCMVSPORT 6.1 using XhoI and NotI, and were electro-

porated into DH10B electrocompetent cells. The resulting library was amplified using Invitrogen's semi-solid agarose technique. The finished library was stored in LB/glycerol at -80°C . P. Ladurner and D. Pfister have prepared another cDNA library for an extension of the EST-analysis of *Macrostomum lignano*.

EST sequencing and the EST-DB database

Aliquots from the *Macrostomum* library were sent to JGI for EST sequencing. Using standard EST protocols, individual clones were isolated, and the resulting cDNAs were sequenced from both directions using both M13F and M13R as primers. The resulting sequences were analyzed using the base-calling program Phred (Ewing et al. 1998; Ewing and Green 1998) and were uploaded into EST-DB for assembly using Cap3 (Huang and Madan 1999) and BLAST analysis (Altschul 1990). Sequences were analyzed using both the BLASTx and BLASTn algorithms, and significance was placed upon a result below a value of $1e^{-04}$. The singlet or contig was then assigned to a functional category based upon its similarity to other genes as determined by the BLAST hit with the lowest significance value. Categories were set up as found in Smed-DB (Sánchez-Alvarado et al. 2002).

In situ hybridization and immunohistochemistry

Macrostomum adults were starved for 2 days, then were relaxed in 7.6% MgCl_2 for 20 min. They were subsequently fixed in 4% PFA for 1 h at RT. The animals were then washed in PBS plus 0.1% Tween 20 (PBT) and were run through a graded alcohol series up to 100% ETOH, and then returned to PBT over the course of an hour. The animals were digested using 1 mg/ml protease K for 17 min at RT, and were processed through a triethanolamine/acetic anhydride wash for 15 min. The worms were refixed using both paraformaldehyde and heat. Hybridization took place over 3 days at 55°C , and the subsequent washes took place according to the Hydra in situ hybridization protocol (Takahashi et al. 2005). RNA probes were produced according to standard methods, and were labeled with digoxigenin (Roche). Visualization of probes was accomplished using an anti-digoxigenin alkaline phosphatase, with either a standard NBT/BCIP substrate (Roche), or using Fast Red (Roche) according to the manufacturer's specifications. If immunohistochemistry was to be performed on in situ processed worms, they were incubated in an anti-muscle antibody (anti-Mmu4; Ladurner et al. 2005a) for 1 h at room temperature. After PBT washes, the specimens were incubated in anti-mouse FITC (Jackson Labs) for 1 h.

For immunohistochemistry, specimens were washed in PBT (for washing, PBT solution was changed 3–5 times over a 10-min period) and incubated overnight in PBT containing the antibody at 1:1,000 dilution (anti-tyrosinated tubulin, Sigma; anti-Mmu4; anti-FMRF, Sigma).

After another washing step in PBT, the preparations were incubated for 4 h in PBT containing the secondary antibody (peroxidase or FITC-conjugated rabbit anti-mouse immunoglobulin, Jackson Labs) at a dilution of 1:800. Muscle fibers were visualized with rhodamin-phalloidine (1:1,000; Sigma).

Fluorescent preparations were viewed and recorded using a Biorad Model MRC1024ES confocal microscope and Laser Sharp version 3.2 software. The confocal stacks were viewed using the program Image J (NIH Image). Non-fluorescent preparations were viewed and recorded using a Zeiss Axiophot microscope equipped with a Sony DKC5000 digital camera. Figures were produced using Photoshop 7.0 (Adobe).

Results and discussion

An introduction to *Macrostomum*: phylogeny, structure, and development

Platyhelminths *s. str.* represent a large and highly diverse taxon, ranging from free-living marine, limnic, and terrestrial forms to parasitic flukes and tapeworms. Cladistic efforts based on morphological, ultrastructural, and molecular criteria have resulted in a widely accepted phylogenetic scheme (Fig. 1b) in which macrostomids occupy a basal position (Ehlers 1985; Westheide and Rieger 1996). Apart from the acoelomorph flatworms, which are now generally considered as a separate taxon, platyhelminths *s. str.* contain the catenulids, whose position appears to be basal, and the monophyletic taxon “Rhabditophora”, which displays a good number of unifying apomorphic characters. Notable among these are the special glands (duo-gland adhesive system) containing ultrastructurally conspicuous, lamellated granules called rhabdites. Further subtaxa among the Rhabditophora were defined on the basis of early developmental characters, the complexity of the pharynx, and the structure of the epidermis, and these taxa have found confirmation through molecular analysis (Baguña et al. 2001; Rieger 2001; Tyler 2001).

At the base of the rhabditophorans are small, free-living species with quartet-spiral cleavage, a pharynx simplex, and a ciliated epidermis. The taxon Macrostromida (including *Macrostomum lignano*) is considered the basal sister group of the remainder of the platyhelminths *s. str.* (“Trepaxonemata”). Macrostromids are microscopic inhabitants of various marine, brackish, and fresh water habitats; further defining characters are non-ciliated sperm and the close association between an anteriorly located pharynx and the brain, with a pronounced post-pharyngeal commissure. The next branch point of the platyhelminth tree separates the Polycladida from all remaining taxa, the Neophora (Fig. 1b). Polyclads, like macrostomids, show a quartet-spiral cleavage of large, yolk-rich blastomeres; they typically grow to much larger sizes than macrostomids and possess a muscular, evertible pharynx.

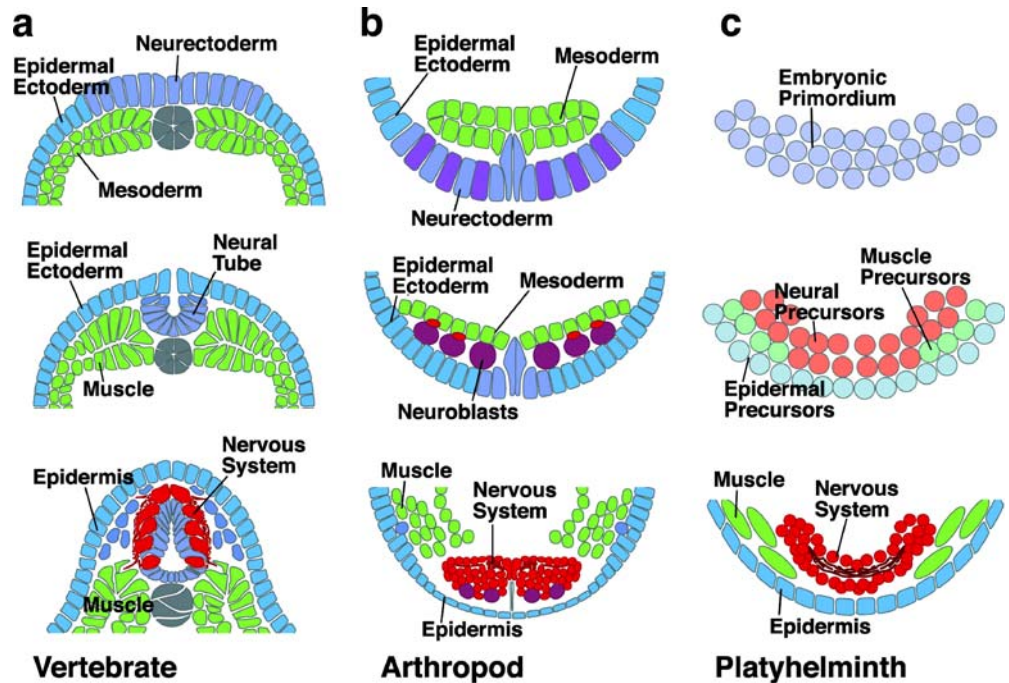
All “higher” platyhelminths (Neophora), including the planarians (members of taxon “Seriata”; Fig. 1b), which are widely used experimental systems for regeneration, are characterized by a unique type of egg that consists of a small oocyte surrounded by separate yolk cells. These ectolecithal eggs show early developmental features that differ fundamentally from the basal, spiralian type of the macrostomids and polyclads (“Archoophora”), although from mid-embryogenesis (stage 6, defined as “phylogenic stage” in Younossi-Hartenstein and Hartenstein 2001) onward, they share many of the defining topological and histological characteristics that can be seen in all flatworm taxa (Thomas 1986; Hartenstein and Ehlers 2000; Younossi-Hartenstein and Hartenstein 2000a,b, 2001; see below).

The early development of many flatworms, including formation of the nervous system, appears different from what has been described in great detail for both vertebrates and arthropods. In these “higher” phyla, populations of neural progenitor cells can be recognized that form part of, or segregate from, a two-dimensional epithelial layer, the neurectoderm (Fig. 2a,b). The neurectoderm represents the ventral (in arthropods) or dorsal (in chordates) subdivision of the ectoderm. Ectoderm, mesoderm, and endoderm form well-delineated tissue layers that arise during the course of gastrulation by invagination and ingression of blastomeres. In embryos of flatworms, separate germ layers are difficult to define based on morphological criteria, especially in neophoran flatworms.

Proliferating blastomeres form a multilayered tissue of mesenchymal cells (Fig. 2c, top), the embryonic primordium that is surrounded by yolk. In the deep layers of this mesenchyme, postmitotic precursor cells of neurons, muscle, and protonephridia appear simultaneously as local cell condensations. Neural progenitors condense into symmetrically arranged brain primordia in the anterior part of the embryo (Fig. 2c, middle). Only after neural differentiation has set in and axons form a small central neuropile (Fig. 2c, bottom) does the outermost cell layer of the embryo form an epithelial epidermal primordium. This “in situ differentiation” mode of development (Younossi-Hartenstein and Hartenstein 2000b) implies that precursors of different cell types are intermingled and sorted out by local cell–cell interaction. Correspondingly, different cell types, including neurons, muscle and gland cells, are found together in the brain of the mature animal.

The brain of the newly hatched juvenile is a compact structure formed by an outer cortex of neural somata surrounding a fibrous neuropile. Based on the diameter and orientation of neurites, the neuropile can be subdivided into a set of discrete compartments from which an invariant pattern of nerve roots emerge (Fig. 3; Morris et al. 2006, submitted). Compartments and their main characteristics are shown on the 3D digital model in Fig. 3a,b. Muscle fibers, presumably accompanied by cell bodies, penetrate the brain at invariant positions (Fig. 3c). Beside the invariant pattern of neurite bundles, these “cerebral muscles” represent a second convenient system of landmarks that help define compartments in the juvenile brain. Other markers are provided by specific transmitters such as

Fig. 2 Structural hallmarks of embryogenesis in vertebrates (a), insects (b), and flatworms (c). All panels show schematic cross-sections of embryos. Panels of *top row* show embryos shortly after gastrulation; *middle row* represents neurulation, *bottom row* organogenesis. For details, see text



FMRamide expressed in distinct compartments of the neuropile (Fig. 3d).

Macrostomum EST database: overview and phylogenetic aspects

The *Macrostomum* LT library was used to generate 7,680 expressed sequence tags (ESTs), sequenced from both directions. The LT library was chosen because in pilot analyses consisting of 100 random sequences, it was found to have the longest clone length (average of 1.5 kb), and the highest clone diversity of any of our *Macrostomum* libraries. Analysis of these 15,360 sequences through the bioinformatics protocol described above led to the discovery of 4,727 cDNA clones, and ~3,000 non-redundant sequences. Of these sequences, 68% showed similarity to the sequences available in Genbank and dbEST. The other 32% showed no similarity to any published gene sequence in these databases (Fig. 4). This percentage corresponds roughly to the results found in both *Schmidtea mediterranea* EST sequencing (Sánchez-Alvarado et al. 2002) and in the proteomes of *Drosophila*, *C. elegans*, and yeast (Rubin et al. 2000).

Based upon the most significant BLAST hit for each sequence, it was determined that 60% of *Macrostomum* sequences were most closely related to the corresponding deuterostome sequence. Only about 11% of the sequences showed highest similarity to lophotrochozoan sequences (Fig. 4). This result, although surprising, was also found in the EST data of *Schmidtea mediterranea* (Sánchez-Alvarado et al. 2002). These data could be attributed to either a close proximity of these two branches as proposed by Tyler (2001), or it could be attributed to the relative paucity of invertebrate sequences in the database (Sánchez-

Alvarado et al. 2002). Since the publication of the Smed EST database, multiple other invertebrate sequences have appeared, in which the skew toward vertebrate sequences is not as pronounced as in the *Schmidtea* and *Macrostomum* EST collection. To solve this fundamental phylogenetic problem, we plan to extend our EST project to include more *Macrostomum* sequences, and to compare them to EST collections from the acoels *Neochildia fusca* and *Convoluta pulchra* that are currently being prepared (in collaboration with Dr. K. Agata, Japan).

Macrostomum EST database: included genes and putative functions

Using the categories set forth in the gene ontology database (<http://www.geneontology.org>), and in the expressed gene anatomy database (EGAD, <http://www.tigr.org>), the sequences were organized into functional categories based upon their BLAST results.

As expected, a majority of these entries fell into the metabolism and structural gene categories (see Table 1 for the developmentally most interesting entries). These genes are predictably expressed at a higher level and/or ubiquitously and should, therefore, be overrepresented in the cDNA libraries used for the EST sequencing.

Cytoskeleton Our EST database includes the genes encoding alpha and beta-tubulin, several different actin genes, and several intermediate filament proteins, among them a cognate of neurofilament protein NF70. Intermediate filament proteins have been identified in a variety of invertebrates (e.g., Falkner et al. 1981; Wang et al. 2002; Karabinos et al. 2003), but they do not generally appear to assemble into the ultrastructurally conspicuous filaments

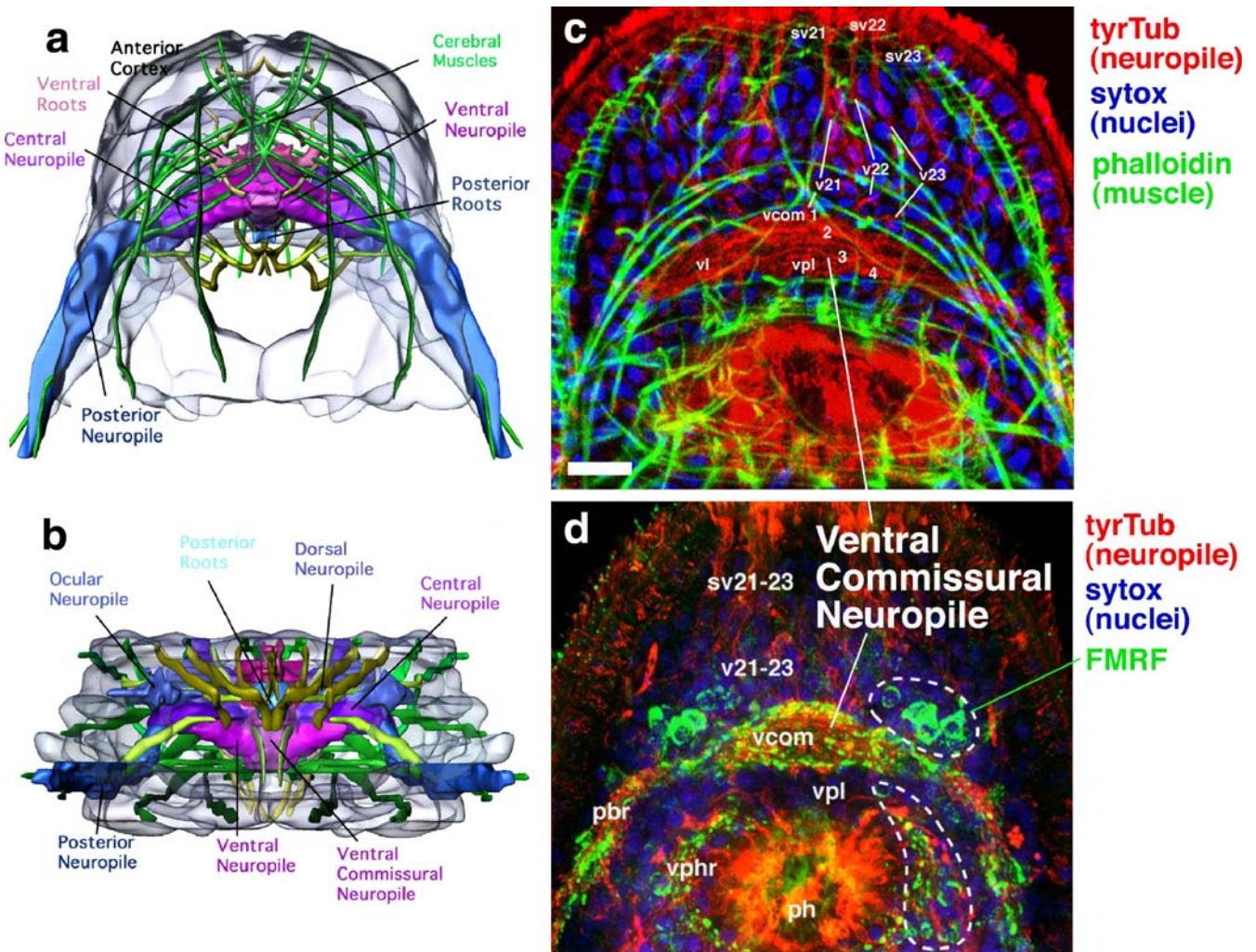


Fig. 3 Structure of juvenile *Macrostomum* brain. **a, b** 3D digital model of the *Macrostomum* brain in ventral view (**a**) and posterior view (**b**). The model is based upon immunohistochemical labeling of neuropile (anti-tyrosinated tubulin), muscles (phalloidin), and cell nuclei (sytox) as represented in panel **c**. In the model, muscle fibers are shown in *green*; parts of the brain neuropile in different variations of *red* and *blue*. The surface of the neuropile is rendered in *gray*. **c, d** Representative horizontal confocal sections (anterior to the *top*) of head of juvenile *Macrostomum*. In both panels, brain

neuropile and cilia of epidermal/pharyngeal cells are labeled in *red* by anti-tyrosinated tubulin; sytox was used as a global marker for nuclei (*blue*). In **a**, muscles are labeled by phalloidin (*green*); in **b**, *green* label visualizes subset of neurons expressing FMRFa-mide. Abbreviations: *pbr* posterior brain, *ph* pharynx, *sv21-23* and *v21-23* ventral anterior roots of neuropile, *vcom 1-4* ventral commissures, *vl* ventro-lateral neuropile compartment, *vphr* ventral pharyngeal nerve ring, *vpl* ventro-posterior roots of neuropile. Scale bar: 10 μm (for **c, d**)

typical of vertebrate cells (e.g., Weaver and Viancour 1991). Recent evidence (e.g., Gregory and Brown 1998; Lee et al. 2000) shows that proteins associated with intermediate filaments in vertebrates bind to microfilaments and microtubules in *Drosophila*, suggesting that the components of the intermediate filament cytoskeleton may have evolved from elements of the phylogenetically more ancient tubulin and actin cytoskeleton.

Addressing this issue in platyhelminths may shed some new light on the evolution of intermediate filaments. Of great interest are also the *Macrostomum* homologs of genes encoding structural proteins that bind to actin or tubulin and modulate the structure of the cytoskeleton. These proteins promote polymerization and depolymerization (profilin, cofilin), bundle actin filaments (fimbrin),

break down filamentous actin (gelsolin), modulate the anchoring of the cytoskeleton to the membrane, or act as motor proteins (myosin, tropomyosin, dynein, kinesin). Insight from model systems makes it abundantly clear that the orchestrated expression of these structural proteins, in specific cell types at specific developmental stages, largely controls morphogenesis.

Cell membrane: adhesion, cell–cell interaction, channels and carriers Beside the cytoskeleton, membrane proteins that mediate cell contacts constitute a major driving force of morphogenesis. The MacEST database includes a number of cadherins, mostly of the “non-classical” (catenin-independent) sort (cadherin 87A, dachsous 2, protocadherin X), as well as components of gap junctions

Table 1 List of developmentally important cognates of EST sequences isolated from *Macrostomum lignano*Cognates of EST sequences isolated from *Macrostomum lignano*

Cytoskeleton	
ANGU1043	Actin 2
ANGU1123	Actin 3
ANGU4214	Myosin-1F
ANGU4083	Myosin VIIa
ANGU694	Myosin cI II heavy chain
ANGU1943	Brush border myosin IB
ANGU1029	Myosin
ANGU2833	Tropomyosin 1
ANGU3072	Thymosin
ANGU1067	Putative fimbrin
ANGU1409	Cofilin
ANGU1712	Gelsolin
ANGU6869	Plectin
ANGU7670	Smoothelin
ANGU4956	Profilin 1B
ANGU4464	Suppressor of profilin
ANGU2069	SDA1 domain containing 1
ANGU4503	ARP 2/3 complex subunit 3
ANGU505	WAS protein fam., memb.2
ANGU5172	Actin interacting protein 1
ANGU5205	Huntingtin interacting prot 1-rel.
ANGU5252	Ciboulot (beta-thymosin)
ANGU6213	N-WASP protein
ANGU6646	Actin-binding prot. fragmin P
ANGU1210	Body wall muscle prot. HR-29
ANGU1014	Alpha-tubulin
ANGU1078	Beta-1-tubulin
ANGU1226	Kinesin-associated protein 3
ANGU1833	Tektin C1
ANGU2030	Axonemal dynein lt chain p33
ANGU2105	Sim. to Dynein interm. chain 2
ANGU1449	Minus agglutinin
ANGU2037	Shippo 1
ANGU1567	Intermediate filament b
ANGU1597	Neurofilament protein NF70
ANGU3569	Lamin
ANGU7330	Cytopl. interm. filament prot.
ANGU7488	Keratin associated prot. 4.15
ANGU968	Growth arrest-specific 8
Membrane: adhesion/cell-cell and cell-matrix interaction	
ANGU4245	Cadherin 87A precursor
ANGU430	Protocadh. gamma B2-alpha C
ANGU7019	Dachsous 2 protocadherin
ANGU2237	Protocadh. gamma subfam. C, 3
ANGU2854	Putative protocadherin X
ANGU6318	<i>Drosophila</i> discs large
ANGU1465	Annexin A13
ANGU1677	Pannexin
ANGU4432	Innexin
ANGU1630	Caveolin-3
ANGU2274	Armadillo repeat cont. 3
ANGU2473	Lethal giant larvae homolog 2
ANGU4806	Deleted in polyposis 1-like 1

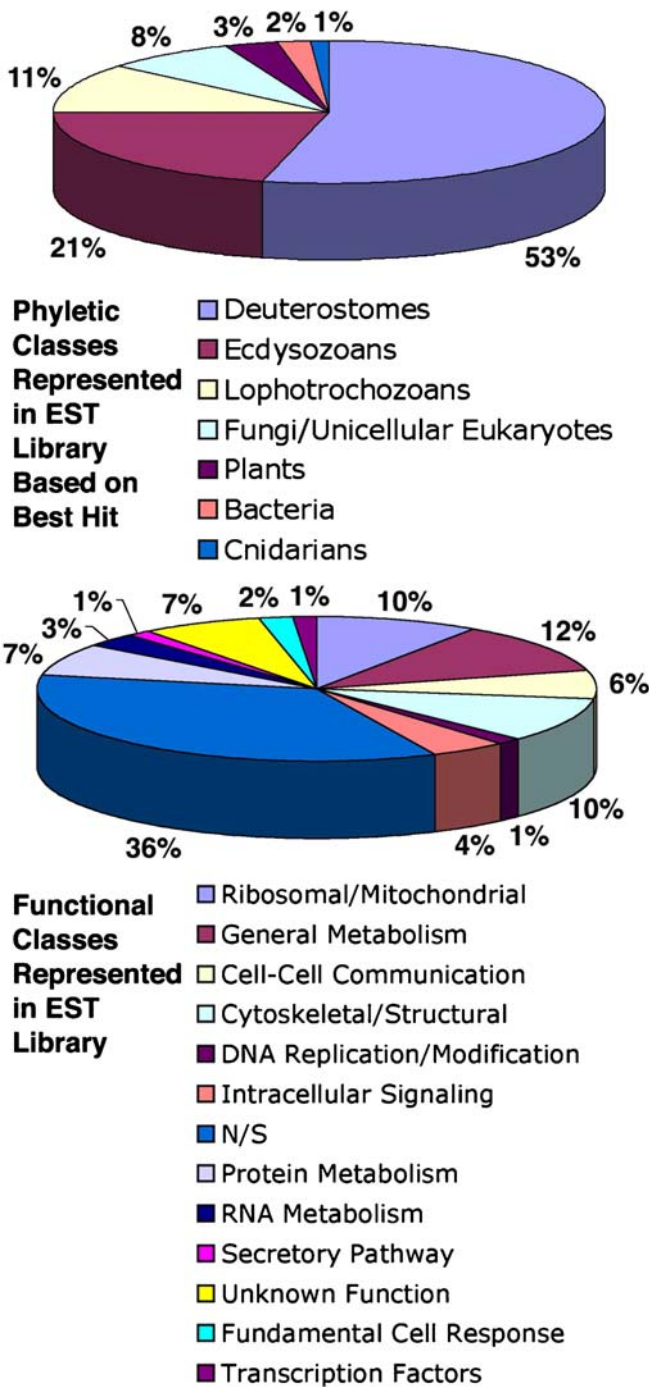


Fig. 4 Diagrams showing the diversity of ESTs in MacEST-DB. **a** Pie chart showing the percentage of best-hit matches for the corresponding animal group. **b** Pie chart showing the percentages of *Macrostomum* cognates of genes belonging to defined functional classes

(innexin, pannexin), and Ig-like adhesion molecules known from the vertebrate nervous system (gliotactin, contactin). Adhesion molecules form part of the membrane-integral protein complex that links the cytoskeleton inside the cell with the extracellular matrix and surrounding cells. Adhesion protein complexes typically form distinctive complexes that ultrastructurally appear as cellular junctions. Cadherins are concentrated in adherens

Table 1 (continued)Cognates of EST sequences isolated from *Macrostomum lignano*

ANGU4830	Retinitis pigmentosa 1-like prot. 1
ANGU5835	Sim. to beta-amyloid bind. Prot.
ANGU6166	Presenilin-like protein 3
ANGU3273	MSF: megakaryocyte stim. factor
ANGU3373	Hematopoietic stem cells 176
ANGU6443	Macrophage expressed gene 1
ANGU6989	Egg receptor for sperm
ANGU2810	nrv3
ANGU3466	SCO-spondin
ANGU4406	VIT
ANGU7084	Flotillin 2
ANGU1682	Otoferlin
ANGU2150	Troponin I
ANGU4383	Calmodulin
ANGU6460	Piccolo protein (Aczonin)
ANGU4777	Tetraspanin 74F
ANGU1235	Tetraspanin 66E
ANGU1375	Neurofascin precursor
ANGU5287	Him-4
ANGU6074	Contactin 6
ANGU6553	Neuroigin 2
ANGU1128	Paxillin-like protein
ANGU7567	Gliotactin
ANGU7098	Anterior pharynx defective 1
ANGU4979	Immediate early prot. Hom.
ANGU1470	Radial spokehead
ANGU798	Kangai 1
Cell membrane: channels and carrier proteins	
ANGU1322	Potassium channel Kv3.2
ANGU4364	Ca activ. chloride channel 1
ANGU4839	ATP dep. transmembr. transp.
ANGU5291	Na ⁺ /K ⁺ -ATPase alpha-subunit
ANGU6506	Acetylcholine transporter
ANGU6801	Glutamate transporter GLT1
ANGU7339	Vibrator
ANGU978	Glycine transporter type 2b
ANGU468	Na pump α subunit
ANGU4677	Odorant binding protein
ANGU6260	Ferritin
ANGU1390	Sorting nexin 5
Extracellular matrix and protein degradation	
ANGU4567	Collagen a1(I)
ANGU1115	Similar to alpha-3 collagen VI
ANGU1213	Similar to papilin
ANGU1349	Matrilin-3 precursor
ANGU2469	Ficolin 3
ANGU3084	Neurocan
ANGU3492	Rh type B glycoprotein
ANGU4064	Myelin Protein (P2)
ANGU453	Versican core protein
ANGU3498	Proteasome alpha 3 subunit
ANGU3895	ADAMTS-like protease
ANGU2238	Metalloproteinase 2
ANGU2945	Cathepsin B

Table 1 (continued)Cognates of EST sequences isolated from *Macrostomum lignano*

ANGU1011	Cathepsin L1
ANGU1104	Cathepsin L
ANGU3111	Calpain
ANGU3181	Cystatin
ANGU1580	Carboxypeptidase A
ANGU1897	Alp1
ANGU1076	Tequila
ANGU1936	Legumain precursor
ANGU2132	Midgut serine proteinase-3
ANGU2356	Sim. to neurolysin
ANGU2481	Effete
Signaling mechanisms: signals	
ANGU1314	TOL-1/takeout
ANGU2050	Secr. frizzled-rel. prot.-2
ANGU2974	Angiopoietin-like 2
ANGU3431	Myomodulin neuropept. Prec.
ANGU4026	XWnt-11
ANGU1679	GABA
ANGU1075	Temptin
ANGU5228	Angiopoietin-like protein 4
ANGU878	Skin secretory protein xP2 precursor
Signaling mechanisms: receptors	
ANGU1562	Acetylcholine receptor
ANGU5915	Neur. nicotinic ACh rec.
ANGU3791	GABA-BR1b receptor
ANGU3184	VEGFR-1
ANGU5871	Insulin-like growth fact. Recept.
ANGU3811	Nuclear Hormone Receptor
ANGU1633	Frizzled-related protein
ANGU6130	Notch receptor protein
ANGU3459	Notch 2
ANGU6785	Toll-6
ANGU4616	Kremen1
ANGU792	Roundabout 1
ANGU1648	Laminin receptor 1
ANGU3916	Thrombospondin
ANGU4480	Lin-9 homolog
ANGU4506	Neurexin
ANGU1972	Endocytic receptor Endo180
ANGU2190	Tyrosine kinase receptor
ANGU1134	HLA-B
ANGU1493	Purinergic Receptor P2X4
ANGU4833	Similar to XMAP4
ANGU788	LAG seven-pass G-type rec.1
Signaling mechanisms: modulators (?)	
ANGU1240	Acetylcholinesterase prec.
ANGU1446	Xolloid
ANGU2139	Fringe
ANGU2178	Formin 2
ANGU2324	Similar to Spred-2
ANGU2559	Calbindin 53E
ANGU2754	Muscle LIM protein
ANGU4212	Small optic lobes
ANGU4897	Hippocalcin like-1

Table 1 (continued)Cognates of EST sequences isolated from *Macrostomum lignano*

Signaling mechanisms: transducers	
ANGU2235	RAS
ANGU1037	Rho2 GTPase
ANGU1124	RAS-like protein
ANGU1735	Guanine-nucl.-releas.prot.
ANGU1788	c-Jun protein
ANGU1451	RACK G-protein
ANGU1378	Dreadlocks
ANGU1571	TOLLIP ;Toll-interact. Prot.
ANGU2092	T-complex expr. gene 1
ANGU3304	ced-6
ANGU4868	MAPK organizer 1
ANGU5343	Notchless protein
ANGU5858	G protein-bind. Prot. CRFG
ANGU1063	TNF rec.-assoc. fact. 5
ANGU5888	Progr. cell death 6 interact. prot.
ANGU6503	Tetraspan NET-5
ANGU6690	PKCq-interact. Prot. PICOT
ANGU6834	Putative ankyrin-kinase
ANGU7002	Diacylglycerol kinase iota-1
Protein kinases and phosphatases	
ANGU4140	cdc-1 related kinase
ANGU1554	cdc-2 related kinase
ANGU6611	cdc-5
ANGU2540	Src tyrosine kinase 1
ANGU2885	Serine/threonine protein kinase
ANGU3198	Adenylate kinase
ANGU3573	Casein kinase 1
ANGU3646	cAMP-dependent prot. kinase
ANGU3919	Protein kinase C-rel. kinase
ANGU4762	Ca/calmodulin-dep. prot. Kin. II
ANGU5852	MAPK kinase kinase 13
ANGU4381	MAPK/ERK kinase 5
ANGU6149	Calmyrin
ANGU7232	p53-rel. prot. kinase
ANGU7481	Slowpoke binding protein
ANGU7644	Target of rapamycin (Tor)
ANGU793	0 insulin receptor-rel. precursor
ANGU1408	ser/thre prot. phosphatase
ANGU1678	Drosophila retinal degraded. C
ANGU8023	Protein phosphatase 1
Transcription factors	
ANGU1217	Bicaudal
ANGU1281	TBRAIN
ANGU4510	Groucho
ANGU4924	Slouch
ANGU5895	Smad4
ANGU1783	Dorsal
ANGU1992	Limpet
ANGU2019	RREB-1
ANGU2295	STAT2
ANGU2717	Yan
ANGU7660	Kruppel-like factor 1
ANGU774	LIM domain only 4

Table 1 (continued)Cognates of EST sequences isolated from *Macrostomum lignano*

ANGU2720	LAG1 longevity assur. hom 2
ANGU1361	Disco-interacting protein 2
ANGU2348	Endothelial diff.-rel. fact. 1
ANGU1138	Wolf-Hirschhorn syndr.cand. 1
ANGU3912	FOXJ3 protein
ANGU6710	C-Rel proto-oncogene protein
ANGU7189	EGF-response factor 2
ANGU1786	Elongation protein 3 homolog
ANGU1933	Spatial-beta protein
ANGU1501	Pur-#945
RNA-binding and RNA-processing proteins	
ANGU7606	Piwi
ANGU3256	Vasa
ANGU1031	ELAV
ANGU1563	XNop56
ANGU5076	Platelet-endoth. tetraspan 3
ANGU7121	Prol-rich Vg1 mRNA-bind.prot.
ANGU2816	DAZ-associated protein 1
ANGU4194	Boule
ANGU2566	Ddx21 protein
ANGU2735	Nucleolar RNA helicase II
Chromatin-associated proteins	
ANGU3150	Histone H1
ANGU1132	Hhistone H3.3
ANGU3668	Histone H2 A.F/Z
ANGU1239	Histone deacetylase
ANGU4299	Histone-binding prot. N1/N2
ANGU4623	Retinoblastoma bind. prot. 7
ANGU3479	Retinoblastoma bind. prot.4
ANGU4714	SIR2-like hist. deacetylase
ANGU4803	MYST histone acetyltransf. 3
ANGU5132	Telomerase-assoc. prot. TP-1
ANGU6212	Superfam. I DNA/RNA helic.
ANGU6333	Suppressor of variegation 205
ANGU2006	Dorsal switch protein 1
ANGU4433	Skippy pol polyprotein
ANGU7238	Chromatin assembly factor 1
ANGU7833	Mariner transposase
ANGU8000	Sirt6
Cell cycle control	
ANGU2876	Cyclin B1
ANGU6499	Cyclin I
ANGU6724	Cyclin M4
ANGU4217	Pescadillo
ANGU2022	Lesswright
Apoptosis	
ANGU3391	Caspase-3
ANGU1141	TSARG2
Immune response	
ANGU1056	Tep-II
ANGU1083	GliPR
ANGU2865	XII secret. phospholipase A2
ANGU6330	Macroph. Migrat. Inhib. factor
ANGU7103	GLI pathogenesis-related 2

junctions, forming a link between membrane and microfilaments (Tepass et al. 2000). Recent investigations have placed neurexin, neurofascin, gliotactin and Na⁺/K⁺ ATPase (all of which are represented in the Mac EST database) in the septate junction, the invertebrate counterpart of tight junctions in vertebrates (Lord and DiBona 1976; Lane 1991; Genova and Fehon 2003). Junctions in basal bilaterians have just begun to be studied. Platyhelminths as well as non-bilaterian phyla possess both adherens junctions and septate junctions. Structurally and developmentally, these junctions differ significantly from their counterparts in arthropods or vertebrates (Hartenstein and Ehlers 2000), which may convey crucial differences in adhesive and dynamic properties upon the tissues in which they appear. Further investigation of the adhesive proteins encoded by genes represented in the Mac EST database will allow one to address these issues experimentally.

Extracellular matrix The third and final class of structural molecules with eminent morphogenetic functions are the secreted proteins that assemble into the extracellular matrix (ECM). The ECM provides the immediate micro-environment to which embryonic cells attach and through which they migrate. Representatives of several classes of ECM proteins (collagens, papilin, neurocan) and ECM modulating enzymes (metalloproteinase 2, ADAMTS-like protease) were identified in the Mac EST project. The isolation of a cognate of myelin protein P2 may provide a tool to address the issue of the origin of glial cells. Myelin-producing glial cells are abundant in vertebrates, but constitute rare exceptions in invertebrates (Radojcic and Pentreath 1979). Glial cells in arthropods, for example, enwrap bundles of axons, but generally do not form concentric sheaths that consolidate into myelin. On the other hand, fast-conducting myelinated axons have been observed in some crustaceans (Lenz et al. 2000). It will be informative to investigate the distribution of *Macrostomum* myelin protein, given that cells with the morphological properties of glial cells have not been identified in flatworms.

Signaling pathways, transcription factors, and RNA-binding proteins

For the purpose of identifying markers for specific cell types, genes falling into these classes are obviously the best candidates. On the other hand, because of their restricted expression, the transcript levels are typically low, which selects against their appearance in EST projects. Our Mac EST collection includes a fair-sized number of putative “developmental regulators”. We identified 61 cognate transcription factors, 47 receptors, and 51 signaling transduction molecules (see Table 1 for developmentally most interesting entries). Among the signals are Xwnt 11 and a secreted frizzled-like protein, as well as a putative neuropeptide precursor and two growth factors (interestingly, similar to vertebrate angiopoietin that is related in the development of the blood/vascular system, an organ not

existent in flatworms). Receptors include several neurotransmitter receptors (acetylcholine and GABA), growth factor receptors (insuline-like growth factor receptor and VEGFR, the latter involved in angiogenesis and hematopoiesis in vertebrates; Hicklin and Ellis 2005), extracellular matrix receptors (laminin receptor, thrombospondin) and receptors for “morphogenetically active” ligands (Notch, Notch2, Frizzled-related protein, Toll-6, Kremen 1, Roundabout 1). A relatively large number of signal transducers and protein kinases in general (Ras, Rho2GTPase, RACK, c-Jun, Toll-interactin protein, cdc-1, cdc-2, cdc2-5, Src-1, Casein kinase 1, MAPKKK 13, p53-related kinase among them) and modulators of signaling (Fringe, Formin 2, Xolloid among them) could also be identified.

Transcription factors as the downstream targets of signaling pathways have played a central role in the analysis of development in model organisms. Activation of a cell-type-specific-transcription factor often constitutes the first step in the developmental pathway that leads to the differentiated cell. Even more, in some cases, the dynamic early expression pattern of a transcription factor can give information about “developmental fields” or “equivalence groups”. For example, the proneural genes first discovered in *Drosophila*, but since then isolated from all other model systems as well, are initially expressed in a so-called “proneural cluster”, a cell population that is competent to become neural progenitor (Campos-Ortega 1995; Bertrand et al. 2002). Subsequently, expression of the proneural gene is restricted to a single or a few cells within the proneural cluster (often by means of Notch signaling), and these remaining proneural-gene positive cells are specified as definitive neural progenitors (Campos-Ortega 1995). A similar expression behavior as that of proneural genes has been shown for transcription factors acting in other lineages, such as GATA factors in endoderm and blood lineages (Huber and Zon 1998; Shivdasani 2002).

The Mac EST database includes cognates of known transcription factors; a short list is provided in Table 1. A number of additional transcription factors included in the list were identified by a PCR approach. Among these are three *Sine oculis/Six* family members (eye development; Oliver et al. 1995; Pichaud and Desplan 2002), *Glass* and *Vsx-1* (both required in the visual system of flies and vertebrates; Liu et al. 1994; Liu and Friedrich 2004), *Mef-2* (muscle and nervous system; Black and Olson 1998; Heidenreich and Linsemann 2004), and *Brn1/3* (nervous system; Schonemann et al. 1998). In the EST collection, we identified several cognates corresponding to the well-described targets of widely active signaling pathways, such as Dorsal, Stat-2, Smad 4, or Yan. The collection also contains additional putative nervous-system-specific cognates, such as T brain. Groucho is a co-factor interacting with bHLH transcription factors (e.g., those encoded by the proneural genes) and are involved in Notch signaling (Fisher and Caudy 1998).

Finally, three highly promising cognates of genes encoding RNA-binding proteins were found: *ELAV*, *vasa*, and *piwi*. *ELAV* is a widely expressed factor in the nervous system of vertebrates and invertebrates (Perrone-Bizzozero

and Bolognani 2002). *Vasa* and *piwi* are highly specific markers for germline cells in *Drosophila*, *C. elegans*, and some vertebrates (Ikenishi 1998; Bosch 2004).

Web-based Mac EST database We have deposited the full complement of Mac EST sequences in a publicly accessible database (<http://www.macest.biology.ucla.edu/macest/>). Besides links to relevant papers on the development, structure, and ecology of *Macrostomum lignano*, the website will have engines that provide access to the following: (1) DNA sequence; (2) NCBI accession number; (3) database accession number; (4) information on the top 10 BLASTx hits including gene name, organism, *E* value, and accession number; (5) information on the clones from which the EST contig was derived; and (6) expression data (if present). The website will also be linked to the NCBI site so that further queries can be directed to a larger database.

Analysis of gene expression in the developing brain

One of our major objectives for generating the EST database was to generate a pool of genes that would allow us to reconstruct embryogenesis, and in particular neurogenesis, in a basal platyhelminth. On one hand, one needs specific markers that will visualize the origin and morphogenesis of neuronal lineages. This has proven to be essential for all developmental models (including vertebrates and *Drosophila*), but is even more important in flatworms where the early embryo lacks overt morphological landmarks. Furthermore, gene expression patterns, aside from their role as markers, can be compared across phylum boundaries, which will help to define the function of the corresponding genes.

Based on conserved sequence analysis, we would expect many ESTs of the present collection to be markers for specific cell lineages, including various subsets of the nervous system. Aside from the regular whole-mount in situ hybridization protocol using alkaline phosphatase, we have adapted a protocol that allows for a combined immunohistochemistry and in situ hybridization labeling for use in adult and juvenile *Macrostomum* (see **Materials and methods**; Fig. 5). Thus far, we have identified multiple ESTs as tissue-specific markers. Genes such as the one encoding a gut cholinesterase specifically labels gastrodermal cells (Fig. 5b); a probe against synaptotagmin labels the entire nervous system (Fig. 5a); Mef-2 appears in muscles and the nervous system (not shown).

Fluorescent probes were generated for the *Macrostomum* homolog of the homeobox genes *lin11/lim1* and *six3/optix*. Lin-11/Lim-1 is a Lim domain and homeodomain containing transcription factor known to be involved in a wide variety of developmental processes across metazoa. In *C. elegans*, *lin11* is required for proper ectodermal cell lineage asymmetry as well as proper olfactory, chemosensory, and thermosensory neuron differentiation (Hobert and Westphal 2000; Sarafi-Reinach et al. 2001). Vertebrate *Lim-1* is expressed and required in the developing CNS

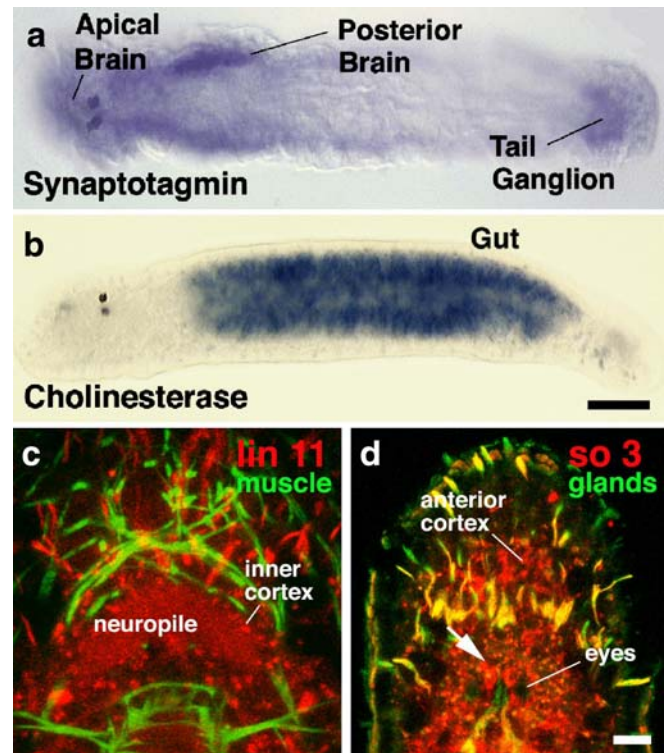


Fig. 5 Representative in situ hybridization results for ESTs found in MacEST-DB. **a** Expression data for *synaptotagmin* (ANGU3678), homologs of which are concentrated at presynaptic sites in the nervous systems of other animals. Note the pan-neural expression of the clone. This is a dorsal view with the anterior to the left. **b** Expression data for cholinesterase (ANGU1277). Note the expression in a subset of gastrodermal cells. This is a lateral view with the anterior to the left. **c** In situ hybridization/immunohistochemistry results for *Mac lin11* and anti-Mmu4 (Ladurner et al. 2005a,b). *Lin-11* is expressed in various parts of the brain, while Mmu-4 labels all muscles. The anterior of the preparation is at the top. **d** In situ hybridization/immunohistochemistry results for *Mac so3/optix*, expressed in subset of neurons in the anterior brain cortex. Highest expression level is seen in cells medially adjacent to the rhabdomic eyes (arrow). Green/yellow label is due to FITC fluorescent secondary antibody that unspecifically labels glands

and kidney (Taira et al. 1994; Shawlot and Behringer 1995; Carroll and Vize 1999). *Macrostomum lin11* is roughly 2 kb, and contains both LIM domains and a homeodomain. Expression was found in the anterior part of the brain cortex. In addition, *Mac lin11* appears in the gonads of *Macrostomum*, a pattern not shared with other animals, except for the bivalve mollusk (Torrado and Mikhailov 2000). This may suggest that *lin11* function in the gonads may be a lophotrochozoan novelty.

All three subfamilies of the *Six* family of homeobox genes were identified in *Macrostomum*. *Macrostomum six* fragments showed significant similarity to their specific subfamilies, and in phylogenetic analysis grouped with their specific subfamily (Bebenek et al. 2004). The *six1/sine oculis* fragment was elongated, yielding a 2-kb full-length gene. *Mac so* is expressed in the anterior cortex of the brain surrounding the pharynx, in both the testes and ovaries, and putatively in the gut (Fig. 5d). The localization of *Mac so* in the brain is reminiscent of the brain expression

of *sine oculis* in *Drosophila* (e.g., Chang et al. 2001) and *Six1/2* in vertebrates (Kawakami et al. 2000), but is quite expanded. Expression of *Mac so* is particularly strong in a small group of cells around the pigmented eyes (Fig. 4d, arrow), similar to the conserved localization in protostome eye primordia (Pineda et al. 2000; Arendt et al. 2002).

Combined with similar projects of EST datasets and genome sequencing projects, the *Mac* EST presented in this paper will provide a useful tool with which to approach development of a basal bilateral organism. Not only will sequence data be useful in resolving phylogenetic questions, but they will also facilitate the generation of specific probes with which to address questions of cell origin, cell fate specification, and morphogenesis.

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