ORIGINAL ARTICLE

Hau-Pax6A expression in the central nervous system of the leech embryo

Ian K. Quigley · Xuanhua Xie · Marty Shankland

Abstract The leech *Helobdella* sp. (Austin) has two genes of the Pax6 subfamily, one of which is characterized in detail. *Hau-Pax6A* was expressed during embryonic development in a pattern similar to other bilaterian animals. RNA was detected in cellular precursors of the central nervous system (CNS) and in peripheral cells including a population associated with the developing eye. The CNS of the mature leech is a ventral nerve cord composed of segmental ganglia, and embryonic *Hau-Pax6A* expression was primarily localized to the N teloblast lineage that generates the majority of ganglionic neurons. Expression began when the ganglion primordia were four cells in length and was initially restricted to a single cell, $n_s.a$, whose descendants will form the ganglion's anterior edge. At later stages, the *Hau-Pax6A* expression pattern expanded to include additional CNS precursors, including some descendants of the O teloblast. Expression persisted through the early stages of ganglion morphogenesis but disappeared from the segmented body trunk at the time of neuronal differentiation. The timing and iterated pattern of *Hau-Pax6A* expression in the leech embryo suggests that this gene may play a role in the segmental patterning of CNS morphogenesis.

I. K. Quigley · X. Xie · M. Shankland (✉)
Section of Molecular Cell and Developmental Biology,
University of Texas at Austin,
Austin, TX 78712, USA
e-mail: hastypig@mail.utexas.edu

I. K. Quigley · X. Xie · M. Shankland
Institute of Cellular and Molecular Biology,
University of Texas at Austin,
Austin, TX 78712, USA

Introduction

The leech CNS has been used to study numerous topics in development and physiology (Kristan et al. 2005). The stereotyped cell lineage of the leech embryo is well characterized (Stent et al. 1992), and phenomena such as neurogenesis (Baptista et al. 1990), axonal pathfinding (Baker and Macagno 2000; Venkitaramani et al. 2004), synaptogenesis (Johnson et al. 2000; Marin-Burgin et al. 2005), and neuronal cell death (Macagno and Stewart 1987; Shankland and Martindale 1989) have been examined in detail. The morphogenesis and segmentation of the central nervous system (CNS) have also been investigated using cellular methodologies (Blair 1982; Stuart et al. 1989; Torrence et al. 1989; Ramírez et al. 1995; Shain et al. 1998, 2000), but little is known about the regulation of CNS morphogenesis at the molecular level.

In the leech, the mature CNS is composed of a segmented ventral nerve cord connected at its anterior end to an unsegmented supraesophageal ganglion (Stent et al. 1992). The segmented tissues arise from a defined set of large, embryonic stem cells called 'teloblasts' (Weisblat and Shankland 1985), each of which undergoes a series of highly asymmetric stem cell divisions to produce a linear column of primary blast cell daughters (Fig. 1). One bilateral pair of M teloblasts generates the mesodermal germ layer, and four bilateral pairs of N, O, P, and Q teloblasts generate the ectoderm. Blast cells are designated by the same letter as their parent teloblast in lower case and function as segmental founder cells for that teloblast lineage.

Early in gastrulation, the five ipsilateral blast cell columns merge to form a germinal band in which the N, O, P, and Q lineages are arrayed in ventral-to-dorsal order over the surface of the M lineage (Fig. 1, inset). The oldest blast cells are the first to enter the germinal band and will eventually contribute their clones to the anteriormost body segments (Fig. 1). As the blastopore closes, the right and left germinal bands meet along the ventral midline and fuse to form the germinal plate (Fig. 1). The ganglia of the CNS develop along this midline.

The segmentation of the leech CNS is implicit in the division pattern of the teloblastic stem cells (Weisblat and Shankland 1985). For example, each N teloblast generates an alternating sequence of daughters designated n_s and n_f blast cells (Bissen and Weisblat 1987). Each n_s cell generates a descendant clone of 70–80 neurons that populate the anterior quadrant of a segmental ganglion or neuromere (Shain et al. 1998). The n_f cell produced by the next N teloblast division generates a descendant clone of similar size that primarily populates the posterior quadrant of that same hemiganglion. Together, the right and left N lineages account for roughly two thirds of the central
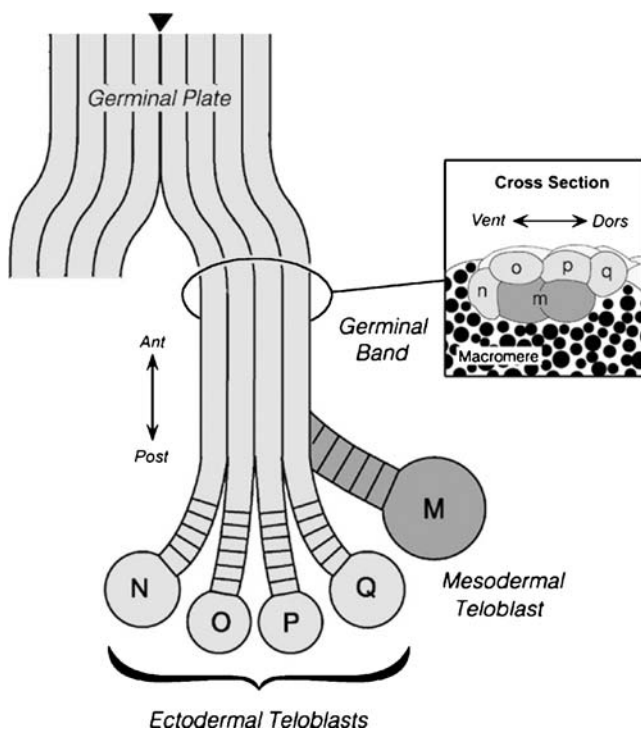
Fig. 1 Schematic overview of the formation of the leech germinal band. The M, N, O, P, and Q teloblasts are a set of bilaterally symmetric embryonic stem cells, here shown for only one side. Each teloblast undergoes an iterated sequence of highly asymmetric cell divisions, producing a linear column of primary blast cell daughters. The five ipsilateral columns merge in parallel to form the germinal band. The individual teloblast lineages adopt specific positions in the band, with the ectodermal N, O, P, and Q lineages arrayed in the ventral-to-dorsal order over the surface of the mesodermal M lineage (*inset*). The right and left germinal bands fuse along the ventral midline to form the germinal plate. The midline is marked with an *arrowhead*

neurons, with additional neurons migrating into the ganglion from the other teloblast lineages. Unlike the N and Q teloblasts, the M, O, and P teloblasts each generate only one blast cell per segment (Weisblat and Shankland 1985).

The developmental cues required for ganglion formation have been studied with embryonic cell ablation. Unilateral ablation of an M teloblast leads to irregular segmentation on the ipsilateral side of the CNS (Blair 1982; Torrence et al. 1989). This ablation eliminates only a few neurons and is thought to influence gangliogenesis by removing a positional framework of mesoderm-derived cues that guide the neural sublineages to appropriate locations (Torrence et al. 1989). A similar although milder disruption of CNS segmentation is produced by knocking down the expression of the *Hau-Pax3/7A* gene in the segmental mesoderm (Woodruff et al. 2007), suggesting that this transcription factor may be involved in the generation and/or deployment of such cues.

Other studies have suggested that the N lineage coordinates its own segmental morphogenesis autonomously. For instance, certain aspects of gangliogenesis still occur in the absence of the mesoderm (Shain et al. 2000). In addition, unilateral ablation of an N teloblast lineage disrupts ganglion formation (Blair and Weisblat 1982; Stuart et al. 1989), and the ablation of a single n_f cell prevents the formation of an interganglionic fissure (Ramírez et al. 1995; Shain et al. 2000). Early reports suggested that the leech's *engrailed* gene might play a critical role in the formation of this fissure (Ramírez et al. 1995), but subsequent work suggests that this is unlikely to be the case (Shain et al. 2000).

We here report the identification of two Pax6 genes from the leech *Helobdella* sp. (Austin) and show that *Hau-Pax6A* is primarily expressed in the N teloblast lineage. Genes of the Pax6 subfamily encode transcription factors with two discrete DNA-binding domains: an upstream paired domain and a downstream homeodomain (Balczarek et al. 1997). Pax6 genes are best known for their widely conserved role in eye development (Callaerts et al. 1997) and have also been implicated in the development of the CNS (Stoykova et al. 1997; Kammermeier et al. 2001; Callaerts et al. 2001). In leech embryos, *Hau-Pax6A* is expressed in a cell population associated with the developing eye primordium and also displays a segmentally iterated expression pattern that precedes and may contribute to the morphological segmentation of the CNS.

Materials and methods

Animals

Embryos were taken from a laboratory breeding colony established in 1997 with leeches collected in Austin, TX. This population was originally described as *H. robusta* on the basis of morphology (Seaver and Shankland 2000), but

molecular phylogenetic analysis suggests they are a distinct and as yet unnamed species (Bely and Weisblat 2006), referred to here as *Helobdella* sp. (Austin).

Adult leeches were maintained in 1% artificial seawater and fed on pond snails collected at the University of Texas Brackenridge Field Laboratory. Embryos were raised at 24°C in a defined saline and staged according to the Stent et al. (1992) system for *H. triserialis.*

Identification and cloning of genes

Hau-Pax6A was isolated from the cDNA by the polymerase chain reaction (PCR) using degenerate primers targeted to conserved amino acid sequences (VSNGCV and FAWEIR) in the paired domain. We extended the mRNA sequence in the 5′ and 3′ directions by the rapid amplification of cDNA ends (RACE) technique, using the Gene Racer kit and gene-specific primers on cDNA synthesized at 63°C with Thermoscript reverse transcriptase (Invitrogen). Primer sequences are available upon request.

A partial transcript *Hau-Pax6B* was isolated from the cDNA using gene-specific primers predicated on a second Pax6 homolog identified from genome sequence traces of the leech *H. robusta*, available at http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml.

The phylogenetic relationship of the leech Pax6 genes was examined with PAUP 4.0b10.

RNA analysis

The size of the *Hau-Pax6A* mRNA was determined by gel electrophoresis and Northern blotting. Total RNA from stage 8–10 embryos was isolated with Tri-Reagent (Molecular Research Center), run on a 1.5% agarose denaturing gel, transferred to nylon, and hybridized overnight with a 1.0-kb digoxigenin (DIG)-labeled riboprobe complementary to Nt 533–1,489. Hybridization was detected by alkaline phosphatase immunostaining using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates.

Accumulation of RNA during embryogenesis was measured with reverse transcription PCR (RT-PCR). Embryos were collected at various times after egg deposition, and maturing oocytes were dissected from the ovary of a gravid adult. For each developmental stage, total RNA was extracted with the RNAqueous kit (Ambion) and reverse transcribed using random primers. A region of the *Hau-Pax6A* transcript (Nt 533–772) was then amplified using gene-specific primers, and an 18 S rRNA sequence was amplified in parallel as a loading control (QuantumRNA, Ambion).

Cellular distribution of RNA was examined by whole mount in situ hybridization (Nardelli-Haefliger and Shankland 1992). DIG-labeled sense and antisense riboprobes were generated from the 1.0-kb clone used for northern blot analysis, and hybridization was visualized with the same reagents. Stained embryos were either cleared in glycerol or dehydrated through an ethanol series and cleared in a 3:2 mixture of benzyl benzoate/benzyl alcohol. Some specimens were embedded in POLYBed 812 plastic (Polysciences) and handcut by razor blade into 0.1-mm sections. All specimens were imaged with a Diagnostic Instruments Spot CCD camera.

Lineage tracing

To trace cell lineages, identified blastomeres were microinjected with a 10-kDa tetramethylrhodamine- or fluorescein-dextran-amine (Molecular Probes) at 50 mg/ml, with 2% Fast Green FCF added to monitor injection volume. Injected embryos were counterstained with 2.5 μg/ml Hoechst 33258 and viewed on a Nikon E800 fluorescence microscope.

Results

Gene isolation

A novel Pax6 gene was identified in *Helobdella* sp. (Austin) by degenerate RT-PCR, and the two ends of the gene product were amplified by RACE. The RACE products predicted a 3,520-bp mRNA with a polyA tail, and Northern blots of embryonic RNA revealed a single band of roughly this size (Fig. 2a). We designated this gene *Hau-Pax6A* and deposited a consensus cDNA sequence in GenBank under accession no. EF375876.

After the isolation of *Hau-Pax6A*, we identified two Pax6 homologs in the online genome sequence of the closely related leech *H. robusta*. We used this sequence information to clone a second *Helobdella* sp. (Austin) Pax6 homolog, which we have designated *Hau-Pax6B*. A partial cDNA sequence of the latter gene has been deposited in GenBank under accession no. EF394359. Characterization and expression analysis will be deferred to a future manuscript.

Molecular characterization

Conceptual translation of the *Hau-Pax6A* cDNA revealed a paired domain at Nt 297–680 (Fig. 3a) and a homeodomain in the same reading frame at Nt 1443–1622 (Fig. 3b). Outside of these two domains, we did not detect any significant similarity to other known protein sequences. Some Pax6 genes encode a proline-, serine-, and threonine-rich transactivation domain in the C-terminal region (Czerny and Busslinger 1995), but the latter motif was not evident in *Hau-Pax6A*. The 3′ RACE products indicated that the RNA was polyadenylated, and a polyadenylation signal sequence was found at Nt 3499–3504.

Splicing and polyadenylation suggested that the *Hau-Pax6A* transcript was translated mRNA, but none of our
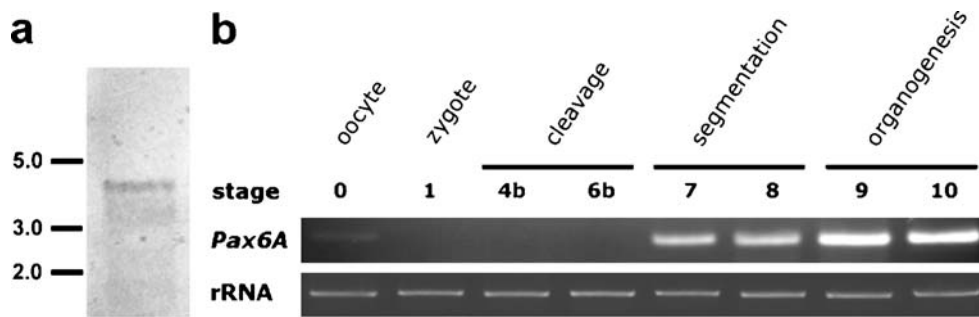
Fig. 2 Expression of *Hau-Pax6A* RNA. **a** Northern blot analysis of total embryonic RNA detected a single prominent band. **b** RT-PCR detected a low level of *Hau-Pax6A* RNA in oocytes but not in zygotes or early cleavage stage embryos. Zygotic expression was first detected at embryonic stage 7 and increased at later stages. Sample loading was normalized by parallel amplification of 18 S rRNA

Hau-Pax6A cDNAs had an upstream ATG codon inframe with the paired domain coding sequence. The five longest 5′ RACE products had stop codons 0.2 kb upstream of the paired domain, with two intervening ATG sequences that were not in the same reading frame as the paired domain. There was an inframe CTG codon at Nt 282–284; if the latter sequence functioned as a noncanonical translation start site (Riechmann et al. 1999), then the consensus cDNA would encode a 933 amino acid protein.

There were sequence variations in our cloned *Hau-Pax6A* cDNAs suggestive of polymorphisms in the leech population. Most involved short indels in stretches of repetitive sequence, i.e., variable numbers of AAT/AAC repeats in three strings of asparagine codons and variable numbers of TCA repeats in a string of serine codons. These putative polymorphisms only affected the predicted protein sequence downstream of the homeodomain.

Phylogenetic analysis

Hau-Pax6A and *Hau-Pax6B* clustered with other known members of the Pax6 subfamily in phylogenetic analyses of
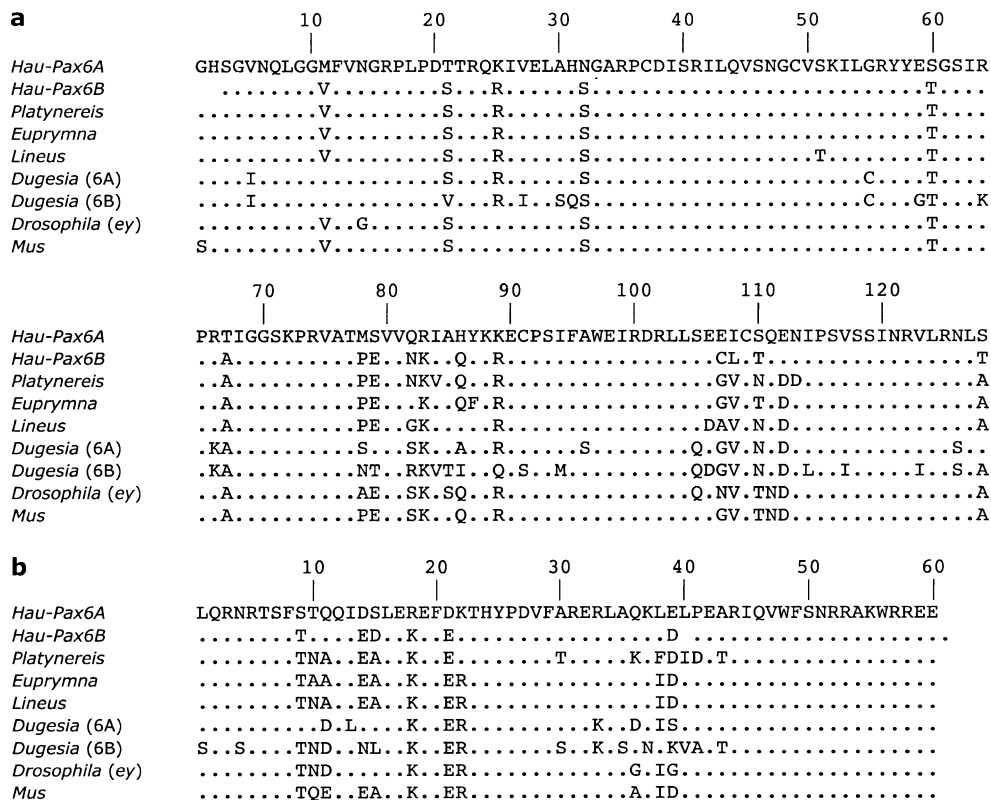
Fig. 3 Sequence alignment of the paired domain (**a**) and homeodomain (**b**) of the leech genes *Hau-Pax6A* and *Hau-Pax6B* with Pax6 homologs from other species. Amino acids identical to *Hau-Pax6A* are marked with a *period*. Sequences were taken from polychaete *Platynereis dumerilii* (AM14770), squid *Euprymna scolopes* (AF513712), nemertean *Lineus sanguineus* (X95594), planarian *Dugesia japonica* (AB017632 and AJ311310), fruitfly *Drosophila melanogaster* (NM_079889), and mouse *Mus musculus* (NM_013627)
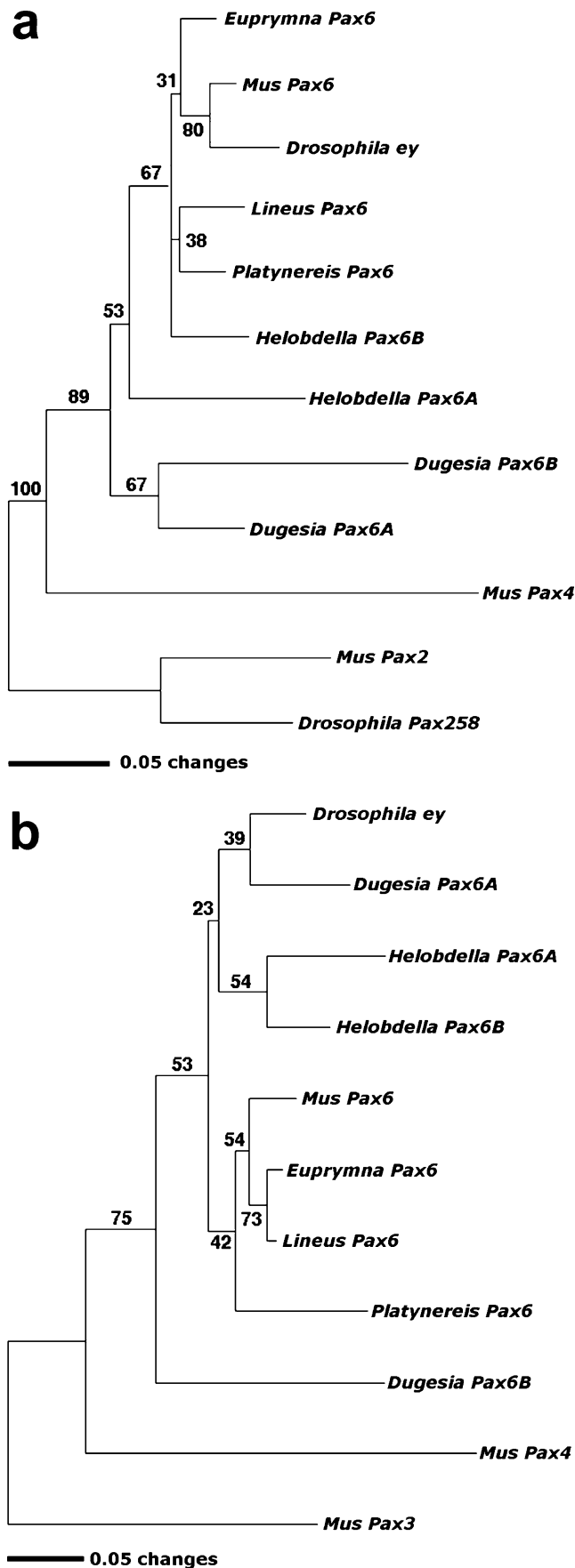
Fig. 4 The *Helobdella* genes *Pax6A* and *Pax6B* clustered with other Pax6 genes in phylogenetic analyses of paired domain (**a**) and homeodomain (**b**) protein sequences. Phylograms were generated by the neighbor-joining method with branch lengths reflecting levels of sequence divergence. Numbers represent bootstrap percentages from 1,000 replicates, and branch nodes incompatible with the 50% majority rule were collapsed. The paired domain tree was rooted with mouse *Pax2* (P32114) and *Drosophila Pax258* (NP_524633) as outgroups. The homeodomain tree was rooted with mouse *Pax3* (AAH48699) as the outgroup. In addition to mouse *Pax4* (NM_011038), other sequences used were the same as for Fig. 3

paired domain and homeodomain sequences (Fig. 4). The *Hau-Pax6A* gene was most similar to *Hau-Pax6B*, with 87% amino acid identity in the paired domain and 90% in the homeodomain (Fig. 3). In contrast, the *Hro-Pax6B* gene product was more similar to Pax6 genes from other species, with 95% amino acid identity to the Pax6 paired domains from two squid species (Tomarev et al. 1997; Hartmann et al. 2003) and 93% identity to a Pax6 homeodomain from the sea urchin (Czerny and Busslinger 1995). Thus, it appeared that *Hau-Pax6B* has retained a protein sequence more characteristic of the Pax6 subfamily, while *Hau-Pax6A* has undergone a higher rate of sequence diversification.

Some evidence suggested that *Hau-Pax6A* and *Hau-Pax6B* arose from a gene duplication event that occurred at a relatively recent time in leech evolution. Only a single Pax6 gene has been reported in the only other annelid species examined, the polychaete *Platynereis dumerilii* (Arendt et al. 2002). In addition, the *Hau-Pax6A* and *Hau-Pax6B* homeodomains clustered with one another (54% of bootstrap replicates) in a neighbor-joining tree that included all known Pax6 genes from the superphylum Lophotrochozoa (Fig. 4b). On the other hand, the two leech Pax6 paired domains did not show as strong a phylogenetic affinity, with the *Hau-Pax6A* paired domain sorting to a more basal position in a neighbor-joining tree (Fig. 4a).

Developmental time course

The time course of *Hau-Pax6A* RNA expression was examined by RT-PCR using primer pairs flanking a known splice site. We detected a low level of *Hau-Pax6A* RNA in oocytes (Fig. 2b) but not in zygotes or early cleavage stages (stages 2–6). This finding suggested that maternal *Hau-Pax6A* RNA is present during oogenesis but degraded by the early stages of embryonic development. A second phase of de novo, presumably zygotic *Hau-Pax6A* expression began at embryonic stage 7 and increased during later stages (Fig. 2b).

In situ hybridization

The cellular distribution of *Hau-Pax6A* RNA was characterized by in situ hybridization. Reproducible staining patterns were observed from stage 7 through the end of
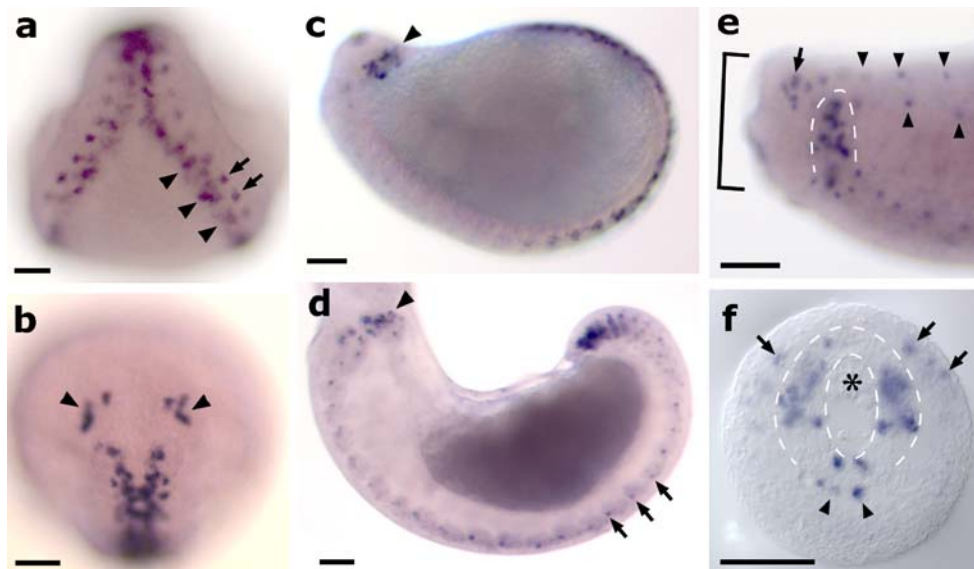
Fig. 5 Expression of *Hau-Pax6A* visualized by whole mount in situ hybridization. **a** Ventral view of stage 8 embryo showing the fusion of the right and left germinal bands (cf. Fig. 1) with anterior to the *top*. At this stage, there was a segmental pattern of *Hau-Pax6A* expression, including numerous cells in the N lineage (*arrowheads*) and a single segmentally repeated cell in the O lineage (*arrows*). **b** Anterior view of a stage 8 embryo with dorsal to the *top*. At this stage, there were bilateral clusters of stained cells (*arrowheads*) in the prostomial head domain. Hybridization was also visible more ventrally in the segmental nervous system. **c, d** Side views of a stage 9 embryo (**c**) and stage 10 embryo (**d**) with anterior to the *left* and dorsal to the *top*. Expression of *Hau-Pax6A* RNA disappeared in an anterior-to-posterior wave during the maturation of the segmental nerve cord but persisted in the supraesophageal ganglion (*arrowheads*) throughout embryogenesis. *Arrows* point to a single mature cell in each

segmental ganglion that also retained *Hau-Pax6A* expression. **e** The head of a stage 11 embryo in the same orientation as parts **c** and **d**. The ventral surface of the rostral sucker is marked by a bracket. At this stage, there were clusters of *Hau-Pax6A*-expressing cells in the supraesophageal ganglion (outlined by *dashes*) and at the site of eye formation (*arrow*). Longitudinal rows of single *Hau-Pax6A*-expressing neurons appeared in the dorsal integument at this stage; the two most dorsal rows are in focus (*arrowheads*). **f** Transverse section of a stage 10 embryo in the plane of the supraesophageal ganglion, which is outlined by *dashes*. The empty proboscis sheath (*asterisk*) passes through the center of the ring-shaped ganglion. *Arrows* mark *Hau-Pax6A*-expressing cells in the dorsal integument; *arrowheads* mark cells located in the anterior segments of the ventral nerve cord. Dorsal is to the *top*. Scale bars–50 μm

embryonic development but not at earlier stages. Control hybridizations with sense probes gave little or no staining at any stage.

Germinal bands and plate. Beginning in stage 7, *Hau-Pax6A* RNA was detected in the anterior, i.e., most mature, portion of the germinal bands (Fig. 5a). Early expression was restricted to blast cell progeny of the N teloblast lineage, and expression persisted in this lineage as the bands fuse to form the germinal plate. A few cells in the O teloblast lineage also began to show a segmental pattern of expression during germinal plate formation (Fig. 5a). We did not detect any *Hau-Pax6A* expression in the M, P, or Q lineages at these stages.

We used fluorescent lineage tracers and nuclear staining to analyze the cellular pattern of *Hau-Pax6A* expression in more detail. The N teloblast lineage is comprised of alternating n_s and n_f blast cells, which undergo stereotyped sequences of asymmetric cell divisions within the germinal band. We found that the sequences of n and o blast cell divisions in *Helobdella* sp. (Austin) were identical to those reported for *H. triserialis* (Bissen and Weisblat 1989) and that *Hau-Pax6A* RNA was first expressed in cell n_s.a, the anterior daughter of the n_s blast cell (Fig. 6a,b).

As blast cell clones matured, the *Hau-Pax6A* expression pattern expanded to other cells of the N lineage including progeny of the n_f blast cell. The dynamic character of the N lineage expression pattern prevented us from individually identifying cells at later stages. Nonetheless, we could see a clear segmental periodicity of *Hau-Pax6A* expression where the N lineages pass from the right and left germinal bands into the germinal plate (Fig. 5a, b). Expression persisted through the early stages of ganglion formation, at which time segmental repeats were composed of an iterated pattern of both *Hau-Pax6A*-expressing and nonexpressing cells (Fig. 6f,g).

In the O teloblast lineage, *Hau-Pax6A* expression began near the point of germinal band fusion and was associated with a single, segmentally repeated sublineage (Figs. 5a, 6d–g). To delimit individual clones of primary o blast cell descendants, we took advantage of the fact that a certain percentage of teloblast injections yield a distinctive faint labeling of the next blast cell produced (Bissen and Weisblat 1987). Focusing on these faintly labeled clones, we found that *Hau-Pax6A* expression first appeared after the primary o blast cell had divided to produce nine clonal
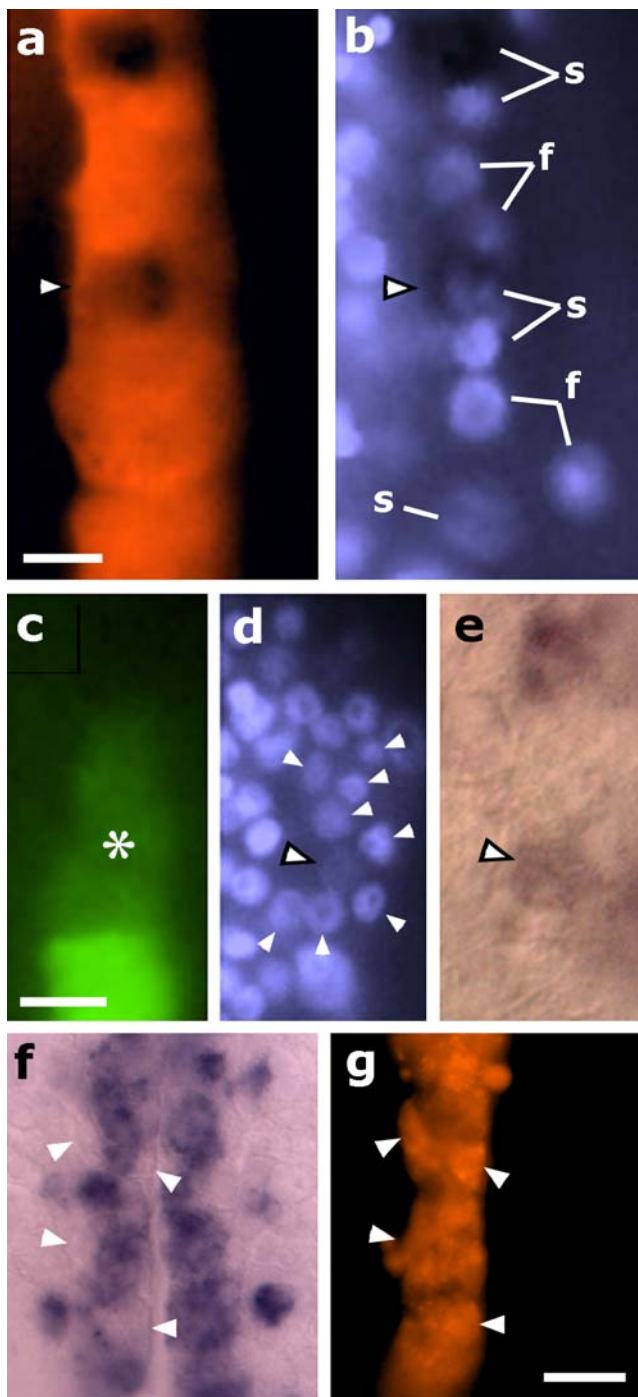
Fig. 6 Colocalization of the *Hau-Pax6A* RNA expression with injected cell lineage tracers. Anterior to the *top*. **a, b** RNA expression (*arrowhead*) first appeared in a segmentally repeated cell of the N teloblast lineage, here labeled with rhodamine dextran (**a**). Individual cells can be identified by nuclear size, seen by Hoechst staining (**b**). The n_s and n_f blast cells have completed their first divisions, and their paired daughter cells are marked. At this stage, the hybridization product was restricted to cell n_s.a, the anterior daughter of an n_s blast cell. An undivided n_s blast cell can be seen at the *bottom*. Future medial to the *right*. **c–e** In this embryo, the O teloblast lineage was injected with fluorescein-dextran in such a way that a single primary blast cell clone showed a distinct pattern of faint labeling (*asterisk*; see Bissen and Weisblat 1987). The clone shown here contained nine progeny cell nuclei (*arrowheads*, **d**), including a single large cell that expressed *Hau-Pax6A* (*outlined arrowhead*) as seen by partial occlusion of Hoechst fluorescence (**d**) and brightfield illumination (**e**). Future medial to the *right*. **f, g** Brightfield view of the *Hau-Pax6A* expression along the ventral midline of the germinal plate (**f**) in a stage 8 embryo whose left N teloblast lineage had been labeled with rhodamine dextran (**g**). By this stage, the labeled cell lineage has adopted a segmental pattern of lateral bulges and was a mosaic of cells that do and do not express *Hau-Pax6A*. Four nonexpressing cells are marked with *arrowheads*. Segmental expression of *Hau-Pax6A* in the O lineage could be seen more laterally on both sides. Scale bars– 10 μm (**a, b**), 20 μm (**c–e**), and 50 μm (**f, g**)

peared from the anteriormost segments during stage 8 and by stage 10 was only visible in the neuromeres associated with the caudal sucker. However, there was one bilateral pair of cells that continued to express *Hau-Pax6A* through the end of embryogenesis at the anterior edge of every segmental ganglion or neuromere (Fig. 5d).

Head development. Expression of *Hau-Pax6A* also appeared during late stage 7 in the unsegmented head domain, where it occurred in a bilateral array of cell clusters associated with the supraesophageal ganglion (Fig. 5b). In contrast to the segmented portion of the CNS, this head ganglion maintained robust *Hau-Pax6A* expression throughout embryonic life (Fig. 5c–f).

Some *Hau-Pax6A*-expressing cells were distributed in peripheral tissues closely associated with the supraesophageal ganglion (Fig. 5e,f). *Helobdella* develops a single bilateral pair of eyes on the dorsal lip of its rostral sucker, and a population of *Hau-Pax6A*-expressing cells was evident at this site during embryonic stages 9–11 (Fig. 5e). Eye pigmentation normally develops in stage 11 (Shankland and Martindale 1989), but our hybridization protocol bleached the eye pigment, and we were unable to use the pigment as an anatomical marker.

Dorsal integument. During the transition from embryonic stages 10 to 11, *Hau-Pax6A* expression appeared in an orthogonal array of isolated cells distributed in the dorsal integument. This integumental staining developed in an anterior-to-posterior wave and consisted of three longitudinal rows of segmentally repeating cells located symmetrically on either side of the dorsal midline (Fig. 5e).

progeny and was restricted to the largest cell in the clone (Fig. 6c–e). Based on size and position, we believe that this *Hau-Pax6A*-expressing cell corresponds to cell o.apapl described by Bissen and Weisblat 1989. The single large cell was later replaced by two small, adjacent *Hau-Pax6A*-expressing cells–possibly the result of cell o.apapl's next division–which appeared to merge medially with the *Hau-Pax6*-expressing cells of the N lineage.

As the CNS matured, *Hau-Pax6A* expression faded in an anterior-to-posterior wave (Fig. 5c,d). Expression disap-

Discussion

We have identified two Pax6 genes in the leech *Helobdella* sp. (Austin) and here characterize the embryonic expression of *Hau-Pax6A*. Like Pax6 genes from a wide range of other animal species (Callaerts et al. 1997, 2001; Stoykova et al. 1997; Arendt et al. 2002; Pineda et al. 2002), *Hau-Pax6A* is expressed during the development of the CNS and in a population of cells associated with the developing eye.

One noteworthy feature of the *Hau-Pax6A* mRNA is the absence of a canonical translation start site upstream of the paired domain coding sequence. The *Hau-Pax6A* mRNA may use a noncanonical start site (Riechmann et al. 1999), possibly a CTG codon (see "Results"). A second possibility is that an inframe AUG is generated by RNA editing before translation (Gott and Emeson 2000). If the latter is the case, we have only been successful to date in cloning the unedited sequence. It is worth noting that a second leech Pax gene, *Hau-Pax3/7B*, also lacks an inframe AUG codon upstream of its paired domain coding sequence (Shankland and Woodruff, unpublished results).

Hau-Pax6A in CNS development

Within the segmented body trunk, *Hau-Pax6A* expression precedes the appearance of overt morphological segmentation or ganglion formation and is largely restricted to the N teloblast lineage, which gives rise to roughly two thirds of the ganglionic neurons (Weisblat and Shankland 1985). Early embryonic expression was also observed in one segmentally iterated descendant of the O teloblast, which we tentatively identify as cell o.apapl. The latter cell is also part of a sublineage known to produce central neurons (Shankland 1987). However, there was no early *Hau-Pax6A* expression in the M, P, or Q teloblast lineages, although they also contribute neurons to the CNS (Weisblat and Shankland 1985).

The observed pattern of *Hau-Pax6A* expression in the leech CNS is suggestive of a developmental role in segmentation and/or ganglion formation. The N teloblast lineage is composed of an alternating sequence of n_s and n_f blast cells whose descendant clones form the structural framework of the ganglia (Shain et al. 2000). Moreover, ablation experiments indicate that the N lineage plays a critical role in ganglion morphogenesis (Blair and Weisblat 1982; Stuart et al. 1989). It has been suggested that proper ganglion formation depends on differences in the adhesivity of certain n blast cell sublineages (Shain et al. 2000), and it is interesting to note that Pax6 regulates cell adhesivity during development of the mammalian brain (Stoykova et al. 1997) and eye (Collinson et al. 2000). The early segmental pattern of *Hau-Pax6A* expression in the n blast cell clones of the leech embryo may lead to changes in adhesivity that facilitate ganglion formation.

Some authors have argued that a small population of N-derived *engrailed*-expressing cells may play a critical role in the formation of fissures between the segmental ganglia of the leech CNS (Lans et al. 1993; Ramírez et al. 1995). This hypothesis was later discounted by Shain et al. 2000, who showed that the formation of interganglionic fissures precedes *en* expression within the N lineage. This is in contrast to *Hau-Pax6A*, whose expression begins at an earlier stage in the N lineage development and clearly precedes the cell rearrangements that bring about ganglion formation.

Hau-Pax6A expression largely disappears from the segmental ganglia before the onset of neurotransmitter expression (Stuart et al. 1987; Shankland and Martindale 1989) or extensive axonal projections (Braun and Stent 1989). This timing suggests that *Hau-Pax6A* has little function in the differentiation of segmentally iterated neurons, although it is still expressed in one bilateral pair of segmentally repeated cells in the mature CNS. *Hau-Pax6A* expression also persists through late embryonic development in neurons of the unsegmented supraesophageal ganglion and could be playing distinct developmental functions in that region of the CNS.

Hau-Pax6A in eye primordia and other peripheral tissues

We also observed the expression of *Hau-Pax6A* outside the CNS. The most prominent peripheral expression surrounded the supraesophageal ganglion, including a bilateral cell population located at the site of eye formation. The expression of a Pax6 gene in the developing eye of the leech *Helobdella* is in no way surprising given the comparable results from many other bilaterian animals (Callaerts et al. 1997; Pineda et al. 2002).

Late *Hau-Pax6A* expression was also observed in an orthogonal array of integumental cells on the dorsal side of the body trunk. The distribution of these cells is similar to that of the sensillae described in adult leeches (Sawyer 1986), and they may represent one particular type of sensillar neuron or neural precursor. Leech sensillae are primarily mechanosensory but can also detect light (Kretz et al. 1976). Additional studies will be needed to ascertain whether the *Hau-Pax6A* expression seen in the dorsal integument of *Helobdella* embryos is associated with the development of extraocular photoreceptors.

Gene duplications in the Pax6 subfamily

The last common ancestor of protostomes and deuterostomes is thought to have had only a single Pax6 gene (Balczarek et al. 1997). However, duplications of Pax6 appear to have occurred in a number of evolutionary lineages derived from

that ancestor. For instance, arthropods have two Pax6 genes, *eyeless* and *twin-of-eyeless*, that play distinct roles in eye development (Kammermeier et al. 2001).

Two lines of evidence suggest that the gene duplication which gave rise to *Hau-Pax6A* and *Hau-Pax6B* occurred after leeches had diverged from polychaete annelids. First, only a single Pax6 gene has been reported in the polychaete *Platynereis dumerilii* (Arendt et al. 2002) and in several other organisms from the superphylum Lophotrochozoa: two species of squid (Tomarev et al. 1997; Hartmann et al. 2003) and a nemertean (Loosli et al. 1996). Planaria have two Pax6 genes (Pineda et al. 2002); however, our phylogenetic sequence analysis failed to reveal any consistent alignment between the planarian and leech Pax6 gene pairs, suggesting that they arose from independent gene duplications.

A second line of evidence consistent with a recent gene duplication is the observation that the *Hau-Pax6A* and *Hau-Pax6B* homeodomains grouped together in our phylogenetic analysis of lophotrochozoan Pax6 genes. However, this grouping was not seen for their paired domain sequences, possibly because the more highly diverged *Hau-Pax6A* paired domain was attracted to a more basal position in that tree. Pax6 genes will need to be characterized in additional lophotrochozoan species before we can reconstruct the history of gene duplications with greater certainty.

References

Arendt D, Tessmar K, de Campos-Baptista MI, Dorresteijn A, Wittbrodt J (2002) Development of pigment-cup eyes in the polychaete *Platynereis dumerilii* and evolutionary conservation of larval eyes in Bilateria. Development 129:1143–1154

Baker MW, Macagno ER (2000) RNAi of the receptor tyrosine phosphatase HmLAR2 in a single cell of an intact leech embryo leads to growth-cone collapse. Curr Biol 10:1071–1074

Balczarek KA, Lai Z-C, Kumar S (1997) Evolution and functional diversification of the Paired Box (Pax) DNA-binding domains. Mol Biol Evol 14:829–842

Baptista CA, Gershon TR, Macagno ER (1990) Peripheral organs control central neurogenesis in the leech. Nature 346:855–858

Bely AE, Weisblat DA (2006) Lessons from leeches: a call for DNA barcoding in the lab. Evol Dev 8:491–501

Bissen ST, Weisblat DA (1987) Early differences between alternate n blast cells in leech embryo. J Neurobiol 18:251–269

Bissen ST, Weisblat DA (1989) The durations and compositions of cell cycles in embryos of the leech, *Helobdella triserialis*. Development 105:105–118

Blair SS (1982) Interactions between mesoderm and ectoderm in segment formation in the embryo of a glossiphoniid leech. Dev Biol 89:389–396

Blair SS, Weisblat DA (1982) Ectodermal interactions during neurogenesis in the glossiphoniid leech *Helobdella triserialis*. Dev Biol 91:64–72

Braun J, Stent GS (1989) Axon outgrowth along segmental nerves in the leech. I. Identi-fication of candidate guidance cells. Dev Biol 132:471–485

Callaerts P, Halder G, Gehring WJ (1997) PAX-6 in development and evolution. Annu Rev Neurosci 20:483–532

Callaerts P, Leng S, Clements J, Benassayag C, Cribbs D, Kang YY, Walldorf U, Fischbach KF, Strauss R (2001) *Drosophila*. *Pax-6/eyeless* is essential for normal adult brain structure and function. J Neurobiol 46:73–88

Collinson JM, Hill RE, West JD (2000) Different roles for *Pax6* in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. Development 127:945–956

Czerny T, Busslinger M (1995) DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for different sequence recognition of Pax-6 and BSAP (Pax-5). Mol Cell Biol 15:2858–2871

Gott JM, Emeson RB (2000) Functions and mechanisms of RNA editing. Annu Rev Genet 34:499–531

Hartmann B, Lee PN, Kang YY, Tomarev S, de Couet HG, Callaerts P (2003) *Pax6* in the sepiolid squid *Euprymna scolopes*: evidence for a role in eye, sensory organ and brain development. Mech Dev 120:177–183

Johnson LA, Kristan WB Jr, Jellies J, French KA (2000) Disruption of peripheral target contact influences the development of identified central dendritic branches in a leech motor neuron *in vivo*. J Neurobiol 43:365–378

Kammermeier L, Leemans R, Hirth F, Flister S, Wenger U, Walldorf U, Gehring WJ, Reichert H (2001) Differential expression and function of the *Drosophila Pax6* genes *eyeless* and *twin of eyeless* in embryonic central nervous system development. Mech Dev 103:71–78

Kretz JR, Stent GS, Kristan WB Jr (1976) Photosensory input pathways in the medicinal leech. J Comp Physiol 106:1–37

Kristan WB Jr, Calabrese RL, Friesen WO (2005) Neuronal control of leech behavior. Prog Neurobiol 76:279–327

Lans D, Wedeen CJ, Weisblat DA (1993) Cell lineage analysis of the expression of an *engrailed* homolog in leech embryos. Development 117:857–871

Loosli F, Kmita-Cunisse M, Gehring WJ (1996) Isolation of a *Pax-6* homolog from the ribbonworm *Lineus sanguineus*. Proc Natl Acad Sci USA 93:2658–2663

Macagno ER, Stewart RR (1987) Cell death during gangliogenesis in the leech: competition leading to the death of PMS neurons has both random and nonrandom components. J Neurosci 7:1911–1918

Marin-Burgin A, Eisenhart FJ, Baca SM, Kristan WB Jr, French KA (2005) Sequential development of electrical and chemical synaptic connections generates a specific behavioral circuit in the leech. J Neurosci 25:2478–2489

Nardelli-Haefliger D, Shankland M (1992) *Lox2*. , a putative leech segment identity gene, is expressed in the same segmental domain in different stem cell lineages. Development 116:697–710

Pineda D, Rossi L, Batistoni R, Salvetti A, Marsal M, Gremigni V, Falleni A, Gonzalez-Linares J, Deri P, Salo E (2002) The genetic network of prototypic planarian eye regeneration is Pax6 independent. Development 129:1423–1434

Ramírez F-A, Wedeen CJ, Stuart DK, Lans D, Weisblat DA (1995) Identification of a neurogenic sublineage required for CNS segmentation in an Annelid. Development 121:2091–2097

Riechmann JL, Ito T, Meyerowitz EM (1999) Non-AUG initiation of *AGAMOUS* mRNA translation in *Arabidopsis thaliana*. Mol Cell Biol 19:8502–8512

Sawyer RT (1986) Leech biology and behaviour. Clarendon, London

Seaver EC, Shankland M (2000) Leech segmental repeats develop normally in the absence of signals from either anterior or posterior segments. Dev Biol 224:339–353

Shain D, Ramirez-Weber F-A, Hsu J, Weisblat DA (1998) Gangliogenesis in leech: morphogenetic processes leading to segmentation in the central nervous system. Dev Genes Evol 208:28–36

Shain DH, Stuart DK, Huang FZ, Weisblat DA (2000) Segmentation of the central nervous system in leech. Development 127:735–744

Shankland M (1987) Differentiation of the O and P cell lines in the embryo of the leech.I. Sequential commitment of blast cell sublineages. Dev Biol 123:85–96

Shankland M, Martindale MQ (1989) Segmental specificity and lateral asymmetry in the differentiation of developmentally homologous neurons during leech embryogenesis. Dev Biol 135:431–448

Stent GS, Kristan WB Jr, Torrence SA, French KA, Weisblat DA (1992) Development of the leech nervous system. Int Rev Neurobiol 33:109–133

Stoykova A, Gotz M, Gruss P, Price J (1997) Pax6-. dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. Development 124:3765–3777

Stuart DK, Blair SS, Weisblat DA (1987) Cell lineage, cell death, and the developmental origin of identified serotonin- and dopamine-containing neurons in the leech. J Neurosci 7:1107–1122

Stuart DK, Torrence SA, Law MI (1989) Leech neurogenesis. I. Positional commitment of neural precursor cells. Dev Biol 136:17–39

Tomarev SI, Callaerts P, Kos L, Zinovieva R, Halder G, Gehring W, Piatigorsky J (1997) Squid Pax-6 and eye development. Proc Natl Acad Sci USA 94:2421–2426

Torrence SA, Law MI, Stuart DK (1989) Leech neurogenesis. II. Mesodermal control of neuronal patterns. Dev Biol 136:40–60

Venkitaramani DV, Wang D, Ji Y, Xu YZ, Ponguta L, Bock K, Zipser B, Jellies J, Johansen KM, Johansen J (2004) Leech filamin and tractin: markers for muscle development and nerve formation. J Neurobiol 60:369–380

Weisblat DA, Shankland M (1985) Cell lineage and segmentation in the leech. Philos Trans R Soc Lond B 312:39–56

Woodruff JB, Mitchell BJ, Shankland M (2007) Hau-Pax3/7A is an early marker of leech mesoderm involved in segmental morphogenesis, nephridial development, and body cavity formation. Dev Biol (in press), DOI 10.1016/j.ydbio.2007.03.002