10 Hirudinida

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10.1 Phylogeny and Systematics

Leech phylogenetic relationships and, consequently, classification of its constituents has seen considerable attention in the last decade particularly as leeches have been the subject of analyses at several taxonomic levels using morphological characters and DNA sequence data. The origin of leeches and other symbiotic clitellate annelids was at one time an issue rather hotly debated by annelid systematists. As with many annelids, leeches are soft-bodied and do not regularly leave a fossil record. There are two putative Jurrasic fossils from Bavarian deposits, *Epitrachys rugosus* and *Palaeohirudo eichstaettensis,* but neither has both the caudal sucker and annular subdivisions that together would definitively suggest a leech (Ehlers 1869; Kozur, 1970). Nonetheless there have long been anatomical clues regarding hirudinidan origins.

Leeches have a constant number of somites and a posterior sucker used for attachment to hosts, but so too do the tiny branchiobdellidan crayfish worms and the Arctic salmon worm *Acanthobdella peledina*. The latter has oligochaete-like chaetae and a constant number of 29 somites but exhibits leech-like coelmic and reproductive structures. In contrast, the branchiobdelidans have a more oligochaete-like reproductive organization, a constant number of 15 body somites and yet lack chaetae altogether. Not surprisingly there have been several historical suggestions of a close relationship amongst these groups (Odier, 1823; Livanow, 1931; Brinkhurst and Gelder, 1989; Siddall and Burreson, 1996) but others worried that the similiarities were mere convergence (Holt, 1989; Purschke et al., 1993; Brinkhurst,1994). Ferraguti and Erséus (1999) suggested several synapomorphies in sperm ultrastructure corroborating a sister-group relationship of leeches and *Acanthobdella*, but they found no evidence in support of an exact position for Branchiobdellida within the Clitellata (but see formation of the preacrosomal vesicle in spermatogenesis below). Siddall et al (2001) demonstrated with nuclear and mitochondrial gene sequences that leeches (Hirudinida), branchiobdellidans and acanthobdellidans are a monophyletic "oligochaete" group that shares a

common ancestor with Lumbriculida (Fig. 1). Consequently each should have equivalent ordinal rank (i.e., Hirudinida, Branchiobdellida and Acanthobdellida) in the class Oligochaeta.

Leeches are themselves subdivided first into suborders based on anatomical adaptations for feeding. "Rhynchobdellida", as the name implies, is a group possessing a muscular proboscis to effect bloodfeeding from vascularized subdermal tissues. The Giant Amazonian Leech, *Haementeria ghilianii*, which grows to a tremendous 16 inches, has a proboscis that is nearly half its body length. Three families of proboscis-bearing leeches are: the strongly dorsoventrally flattened freshwater Glossiphoniidae (Fig. 2); the mostly marine Piscicolidae feeding seasonally on fishes, and the Ozobranchidae specializing on (usually) marine turtles (Fig. 3). Arhynchobdellida, of which *Hirudo medicinalis* (Fig. 4) is typical, are larger, vermiform and typically have three muscular jaws, each of which may be armed with a row of teeth creating a serrated cutting edge allowing them to feed through the skin on capillary-rich tissues. The large aquatic Hirudinidae ("medicinal leeches") and the smaller terrestrial Haemadipsidae ("jungle leeches" Fig. 5) are perhaps the best known blood feeding arhynchobdellids. Both of these groups are equipped with a parabolic arc of 10 eyespots that detect movement in 3 dimensions. Terrestrial leeches have the additional adaptation of respiratory auricles near their caudal sucker permitting gas exchange without excessive loss of fluid and well-developed sensory systems for detecting vibrations, carbon dioxide and heat. As well there are several predatory arhynchobdellids like the slender Erpobdelliformes (families Erpobdellidae, "Salifidae" and Americobdellidae), the larger amphibious Haemopidae and several other poorly understood families like the Cylicobdellidae and Semiscolescidae.

The evolutionary relationships of leeches have been investigated using morphological data (Siddall and Burreson, 1995), life history characters (Siddall and Burreson, 1996), nuclear and mitochondrial gene sequences (Siddall and Burreson, 1998; Trontelj et al., 1999; Siddall et al., 2001), as well as combinations of these data sets at the familial level (Apakupakul et al., 1999; Light and Siddall, 1999; Siddall, 2002; Borda and Siddall, 2004) and for individual genera (Siddall and Borda, 2003). A concatenation of recent phylogenetic datasets (Fig. 6) reveals the artificiality of many traditional groupings. Accepting the presence of a proboscis as an unreversed synapomorphy for the Rhynchobdellida (e.g., Siddall and Burreson, 1995, Livanow, 1931; Mann 1962; Sawyer 1986) had not been controversial. However, analyses that have used DNA sequence data consistently suggest these are paraphyletic (Siddall and Burreson, 1998;

Apakupakul et al., 1999; Trontelj et al., 1999) with the Glossiphoniidae diverging from other leeches first leaving the Piscicolidae as sister to the arhynchobdellids (Fig. 6). In retrospect there previously have been several suggestions of loss of the proboscis en route to the more "advanced" medicinal leeches (e.g., Apathy 1888) as well as a basal position for the Glossiphoniidae (Selensky, 1907; Autrum, 1939). Moreover there are several aspects of development and developmental regulation that indicate a suite of plesiomorphies retained in the Glossiphoniidae (see section 10.6).

Among the various families of leeches (Fig. 6), characteristics that typically have been considered diagnostic often prove to not be so. The notion that five pairs of eyespots arranged in a parabolic arc is a synapomorphy for the Hirudiniformes is refuted by its presence in *Linta adrianampionimarinai* and (possibly) *Americobdella valdiviana* that constitute the basal most clade of Erpodeblliforrmes. Small terrestrial jungle leeches likewise form two independent clades corroborating the notion that a portion of the Haemadipsidae stands as a family in its own right: Xerobdellidae. Even the so-called Medicinal leeches are not monophyletic. The predominantly new world Macrobdellidae stands apart from an exclusively old-world Hirudinidae, the latter of which is more closely related to the non-bloodfeeding genus *Haemopis* (for which, however, the parent family, Haemopidae, is polyphyletic insofar as the Semiscolescinae group with the new world hirudinids). Obviously reliance on bloodfeeding bahaviour has muddled leech systematics for some time as the cladogram in Figure 6 requires the loss of sanguivory at least six times. Awkwardly, other characteristics that were deemed unreliable in the past prove to be extremely consistent in analyses of combined data sets. For example, the possession of two pairs of compact salivary cells at the base of the proboscis unites *Haementeria* species with *Placobdella* species within the Glossiphoniidae notwithstanding their previously having been placed in separate subfamilies (Sawyer, 1986). Similarly the arrangement of eyespots in erpobdellids was the only consistent morphological character for that group (Siddall, 2002).

Evolutionary relationships among leeches (Fig. 6) demonstrate that the ancestral hirudinid was a blood feeder in a freshwater environment suggesting that they are no older than vertebrates and probably are no older than the amphibian lineage. Corroborating this are the dietary preferences of basal lineages in Glossiphoniidae and Piscicolidae with *Marsupiobdella africana* still feeding on pipid frogs and ozobranchids on turtles. Moreover, several leech species

have been examined for anticoagulants and, in terms of the phylogeny of the group (Fig. 6), it is clear that three coagulation inhibitors must have been inherited from the common ancestor. That is, a broad range of leeches have all inherited from the ancestral leech, genes coding for anti-platelet, anti-thrombin and antimetastatic anti-Xa factors. Probably many of the lineages that later gave up blood feeding have as well. Already this has been corroborated by the discovery of anti-Xa guamerins in the macrophagous haemopid, *Whitmania edentula*. What lies undiscovered in other non-bloodfeeding groups remains an exciting prospect.

Notably, even though the well-known medicinal leeches are associated with freshwater habitats, Figure 6 implies that that the ancestral hirudiniform unequivocally was terrestrial. Aquatic hirudinid sexual biology would seem to corroborate this terrestrial ancestry. Unlike most rhynchobdellid and erpobdellid leeches that mate by way of traumatic insemination (hypodermic implantation of a membrane-bound spermatophore that injects sperm in response to an osmotic pressure change), the haemadipsids and other hirudiniforms are characterized by internal fertilization (gonopore to gonopore copulation with a protrusible penis and a compensatory vagina). As well, aquatic hirudinids and macrobdellids still deposit their cocoons on land and hatchling leeches must find their way to nearby water when they emerge.

In terms of biogeography, the evolutionary history of leeches is perhaps most remarkable for the anomalies implied by Figure 6, though several items offer easy interpretation. The majority of the basal-most members of Glossiphoniidae are African (i.e., *Marsupiobdela africana*, *Placobdelloides multistriatus Batracobdelloides tricarinata Oosthuizobdella garoui* and *Placobdelloides jagerskioeldi*). Furthermore, subsequent diversification, proximal in time, involves the overwhelmingly South American genus *Helodbella* (though many North American taxa are known) and the exclusively South American *Haementeria*. The foregoing suggests a Gondwanan origin for that family, and this is somewhat consistent elsewhere with finding the Malagasy *Linta* species and South American *Cylicobdella* species at the base of their respective groups (i.e., Erobdelliformes and Hirudiniformes). However, several arrangements mitigate against such an easy interpretation. The North American *Placobdella* radiation stands wholly as sister to the South American *Haementeria* group. A post-Gondwanan Panamanian isthmus explanation for the origin of the former would only be sensible only if *Placobdella* were nestled within *Haementeria* (much as how *Macrobdella* species are few and nestled within a paraphyletic South American clade of *Oxyptychus*, *Semiscolex* and *Patagoniobdella* species, and

that the North American species of *Helobdella* arise in several places from a basal South American stock); and would require the complete speciation of the group plus a leap to Europe (for *Placobdella costata*) in a very short 3.5 million years.

There are in fact several relatively recent sister group relationships consistent with a Laurasian connection. The European pair *Haemopis sanguisuga* and *Haemopis caeca* are sister to the North American clade of *Haemopis* comprising the balance of that genus. The European pair *Erpobdella lineata* and *Erpobdella mestrovi* are sister to a North American *Erpobdella* clade. Similarly, the balance of that genus comprises only North American, European and Asian taxa. Also, the North American *Glossiphonia elegans* (frequently misnamed *G. complanata* which is European) is sister to the Eurasian fauna in this genus. If this is to be believed, on might find it awkward to postulate a series of Laurasian associates grouping within (as opposed to adjacent to) Gondwanan associations in so far as both land masses are supposed to have been late Paleozoic contemporaries.

Other relationships defy explanation at all, save perhaps through ad hoc invocations of massive extinction or extremely long distance dispersal by leeches that do not seem capable of such a feat. In the Xerobdellidae in particular, taxa included in this analysis are the Alpine *Xerobdella lecomtei* and the Chilean *Mesobdella gemmata*. Lest taxon sampling alone be thought to explain this extreme disjunction, the remaining taxa in the family are known only from Mesoamerica or the Seychelles! Similarly awkward is a sister-group relationship for the Malagasy *Linta andrianampoinimerinai* and Chilean *Americobdella valdiviana*.

10.2 Anatomy with reference to the reproductive system

Among the various diagnostic morphological characteristics for leeches, the reproductive system has been particularly important in leech taxonomy and phylogenetics (Richardson, 1969, 1976; Ringuelet, 1985; Sawyer, 1986; Siddall and Burreson, 1995; Apakupakul et al., 1999; Siddall, 2001a, b; Siddall and Borda, 2003; Borda and Siddall, 2004). The reproductive anatomy of leeches is variable, but the degree of variability is group specific and therefore can be used to classify higher taxa (i.e. Families) of Hirudinida and is even species-specific in many cases.

Like other clitellate oligochaetes, leeches possess a clitellum, the saddle-like glandular region associated with cocoon deposition in the anterior portion of the body. The prominence of the clitellum is variable in leeches (usually somites X to XIII; that is, the eighth through eleventh

true segments, or M4-M7) and often is not evident externally in most species, compared to the pronounced swollen clitellar region typified by the lumbricid oligochaetes (Figs. 7-9). Leeches are hermaphrodites, with simultaneous possession of independent male and female reproductive systems. The male median reproductive apparatus is found anterior to the female median reproductive apparatus and each possesses a separate ventral opening to the exterior, or gonopore, on clitellar somites XI and XII respectively. In general, the male reproductive system consists of an atrium (or bursa), either fused (unpaired) or paired (bilobed), and sperm ducts of varying thickness (inappropriately called vasa deferentia, etc). Some species are characterized by the presence of ejaculatory bulbs, coiled "epididymes", and a protrusible penis. The testisacs are derived from coelomic epithelial sacs and usually are paired posteriorly along distal sperm ducts (one testisac on each side per somite). The female reproductive system consists of a pair of ovisacs. In some groups this is elaborated into a vagina and oviducts that connect the ovisacs to the vagina.

Glossiphoniidae. The reproductive anatomy of glossiphoniid leeches (e.g. *Helobdella* spp., *Haementeria* spp., *Placobdella* spp.) is relatively simple in form (Figs. 10, 11), typically with laterally directed bilobed (paired) atria, which give rise to simple descending and ascending sperm ducts. Some glossiphoniids (i.e. *Theromyzon* spp.) have a male apparatus with a fused (unpaired) atrium and elongation of an eversible atrium (Wilkialis and Davies, 1980; Siddall and Burreson, 1995). The sperm ducts descend from the atrial lobes then ascend towards the atria before entering the parenchyma with a posterio-lateral arrangement of one pair of testisacs per somite (five to eight pairs total). The female reproductive system only has a pair of posteriorly directed tubular ovisacs. The posterior extents of ovisacs and sperm ducts in the central coelomic space are species specific.

Piscicolidae and Ozobranchidae. As with the Glossiphoniidae, the fish leeches (freshwater and marine piscicolids) and the turtle leeches (ozobranchids) also have a relatively simple reproductive apparatus (Fig. 12). Their male reproductive systems generally have an anteriorly directed bilobed bursa, with sperm ducts and ejaculatory bulbs curving posteriorly revealing one pair of testisacs per somite in a manner similar to the glossiphoniids. The female system also is similar to that of the glossiphoniids in having long tubular ovaries. Most notably, piscicolids possess parenchymal conducting (vector) tissue that is utilized for the passage of sperm from the copulatory area to the female reproductive tissue (See Fig. 12; Sawyer, 1986;

Siddall and Burreson, 1995), whereas the Ozobranchids have bilateral coelomic tubes connecting the male and female reproductive systems (MacCallum and MacCallum, 1918; Raj and Penner, 1962)

Erpobdelliformes. The reproductive anatomies of erpobdellid (Erpobdellidae) and salifid (Salifidae) leeches are similar to those already discussed for the Glossiphoniidae, Piscicolidae, and Ozobranchidae. The male atrium also is bilobed and can have sperm ducts with or without a preatrial loops ('U' shaped) that extend anteriorly from the atria, then descend posteriorly; ejaculatory bulbs are not present. As for the female system, they too have simple tubular ovaries. What is unique to the Erpobdellidae and Salifidae, is testisac arrangement. Contrasting the typically discrete paired arrangement of the testisacs, salifid species have testisacs that are discretely arranged, but in somatic tetrads (two pairs of testisacs on each sperm duct) per somite, while erpobdellid leeches have an atypical arrangement of the testisacs that are in multiple grape-like clusters profusely arranged along the length of the sperm ducts (Sawyer, 1986; Siddall and Burreson, 1995; Siddall, 2002; Borda and Siddall, 2004).

Americobdellidae. Some of the problems in classifying the anomalous *Americobdella valdiviana* were due to similarities in reproductive structures to erpobdellids, glossiphoniids and piscicolids (Moore, 1924; Sawyer, 1986; Siddall and Burreson, 1995), though the leech is hirudiniform both in gross morphology and in feeding habit. *Americobdella valdiviana* has a reproductive anatomy that is unique, in its own right (Fig 13). *Americobdella valdiviana* has a fused male atrium with an anterior seminal receptacle, awkwardly referred to as the "female" bursa (Moore, 1924; Sawyer, 1986). The sperm ducts are preceded by tightly coiled epididymes, followed by pairs of testisacs in nine somites. The female ovaries are tubular, much like in erpobdellids and the rhynchobdellid leeches. The most striking feature, however, are intergonadal coelomic tubules connecting the ovisacs with the female bursa (copulatory area), which may facilitate the passage of sperm. These have been considered homologous in form and function to the vector tissue known from piscicolid leeches (Moore, 1924; Sawyer, 1986; Siddall and Burreson, 1995) and approach a morphology very similar to those in the Ozobranchidae. Predictably, the monotypic Americobdellidae is the basal-most lineage of erpobdelliform leeches, sister to which are the piscicolids and ozobranchids.

Hirudiniformes. The so-called 'medicinal' leeches (e.g. *Hirudo medicinalis* and *Macrobdella decora*), other aquatic sanguivorous species (e.g. South American *Oxyptychus* spp.)

and several macrophagous groups (species of *Haemopis* and *Patagoniobdella*) have all been classified under the Family Hirudinidae (Blanchard, 1896). Similarly, all terrestrial leeches were once placed in the Haemadipsidae. This classification was primarily based on feeding habit, habitat preference and/or gross external morphology. Dissection and examination of the reproductive anatomy of members of these families initiated a re-examination of their relationships, as well as re-evaluation of their taxonomic status (Moore, 1927; Richardson, 1969, 1971, 1976; Ringuelet, 1985; Sawyer, 1986; Siddall and Burreson, 1985; Apakupakul et al., 1999; Borda and Siddall, 2004). Compared to the relatively simple reproductive anatomies already discussed, the hirudiniformes (Haemadipsidae, Haemopidae, Hirudinidae, Macrobdellidae, Semiscolecidae, and Xerobdellidae) have a considerably more 'complex' anatomy, coincident with their copulatory mating behaviour and with as much variation in structure as they are ecologically diverse. The male reproductive apparatus of hirunidform leeches generally has a fused atrium with posterior elongation of the atrium into a protrusible penis that recurves and ascends towards the male gonopore. The sperm ducts usually are equipped with bilateral ejaculatory bulbs adpressed to which are coiled epididymes. Typically there is one pair of testisacs in each of nine somites though species in the Semiscolecinae may have two pairs of testisacs per somite (Fig 14a) much as was described for the Salifidae. The female reproductive apparatus is equipped with a pair of small bulbous ovisacs subtended by oviducts to the vagina, either as independently (e.g. *Mesobdella gemmata* Fig. 15) or as a single common duct from the ovisacs (eg. *Patagoniobdella variabilis*; Fig. 14b).

Cylicobdellidae. As hirudiniforms, cylicobdellid leeches have a remarkably plesiomorphic reproductive anatomy. Borda and Siddall (2004) found that Cylicobdellidae was the basal-most lineage to the Hirudiniformes and their anatomy seems to be consistent with this phylogenetic position. Cylicobdellid leeches appear to retain the reproductive anatomy found in the Erpobdellidae. They have anteriorly directed, paired male atria with cornua (Fig. 16), and primary coiling of the sperm ducts that extend posteriorly looping anteriorly towards the atria before descending laterally. Unlike erpobdellid leeches (which this arrangement resembles) they have a single pair of testisacs in nine to twleve somites (much as described for the Glossiphoniidae above). The female apparatus consists of a pair of simple tubular ovisacs as is typical for Erpobdelliformes and "Rhynchobdellida."

10.3 Gametogenesis

Ooogenesis and spermatogenesis in leeches each begins with the release of epithelial cells from the walls of specialized coelomic sacs. Testisacs are found on either side intersegmentally. Ovisacs are typically ventrally arranged along the midline. The released spermatogonia and oogonia float freely in the lumen of the coelomic sacs wherein they undergo mitotic divisions followed by meiosis and the haploid nuclei eventually are found arranged around the periphery of an anucleate cytophore to which the gametes remain connected.

Unlike many other annelids, hirudinidan meiosis reportedly produces only a single viable oocyte receiving the bulk of the cytoplasm and remaining associated with three adjacent cells (Aisenstadt et al., 1967). The latter are true "nurse cells" inasmuch as there is considerable passage both of rRNA and proteinaceous material from them to the oocyte first by cytoplasmic streaming prior to cytokinesis and later pinocytically (Aisenstadt et al., 1967).

Hirudinidan meiosis to spermatocytes typically follows six mitotic divisions resulting in 256 immature spermatocytes attached to the cytophore (Malécha 1970; Lechenault and Pastisson, 1973). There appears to be an additional mitotic event among erpobdelliformes (Bonet and Molinas, 1988) though the precise number is unknown for most taxa. Maturation of the sperm in leeches, *Acanthodbella* and branchiobdellidans reflects their close phylogenetic affinities. All three groups possess an unusual dextrogyrously twisted acrosomal tube, the leading edge of which protrudes to form a marginal ridge (Ferraguti and Gelder, 1991; Franzen, 1991; Westheide and Purschke, 1996; Malécha, 1975; Bonet and Molinas, 1988). Branchiodebllidans have either seven or four mitochondria whereas leeches and *Ancanthodbella* each have only one per spermatozoon.

Among leeches there is considerable consistency in the shape and maturation of the spermatozoon (Malécha, 1975; Sawyer, 1986, Bonet and Molinas, 1988). In addition to the helical acrosome, all leeches have a dextrogyrously twisted nucleus with two or more helical ridges a well as a helical acrosomal tip (or anterior acrosome) that forms anterior to the invagination point of the proacrosomal vesicle. Following the second meiotic event, the large multilaminate U-shaped Golgi apparatus (from which the proacrosomal cap forms) is positioned between the nucleus and the cytophore with a single large mitochondrion and basal body at the distal pole. Elongation of the flagellum and the single mitochondrion proceeds unremarkably while the acrosomal protube begins to form in the collar region between the cap and the nucleus

attachment zone. The protube elongates in association with a palisade of microtubules extending from a distinctive plate region where the tube contacts the nucleus. At the point in which the acrosomal tube is fully formed, several transformations unique to leeches occur. The terminal electron-dense cap is forced to one side as a fibrous sheath extends anteriorly to form the anterior acrosome while the surrounding microtubules change conformation inducing a rotational force. This rotation ultimately twists each of the acrosomal tip, the acrosome itself, the nucleus and, in some taxa (Wissocq and Malécha 1975; Bonet and Molinas, 1988), the mitochondrion as well. The proacrosomal vesicle appears to consist of a single highly coiled Golgi tubule (Figs. 17, 18) both in leeches and in branchiobdellidans. The vesicle then invaginates into the acrosomal tube posteriorly with the acrosomal tip not receiving any acrosomal material. Finally a perforatorium-like structure assembles at the base. Maturation of the nucleus entails condensation of chromatin followed (at the rotational stage) by the longitudinal formation of two or more ridges such that the mature nucleus is either a double helix in the case of Hirudinidae and Erpobdelliformes or a triple helix among piscicolids (Figs.17, 19) whereas the mature glossiphoniid spermatozoon has a more complex and geometric unequal double helical nucleus (Fig. 20).

Oocytes generally remain in an arrested state until fertilized while still in the ovisacs. Spermatozoa travel up the sperm ducts towards the male median structures where they are bundled with agglutinating secretions from associated glands and are packaged into spermatophores.

10.5 Mating, fertilization and parental care

The most common form of sex in leeches (observed for more Glossiphoniidae and Erpobdelliformes) is traumatic insemination by way of packaged spermatophores implanted indiscriminately through the cuticle of a recipient mate (Fig. 21). Presumably hydrostatic changes induce the spermaotphore to empty its contents whereupon the spermatozoa cork-screw their way through the parenchyma and the coelomic spaces ultimately fertilizing oocytes in the ovisacs. Among piscicolids, spermatophore implantation is often site-specific to the ventral region of the clitellum where conducting (or "vector") tissues (Fig. 11) guide the sperm directly to the ovisacs. Among Hirudinformes (with the exception of the basal-most lineage Cylicobdellidae) mating is by copulation and insertion of a protrusible penis into a vaginal sac, the sizes and shapes of which tend to be species-specific (Figs. 14, 15).

Parental care has two basic forms among leeches. The fish leeches, or Piscicolidae, exhibit an adaptation that promotes their offspring achieving an early blood meal. Rather than abandoning a secreted "cocoon" as the oligochaetes and arhynchobdellid leeches do (Fig. 22), the piscicolids cement their egg cases to the surface of crustaceans (Fig. 23, 24). When that animal is later eaten, young leeches readily attach to the fish host's buccal surfaces and migrate to the gills. The Glossiphoniidae, like *Haementeria ghilianii*, are broad and flattened, and normally found feeding on turtles or amphibians. Species in this family secrete a membranous bag holding their eggs on their underside in a brooding position underneath rocks and other debris (Fig. 25). When the brood hatches, the young will turn and attach to the venter of their parent and, when the parent finds its next blood meal, they are carried to their first.

10.6 Development

Leeches stand out for being the best characterized annelid group, and arguably the best characterized lophotrochozoan group, with respect to cellular and molecular aspects of embryogenesis. As such, leeches have featured prominently in recent discussions of the evolution of development (Shankland and Seaver 2000). Clitellate embryogenesis is clearly derived in many respects, so it is fortunate that additional annelid groups are attracting the attention of a growing number of molecular and experimental developmental biologists (see Chapter **INSERT ED**). Still, the currently unparalleled experimental tractability of leech model systems and the deep base of knowledge already available for this group will doubtless keep leeches in the developmental spotlight for years to come.

Leeches, like all other clitellates, develop directly from yolky eggs: there is no trace of a trochophore larvum. All thirty-two segments are formed during embryogenesis and after hatching juveniles grow only in segment size, not segment number, in contrast to many other annelids which continue to add segments throughout their lives. (Note that the prostomium and peristomium are traditionally denoted somites I and II by systematists even though they do not constitute true developmental segments.) Embryonic development in the large yolky embryos of glossiphoniids (e.g., *Helobdella*, *Theromyzon*) closely resembles that in the large yolky embryos of several oligochaete groups (e.g., tubificids, lumbriculids), suggesting that glossiphoniid embryogenesis may resemble ancestral hirudinid embryogenesis in many respects. Albumenotrophy, in which embryos ingest the albumen provisioned in the cocoon rather than

relying solely on yolk stores of the egg, occurs in several groups including the hirudiniforms, erpobdellids, and piscicolids. Early development in albumenotrophic species involves the formation of temporary embryonic feeding structures and other modifications that are clearly derived. However, later development in these groups is largely similar to development in yolky-egged species.

The small non-blood feeding glossiphoniid leeches in the genus *Helobdella* produce large (~450 µm diameter) embryos which are amenable to a range of manipulations (e.g., intracellular injections of lineage tracers, intracellular injections of drugs and reagents to manipulate gene-expression, targeted cell ablations) offering tremendous advantages for experimental investigations of development. Studies of leech development have focused heavily on species of *Helobdella*, though considerable work has also been done on glossiphoniid *Theromyzon* species and on the hirudiniform *Hirudo medicinalis*.

10.6.1 Cellular aspects of leech development: cell lineages and cell fates

As in other annelids, cleavages in leeches are highly stereotyped with respect to timing, orientation, and cell size, making it possible to reproducibly identify many cells of the embryo and follow many of their cleavages. Cell lineages and cell fates have been most extensively investigated in species of *Helobdella*, primarily using injections of intracellular lineage tracers (see Weisblat and Huang 2001 for a recent review).

After fertilization, meiosis of the egg is arrested at metaphase I until the zygote is deposited into the cocoon. Meiosis then resumes, two polar bodies are produced, and the two pronuclei fuse. Prior to the first cleavage, a series of cytoplasmic reorganizations assemble regions of yolk-free cytoplasm, called teloplasm, at the animal and vegetal poles of the zygote. This teloplasm contains cell-fate determinants and is enriched in maternal mRNAs, mitochondria and ribosomes (Astrow et al. 1987; Fernandez et al. 1987; Nelson and Weisblat 1992; Holton et al. 1994).

Early cleavages in *Helobdella* give rise to three classes of cells: 3 large, yolk-rich vegetal cells called macromeres (the main endodermal precursors), 25 small animal cells called micromeres (which contribute primarily to asegmental head structures and a provisional embryonic epithelium), and 10 large cells called teloblasts (the precursors of all segmental mesoderm and ectoderm) (Figs. 26, 27).

The spiralian cell division pattern is discernable in leech embryos through the first few cleavages, although important modifications are apparent even early on. The first two cleavages are roughly meridional and orthogonal to each other, dividing the animal into four large cells, A, B, C, and D, of which D is the largest and inherits the bulk of the teloplasm (Fig. 28). The third cleavage is equatorial and highly unequal in all four quadrants, generating four large vegetal macromeres and four small animal micromeres (Fig. 26). Although this and several subsequent cell divisions show the spiralian pattern of alternation of sinistral and dextral cleavages, with micromeres produced towards the animal pole, glossiphoniid leech embryos show an important deviation beginning at this third division: divisions in the B quadrant are in the opposite direction from the typical spiralian pattern (Sandig and Dohle 1988; Weisblat and Huang 2001). Thus, the third and fourth B-quadrant divisions are sinistral and dextral, respectively, while they are respectively dextral and sinistral (the typical spiralian pattern) in the A, B, and C quadrants. The A and B quadrants are therefore mirror images of each other.

Endoderm. The macromeres of the A, B, and C quadrants are the main precursors of the endoderm. In *Helobdella*, these three macromeres produce three micromeres each and then cease cleaving, arresting in the G2 phase of the cell cycle (Bissen and Weisblat 1989). Later in development, these macromeres fuse together in a stepwise manner to form a syncytial yolk cell (Fig. 27) (Liu et al. 1998), a process at least partly dependent on signals from cells in the D quadrant (Isaksen et al. 1999). Late-stage teloblasts and some of their recent progeny (supernumerary blast cells) also eventually fuse with the syncytial yolk cell (Liu et al. 1998; Desjeux and Price 1999). The syncytial yolk cell ultimately cellularizes to form the midgut endoderm (crop, intestine, and rectum) surrounding the remaining yolk (Whitman 1878; Nardelli-Haefliger and Shankland 1993), which is digested prior to the juvenile's first meal. Local signals from the mesoderm to the endoderm are critical for normal leech gut morphogenesis (Wedeen and Shankland 1997), as they also appear to be in the oligochaete *Eisenia* (Devriès 1974). Unlike the situation in *Helobdella*, however, in several other leeches and in some oligochaetes the D macromere contributes substantially to the presumptive endoderm (in addition to generating the teloblasts), and the macromeres do not cease cleaving early in development but rather continue dividing to form a multi-celled endodermal mass (Anderson 1973; Shimizu 1982). Thus, *Helobdella* may represent a derived condition in these two aspects of gut morphogenesis.

Segmental ectoderm and mesoderm. All segmental ectoderm and mesoderm is derived from 10 large embryonic stem cells, called teloblasts, which are produced by the large D' macromere (Fig. 27). D' generates the 10 teloblasts and 15 additional micromeres through a unique series of cell divisions which are teloplasm-dependent (Astrow et al. 1987; Nelson and Weisblat 1991). The division of D' is obliquely equatorial and forms a (more vegetal) mesodermal precursor cell (DM) which ultimately produces a left/right pair of mesodermal teloblasts called mesoteloblasts, and a (more animal) ectodermal precursor cell (DNOPQ) which ultimately produces four left/right pairs of ectodermal teloblasts called ectoteloblasts. These five pairs of teloblasts form the left and right halves of the leech body, each half developing largely independently of the other. The sequence of divisions producing the ectoteloblasts and the relative positions of teloblasts vary among leech species (Fernandez and Stent 1982), but those of *Helobdella* resemble those of other glossiphoniids and the oligochaete *Tubifex* (Shimizu 1982; Sandig and Dohle 1988), suggesting that these may represent the ancestral patterns for leeches, a notion consistent with the relationships in Fig. 6.

On each side of the embryo, then, there is one mesodermal teloblast, designated M, and four ectoteloblasts, designated N, O/P, O/P, and Q. Each teloblast repeatedly divides asymmetrically producing small progeny cells, called primary blast cells, which are organized into a discrete column, or bandlet (Figs. 26, 30A). The M, N, and Q teloblasts generate blast cells that are from birth fated to be of the M, N, or Q type, respectively, but the O/P teloblasts are equivalent (Weisblat and Blair 1984; Zackson 1984). They generate bandlets which are only secondarily induced to adopt an O or P fate through interactions with neighboring bandlets and the micromere-derived provisional epithelium (Shankland and Weisblat 1984; Ho and Weisblat 1987; Huang and Weisblat 1996).

Segment formation occurs in an anterior-to-posterior progression: the first blast cells produced give rise to the most anterior segmental tissue and subsequent blast cells give rise to progressively more posterior tissue. The segmental identities of blast cells are largely established at birth (Martindale and Shankland 1990; Gleizer and Stent 1993; Nardelli-Haefliger et al. 1994). Possibly reflecting the ancestral clitellate phenomenon of indeterminate segment addition, leech teloblasts generate more than the number of blast cells required to build the 32 segments of the leech. These "supernumerary" blast cells fuse with the syncytial yolk cell late in development (Desjeux and Price 1999).

The five bandlets on each side of the embryo come together to form left and right germinal bands, with the n, o, p, and q bandlets lying side by side and overlying the m-bandlets. The left and right germinal bands progressively move ventrally over the surface of the macromeres and coalesce to form the germinal plate (Figs. 26, 29), with the two n-bandlets straddling the future ventral midline. Finally, the lateral edges of the germinal plate extend dorsally and the left and right q-bandlet progeny fuse at the dorsal midline. The mass of endoderm is thus internalized, completing the formation of the leech's body tube. Germ band formation, germinal plate formation, and dorsal closure appear to be quite similar among various clitellate annelids (Anderson 1973).

Injections of cell lineage tracers into teloblasts have beautifully revealed the ultimate fates of cells in each teloblast lineage (e.g., Weisblat et al. 1978; Weisblat et al. 1980; Weisblat and Shankland 1985). Cells in each of the five types of bandlets divide in a unique stereotypical fashion and give rise to segmentally iterated sets of definitive progeny (Fig. 30B). Each blast cell in the m, o, and q bandlets produces one hemisegmental compliment of definitive progeny, while in the n and q bandlets a pair of consecutive blast cells (n_f/n_s and q_f/q_s) together produces one hemisegmental complement (Fig. 30B,C). The M lineage gives rise to body wall muscle, visceral mesoderm, septa walls (which are later lost), nephridia, a few ganglionic neurons, and probably the germ line. Each of the four ectodermal lineages gives rise to neurons and epidermis, the N lineage contributing mostly to ventral tissue (including most of the ventral nerve cord neurons) and the O, P, and Q lineages contributing primarily to progressively more dorsal tissue.

Asegmental tissue and provisional epithelium. Initially restricted to the "micromere cap" at the embryo's animal pole, the micromeres produced during early cleavages ultimately give rise to asegmental tissues, primarily of the head, and a provisional embryonic epithelium (Weisblat et al. 1984; Nardelli-Haefliger and Shankland 1993; Smith and Weisblat 1994; Huang et al. 2002). Definitive micromere-derived structures include the proboscis (foregut) epithelium, proboscis muscles (Fig. 31), proboscis sheath, neurons and connective tissue of the cerebral (supraesophageal) ganglion, putative glial cells and connective tissue of the fused rostral (subesophageal) ganglia, and anterior and posterior sucker epithelium. Interestingly, a recent detailed cell-lineage analysis of micromeres reveals that some micromeres give rise to different definitive progeny in two closely related *Helobdella* species, attesting to the evolutionary lability

of micromere cell fates (Huang et al. 2002). The micromere-derived provisional epithelium initially covers the animal pole and progressively spreads over the germinal bands (Fig. 29). Although it does not produce any definitive structures, it sends critical signals to the underlying developing germinal bands and is required for proper germ band migration over the endoderm surface (Ho and Weisblat 1987; Smith et al. 1996). The provisional epithelium expands ventrally as the germinal bands coalesce ventrally, and then contracts dorsally and ultimately disappears during dorsal closure.

10.6.2 Molecular aspects of leech development: developmental regulatory genes

Since the late 1980's, when the first molecular studies of leech development were published, a wide range of developmental regulatory genes have been isolated from and characterized in leeches (Table 1). These genes include members of many gene families, including homeodomain transcription factors (Hox, ParaHox, *engrailed*, *even-skipped*, *msx*, NK-2, *orthodenticle*), zinc-finger transcription factors (*hunchback, snail*), basic helix-loop-helix transcription factors (*hairy/Enhancer of split*, *twist*), a *rel*-domain transcription factor (*dorsal*), secreted signaling molecules (*wnt*, *hedgehog*, *netrin*), a zinc-finger RNA-binding protein (*nanos*), a phosphatase (*cdc25*), and a cyclin (*cyclin A*) and investigations of many other genes are in progress. Several techniques to investigate mRNA and protein expression are now routine in leech studies (RT-PCR, in situ hybridization, immunolocalization, developmental Northern analyses) and techniques for manipulating normal expression of target genes are also becoming available (Nardelli-Haefliger et al. 1994; Pilon and Weisblat 1997; Baker and Macagno 2000; Song et al. 2002).

Gene expression during early development. Maternal genes whose products are present in leech oocytes and in the early embryo include *Hro-wnt-A*, *Hro-nos*, *Lzf2*, *Le-msx*, *Hro-twi*, *cdc25* and *cyclin A*, and two additional genes, *Hro-eve* and *Hro-hes*, are expressed beginning in early cleavage stages, although they may not be maternally deposited (Table 1). *Hro-nos*, *lfz2*, and *Le-msx* show a similar early pattern of early mRNA localization: transcripts are initially present throughout the oocyte and uncleaved zygote, become segregated to the pools of teloplasm that form prior to first cleavage, and ultimately become localized primarily to the D' macromere, the precursor of all segmental tissues which inherits the bulk of the teloplasm. In line with these gene-specific findings, earlier studies showed that the majority of polyadenylated

RNAs in leech oocytes become concentrated into the teloplasm (Holton et al. 1994). *Hro-eve*, *Hro-hes*, *cdc25* and *cyclin A* are expressed throughout the embryo beginning in early cleavage stages. In some or all embryonic lineages they are regulated, or become accessible, in a cell-cycle dependent manner suggesting that their expression helps regulate or responds to embryonic cell cycles.

Hro-Wnt-A is one of the earliest genes detected in leeches and its protein product, HRO-WNT-A, displays a highly unexpected expression pattern for a species with a fixed cell lineage: stochastic expression between non-equivalent cells (Huang et al. 2001). Despite the many clear differences between cells AB and CD (e.g., in developmental potential, volume, cytoplasmic inheritance, and cell cycle duration), shortly after first cleavage HRO-WNT-A is expressed stochastically in either the AB *or* the CD cell. Experimental manipulations suggest this gene is involved in signalling from one cell to the other to regulate cell-cell adhesion, and is not involved in cell fate decisions at this stage. HRO-WNT-A is expressed at later stages as well, stochastically at the 4-5 cell stage, during micromere production, and apparently stochastically in provisional epithelium cells (Kostriken and Weisblat 1992; Huang et al. 2001). HRO-WNT-A is expressed in a similar way in micromeres and the provisional epithelium in several glossiphoniid species (Kostriken and Weisblat 1992).

Another gene expressed early in leech development, *Hro-nos*, is a strong candidate for being involved in the cell-fate decision between segmental ectoderm and segmental mesoderm (Pilon and Weisblat 1997). Its protein is expressed primarily in cells which inherit teloplasm and its levels peak when macromere D' divides to generate the ectodermal and mesodermal precursor cells. At this cleavage, although both daughter cells inherit teloplasm, the transcripts and protein of *Hro-nos* are preferentially segregated to the ectodermal precursor (DNOPQ) relative to the mesodermal precursor (DM). *Hro-nos* apparently plays no role in anterior/posterior axis specification, in contrast to the important role the *Drosophila* homolog *nanos* plays in this specification (St. Johnston and Nüsslein-Volhard 1992).

Lzf2, *Hro-wnt-A*, and *Hro-nos* (Table 1 and S. J. Agee and D. A. Weisblat, unpublished data on *Hro-nos*) are strongly and transiently expressed in subsets of micromeres shortly after their birth. It seems possible that *Hro-wnt-A* plays a role in cell adhesion during micromere production, but the role of *Lzf2* and *Hro-nos* in micromeres at this stage is unclear.

Gene expression during mid development. Despite great interest in understanding the molecular basis of the leech segmentation, this aspect of leech embryogenesis remains largely obscure. Teloblasts clearly differ in size and developmental potential, and blast cells exhibit lineage-specific stereotypical cleavage patterns, possess lineage-specific developmental potentials, and inherit different axial identities. Nevertheless, nearly all of the characterized genes expressed in segmental precursor cells are expressed broadly and uniformly, with no obvious expression differences between teloblast lineages, blast cell types, or along the anterior/posterior axis. A central puzzle in leech development continues to be how, at the molecular level, differences between teloblasts and between blast cells are established. Genes broadly expressed in developing segmental tissue (i.e., in teloblasts, blast cells, germinal bands, and/or the early germinal plate) include *Le-msx*, *Lzf2* (transcript though not protein), *Hro-nos*, *Hro-eve*, *Hro-hes*, *Hro-dl*, and *Hro-sna1/2* (Table 1).

A homolog of the *Drosophila* segment polarity gene *engrailed* was one of the earliest genes characterized in leeches, and remains the only gene known to exhibit clear expression differences between teloblast lineages during germ-band stages of development. The protein of *ht-en* is expressed transiently in each of the five teloblast lineages, in an iterated, lineage-specific pattern in each blast-cell clone (Wedeen and Weisblat 1991; Lans et al. 1993). Although the *ht-en* expression pattern initially prompted speculation that it may be involved in establishing segmental boundaries or in compartmentalizing the ventral nerve tissue into ganglia (Wedeen and Weisblat 1991; Lans et al. 1993; Ramirez et al. 1995), further studies including targeted ablations of *ht-en* expressing cells or their precursors have clearly demonstrated that neither of these hypotheses is correct (Shain et al. 1998; Seaver and Shankland 2000; Seaver and Shankland 2001). Particularly telling, primary blast cell clones can develop normally even when anterior and/or posterior clones are experimentally removed (Seaver and Shankland 2000), indicating that signalling between neighboring clones (by *ht-en* or any other gene) is not required for normal segment polarity or delineation, in sharp contrast to the method of segment formation in arthropods (Kornberg and Tabata 1993; Davis and Patel 1999).

Mid-stage leech embryos also exhibit expression of several genes in developing non-segmental tissue. The protein products of *Hro-wnt-A* and *Lzf2* are both expressed in the provisional epithelium, although in quite different patterns: HRO-WNT-A is expressed in an apparently stochastic pattern that varies from embryo to embryo (Kostriken and Weisblat 1992),

while LZF2 is expressed in a bilaterally symmetrical pattern (Iwasa et al. 2000). In the anterior asegmental tissue (prostomium), LZF2, *Lox10*, and *Lox22-Otx* are expressed, each in a unique pattern (Table 1). Of note, *Lox22-Otx* is expressed at the extreme anterior end of the germinal plate, ultimately forming a circle of expression surrounding the developing mouth. *Otx* homologs are expressed in the developing head of many organisms including other annelids (Arendt et al. 2001; Bely and Wray 2001), suggesting that this domain of expression in leeches represents a very conserved aspect of animal embryogenesis.

Gene expression during late development. In the final stages of embryogenesis, during organogenesis and tissue differentiation, a whole suite of genes are expressed in the leech embryo. Leech neurogenesis is marked by particularly extensive expression of developmental regulatory genes: *Ht-en*, *Hro-eve*, *Lox22-Otx*, *Hro-hh*, *Lzf2*, *Le-msx*, LNET-1, and all characterized Hox gene homologs are expressed in subsets of ventral nerve cord neurons and peripheral neurons (Table 1). Most of these genes are expressed in many contiguous segments or in all segments, in segmentally iterated subsets of neurons that are likely to be (or known to be) segmental neuronal homologs (many of which are identified in *Hirudo*). These data suggest that specific neuronal phenotypes may be established and/or maintained by a large number of regulatory genes possibly acting in a combinatorial way. In addition to genes expressed neuronally, LNET-1 is expressed by ventral longitudinal muscles which just lie below the ventral nerve cord, and *Lox18* and LZF2 are expressed in interganglionic longitudinal connective tissue. As a member of the *netrin* gene family, LNET-1 is expected to direct axonal growth, and the leech muscle expression likely serves as a guide for peripheral innervation. Expression in the central nervous system midline and in muscles appear to be ancient and conserved features of *netrin* in metazoans (Arendt and Nübler-Jung 1999). As for asegmental neuronal expression, peripheral neurons associated with sensillae and eyes express *Lox6* in *Hirudo* (though not in *Helobdella*), and neurons of the asegmental, micromere-derived cerebral (supraesophageal) ganglion express *Lox22-Otx* and *Lox10*.

With two exceptions (*Lox18*, which is one of two *Dfd* homologs, and *Lox1*, of uncertain affinity), all leech Hox genes display nested anterior boundaries of expression relative to one another in the segmental ganglia (reviewed in Kourakis et al. 1997), conforming to the Hox colinearity rule that has been observed in most animals investigated. The *labial* homolog *Lox7* is the most anterior Hox member and is expressed in all segmental ganglia, the *Dfd* homolog *Lox6*

is the next most anterior Hox gene and is expressed in all but the anterior-most ganglion, the *Scr* homolog *Lox20* is expressed beginning in the third rostral ganglion, the *Antp* homolog *Lox5* is expressed beginning in the fourth rostral ganglion, and the two *Ubx*/*abd-A* homologs *Lox2* and *Lox4* are expressed primarily in the midbody region, in segments 10-25 (somites XII-XXVII) and segments 14-25 (somites XVI-XXVII), respectively. Anterior limits of expression tend to be sharp, while posteriorly expression generally fades gradually over multiple segments. All leech Hox genes are expressed considerably after segmental identities are conferred to the segmental ganglia (Martindale and Shankland 1990; Nardelli-Haefliger et al. 1994), and thus cannot be involved in establishing segmental identities, in contrast to the role of Hox genes in *Drosophila* (McGinnis and Krumlauf 1992). Instead, most leech Hox genes may be involved only in late stages of segmental diversification by helping to establish and maintain different terminal cell fates of neurons. Hox genes in a polychaete (*Chaetopterus*) *are* expressed during segment formation and thus could be involved in conferring segmental identity (Irvine and Martindale 2000). Broader sampling within the annelids is needed to determine the ancestral expression patterns and inferred functions of annelid Hox genes.

Several genes are also detected in developing mesodermal structures of late-stage embryos (Table 1). *Lox1*, *Lox2*, and *Le-msx* are expressed in developing nephridia and *Lox20* and *Lox5* are expressed in developing segmentally iterated mesodermal structures, such as septa. Dorsoventral flattener muscles (which cause segmental constrictions of the gut) in the posterior two-thirds of the body express *Lox2* (the same body segments that express *Lox2* in the ventral nerve ganglia), and as mentioned previously ventral longitudinal muscles express LNET-1, presumably to direct peripheral nerve axonal growth. The developing gonads express *Lox2* and *Hro-hh*, and putative primordial germ cells express *Hro-nos*. Expression of *nanos*-class genes in germ cells appears to be a widely conserved feature of metazoans (Matova and Cooley 2001). Several genes, including *Lzf2*, *Hro-dl* and *Hro-sna1/2*, are expressed in segmentally iterated domains which may be mesodermal, but the exact origin and ultimate fates of these expressing cells are not known. (Expression in visceral mesoderm is described below, along with expression in the gut lining.)

Although a number of genes are expressed in the body wall epidermis, expression tends to be sparse, occurring in only a few, mostly unidentified, cells per segment. A notable

exception is *Lzf2*, for which transcripts, though not protein, are detected specifically in the caudal sucker.

The developing gut expresses several genes, either in the true gut lining or in the visceral mesoderm (Table 1) The endodermal gut lining initially appears unsegmented, but the crop and intestine (though not the rectum) ultimately develop segmentally iterated bulges and constrictions, the caeca of the crop and intestine. Interestingly, two genes, *Lox10* and *Lox3*, are expressed in the endodermal gut lining in segmentally iterated patterns of spots or stripes that largely prefigure the segmental periodicity and regionalization of the leech gut architecture (Nardelli-Haefliger and Shankland 1993; Wysocka-Diller et al. 1995). The expression of at least one of these (*Lox3*) is dependent on signals from the tightly apposed visceral mesoderm (Wedeen and Shankland 1997). Both ParaHox and *NK-2*-class genes are expressed in the developing guts of disparate animals, suggesting that these roles may be widely conserved (Brooke et al. 1998; Ristoratore et al. 1999; Venkatesh et al. 1999). *Lzf2* transcripts and protein are also expressed in the intestine and rectum, but in broad, aperiodic patterns. At late embryonic stages *Lox22-Otx* continues to be expressed in a ring around the developing mouth and also becomes highly transcribed in the developing foregut, specifically in muscles of the proboscis (Bruce and Shankland 1998). *Hro-hh* is also expressed in proboscis muscles, as well as in all three regions of the midgut (crop, intestine, and rectum), including in rings of segmentally iterated visceral mesoderm (Kang et al. 2003). Experimental disruptions of *Hro-hh* expression demonstrate that *Hro-hh* signalling is required for normal gut morphogenesis. Together, molecular studies of *Lox3* and *Hro-hh* reinforce findings from cell ablation studies in implicating endoderm - visceral mesoderm interactions as critical for normal gut morphogenesis.

While the number of genes investigated during leech embryogenesis is still relatively limited, at least a few genes involved (or likely to be involved) in many aspects of development have now been identified. Important similarities between leeches and other animal models (*Drosophila*, vertebrates) have helped to highlight the extreme conservation of the involvement of some of these genes in specific processes: Hox genes in generating axial diversity (especially of the central nervous system), ParaHox genes, *NK-2*-class genes, and *hedgehog*-class genes in gut development and regionalization, *Otx*-class genes in head development, *nanos*-class genes in germ line development, and *wnt*-genes in cell adhesion. However, it is clear that the molecular basis of many aspects of leech development differs profoundly from that in other developmental

model systems. In sharp contrast to *Drosophila*, for example, in leeches the establishment of the anterior-posterior axis does not appear to involve *nanos* or *hunchback* homologs, establishment of the dorsal-ventral axis and specification of the mesoderm does not appear to involve *dorsal* or *snail* homologs, and leech segmentation apparently does not involve homologs of several important *Drosophila* segmentation genes, including *engrailed*, *even-skipped*, *hairy/Enhancer of split*, *hunchback*, and *hedgehog*. Clearly, though much has been learned, leech development still presents many puzzles which remain to be solved.

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Tables

Table 1. **SEE SEPARATE FILE NAMED "TABLE 1"**

Figure Legends

Figure 1. Ordinal level phylogeny of the Oligochaeta with symbiotic lineages represented by thick lines (after Siddall et al., 2001 and Jameison et al. 2002).

Figure 2. The glossiphoniid leech *Placobdelloides jaegerskioeldi* feeding from its preferred attachment site, the rectal tissues of *Hippopotomus amphibious*.

Figure 3. The marine turtle leech *Ozobranchus margoi* sporting lateral appendages of uncertain function.

Figure 4. The very colorful *Hirudo medicinalis* of blood-letting infamy engaging in that most infamous act on the third author's finger.

Figure 5. A Malagasy terrestrial haemadipsid in the genus *Malagobdella* making an incision on the first author's hand.

Figure 6. Phylogeny of leeches using the combined information from morphology and the genetic loci 28S rDNA, 18S rDNA, mitochondrial 12S rDNA CO-I, and ND-I, in which black lineages are most parsimoniously optimized as historically blood-feeding.

Figure 7. A large glossoscolescid oligochaete from the Andes in Boliva equipped with a large and prominent clitellum.

Figure 8. The erpobdelliform leech *Barbronia gwalagwalensis* from South Africa with a noticeable clitellum.

Figure 9. *Hirudo medicinalis* in which the clitellum is cryptic as it is in most species of leech.

Figure 10. Dissected *Helobdella wodzickiorum* revealing typical glossiphoniid anterior paired ejaculatory atria from which descend and loop the male sperm ducts along the midline of the coelomic cavity.

Figure 11. Dissected *Helobdella wodzickiorum* with the male sperm ducts removed revealing typical elongate glossiphoniid ovisacs (arrows).

Figure 12. Diagrammatic representation of the median reproductive structures of piscicolids with conducting tissues (arrows) between the anterior bursa and the paried ovisacs.

Figure 13. Dissected *Americobdella valdiviana* revealing the pair intergonadal coelomic tubules (arrows) connecting the ovisacs (to the right) with the female.

Figure 14. Median reproductive anatomy of *Aliolimnatis africana* exhibiting globular oviscacs (o) terminal to the paired and common oviduct, and the typically hirudiniform "epydidymes" (e) one either side of the protrsuible penis (p).

Figure 15. Median reproductive anatomy of the terrestrial *Mesobdella gemma* exhibiting the left globular oviscac (o) paired "epydidymes" (e) and protrsuible penis (p).

Figure 16. Median male atria of Cylciobdella coccinea.

Figures 17-21. Hirudinidan-like spermatogenesis. 17. Spermatozoa of *Malmiana scorpii* in mid-stage of maturation arranged around a central cytophore (c) and exhibiting a coiled nucleus, an empty acrosomal tube, an early acrosomal tip as well as the highly coiled proacrosomal vesicle (pv) prior to invagination. 18. Coiled proacrosomal vesicle of the branchiobdellidan Cronodrilus ogygius [courtesty of Marco Ferraguti]. 19. Transverse section of spermatozoa of *Malmiana scorpii* in mid-stage of maturation exhibiting the flagellum, single mitochondrion, trihelical nucleus and empty acrosomal tube. 20. Mature spermatozoa of *Theromyzon tessulatum* in longitudinal section revealing the complex geometry of the coiled nucleus (n) [courtesty of Marco Ferraguti]. 21. Diagrammatic representation of the relative structure and sizes of the five principal zones of a mature leech spermatozoon.

Figure 22. Spongy cocoon of the hirudinid *Hirudo medicinalis* which that species deposits on land usually in damp vegetation.

Figure 23. Scanning electron micrograph of the spheroid coccon of the piscicolid *Oxytonostoma typica* showing the posterior operculum.

Figure 24. Scanning electron micrograph of the flattened coccon of the piscicolid *Oceanobdella microstoma* showing both opercula (arrows).

Figure 25. Scanning electron micrograph of *Helodbella elongata* revealing the membranous cocoon brooded on the venter.

Figure 26. Diagram of glossiphoniid leech development. (A) Eight-cell embryo (animal view). (B) 20-cell embryo (animal view). (C) Beginning of germinal-plate formation (animal view). The left and right germinal bands coalesce along the future ventral midline to form the germinal plate in an anterior to posterior direction. The syncytial yolk cell

(prospective endoderm) is shaded light gray; the micromere-derived provisional epithelium which covers the germinal bands and germinal plate is shaded dark gray. (D) Completion of germinal band and beginning of segment morphogenesis (lateral view). (E) Juvenile (dorsal view). The midgut (endoderm) is shaded gray; the foregut (micromere-derived) is outlined in gray.

Figure 27. Summary of early cell divisions and ultimate cell fates in the glossiphoniid *Helobdella robusta*. For diagram clarity, cell divisions of only one of the two DNOPQ cells is included. O/P teloblasts produce o/p blast cells which acquire O and P fates through interactions with other bandlets. Dotted lines represent continued blast cell production by teloblasts. Supernumerary blast cells, like teloblasts, fuse with the syncytial yolk cell (not shown). The relative timing of fusion of teloblasts O, P, and Q and of supernumerary blast cells with the syncytial yolk cell is not known. Divisions of micromeres and blast cells are not included in this diagram. Leech cell nomenclature is used throughout; standard spiralian notation is indicated in parentheses for the first few cleavages. Diagram based on Weisblat and Huang (2001).

Figure 28. *Helobdella* embryos at the 4 cell stage. Note the large D macromere. Photo courtesy of Marty Shankland.

Figure 29. *Helobdella* embryo during germinal plate formation silver-stained to highlight superficial cell outlines. This is a ventral view, with anterior up. The provisional epithelium covers much of the embryo at this stage, but the left and right germinal bands and the germinal plate (formed by the coalescence of left/right germinal bands) are apparent as bulges below this epithelium. Photo courtesy of Françoise Huang.

Figure 30. Teloblast contributions (A) Fluorescently labeled O teloblast and bandlet of a *Helobdella* embryo. The labeled teloblast has produced a column of approximately 20 primary blast cells since being injected. Photo courtesy of Marty Shankland. (B) Late-stage *Helobdella* embryo (dissected away from yolk) in which two teloblasts (M on left, N on right) were injected with fluorescent tracers early in development. This is a

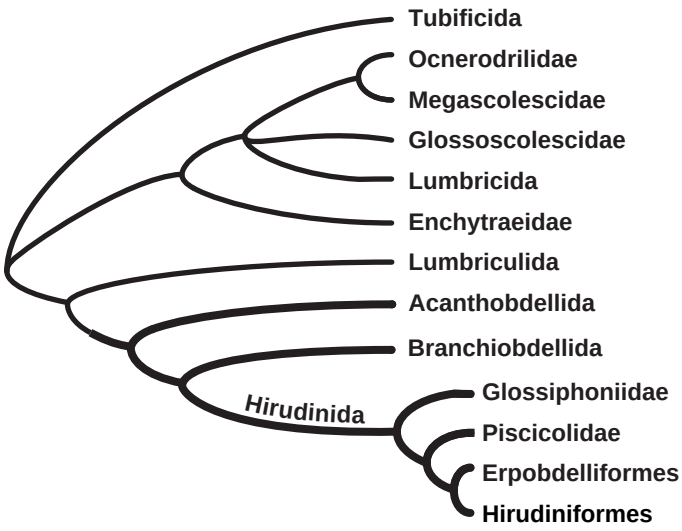
ventral view, with anterior up. The M teloblast gives rise to extensive mesodermal tissues (red) and the N teloblast gives rise primarily to the ventral nerve cord ganglia (green) which lie along the ventral midline. The M and N teloblasts were injected at the same time and produce primary blast cells at approximately the same rate. However, the anterior boundaries of labeled M progeny and labeled N progeny differ because one m primary blast cell produces one M-lineage hemisegmental complement, while two consecutive n primary blast cells are required to produce one N-lineage hemisegmental complement. Photo courtesy of Marty Shankland. (B) Late stage *Helobdella* embryo (dissected away from yolk) with one hemisegmental complement of the o lineage fluorescently labeled (red). This is a ventral view, with anterior up. A single o primary blast cell was injected with tracer four days earlier. Nuclei are labeled with Hoechst 33258 stain (blue).

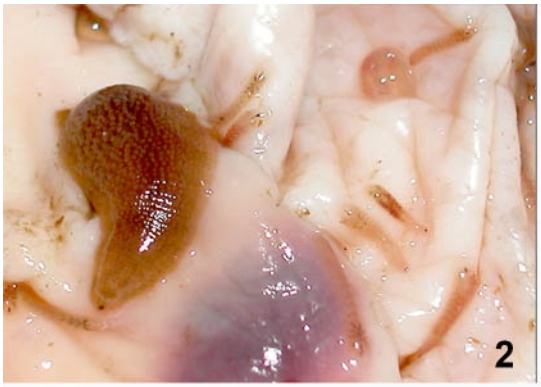
Figure 31. Late stage embryo in which two micromeres, dm’ and c’’’, were injected at an early stage. Anterior is to the left, and dorsal is up. Progeny of dm’ (red) and c’’’(green) interdigitate to give rise to probable muscle fibers of the proboscis as well as to a temporary fiber network extending throughout the embryo.

Table 1. Developmental regulatory genes investigated in leeches and their primary domains of expression. Where more than one species of *Helobdella* has been investigated, this is indicated by "spp.".

gene family	leech gene(s)	species	expression (mRNA/protein)	references
cyclin	*cyclin A*	*Helobdella* spp.	all cells, mRNA accessibility invariant in early blastomeres and cell-cycle dependent in teloblasts and probably micromeres (mRNA)	Chen and Bissen (1997)
cdc25	*cdc25*	*Helobdella* spp.	all cells, constitutive expression in blastomeres, macromeres, and teloblasts, cell-cycle dependent expression in micromeres	Bissen (1995)
***dorsal* (*dl*)**	*Hro-dl*	*Helobdella robusta*	all primary blast cells and their progeny; unidentified segmentally iterated stripes of cells (protein)	Goldstein et al. (2001)
***engrailed* (*en*)**	*ht-en*	*Helobdella* spp. *Theromyzon rude*	specific cells of young blast cell clones in all teloblast lineages; neurons of segmental ganglia (protein)	Wedeen and Weisblat (1991); Lans et al. (1993); Ramirez et al. (1995)
***even-skipped* (*eve*)**	*Hro-eve*	*Helobdella robusta*	most (all?) cells in mitosis; neurons of segmental ganglia (mRNA)	Song et al. (2002)
***hairy/Enhancer of split* (*hes*)**	*Hro-hes*	*Helobdella robusta*	most (all?) cells in mitosis (mRNA); most (all?) cells in interphase (protein)	Song et al. (2004)
***hedgehog* (*hh*)**	*Hro-hh*	*Helobdella* spp.	foregut and midgut; reproductive organs; body wall; neurons of segmental ganglia (mRNA)	Kang et al. (2003)
Hox: *labial* (*lb*)	*Lox7*	*Helobdella* spp.	neurons of segmental ganglia, all segments (mRNA)	Kourakis et al. (1997)
Hox: *Deformed* (*Dfd*)	*Lox6*	*Helobdella* spp. *Hirudo medicinalis*	neurons of segmental ganglia from segment 2 posterior (strongest in segment 3); peripheral nervous system (sensillae and eyes) in *Hirudo* only (mRNA)	Kourakis et al. (1997); Wong and Macagno (1998)
Hox: *Deformed* (*Dfd*)	*Lox18*	*Helobdella triserialis*	longitudinal connectives and lateral nerve roots of all segmental ganglia (mRNA)	Kourakis and Martindale (2001)
Hox: ? *(Scr?Antp?)*	*Lox1*	*Hirudo medicinalis*	neurons in all (early) or most (later) segmental ganglia, strongest from segment 3 posterior; nephridia; unidentified body wall cells (mRNA, protein)	Aisemberg et al. (1993); Aisemberg and Macagno (1994)
Hox: *Sex combs reduced* (*Scr*)	*Lox20*	*Helobdella* spp.	neurons of segmental ganglia from segment 3 – 6 (mRNA)	Kourakis et al. (1997)
Hox: *Antennapedia* (*Antp*)	*Lox5*	*Helobdella* spp.	neurons of segmental ganglia from segment 4 posterior (mRNA)	Kourakis et al. (1997)
Hox: *Ultrabithorax/abdominal*-A (*Ubx/abd-A*)	*Lox2*	*Helobdella* spp. *Hirudo medicinalis*	neurons of segmental ganglia from segment 10 posterior (posterior 2/3 of nerve cord); ovaries (segment 10); musculature (dorsoventral flattener muscles in posterior 2/3 of midbody); nephridia; body wall; digestive tract (mRNA, protein)	Wysocka-Diller et al. (1989); Nardelli-Haefliger and Shankland (1992); Aisemberg et al. (1993); Nardelli-Haefliger et al. (1994)
Hox: *Ultrabithorax/abdomin*	*Lox4*	*Hirudo medicinalis*	neurons of segmental ganglia strongest from segment 14 posterior (though anterior limit is at segment 7); peripheral tissues from	Wong et al. (1995)

***al*-A (*Ubx/abd-A*)**			segment 17 – 25 (mRNA, protein)	
***hunchback* (*hb*)**	*Lzf1*	*Helobdella triserialis*	not expressed? undetectable at any stage (mRNA)	Savage and Shankland (1996)
***hunchback* (*hb*)**	*Lzf2*	*Helobdella triserialis*	oocytes; micromeres; macromeres (especially in teloplasm); throughout germinal bands and germinal plate; micromere cap; unidentified stripes lateral to ganglia; caudal ganglion of rear sucker; rostral region of ventral nerve cord; proboscis sheath; gut (intestine/rectum/anus); subesophageal ganglion; rear sucker (mRNA); one cell stage; micromeres; provisional epithelium; neurons of segmental ganglia; prostomium; intestine (caeca) and rectum (protein)	Savage and Shankland (1996); Iwasa et al. (2000)
msx	*Le-msx*	*Helobdella* spp.	oocyte; D quadrant (primarily in teloplasm); all teloblasts, blast cells, bandlets, and the germinal plate; neurons of segmental ganglia; nephridia (mRNA)	Master et al. (1996)
NK-2	*Lox10*	*Helobdella triserialis*	midgut, in segmentally iterated spots/stripes in the crop and intestine, and throughout rectum; supraesophageal ganglion (mRNA)	Nardelli-Haefliger and Shankland (1993)
***nanos* (*nos*)**	*Hro-nos*	*Helobdella robusta*	oocyte; D quadrant (primarily in teloplasm); strong in ectodermal precursor (DNOPQ) and low in mesodermal precursor (DM); primordial germ cells (putative) (mRNA, protein)	Pilon and Weisblat (1997); Kang et al. (2002)
netrin	*LNET-1*	*Hirudo medicinalis*	neurons of segmental ganglia; ventral longitudinal muscles (mRNA, protein)	Gan et al. (1999); Aisemberg et al. (2001);
***orthodenticle* (*otx*)**	*Lox22-Otx*	*Helobdella triserialis*	anterior surface ectoderm, eventually encircling developing mouth; foregut; supraesophageal ganglion; neurons of segmental ganglia (mRNA)	Bruce and Shankland (1998)
ParaHox: *Xlox*	*Lox3 (A/B/C)*	*Helobdella triserialis* *Hirudo medicinalis*	midgut, in segmentally iterated stripes of segment 6 – 17 corresponding to crop constrictions and intestinal caeca, and throughout rectum (mRNA)	Wysocka-Diller et al. (1995); Wedeen and Shankland (1997)
***snail* (*sna*)**	*Hro-sna1* *Hro-sna2*	*Helobdella robusta*	all primary blast cells and their progeny; unidentified segmentally iterated strips of cells (protein)	Goldstein et al. (2001)
***twist* (*twi*)**	*Hro-twi*	*Helobdella robusta*	oocyte; throughout development (no spatial distribution information) (mRNA)	Soto et al. (1997)
wnt	*htr-wnt-A* *Hro-Wnt-A*	*Helobdella* spp.	oocyte; dynamic expression in 2-cell stage, including stochastic expression; micromeres; provisional epithelium (mRNA, protein)	Kostriken and Weisblat (1992); Huang et al. (2001)


Tubificida
Ocnerodrilidae
Megascolescidae
Glossoscolescidae
Lumbricida
Enchytraeidae
Lumbriculida
Acanthobdellida
Branchiobdellida
Hirudinida
Glossiphoniidae
Piscicolidae
Erpobdelliformes
Hirudiniformes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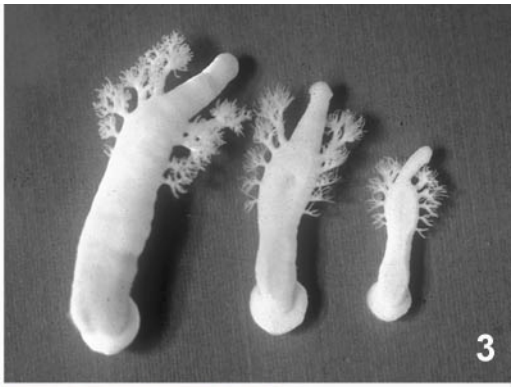
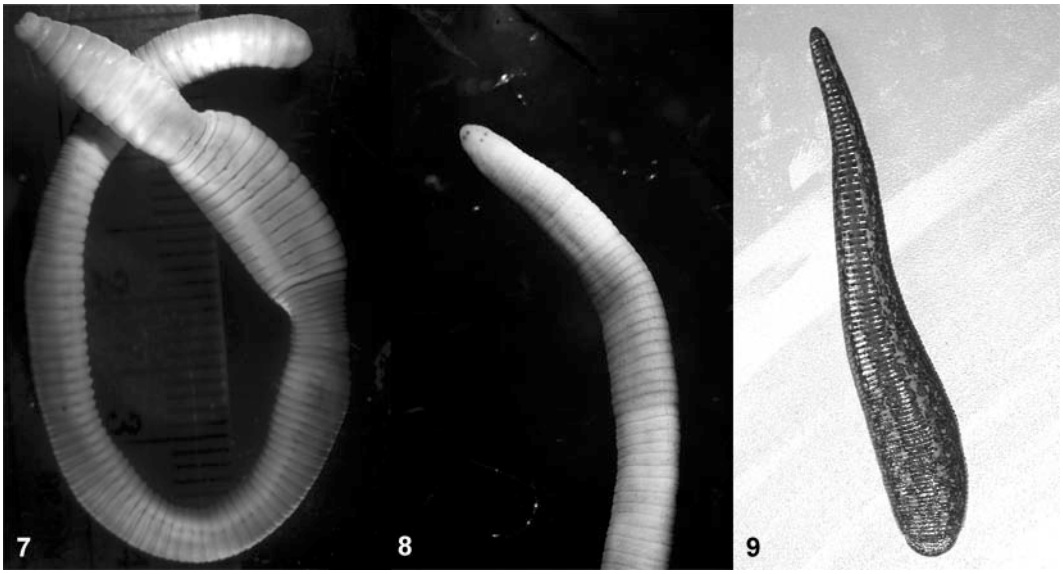

3


4


5

Marsupiobdella africana
Oosthuizobdella garoui
Theromyzon trizonare
Theromyzon tessulatum
Theromyzon bifarium
Hemiclepsis marginata
Batracobdelloides tricarinata
Placobdelloides multistriatus
Placobdelloides siamensis
Placobdelloides jagerskioeldi
Glossiphonia weberi
Alboglossiphonia heterocilita
Glossiphonia elegans
Glossiphonia complanata
Torix baicalensis
Glossiphonia concolor
Glossiphonia verrucata
Haementeria lutzi
Haementeria gracilis
Haementeria ghilianii
Haementeria tuberculifera
Haementeria molesta
Placobdella picta
Placobdella biannulata
Placobdella montifera
Placobdella pediculata
Placobdella costata
Placobdella phalera
Placobdella transluscens
Placobdella parasitica
Placobdella ornata
Placobdella papillifera
Placobdella multilineata
Helobdella ringueleti
Helobdella bolivianita
Helobdella michaelseni
Helobdella modesta
Helobdella stagnalis
Helobdella elongata
Helobdella lineata
Helobdella paraguayensis
Helobdella nununununojensis
Helobdella sorojchi
Helobdella fusca
Helobdella lineata
Helobdella transversa
Helobdella papillata
Helobdella cordobensis
Helobdella triserialis
Helobdella papillomata
Helobdella europaea
Pontobdella muricata
Stibarobdella macrothela
Branchellion torpedinis
Calliobdella vivida
Piscicola geometra
Ozobranchus margoi
Americobdella valdiviana
Linta andrianampoinimerinae
Barbronia gwalagwalensis
Barbronia formosana
Barbronia weberi
Erpobdella dubia
Erpobdella obscura
Erpobdella testacea
Erpobdella japonica
Erpobdella octoculata
Erpobdella mestrovi
Erpobdella lineata
Erpobdella punctata
Erpobdella melanostoma
Erpobdella bucera
Erpobdella costata
Cylicobdella coccinea
Chtonobdella bilineata
Haemadispa sylvestris
Haemadipsa picta
Haemadipsa sumatrana
Limnatis nilotica
Semiscolex similis
Patagoniobdella variabilis
Patagoniobdella fraterna
Macrobdella decora
Macrobdella diteta
Oxypychus brazilensis
Oxypychus striatus
Mesobdella gemmata
Xerobdella lecomtei
Hirudo nipponia
Aliolimnatis africana
Aliolimnatis michaelseni
Hirudinaria manillensis
Hirudo medicinalis
Haemopis caeca
Haemopis sanguisuga
Haemopis kingi
Haemopis marmorata
Haemopis grandis
Haemopis lateromaculata
Glossiphoniidae
Piscicolidae
Erpobdelliformes
Hirudiniformes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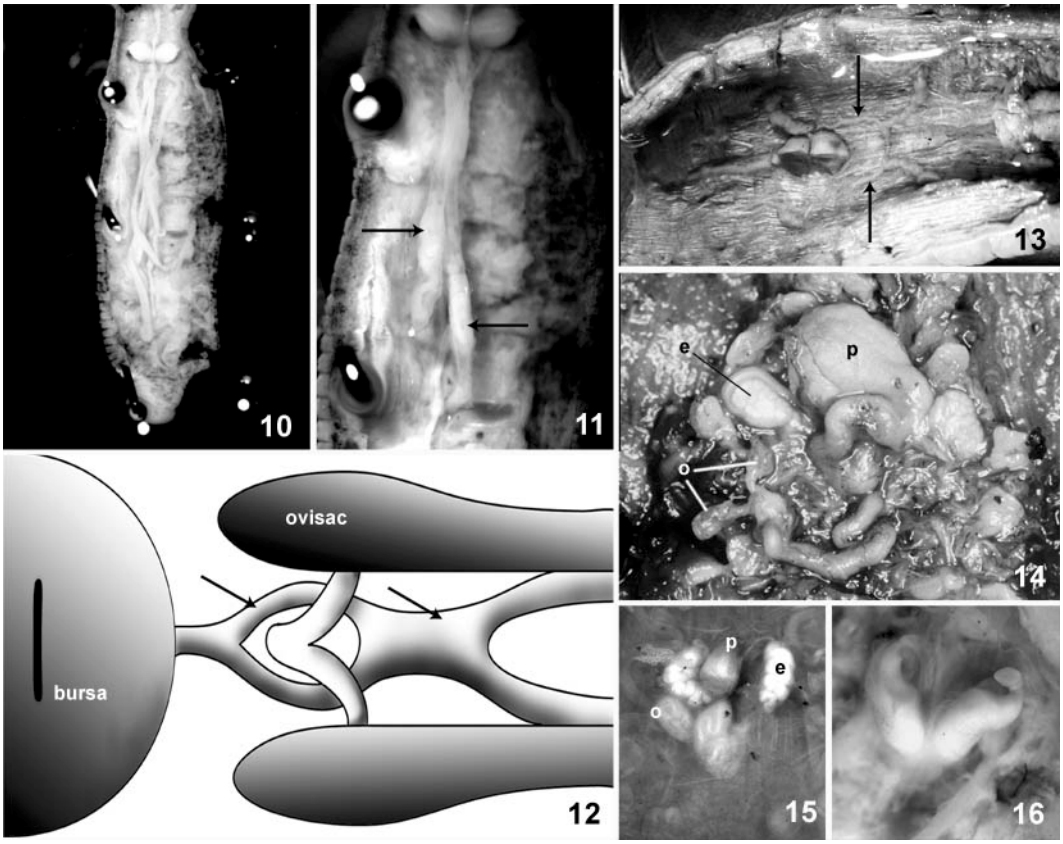
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11
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e
p
o
14
ovisac
bursa
12
p
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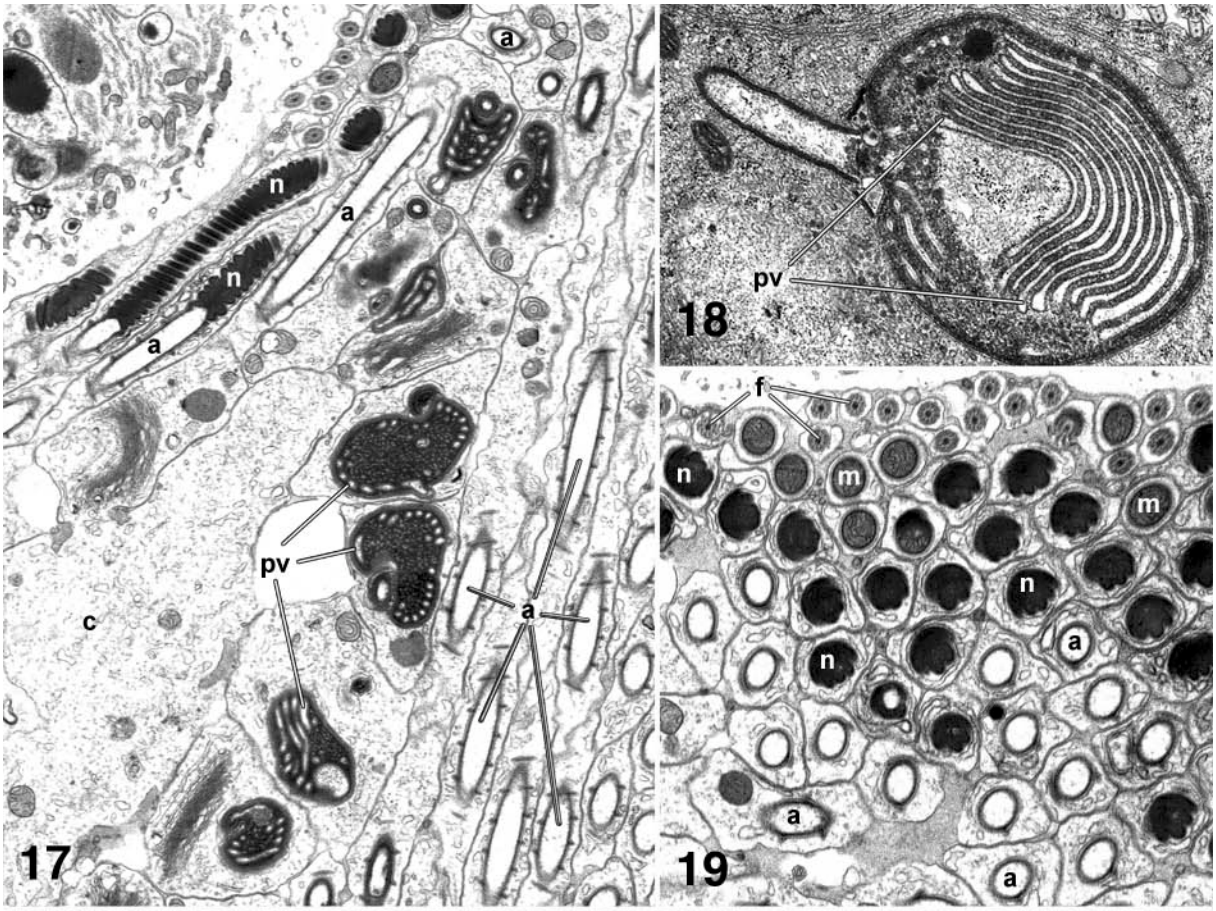
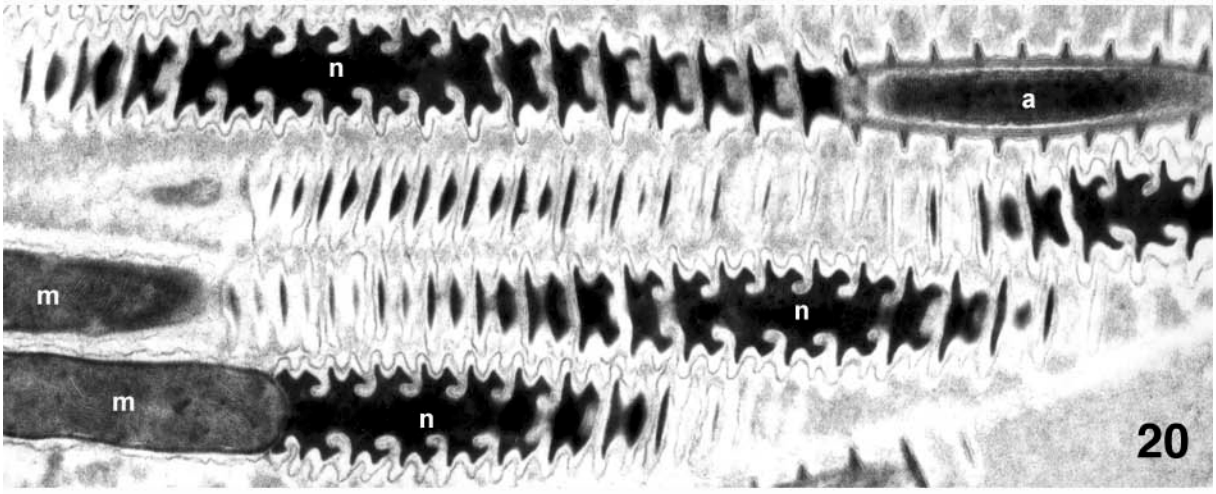
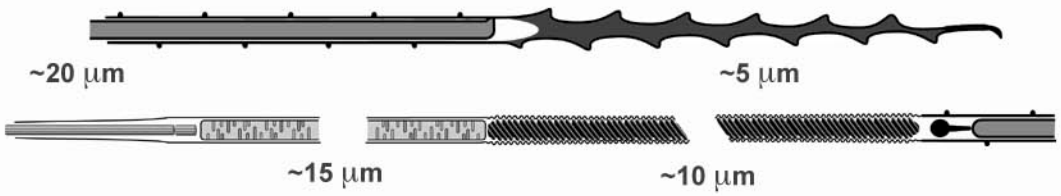
16







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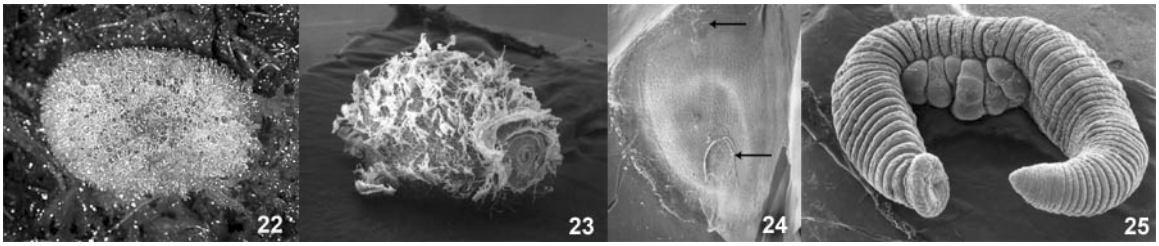
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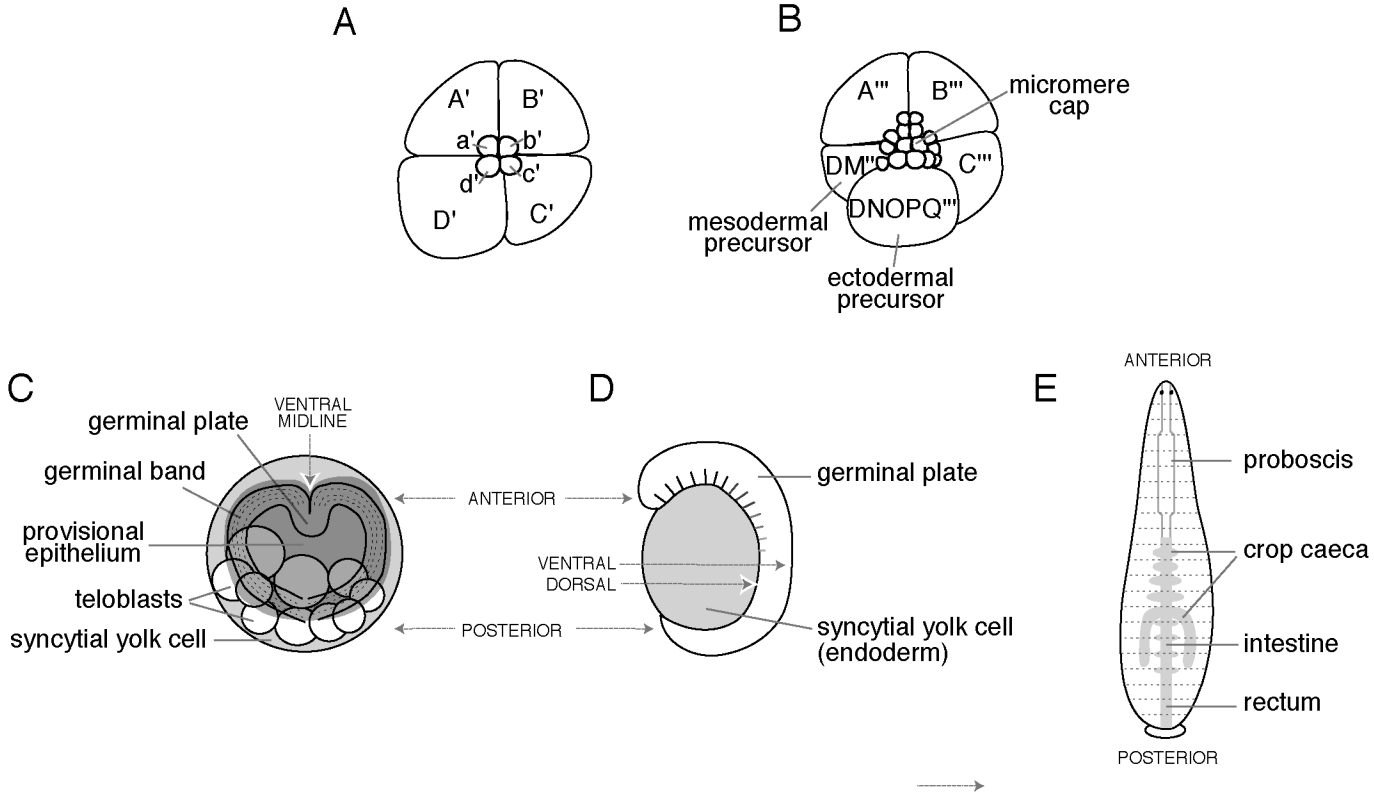
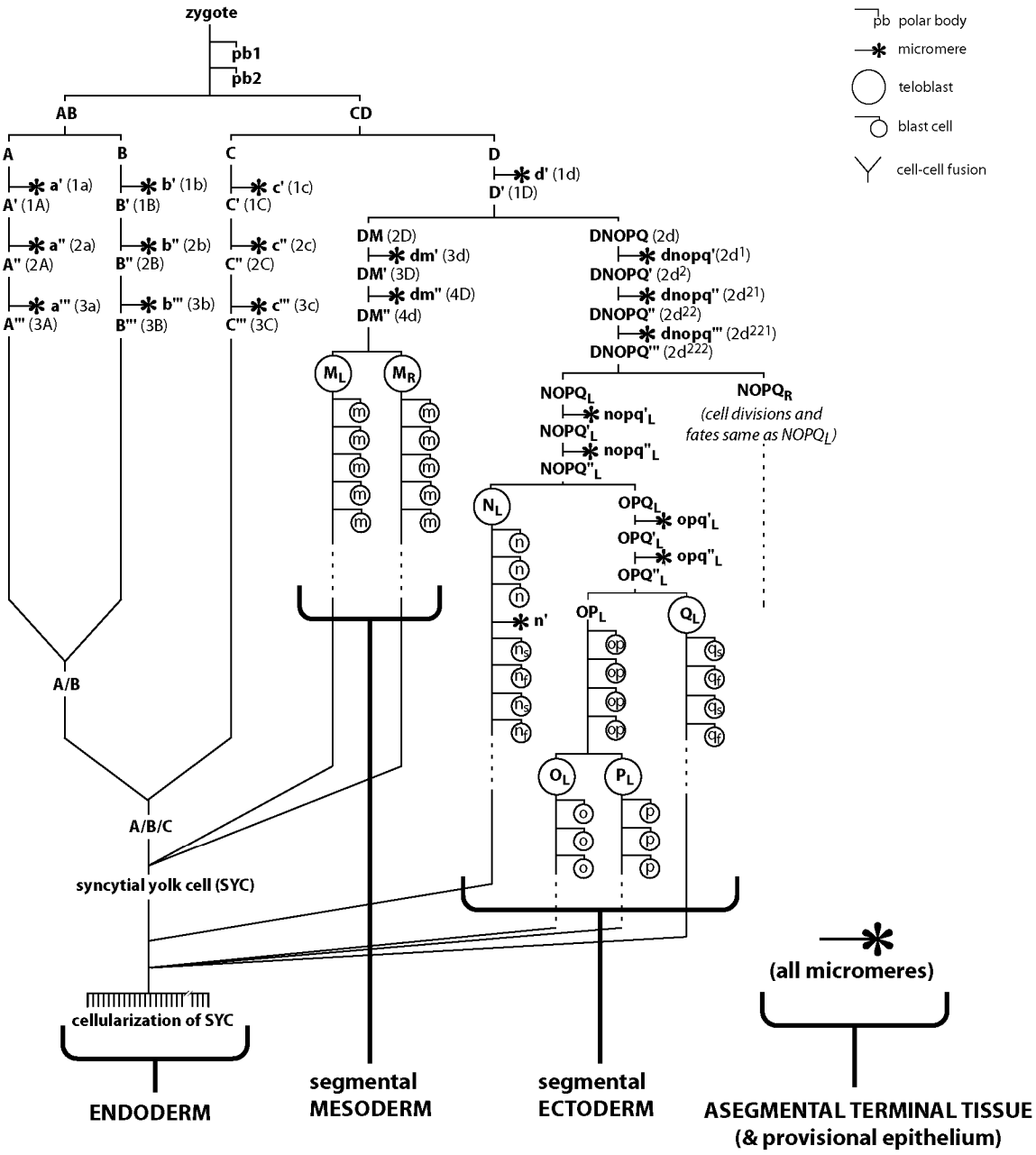

A
A'
B'
a'
b'
c'
d'
D'
C'
B
micromere cap
A'''
B'''
DM'''
C'''
DNOPQ'''
mesodermal precursor
ectodermal precursor
C
germinal plate
VENTRAL MIDLINE
germinal band
provisional epithelium
teloblasts
syncytial yolk cell
ANTERIOR
POSTERIOR
D
germinal plate
VENTRAL
DORSAL
syncytial yolk cell (endoderm)
E
ANTERIOR
proboscis
crop caeca
intestine
rectum
POSTERIOR


zygote
pb1
pb2
AB
CD
A
B
C
D
a' (1a)
b' (1b)
c' (1c)
d' (1d)
A' (1A)
B' (1B)
C' (1C)
D' (1D)
a" (2a)
b" (2b)
c" (2c)
A" (2A)
B" (2B)
C" (2C)
a'" (3a)
b'" (3b)
c'" (3c)
A'" (3A)
B'" (3B)
C'" (3C)
DM (2D)
dm' (3d)
DM' (3D)
dm" (4D)
DM" (4d)
DNOPQ (2d)
dnopq' (2d1)
DNOPQ' (2d2)
dnopq" (2d21)
DNOPQ" (2d22)
dnopq'" (2d221)
DNOPQ'" (2d222)
NOPQL
nopq'L
NOPQ'L
nopq"L
NOPQ"L
NOPQR
(cell divisions and fates same as NOPQL)
NL
OPQL
opq'L
OPQ'L
opq"L
OPQ"L
n'
OPL
QL
OL
PL
A/B
A/B/C
syncytial yolk cell (SYC)
cellularization of SYC
ENDODERM
segmental MESODERM
segmental ECTODERM
(all micromeres)
ASEGMENTAL TERMINAL TISSUE (& provisional epithelium)
pb polar body
micromere
teloblast
blast cell
cell-cell fusion

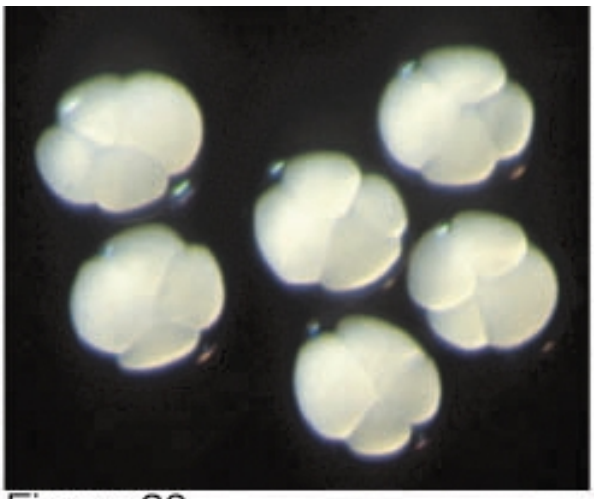


Figure 28

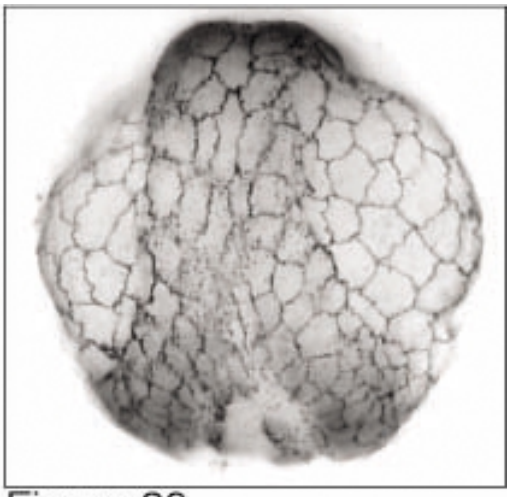


Figure 29

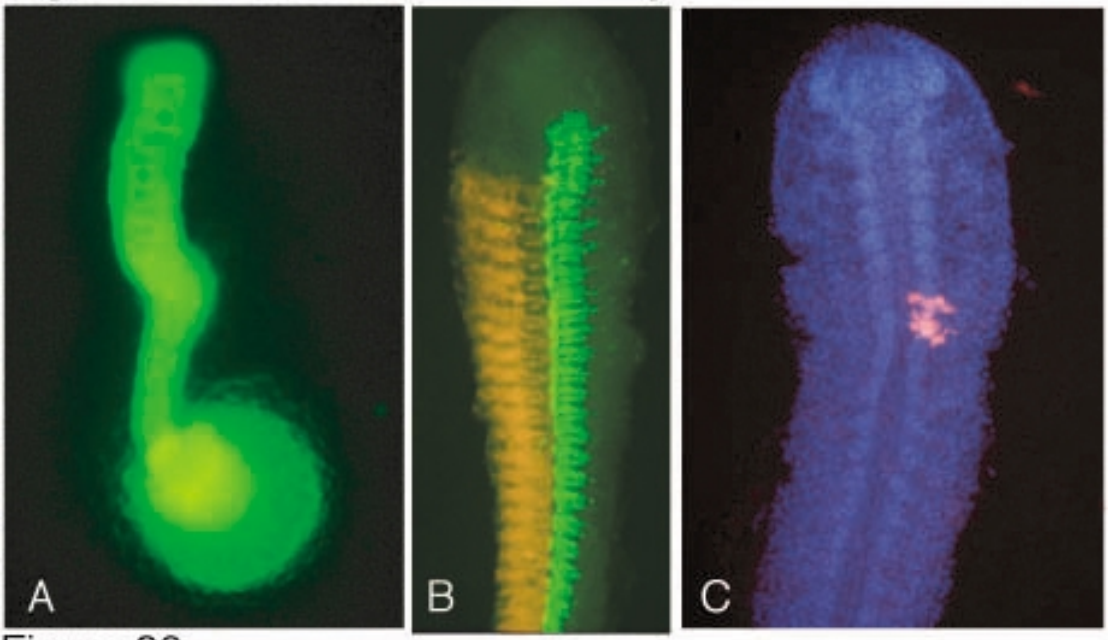


Figure 30

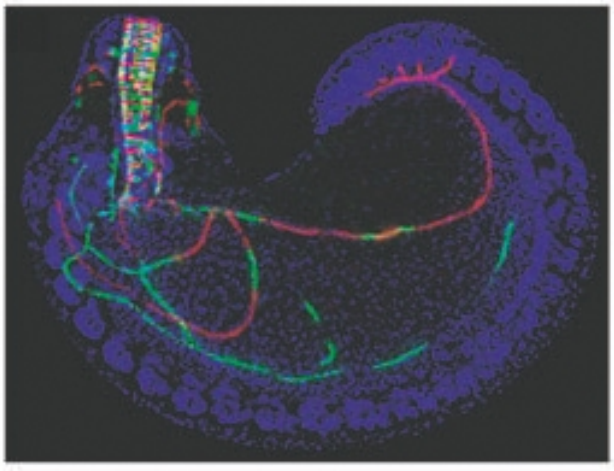


Figure 31