

Embryonic origins of hull cells in the flatworm *Macrostomum lignano* through cell lineage analysis: developmental and phylogenetic implications

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Abstract The development of macrostomid flatworms is of interest for evolutionary developmental biology research because these taxa combine characteristics of the canonical spiral cleavage pattern with significant deviations from this pattern. One such deviation is the formation of hull cells, which surround the remaining embryonic primordium during early development. Using live observations with a 4D microscope system, histology, and 3D reconstructions, we analyzed the ontogeny of these hull cells in the

macrostomid model organism *Macrostomum lignano*. Our cell lineage analysis allowed us to find the precursors of the hull cells in this species. We discuss the relation between macrostomid development and the development of other spiralian and the question of whether hull cells are homologous within rhabditophoran flatworms.

Keywords Embryo · 4D microscopy · Cell lineage · Hull cells · Ontogeny · Rhabditophora

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Introduction

Recent studies suggest that the taxon Platyhelminthes is polyphyletic. Acoela and Nemertodermatida are basal offshoots within the Bilateria, whereas, the Catenulida and Rhabditophora are part of the spiralian clade at the base of the Lophotrochozoa (Fig. 1; e.g. Bagaña and Riutort 2004; Philippe et al. 2007; Sempere et al. 2007; Dunn et al. 2008; Egger et al. 2009). Spiralian are characterized by a typical pattern of cleavage during embryonic development known as spiral cleavage. However, the embryological development of rhabditophorans and catenulids is poorly understood. The main reason behind this lack of knowledge is that catenulids rarely, if ever, reproduce sexually and that most of the rhabditophorans studied are freshwater triclads (planarians). Triclads, as most rhabditophorans, are neophorans; their eggs consist of an oocyte produced by the germarium, which is surrounded by vitellocytes or yolk cells produced by vitellaria. Moreover, triclads show a typical and unique type of embryonic development, known as blastomere anarchy. As such, triclad development is difficult to compare with the development of other spiralia or even Metazoa. More basal archoophoran turbellarians,

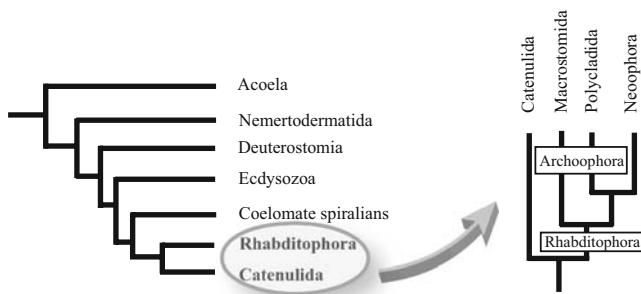


Fig. 1 Interrelationships between the major flatworm taxa and within rhabditophora (adapted from Wallberg et al. 2007, Schockaert et al. 2008 and Egger et al. 2009). Turbellarians are a paraphyletic grouping uniting all flatworms except the parasitic taxa (the latter grouped into the neodermata). Flatworms are polyphyletic: acoela and nemertodermatida are basal offshoots within the bilateria, whereas, the catenulida and rhabditophora are a sister group to the remaining spiralian (e.g., mollusca, annelida) at the base of the lophotrochozoa. Archophora is also a paraphyletic grouping of those taxa (polyclads and macrostomorphans) that feature entolecithal eggs, whereas, all remaining rhabditophorans (e.g., tricladida,) can be grouped into the neoophora (ectolecithal eggs). For alternative views on the phylogeny of flatworms see Phillippe et al. (2007), Dunn et al. (2008) and Egger et al. (2009)

which produce yolk-rich egg cells, are therefore better suited for comparative embryological studies. Typical archophorans are polyclads, which show some characteristics of a typical spiral cleavage, and macrostomids, the embryonic development of which was only very recently studied by Morris et al. (2004).

Morris et al. (2004) described the full embryogenesis of *Macrostomum lignano*, a macrostomid that was introduced as a new model organism for studying the development and evolution of “lower” Bilateria (e.g. Ladurner et al. 2000, 2005a; Peter et al. 2001, 2004; Salvenmoser et al. 2001; Nimeth et al. 2002, 2004; Schärer and Ladurner 2003; Bebenek et al. 2004; Morris et al. 2004, 2006; Schärer et al. 2004a, b, 2005; Egger and Ishida 2005; Bode et al. 2006; Egger et al. 2006; Pfister et al. 2007). Early cleavage (up to the eight-cell stage) could be observed in vivo and was described as a typical spiral cleavage. In later stages (15–30 h-old embryos), development starts to deviate from the typical spiral pattern observed in polyclads. Yolk-rich, presumably vegetal blastomeres with large nuclei (the so-called hull cells) expand and surround the other blastomeres, forming a sort of yolk mantle. Within this yolk mantle, the remaining blastomeres form a proliferating mass, which is referred to as the embryonic primordium.

Despite the many studies on macrostomid development, the origin of hull cells from blastomeres is still unknown, as is the evolutionary significance of the formation of these cells (Seilern-Aspang 1957; Ax 1961; Thomas 1986; Morris et al. 2004; Cardona et al. 2005, 2006). As the presence of hull cells does not occur solely in macrostomids

but in almost all major flatworm taxa, a better insight in the ontogeny of the hull cells is an important key in understanding how archophoran development is related to that of the neoophorans and how this development can be compared to the spiral cleavage pattern observed in polyclads and other spiralian.

In this paper, we investigate the embryonic origin of hull cells in *M. lignano* using cell lineage analysis based on a 4D microscopy system (3D time-lapse recordings) (Schnabel et al. 1997). This technology has been successfully used for studies of nematode and tardigrade embryos (Schnabel et al. 1997; Dolinski et al. 1998; Houthoofd et al. 2003; Hejnl and Schnabel 2005, 2006), the analysis of brain development in *Drosophila* (Urbach et al. 2003) and cell lineage analyses in crustaceans (Hejnl et al. 2006; Wolff and Scholtz 2006). Our results apply to the evolution of spiral cleavage within rhabditophorans.

Material and methods

Cultures

Cultures of *M. lignano* were reared in Petri dishes following the protocol of Rieger et al. (1988) and fed with the diatom *Nitzschia curvilineata*. They were maintained in a temperature-controlled chamber at 20°C, 50% humidity, and a photoperiod of 14 h light and 10 h dark.

Semithin sections and 3D reconstructions

Early stages of embryos were punctured with electrolytically sharpened tungsten needles and immediately fixed using the weak osmium cocktail method as described by Eisenman and Alfert (1982). After dehydration in standard acetone series, specimens were embedded in Spurr’s low viscosity resin (Spurr 1969). Blocks were sectioned with a Reichert Jung 2040 microtome. Semithin sections (1 or 0.5 μm) were stained with a 1:1 mixture of methylene blue and toluidine blue/borax (Ashburner 1989). The serial sections were digitized and used for 3D reconstruction using Amira 3.1.1 (TGS Europe, France).

4D microscopy

One-cell embryos were selected under a dissecting microscope, covered with a cover slip, and sealed with Vaseline, following a minor modification of the protocol developed for *Caenorhabditis elegans* (Sulston and Horvitz 1977).

The 4D microscopy—established by Schnabel et al. (1997)—is a computer-aided system, which facilitates cell lineage tracing within developing embryos. Recordings were made with a multifocal plane, time-lapse recording

system. Records were analyzed as described by Schnabel et al. (1997). Only data from embryos that developed normally (e.g., consistent cell divisions, no loss of blastomeres) and produced normal juveniles were used.

SIMI BioCell software (SIMI, Germany) was used to manage the large quantity of digital image data generated and to document cell positions, migrations, and mitoses during analysis. The data are illustrated as a cell genealogy tree, and the positions of the nuclei can be viewed as 3D representations using colored spheres.

Thirty-five recordings were made of early development, six of which extended until after hull cells were formed and positioned, which allowed us to analyze their origin and formation.

Cell lineage analysis: nomenclature

Blastomeres were named according to standard nomenclature for spiral cleaving embryos (see Robert 1903).

In most cases, cells at the two-cell stage were unequal in size, in which case we designated the larger as CD and the smaller as AB. In other cases, however, cells could not be distinguished based on their relative size. The polar bodies were very motile and therefore could not be used to assess the orientation of the embryo. When the difference between macromeres and micromeres was not obvious, cells were named in accordance to their relative position within the embryo based on the system established for the polyclad *Hoploplana inquilina* by Henry et al. (1995) and Boyer et al. (1996); at the four-cell stage, the cross-furrow blastomeres were defined as B and D, and the cross furrow was taken as the vegetal pole.

After the third cleavage, *M. lignano* no longer followed the spiral cleavage pattern. To avoid misinterpretations and documentation of false homologies, we applied an alternative nomenclature from this point. The cells located more towards the vegetal pole were designated as the macromeres; the cells lying more towards the animal pole were designated as the micromeres; or the more vegetal-lying blastomere was considered 1d*2 and the more animal-lying blastomere was considered 1d*1. The asterisk is meant to discriminate from normal spiralian lettering system. We used the vegetal pole as a marker for the macromeres. Plates were mounted using Adobe Photoshop CS2.

Lineage tracing

Two types of developmental stages were selected: (1) a stage where the hull cells (HC) were fully formed (approximately 25% of total development time, i.e., 30 h after hatching; HC injection) and (2) a stage where the gut primordium (G) was clearly visible (approximately 50% of total development time; G injection). These developmental

stages were transferred onto a droplet of ASW and injected using an Eppendorf Femtojet microinjection system. Dil injections were performed as previously described by Boyer et al. (1996a). After injection, each embryo was transferred to a single well of a multiwell plate and filled with ASW, where it continued to develop until hatching (HC injection: four hatchlings analyzed; G injection: five hatchlings analyzed). Injected and uninjected embryos developed over the same time course. Negative controls were effected by injecting individuals with oil only. Hatchlings were relaxed in 1:1 7.14% ASW-MgCl₂ (5 min) and 7.14% MgCl₂ (undiluted, 5 min) and fixed for 60 min in 4% paraformaldehyde followed by several rinses with phosphate buffer saline. Finally, hatchlings were mounted in Vectashield (Vector laboratories). Slides were examined using either a Leica SP5 or a Nikon EZ1 confocal microscope in different focal planes.

Results

Formation of hull cells in macrostomid development has been previously reported (Reisinger 1923; Bogomolow 1949, 1960; Papi 1953; Seilern-Aspang 1957; Ax and Borkott 1968a, b; Morris et al. 2004), but little is known about their embryonic origin. Our focus in this work was to determine the origin and fate of hull cells.

Early cleavage: two types of blastomere arrangements

A general characteristic of early *M. lignano* development is the occurrence of amoeboid bulges (a process called blebbing) of the embryonic cells. During this process, visible cytoplasmic protuberances are accompanied by deformations of the cell surface. In 20% of cases at the two-cell stage in this study (7/35 recordings), the two blastomeres were of equal size. In 80% of the cases, the first two blastomeres differed in size, the CD cell being the largest. In some recordings, a polar lobe was observed in association with the formation of this larger blastomere.

The second cleavage, generally, took place about 1 h after the first cleavage. In cases of equal cleaving embryos, AB and CD blastomeres were of equal size and cleaved synchronously. In unequal cleaving embryos, the bigger blastomere CD divided asynchronously some minutes earlier than the smaller AB blastomere (Fig. 2). The cleavage spindle of CD was, generally, perpendicular to the first cleavage spindle. The two daughter cells were usually of different sizes, D being larger than C. The plane of blastomeres, A and B, the latter cell being smaller, did not lie in the same plane as C and D, but was slightly inclined. Particularly, blastomere A tended to lie in a

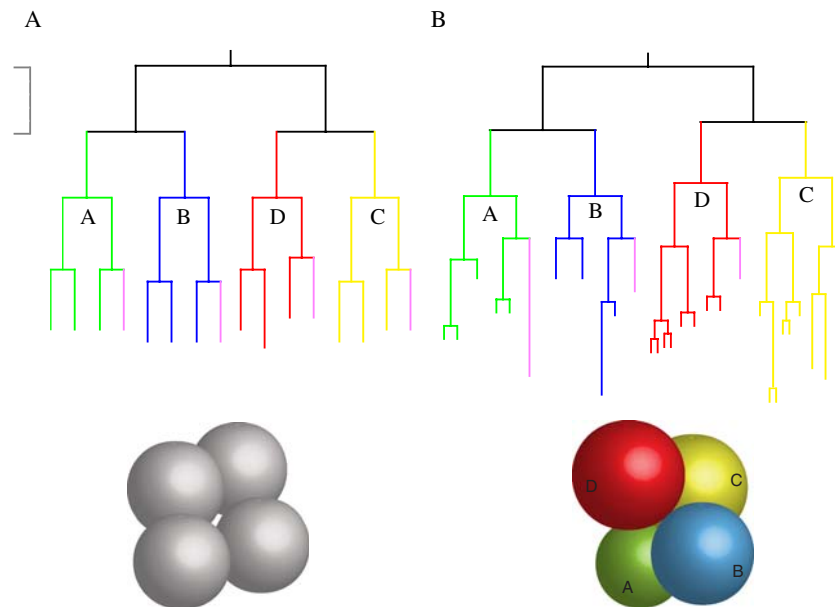


Fig. 2 Two types of cleavage. **a**, **b** Represent cell lineages of two different embryos, one with **a** equally and one with **b** unequally sized blastomeres at the two-cell stage. The cell lineages of equal and unequal cleavage are different; in unequally cleaving embryos, blastomere CD divided before blastomere AB, whereas, this was not the case for equally divided blastomeres. This resulted in two different types of blastomere arrangement in a four-cell stage (schematically depicted under their respective lineage). Each sublineage is color

coded: *green* representing the *A* lineage; *blue*, *B* lineage; *yellow*, *C* lineage; and *red*, *D* lineage. The blastomeres of the equally cleaving embryo are color coded *gray* as it is difficult to assign their identity based on size or origin. *Pink* lineages represent hull cells (HC). The *time axis* differs between **a** and **b** because of the different time intervals that can be taken between two pictures with the 4D-microscope system. Time scale bar=1 h

deeper, more animal position. Thus, two different types of configurations existed at the four-cell stage (Fig. 2).

The transition from the four- to the eight-cell stage (third cleavage round) occurred in a dextrotropic (right-handed) fashion, resulting in a typically spiralian macromere quartet and the first micromere quartet. This cleavage round occurred in two steps, passing through a six-cell stage with blastomeres C and D usually dividing before A and B (Fig. 2).

Hull cell formation

Using the technique of 4D microscopy, we were able to analyze cleavage *in vivo* until the fourth and fifth division round (16–32 cells) for all blastomeres and for specific sublineages until the sixth and even seventh division round (64–128 cells). This allowed us to follow hull cell formation and morphogenesis.

Equally and unequally, cleaving embryos at the eight-cell stage exhibited macromeres and micromeres of approximately the same size and could only be distinguished by the fact that the micromeres (1b and 1d) met in a slightly skewed angle in comparison to the vegetal cross-furrow cells (blastomere 1B and 1D). The transition from the eight-cell stage to the 16-cell stage occurred in a laeotropic direction, i.e., counterclockwise with respect to

the animal pole. This is the last cleavage round that occurred in a typical spiral fashion.

In each of the six movies in which hull cell formation was observed, we found that each of the macromere lineages (1A, 1B, 1C, and 1D) gave rise to exactly one hull cell (Figs. 3 and 4; supplementary movie S1).

The blastomeres of both cleavage types at the 16-cell stage were all of the same size. At this stage, the four peripheral cells, being cells 2A–2D, flattened and covered the outside of the embryo (Fig. 4). These were the hull cells. The remaining 12 cells formed the embryonic primordium, which lies in the center of the embryo. From this point in development, the hull cells remained in position without further divisions (Fig. 3).

To assess the fate of the hull cells in later development, hull cells of stage two embryos (15–30% of total development time) were injected with DiI and the injected embryos were cultured to hatchlings (5 days). DiI label was observed in the gut of the freshly hatched worms (Fig. 5), suggesting that hull cells were possibly incorporated into the gut during embryogenesis. This was confirmed by observations made with the 4D microscope. During development, at the time when the ciliated ectoderm is differentiated, an inward migration of the hull cells or remnants thereof into the gut was visible (Supplementary Movie S2).

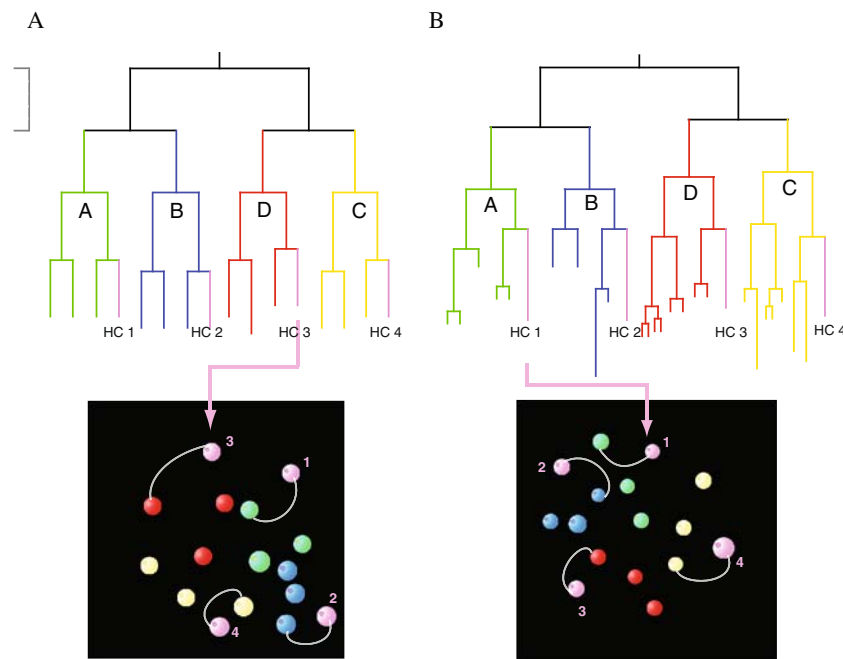


Fig. 3 Hull cell origin. **a** Equal and **b** unequal cell lineages combined with 3D embryonic configuration. The Y-axis represents the time of embryonic development. Lineages were linked to 3D reconstruction based on the 4D position in the lineage as indicated by a pink arrow. Every sublineage gives rise to a progenitor of a hull cell and right daughter cell is always at the fourth cleavage. Some embryos could be

followed further than others, depending on the quality of the recording. Therefore, more cell divisions (*branches*) are visible in **b**. Note that, unlike the other cells, hull cells no longer divide at this point (**b**). Each sublineage is color coded: green representing the *A* lineage; blue, *B* lineage; yellow, *C* lineage; and red, *D* lineage. Pink lineages represent hull cells (HC). Time scale bar=1 h

The cells of the embryonic primordium divided with randomly oriented cleavage spindles and seemed to move around in no distinct pattern. Divisions were not synchronous and the cleavage planes were not always oblique, as is the case in spiral cleavage: left–right, dorso ventral, as well as oblique division planes could be observed after the 16-cell stage. This was also reflected in the 3D arrangement of the cells from the 16-cell stage on, where no spiral pattern could be found in the embryo.

Discussion

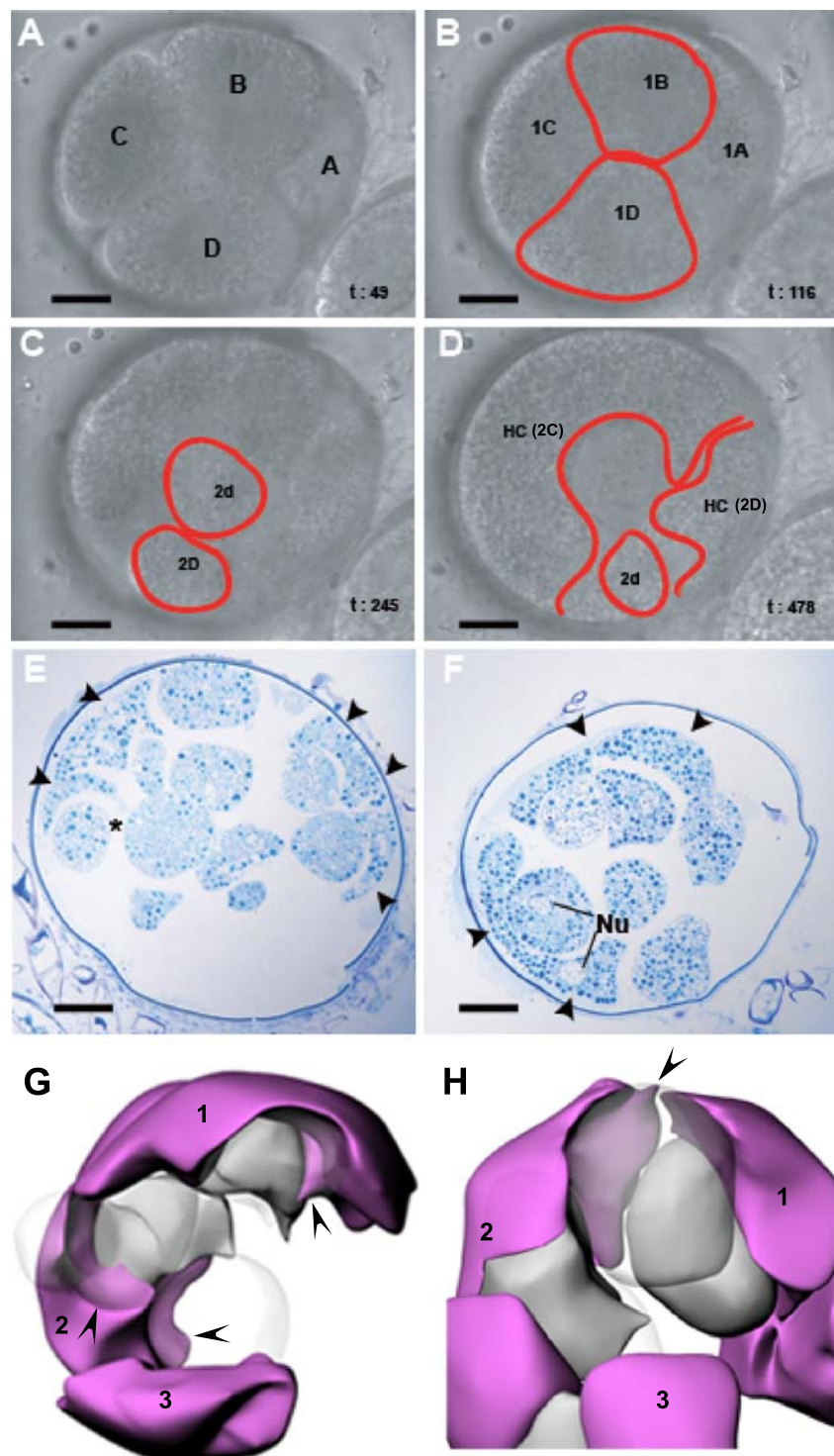
Developmental implications of hull cells: derived spiral cleavage

The early development of *M. lignano* shows some interesting deviations from the typical spiral cleavage, which is normally considered to be highly conserved (Henry and Martindale 1999). One deviation is the fact that *M. lignano* shows an intraspecific variation as to the relative size of the first two cells. In some specimens, the AB and CD cells are of equal size; whereas in others CD is larger. This observation bears on the general idea that within spiralia the relative cell size in early development is fixed within taxa. A taxon either has cells of equal size in the

four-cell stage, resulting in an equal cleavage pattern, or it has cells of unequal size, resulting in an unequal cleavage pattern. In *M. lignano*, both situations occur. Because Baguñà and Boyer (1990) suggested that equal cleavage is the primitive condition, the presence of unequal spiral cleavage in the basal Macrostomida can be considered derived and resembling unequal cleavage types found in the more derived neophoran taxa. Furthermore, in *M. lignano*, an asynchronous division of the blastomeres was observed in 80% of the cases where the blastomeres are unequal. The intraspecific variation of cell arrangements and cleavage observed in *M. lignano* shows that the spiral cleavage is not always as conserved within taxa as is generally thought.

The most striking deviation from the typical spiral development pattern is the presence of hull cells. Our cell lineage study clearly shows that these hull cells are derived from macromeres (2A–2D) at the 16-cell stage, a result that corroborates the previous suggestion by Seilern-Aspang (1957) that hull cells are derived from all four macromeres, or part of the macromeres plus part of the third quartet micromeres. Apparently, a viable juvenile can develop from an embryo without these 2A–2D macromeres. The direct descendants of these 2A–2D macromeres, the 4A–4D macromeres, and 4a–4d micromeres contribute to the formation of the endoderm and mesoderm in most spiralian. However, in the polyclad flatworm *H. inquilina*,

Fig. 4 Hull cell formation. **a–d** Animal view of early developmental stages of *M. lignano* embryo. **a** Four-cell stage, **b** eight-cell stage, **c** 16-cell stage prior to and **d** after hull cell formation. The lineage name corresponding to the visible hull cells are given in *parentheses*. The *borders* of the blastomeres are *highlighted* with *curved red lines*. In **d**, two hull cells contact each other, making it difficult to discern the boundary between them. **e–f** Semithin cross section showing blastomeres and hull cells. *Arrowheads* indicate hull cells. Blastomeres, as well as some of their descendants, are named according to the spiralian nomenclature. **g** 3D rendering based on semithin cross sections of 20-cell stage embryos. For the sake of clarity, not all blastomeres are rendered. Some are rendered as slightly transparent to allow the visualization of underlying cells. Hull cells (three visible) are rendered *pink*, while other blastomeres are rendered *light gray*. Note how the hull cells precisely surround the remaining blastomeres of the embryonic primordium at different points within the embryo (*arrowheads*). **h** Detailed view of (**g**) showing one blastomere (*slightly transparent*) surrounded by two hull cells. Time stages (in min since start of observation) are indicated in the *lower right corner*. *HC* hull cells, *Nu* nucleus. Scale bar=20 μ m



these 4A-4D cells degenerate, while the 4a-4c are used as food for the developing embryo (Surface 1907; Boyer et al. 1998). The facts that we did not observe any mitosis in fully developed hull cells (Fig. 3), that DiI was observed in the juvenile gut after injection of hull cells (Fig. 5) and that hull cells seem to migrate towards the embryonic primordium (supplementary movie S2), all suggest that hull cells

in *M. lignano* at least degenerate in the gut, and are probably used as nutrition. The observations of Tyler (1981) that in species of *Macrostomum* no mitoses occur in the yolk mantle and that the epidermis is formed other than the yolk mantle (formed by the hull cells) suggest that hull cells indeed do not contribute to the embryonic body, at least not in the taxon *Macrostomum*. Because in

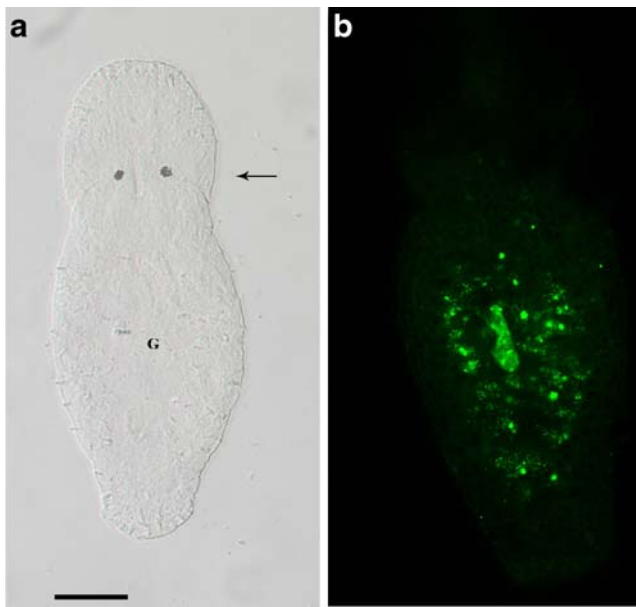


Fig. 5 Representative example of the fate of a hull cell injected with DiI in an earlier stage. **a** DIC photo of the juvenile. *Arrow* indicates eye level. **b** The same animal with induced fluorescence of DiI (false-colored in *green*). The remnants of the marked hull cell are exclusively within the gut (G). Scale bar=50 μ m

spiralian, mesoderm develops from the 4d mesoderm precursor, the question that remains is how mesoderm is formed in *Macrostomum*. This would imply that, although mesoderm formation is highly conserved in spiralian (Lambert 2008), in *M. lignano*, the progenitor of the mesoderm precursor (4 day) forms a hull cell and is then ultimately lost; yet, mesoderm is still produced. This would imply that mesoderm is formed differently in this taxon. This issue clearly needs to be investigated further by, for example, fate mapping the derivatives of the mesodermal precursor in *M. lignano*.

Phylogenetic implications of hull cells: on homology and exaptation

The term hull cell is used for a cell that surrounds the developing embryo in rhabditophoran flatworms. They are lacking only in polyclads, which show a typical spiral cleavage pattern (Surface 1907; Boyer et al. 1996, 1998; Younossi-Hartenstein and Hartenstein 2000). However, the results of our study and data from literature show that the hull cells of different taxa can have different ontogenetic origins (see Fig. 6). They may either originate from (1) vegetal macromeres as in *Macrostomum* (this paper); (2) animal micromeres as in proseriates, lecithoepitheliates, and *Bothrioplana semperi*; or (3) yolk cells (derived from the vitellaria) as in triclads, prolecithophorans, and rhabdocoels. Hull cell formation in rhabdocoels is especially puzzling. Indeed, in some taxa, a so-called hull membrane

appears to be absent and in others (*Mesostoma ehrenbergii*), the formation of hull cells is linked to the seasonal cycle: in summer eggs, hull cells are formed while in winter eggs, they are not (Bresslau 1904). In the classical definition of homology, the two structures are called homologous only if these structures have a common evolutionary origin. Based on our cell lineage analysis of *M. lignano* and data from other rhabditophoran taxa, we think that the homology of hull cells between flatworm taxa is highly questionable. However, in recent years and especially in evolutionary developmental biology research, the term homology is used in a somewhat “dissident” meaning. According to Hejnal et al. (2006; see also Dohle et al. 2004; Scholtz 2005), early developmental stages can be modified during evolution without affecting later stages, and neither are homologous developmental stages necessarily based on homologous earlier stages nor are they necessarily followed by homologous later stages. In other words, considering this view, even if the origin of hull cells is not homologous, the hull cells themselves can be homologous. In our opinion, since hull cells do not arise from the same founder cells, they are not homologous, so it is difficult and possibly wrong to draw any conclusion concerning their evolutionary or developmental history. In

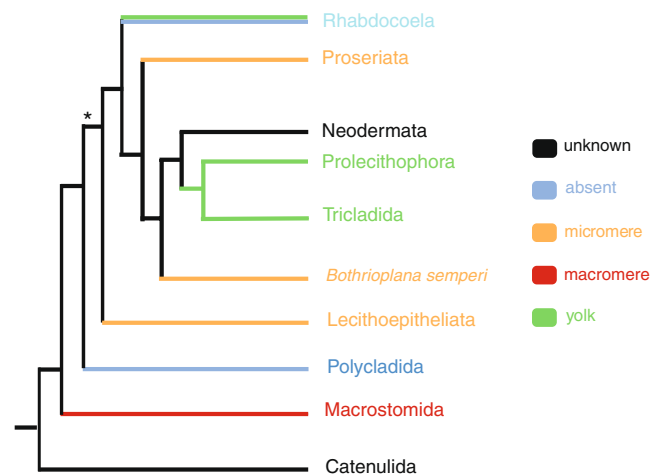


Fig. 6 Origin of hull cells superimposed on a phylogenetic tree of the rhabditophora + catenulida, reconstructed based on the existing trees published by Willems et al. (2006) and Baguñà and Riutort (2004a). Absence (*blue*), presence, and origin(s) of hull cells (see color code) are mapped on the phylogenetic tree based on our own data (*M. lignano* for macrostomida) as well as what is known from the literature. It is not implied that these specific patterns of hull cell origin (or absence) are characteristic for the entire taxon. We consider it probable that with data on more taxa, additional variations within branches will be discovered. Note that in rhabdocoels, hull cells can be absent and originate from yolk cells. The *asterisk* represents the transition to the neophoran clade of flatworms. Color code: *black*—unknown; *blue*—hull cells absent; *orange*—hull cells originate from micromeres; and *red*—hull cells originate from macromeres; *green*—hull cells originate from yolk cells

addition, interspecific variability in the origin of hull cells as well as intraspecific variability as to whether a hull membrane is formed exists (see examples above). Thus, we consider the potential of hull cell ontogeny to resolve phylogenetic relationships weak. Although hull cells look similar, occupy the same position within the embryo, and probably, perform the same function across taxa, their different origins imply a different structural composition. Moreover, the absence of intermediate states, in part due to the “invention” of ectolecithality, makes their homoplastic nature more probable. It has been shown in sea urchins that evolutionary change from indirect to direct development evolved independently multiple times and in such a way that even closely related species exhibit major differences in their embryonic development patterns and that such modifications are closely tied to an increase in maternal factors such as yolk and lipids (Wray and Raff 1990; Wray and Bely 1994; Byrne et al. 1999). The “invention” of hull cells and their different origins in closely related taxa is, in our opinion, an additional example of how a conserved cleavage pattern can be drastically modified independently during the course of evolution. How these changes are expressed in the juvenile fate map remains to be seen and is the next step to understanding spiral cleavage evolution.

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