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Differences in embryonic pattern formation between *Caenorhabditis elegans* and its close parthenogenetic relative *Diploscapter coronatus*

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ABSTRACT In order to evaluate the evolutionary preservation of developmental programs during nematode embryogenesis, we searched for close relatives of the model system *Caenorhabditis elegans* with deviant patterns. The parthenogenetically reproducing species *Diploscapter coronatus* shows prominent differences to *C. elegans*. While in the 2-cell stage of *C. elegans* a rotation of the nuclear/centrosome complex is found only in the posterior P_1 cell, in *D. coronatus* cell isolation indicates that rotation takes place in a cell-autonomous manner in both blastomeres, resulting in a linear 4-cell array. In *C. elegans,* the ABp cell becomes different from its ABa sister via a germline-induced induction. In *D. coronatus,* AB daughters do not touch the germline but nevertheless execute different fates, suggesting a cell-autonomous mechanism or signaling over distance. Laser ablation experiments revealed that active migration of the EMS cell is required to transform the linearly ordered blastomeres into a 3-dimensional embryo, and the difference can be most easily explained with a heterochronic shift with respect to cell mobility. In *D. coronatus,* reversal of cleavage polarity in the germline, typical for *C. elegans*, is absent. This results in four different transient variants of posterior blastomeres which eventually merge into a single pattern prior to the onset of gastrulation. This merging includes primordial germ cell migrations of variable extent toward the gut precursor cell and suggests a specific cell-cell recognition mechanism. Cell distribution in advanced embryos is essentially indistinguishable between both species.

KEY WORDS: *nematode, polarity, cell specification, pattern formation, evolution*

Introduction

In the animal kingdom different early embryonic cell division patterns are found that lead to characteristic early arrangements of blastomeres, e.g., radial, spiralian or bilateral symmetric cleavage. The typical cleavage pattern in nematodes does not fit any of the above schemes. It includes a series of early asymmetric cleavages sequentially separating a small number of somatic lineage founder cells from the germline. This process had been originally identified in *Ascaris* (Boveri, 1899; clade 8, Fig. 1) and has been studied in much detail in *Caenorhabditis elegans* (clade 9). In the 2-cell stage the first somatic founder cell AB divides with a transverse orientation of the cleavage spindle, generating two daughters of equal developmental potential (Priess and Thomson, 1987). In contrast, its sister, the germline cell P_1 undergoes a rotation of the nuclear/centrosome complex (Hyman and White, 1987; Hyman, 1989) leading to a longitudinal orientation of the

spindle and subsequently to the generation of the somatic cell EMS and a new germline cell P_2. In the resulting rhombic 4-cell embryo essential cell-cell interactions take place, in which P_2 induces both of its neighbors via the Delta/Notch (ABp) and the Wnt/Frizzled (EMS) pathways, respectively. In this way ABp becomes different from ABa, in losing its ability to react to certain later signals, and EMS is polarized such that it will divide into a muscle/pharynx percursor MS and the gut founder cell E (Mello *et al.*, 1994; Rocheleau *et al.*, 1997; Thorpe *et al.,* 1997; for reviews, see Eisenmann, 2005; Greenwald, 2005). Signal transduction generally requires physical contact between signaling cells (Goldstein, 1993).

Comparative studies have shown that not all nematodes follow

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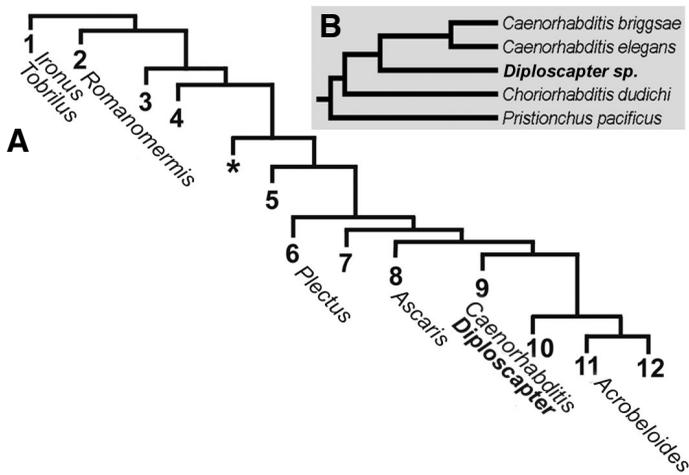
Fig. 1. Phylogenetic relationships among nematodes based on SSU rDNA sequences. (A) *Phylogenetic tree with twelve clades (1-12) and one unresolved branch (*), after Holterman* et al. *(2006). Branch lengths reflect substitution rates and affiliations of selected representatives are shown.* **(B)** *Detail of phylogenetic tree (modified after Kiontke* et al., *2007) indicating close relationship between* Caenorhabditis *and* Diploscapter.

the *C. elegans* scheme, i.e., variants exist during the initial phase of development (Skiba and Schierenberg, 1992; Malakhov, 1994; Dolinski *et al.*, 2001; Schierenberg, 2001; Schierenberg, 2005; Schulze and Schierenberg, 2008). A classic example is chromatin diminution in *Ascaris* accompanying the above-mentioned soma-germline separation (Boveri, 1899). In parthenogenetic species variations in meiosis and the establishment of embryonic axes have been described (Goldstein *et al.,* 1998; Lahl *et al.*, 2006). Another cellular event that is found in many but not in all nematodes is a reversal of cleavage polarity in P$_2$ (zur Strassen, 1959; Schierenberg, 1987; Skiba and Schierenberg, 1992; Laugsch and Schierenberg, 2004) which ensures that gut primordium and germline stay in physical contact. Such an association is found in many organisms and seems to be essential for normal development (Wylie, 1999). Experimental analysis revealed that in another nematode, *Acrobeloides nanus* (clade 11; Fig. 1), in contrast to *C. elegans* early blastomeres are multipotent and can replace lost cells (Wiegner and Schierenberg, 1998; 1999). In two members of clade 10, cell lineages and arrangements during gut formation were found to differ from *C. elegans* (Houthoofd *et al.*, 2006).

Instead of a diamond-shaped blastomere arrangement in the 4-celled embryo, some nematode species show a linear grouping along the anterior-posterior axis (Malakhov, 1994; Dolinski *et al.*, 2001). Such an arrangement is also found in a few close relatives of *C. elegans*, e.g., *Diploscapter coronatus* (clade 9; Tahseen *et al.*, 1991). This unusual pattern prompted us to study early development of this species in more detail. Firstly, we wanted to know how this arrangement of cells is achieved and whether it eventually merges into a *C. elegans*-like pattern as was found earlier for other variants of nematode embryogenesis (Skiba and Schierenberg, 1992; Lahl *et al.,* 2006). Secondly, we explored whether this pattern is compatible with the early inductions as found in *C. elegans* (see above) or whether alternative mechanisms have to be postulated to allow proper cell specification in

this species.

Based mainly on molecular sequence data, a modern nematode phylogeny was suggested by Blaxter *et al*. (1998), extended and modified by De Ley and Blaxter (2002). Recently, from a larger set of species 339 nearly full-length small-subunit rDNA sequences were analyzed and revealed a backbone of twelve consecutive dichotomies that subdivide the phylum Nematoda into twelve clades (Holterman *et al.,* 2006; Fig. 1). The clade numbers we use below refer to this work. A detailed modern phylogeny of rhabditid nematodes (including *Diploscapter*) can be found in Kiontke *et al.* (2007).

Results

Diploscapter coronatus*, a close relative of* C. elegans *shows a variant early development*

Looking for nematodes with early embryogenesis that differs significantly from *C. elegans*, a close relative, *Diploscapter coronatus* (Fig. 1), constitutes an interesting case, an initial analysis of which will be presented here. To start with, some basic features of this species are given.

Adults of *D. coronatus* are less than half the size of *C. elegans* (Fig. 2) while eggs are only about 25% shorter (average size: 42 x 20 µm; n=52), however, to a variable degree more elongated (37-48 µm x 17-25 µm). In contrast to *C. elegans* this species reproduces parthenogenetically. Despite the absence of sperm an early axis polarity is established, but this seems to be independent of an external signal and determined randomly by chance (Lahl *et al.*, 2006). At 20°C embryogenesis in *D. coronatus* takes 4-5 times longer than in *C. elegans* (Fig. 3). However, it can tolerate about 6°C higher temperature (i.e., 32°C) and under these conditions develops only 2-3x slower.

Our 4-D lineage analysis (n=22) revealed that early development of *D. coronatus* is reproducible and similar enough to *C. elegans* to assign cells to specific lineages and name them according to the same standard nomenclature (Sulston *et al.*, 1983). The sequence of early cleavages is essentially invariant, independent of temperature, but differs reproducibly from *C. elegans*. Particularly obvious are premature germline divisions, the absence of polarity reversal in the germline (see below), aspects also found in other slow developing nematodes (Skiba and Schierenberg, 1992), and a delayed cleavage of the gut founder cell E (Fig. 3).

Characteristic for the *C. elegans* 2-cell stage is the initially transverse orientation of centrosome pairs and the subsequent rotation of the nucleus/centrosome complex in the germline cell P$_1$, finally resulting in a rhombic 4-cell stage (see Introduction). However, this process is difficult to observe with Nomarski optics because of the inconspicuous centrosomes, rapid progression

Fig. 2. Adults with eggs of *Caenorhabditis elegans* (top) and *Diploscapter coronatus* (bottom). *Scale bar, 100 µm.*
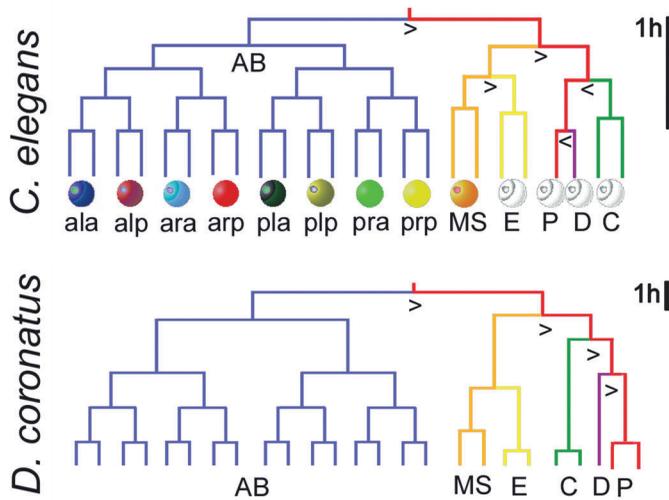
Fig. 3. **Early cell lineages of *C. elegans* and *D. coronatus*.** *Arrowheads indicate unequal divisions, with tip pointing toward the smaller sister cell. Note polarity reversal in the germline (red lineage branches; for definition, see text) of* C. elegans *which is absent in* D. coronatus. *Spheres indicate color coding of cells originating from a specific lineage branch.*

and cell deformation. In another so far unnamed *Rhabditis* species in our collection (*Rhabditis* sp., ES104) where the first two blastomeres divide simultaneously, this rotation is very obvious (Fig.4 B-D) as the cleavage spindle is fully developed at that time. While here positioning of centrosomes follows initially the «centriolic principle», according to which they form the mitotic spindle perpendicular to the orientation in the previous division (Costello, 1961), this principle is breached in P_1.

In contrast to this pattern, in *D. coronatus* the first four blastomeres are ususally ordered in a linear sequence (Fig. 4d). Because of variable egg shape frequently even 5 cells (Fig. 4f) or only 3 cells form a single row before blastomere rearrangement starts (see below). Further analysis made clear that this linear order corresponds to the anterior-posterior (a-p) axis of the embryo.

We wanted to know how this pattern is achieved and what consequences this has for axis formation, cell patterning and cell specification, which in *C. elegans* requires a rhombic 4-cell embryo for proper cell-cell interactions to occur (for references, see introduction).

A cell-autonomous rotation of the nucleus/centrosome complex takes place in P_1 and AB

Different from *C. elegans* and *Rhabditis* sp. (Fig. 4 A-F), in *D. coronatus* a rotation of the nucleus/centrosome complex takes place not only in P_1 but also in AB, leading to the same longitudinal spindle orientation in both blastomeres (Fig.4 a-d).

In conjunction with the rotation of the nuclear/centrosome complex in P_1, Hyman (1989) reported the formation of a small clear cortical region («cortical invagination») in the anterior cortex of the germline cell, in the area where microtubules compete for an attachment site. If such a structure is a prerequisite or a side effect of centrosome rotation, we should expect to find it in both blastomeres of the *D.coronatus* 2-cell embryo. This is in fact what we observed. Clear cortical regions (CCR) form adjacent to the anterior plasma membrane in P_1 and the posterior membrane in
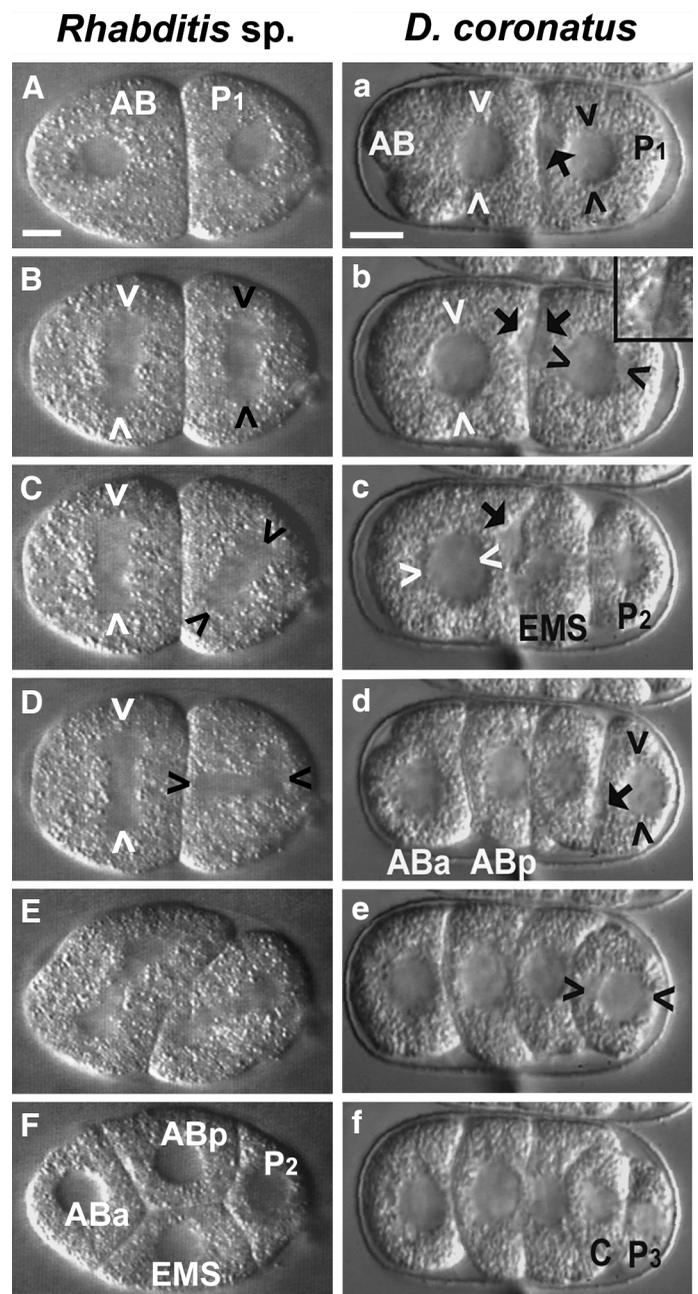

Fig. 4. **Early cleavage patterns of *Rhabditis* sp (A-F) and *D. coronatus* (a-f).** **(A)** *2-cell embryo, both blastomeres in interphase.* **(B)** *Simultaneous cleavage of AB and P_1 with transverse orientation of cleavage spindles in both blastomeres.* **(C, D)** *Rotation of the nucleus/ centrosome complex in P_1.* **(E, F)** *Subsequent skewing of cells caused by mitotic forces and constraints of the eggshell leads to a rhombic arrangement. Arrowheads indicate orientation of cleavage spindles.* **(a)** *2-cell embryo, start of mitosis and clear cortical region (CCR) in P_1 (arrow).* **(b)** *Centrosomal rotation essentially completed in P_1. CCR in both blastomeres (arrows); inset, magnification of CCR.* **(c)** *Centrosomal rotation essentially completed in AB with prominent CCR (arrow).* **(d)** *Linear 4-cell stage, CCR in P_2 (arrow).* **(e)** *Centrosomal rotation essentially completed in P_2.* **(f)** *Linear arrangement of five blastomeres. Arrowheads, orientation of centrosomes/spindles. Orientation of embryo: anterior, left; dorsal, top. Scale bar, 10 µm.*

AB prior to centrosome rotation (Fig. 4b). We identified straight running cell borders in this area indicating that these structures are intracellular and not indentations of plasma membranes (Fig. 4b, inset). Initially, these CCRs persist after the division of P_1 (Fig. 4c), but then they disappear (Fig. 4 d-f). The same process takes place in P_2 (Fig. 4 d-f) but not in the AB cells (Fig. 6 b', c'), i.e., formation of a CCR, rotation of the nuclear/centrosome complex and subsequent division with a-p spindle orientation.

As in *C. elegans* an induction by P_1 is required to ascertain a longitudinal spindle orientation in AB and an a-p polarity in AB descendants (Laufer *et al.*, 1980; Bischoff and Schnabel, 2006), it
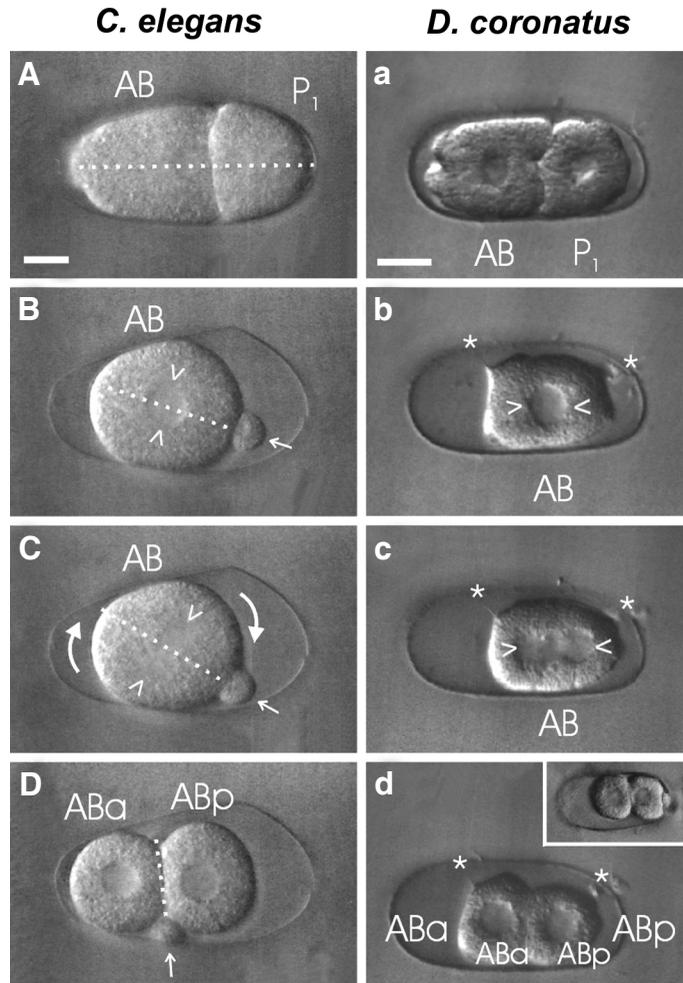

Fig. 5. Cell-autonomous spindle orientation in AB. (A-D) C. elegans. **(a-d)** D. coronatus. *After removal of P_1 from an early 2-cell stage* **(A,B)**, *the mitotic spindle in AB forms in a transverse position. Due to a rotation of the complete blastomere* **(C)**, *both daughter cells occupy an anterior-posterior (a-p) position in the eggshell* **(D)**. *The position of the small P_1 fragment marks the posterior pole of AB. In* D. coronatus, *the isolated AB cell* **(b)** *undergoes a division with a strict a-p position of the cleavage spindle* **(c)** *and consequently daughter cells occupy a-p positions in the eggshell* **(d)**. *The same result was obtained when a P_1 fragment remained as a marker for the posterior pole of AB* **(d)** *(see inset). Arrowheads, orientation of centrosomes/cleavage spindle; small arrow, remnant of P_1; large arrows, rotation of complete cell; dashed line, orientation of a-p axis; asterisks, cytoplasmic filament and a tiny remainder of P_1, respectively, as markers for anterior and posterior regions of AB. Orientation of embryo: anterior, left. Scale bar, 10 µm.*

seemed plausible that in *D. coronatus* the spindle rotation in AB may be induced by the germline cell. To investigate this, we ablated P_1 in *D. coronatus* (n=27) and found that nevertheless the AB cleavage spindle occupied an a-p position. To exclude that this was due to a residual influence of the ablated cell, in a second series of experiments we physically removed P_1 through a laser-induced hole in the eggshell immediately after its birth (Fig. 5b). Isolated AB cells in *D. coronatus* (n=8) performed divisions with a-p spindle orientations independent of whether P_1 had been removed completely (Fig. 5d) or a small cytoplasmic fragment had remained attached to AB as a marker for the posterior pole (Fig. 5d, inset). Isolated AB cells of *C. elegans* (n=7) executed cleavages with a transverse spindle orientation like in intact embryos (Fig. 5 B,C). However, because during division the AB cell rotated completely inside the eggshell, due to spatial restrictions of the eggshell, its daughters ultimately occupied anterior and posterior positions (Fig. 5 C,D). From these data we conclude that the longitudinal spindle orientation in the AB cell of *D. coronatus* as well as the transverse orientation in *C. elegans* are cell-autonomous processes that do not require an induction by P_1.

EMS activity leads to disruption of linear cell arrangement and establishment of the dorsal-ventral axis

To determine to what extent the linear 4-cell stage in *D. coronatus* subsequently results in an embryogenesis that differs from *C. elegans*, we analyzed further development, in particular with respect to axis formation, lineage-specific patterns and initiation of gastrulation.

In the 5-cell stage a dramatic rearrangement of cells takes place in *D. coronatus*, whereby ABp and EMS leave their positions in the linear sequence, thus establishing the dorsal-ventral axis, i.e., ABp represents the dorsal and EMS the ventral side of the developing embryo (Fig. 6 A-D). Despite differences in the order of cleavages (Fig. 3), from this time onward the cellular pattern resembles that of the *C. elegans* wildtype and other related nematodes (Skiba and Schierenberg, 1992; Malakhov, 1994; Houthoofd *et al.*, 2003). In contrast to *C. elegans* all nuclei contain prominent nucleoli, indicating synthetic activity (Fig. 6 A-D).

To investigate which of the blastomeres are actively involved in the early rearrangements in *D. coronatus*, we ablated individual blastomeres. After ablation of ABp (Fig. 6a; n=3), this cell was soon translocated from its original position (Fig. 6 b,c) and usually ended up ectopically at the posterior pole of the embryo (Fig. 6d). In such experimental embryos the proper movement of EMS to the ventral side and its asymmetric nuclear positioning were not affected (Fig. 6c). In contrast, ablated EMS cells were found to remain in their original position for a long time and the typical early cell rearrangements did not occur (Fig. 6 a'-d'; n=3). From these results we conclude that an active movement of the EMS cell is required to allow correct pattern formation in the early *D. coronatus* embryo. We also ablated P_2 (n=3) to test its effect on the migration behavior of EMS and ABp. We found that cellular rearrangements took place in the normal way (Fig. 6 a*-d*) giving no indication for a germline influence on this process.

Absence of cleavage polarity reversal is associated with transiently variable patterns of posterior cells

In *C. elegans* a reversal of cleavage polarity takes place in the germline cell P_2 (see Introduction) leading to an invariant arrange-
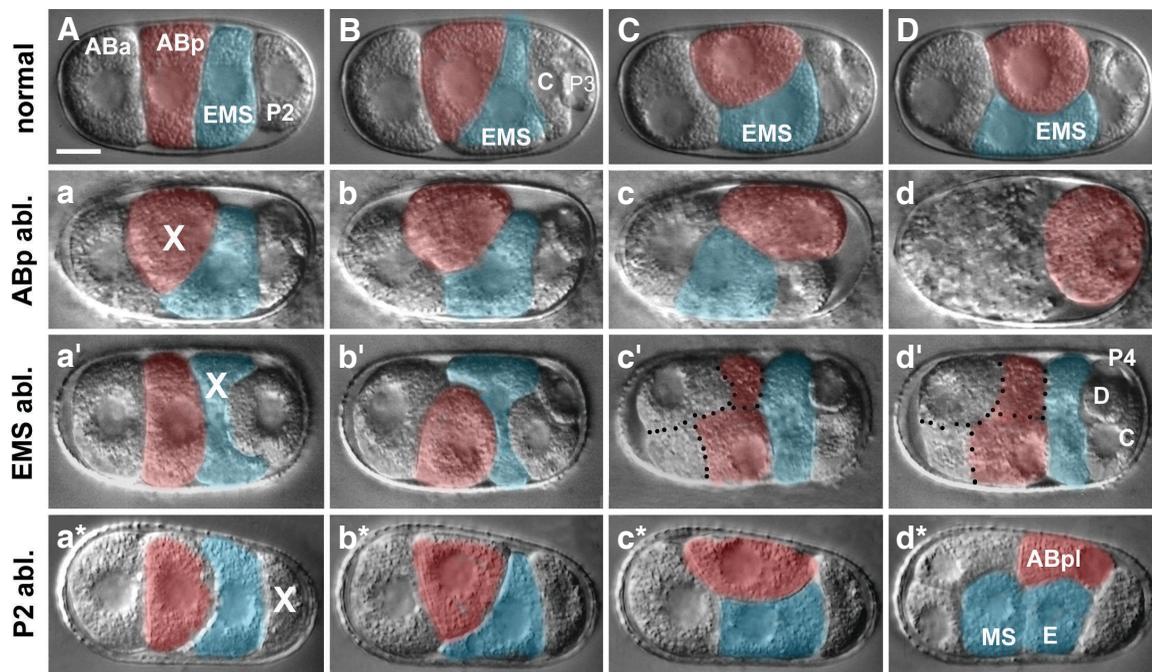
Fig. 6. Rearrangement of *D. coronatus* blastomeres. (A-D) *Untouched embryos.* **(a-d)** *After ablation of ABp.* **(a'-d')** *After ablation of EMS.* **(a*-d*)** *After ablation of P₂.* **(A)** *Four blastomeres in one linear sequence.* **(B)** *5-cell stage; P₂ has divided into C and P₃; the EMS has changed shape.* **(C)** *EMS has made contact to ABa.* **(D)** *EMS occupies a ventral and ABp a dorsal position in the embryo.* **(a)** *5-cell embryo after ablation of ABp (red; crossed).* **(b,c)** *5-cell stage; translocation of ABp and EMS (blue).* **(d)** *Terminal phenotype; posterior position of ABp.* **(a')** *4-cell embryo after ablation of EMS (blue, crossed).* **(b')** *5-cell stage; central position of EMS.* **(c')** *7-cell stage; two AB nuclei out of focus.* **(d')** *8-cell stage; dotted lines mark contours of the four AB descendants.* **(a*)** *4-cell stage after ablation P₂.* **(b*,c*)** *Rearrangements of EMS and ABp.* **(d*)** *7-cell stage. For better visibility of ABp and EMS images were colored. Orientation of embryo: anterior, left. Scale bar, 10 μm.*

ment of early blastomeres. This polarity reversal was found to be absent in *A. nanus* (clade 11) but this is compensated by early cell migrations (Skiba and Schierenberg, 1992). In *D. coronatus* we made a similar observation. Comparing a larger number of individual embryos (n= 22) we found major variations in the positions of C, D, P₃ and P₄ (Fig. 7). In approximately half of the analyzed 5-cell stages P₃ had no contact to ABp (Fig. 7 B,C). Essentially equal numbers could also be attributed to the four versions of 8-cell stages (Fig. 7 B1-C2; although cell positions were somewhat variable between embryos, with respect to cell contacts of P₄ each embryo could be assigned to one specific pattern). Dependent on their positions relative to each other, cells migrate and eventually merge into a single common pattern prior to the onset of gastrulation (Fig. 7D). The behavior of P₄ appears to involve several phases. Initially, it loses its firm attachment to its sister D, shifts around, and only after the division of EMS shows a quite directed movement toward the E cell independent of whether it is close or further away from it. This suggests that at least the germline cell is able to sense its actual position and responds to signals from other cells in order to find its final position.

Despite the delayed division of the gut founder cell E relative to *C. elegans* (Fig. 3), gastrulation in *D. coronatus* starts after the 24-cell stage, with the immigration of the two E cell daughters essentially as found in *C. elegans*. However, before that, P₄ divides again around the 23-cell stage (Fig. 3) while in *C. elegans* this division happens only later around the 100-cell stage (Deppe *et al.*, 1978). Nevertheless, in both species P₄ generates descendants of similar size. The P₄ daughters in *D. coronatus* behave like

those in *C. elegans* (where they give rise to germ cells only; Sulston *et al.*, 1983) in that both do not divide as far as we followed them (well beyond the 200-cell stage). In addition, they contain hardly any yolk granules and thus are much more transparent than somatic cells (not shown). Attempts to visualize germline with antibodies against *C. elegans* P granules failed.

Besides this case of premature germline division, we observed additional examples which can be interpreted as heterochronic shifts. These include the presence of 32 gut cell nuclei at hatching in *D. coronatus*, while *C. elegans* has only 20 at that time but adds another 10-14 nuclei during postembryonic development (Sulston and Horvitz, 1977). Preliminary observations indicate that the additional nuclei in *D. coronatus* reflect actual cell divisions rather than dinucleic cells.

AB daughters execute different developmental programs

In *C. elegans* the two daughters of the AB cell are originally of equivalent developmental potential and only become different from each other after an inductive signal from P₂ has been received by ABp (see introduction).

In *D. coronatus* P₂ never touches ABp (Fig. 7A), and P₃ contacts AB daughters only in about half of the analyzed embryos (Fig. 7 B,C) without any effect on normal development. Thus, in contrast to *C. elegans*, in *D. coronatus* this crucial interaction seems either be dispensable or to work without visible cell-cell contact. We therefore addressed the question whether here the developmental programs of ABa and ABp remain similar to each other or nevertheless become different. To do this we (1) studied
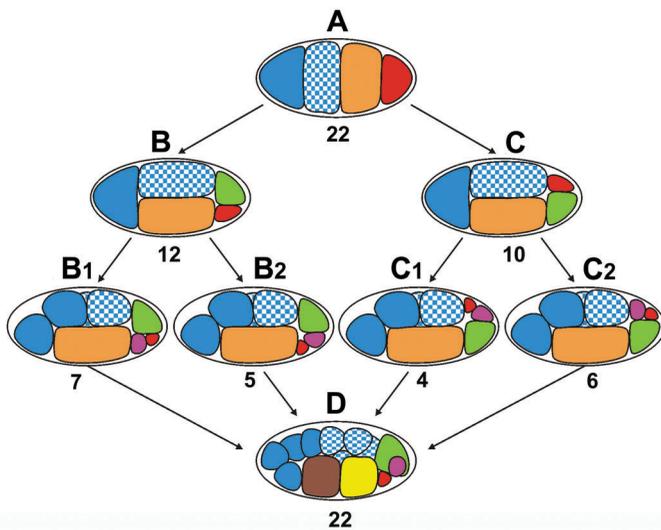
Fig. 7 (Left). Variable arrangement of blastomeres in 5- and 8-cell embryos of *D. coronatus*. *After division of the 4-cell stage **(A)**, two 5-cell variants **(B,C)** can form where P_3 touches either EMS **(B)** or ABp **(C)**. With the division of P_3 four different variants **(B1-C2)** are generated, which eventually merge into a single pattern **(D)**. Cell stage when P_4 touches E may vary. Observed numbers of variants are given below embryos. Color code: ABa (and descendants), solid blue; ABp (and descendants), cross-hatched blue; EMS, orange; C, green; D, purple; germline (P_2-P_4), red; MS, brown; E, yellow. Orientation of embryo: anterior, left; dorsal, top.*
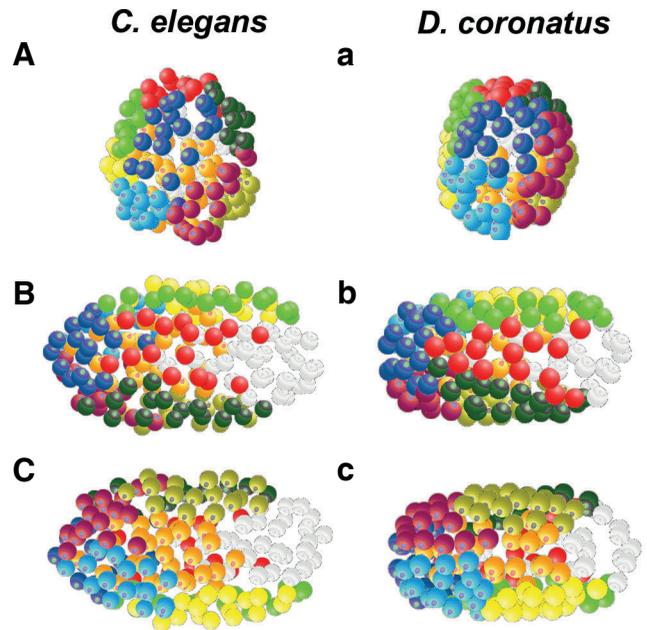
Fig. 8 (Right). Essentially identical arrangement of 128 AB cells in *C. elegans* (A-C) and *D. coronatus* (a-c). (A,a) *Anterior view; dorsal, top.* **(B,b)** *Dorsal view; anterior, left.* **(C,c)** *Ventral view; anterior, left. For color code of 8 AB clones and MS descendants, see Fig. 3.*

spatial pattern formation in the clones derived from these two cells and (2) compared the fates of selected blastomeres.

At a stage when 128 AB cells are present (172 cells in total) the spatial arrangements are essentially indistinguishable between both species (Fig. 8) including the prominent splitting up of ABar into two separate clones in *C. elegans* and *D. coronatus* (Fig. 8A, a; light blue and bright red spheres). Descendants of ABa and ABp form reproducible spatial patterns that differ considerably from each other (n=3). Our observations indicate that, like in *C. elegans*, AB cells in *D. coronatus* also contribute to hypodermis, neurons and pharynx (data not shown).

Moreover, we analyzed the fate of four selected ABa descendants (ABalapapaa, ABalappaaa, ABalppaapa and Abarpaaapp) which in *C. elegans* undergo early programmed cell death (i.e., in the 256 AB-cell stage), while the corresponding ABp descendants (i.e., ABplapapaa, etc.) execute different fates (Sulston *et al.*, 1983). We found that in *D. coronatus* the situation is like in *C. elegans*, i.e., the four descendants of ABa die (n=3) but those derived from ABp do not (n=2). Our data demonstrate that also in *D. coronatus* descendants of ABa and ABp execute different fates.

Cell ablation experiments indicate non-regulative development

In *C. elegans* blastomeres are fixed early to a specific, essentially invariant lineage program (see Introduction), and eliminated cells are not replaced by others. In contrast, in *A. nanus* early blastomeres compete for a primary fate and the loss of one cell may cause a switch in the developmental program of another. For example, if EMS is ablated, its neighbor C takes over the EMS

program (Wiegner and Schierenberg, 1999). As *D. coronatus* develops slowly, is parthenogenetic and does not show a reversal of cleavage polarity, thus behaving like *A. nanus* in this regard (see above), we asked whether this species may show such regulative behavior as well. For this we ablated EMS as soon as it was born and analyzed the development of the C cell (n=8). In no case did we find indications for a lineage transformation, i.e., no separation into two founder cells with different cleavage rhythms took place, none of the early C descendants immigrated, and no differentiated gut cells were detected with the tissue-specific antibody ICB4 and by looking at birefringent gut granules.

Hence, with respect to early embryonic plasticity *D. coronatus* appears to be similar to *C. elegans*, indicating that regulative potential does not depend on developmental tempo or the mode of reproduction but may rather be correlated with phylogenetic position.

Discussion

In *D. coronatus* (clade 9; Fig. 1) a linear 4-cell embryo is formed. As such an arrangement has also been described for a variety of other nematodes in the classical literature (for references, see Malakhov, 1994), it can be asked whether it represents a more original pattern or is a derived character. Using outgroup comparisons, Dolinski *et al.* (2001) concluded that within Rhabditida (clades 7-12) the rhombic 4-cell pattern as found in *C. elegans* is ancestral to the tandem pattern, which thus must have evolved more than once independently in individual nematode branches. The latter view is supported by our observations of linearly ordered blastomeres in the phylogenetically very

distant *Ironus* (clade 1; E.S., unpublished data), while other representatives of this taxon do not show such a pattern (Malakhov, 1994).

The process of nuclear/centrosome rotation was studied in detail by Hyman and White (1987) and Hyman (1989). It requires the activity of spindle microtubules that compete for attachment to a defined site at the anterior cortex of P_1. In *D. coronatus* the same process seems to takes place in both blastomeres of the 2-cell stage. This assumption is supported by our observation that in *Diploscapter* a prominent clear cortical region (CCR) appears in the cortex of both 2-cell stage blastomeres (Fig. 4b). It seems that this variant cell behavior between both species can best be attributed to an altered distribution of maternal factors responsible for capturing spindle microtubules.

A cortical structure resembling the CCR in *D. coronatus* was found in early ascidian embryos (Hibino *et al.*, 1998). Orientation of mitotic spindles and their asymmetric translocation resulting in unequal divisions are induced by this structure called CAB ("centrosome-attracting body"). It contains a complex of specific polarity proteins including PAR-6 and PAR-3 and appears to capture plus ends of spindle microtubules (Patalano *et al.*, 2006). The way in which cellular asymmetries are generated there shows obvious similarities to *C. elegans* but also to other systems (Nishikata *et al.*, 1999). A comparable effect on spindle behavior appears to be exerted by the RFM («region of the first midbody») in early embryos of the basal nematode *R. culicivorax* (clade 2; Schulze and Schierenberg, 2008).

What underlying mechanisms may have led during evolution from a transverse to a longitudinal spindle orientation in AB or vice versa? One option is an altered expression of *par* genes (Nance, 2005). A single mutation in the gene *par-3* of *C. elegans* leads to a linear *D. coronatus*-like 4-cell pattern (Kemphues *et al.*, 1988). However, this mutant has an embryonic lethal phenotype and therefore the differences in the genetic program between both species must be more complex. In the *par-2/par-3* double mutant where proteins are affected which are normally located at opposite poles of the zygote, the first mitotic spindle is longitudinally oriented (Cheng *et al.*, 1995), indicating that neither gene is required for spindle rotation in AB. It may be interesting to explore whether an RFM-like mechanism as found in *R. culicivorax* (Schulze and Schierenberg, 2008), which prevents a cell from following the centriolic principle (see above) may be present in *C. elegans* as well but then be normally overruled by *par* gene activity.

We found that in *D. coronatus* both AB daughters become different from each other despite the lack of contact between ABp and P_2, which in *C. elegans* is required for proper cell signaling (Priess and Thomson, 1987; Priess 2005). One alternative is that fate specification in AB cells is fundamentally different between the two species, despite their close phylogenetic relationship. In such a scenario the a-p polarity (which in *C. elegans* but not in *D. coronatus* and other parthenogenetic species is initiated by the entrance of the sperm; Goldstein and Hird, 1996; Lahl *et al.*, 2003; Cowan and Hyman; 2004), leads to a graded distribution of cytoplasmic factors in AB. Spindle rotation takes place not only in the germline resulting in differential segregation of maternal gene products (Strome and Wood, 1983; Strome and Lehmann, 2007) but also between ABa and ABp, making an induction dispensable. Alternatively, germline signals may be transmitted over distance.

In a series of elegant recombination experiments Bischoff and Schnabel (2006) showed that in fact P_2 in *C. elegans* can polarize cells that it does not touch. Their data support a transmission mechanism whereby the germline signal is relayed, i.e., renewed in each cell it travels through before reaching its final target.

We have initiated experiments to discern between these two options. So far, our approach is hampered by a high sensitivity of *D. coronatus* blastomeres to experimental interference and limited transparency of embryos to determine terminal cell fates.

The variable relative positions of C, D and P_4 in *D. coronatus* require more or less extensive migrations to reach the standard pattern prior to the onset of gastrulation (Fig. 7). This is different to *C. elegans* where during the early cleavage phase these cells are «automatically» placed at their correct position. The most plausible explanation for the directed migration of P_4 to the gut founder cell E is that the latter sends a signal which the germline cell can sense. Cell isolation and recombination experiments in *C. elegans* have demonstrated the existence of a cell surface recognition mechanism between gut precursor and germline cells (Goldstein, 1993). As in *D. coronatus* P_4 is often placed quite far away from its terminal position adjacent to the gut founder (Fig. 7 C1,C2), signaling must work over distance. Here a graded substance guiding the germline cell along one or more blastomeres to its final destination could be involved. This would constitute a so far undescribed way of cell-cell interaction in nematode embryos. It was shown that lysophospholipids play important roles in the migration of germ cells in vertebrates and invertebrates and a guiding function of a phospholipid gradient has been suggested (Renault and Lehmann, 2006). Recently Schnabel *et al.* (2006) proposed a model explaining migrations of AB descendants in *C. elegans*. They suggest that blastomeres are neither guided by graded signals nor by discrete guide posts but are sorted by a mechanism coupled to cell identity.

Based on our limited light microscopical analysis, we cannot exclude that an initial contact between cells (i.e., EMS and P_2) is perpetuated, e.g., in the form of filopodia or tunneling nanotubes (Rustom *et al.*, 2004), is remembered in some other way through consecutive cell divisions, until E and P_4 are present, or the proper contact partner is found via a «trial and error» mechanism (Hardin and McClay, 1990). While in a study using EM serial sections from early *C. elegans* embryos (Krieg *et al.*, 1978) no such connecting structures between non-adjacent cells were identified, it may be worthwhile to search more specifically for a visible structural basis of cell-cell communication between germline and gut precursors in *D. coronatus* and other species.

Our data indicate that the activity of the EMS cell initiates the rearrangements in the 5-cell stage resulting in a *C. elegans*-like pattern (Fig. 6). The ability of EMS to change its shape and then to move may be explained with a shift in the timing of events. In *C. elegans* the two granddaughters of EMS elongate and then migrate into the center of the embryo during the initial phase of gastrulation (von Ehrenstein and Schierenberg, 1980; Junkersdorf and Schierenberg, 1992; Nance and Priess, 2002). In several members of the taxon Plectida (clade 6) gastrulation starts one cell generation earlier with the immigration of the single gut founder cell E (Lahl *et al.*, 2003). In *D. coronatus* already EMS becomes competent to migrate or neighboring cells exhibit prematurely necessary cell surface molecules to allow it.

Heterochrony is often considered the single most important

process of evolutionary change (for discussion, see Raff, 1996). While prominent examples concern postembryonic events (e.g., neoteny or larval vs. direct development), several early embryonic modifications we have found in *D. coronatus* can be interpreted as heterochronic shifts (e.g. premature generation and division of the primordial germ cell; Fig. 3). It remains to be determined whether the movement of the EMS cell can be interpreted along the same line. A more detailed comparative analysis of nematode embryogenesis on a cellular and molecular level should give us a better estimate as to which of the developmental differences between species cannot be explained with heterochronies and to what extent variances like the ones described here may have an adaptive value, i.e., affect fitness of the hatched larva.

Materials & Methods

Nematode culture and strains

All strains were cultured on agar plates with the uracil-requiring strain of *E. coli* OP50 as a food source, essentially as described by Brenner (1974) except that, to reduce contamination with other bacteria, we used minimal medium plates (Lahl *et al.*, 2003). *Diploscapter coronatus* was kindly provided by Paul De Ley, University of California, Riverside. *Rhabditis* sp. (strain designation ES104) was isolated from the local greenhouse and is reminiscent of *Rhabditis dolichura*.

Microscopical analysis and 3-D reconstructions

Development was studied with Nomarski optics using a 100x objective. For cell lineage studies early stage embryos were collected from agar plates with a drawn-out pasteur pipette as early as the the 1-cell stage. Specimens were placed on microscope slides carrying a thin agar layer as a mechanical buffer and covered with a coverslip sealed on the edges with petroleum jelly. Depending on the species, development was either recorded on video tape, as described in Lahl *et al.*, 2003, or stored in digital form using a 4-D microscope with 15-25 optical sections/embryo and 15-60 sec time intervals between scans (Schnabel *et al.*,1997). Supported by specific software programs (Simi Biocell, München), cell behavior was traced and cell lineages were analyzed. 3-D reconstructions of nuclear positions were generated which can be freely rotated in all three dimensions. Antibodies K76 and L416 against germline specific cytoplasmic granules in *C. elegans* were used as described in Strome and Wood (1983) and antibody ICB4 (Okamoto and Thomson, 1985), which visualizes differentiated gut cells in both species.

Laser micromanipulation

For cell ablation and extrusion experiments embryos were mounted on polylysine-coated slides in distilled water which was then replaced by an embryonic growth medium (Edgar, 1995) as described by Wiegner and Schierenberg (1998). Cells were ablated by repeated short pulses of an N_2-pumped laser microbeam coupled to a microscope via glassfiber optics (Photonic Instruments, St. Charles, Ill.; laser dye, Coumarin; absorption maximum 440 nm). For extrusions the eggshell was perforated with the laser and individual blastomeres were squeezed out by gentle pressure on the coverslip.

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