Asymmetric Cell Divisions in the Early Embryo of the Leech *Helobdella robusta*

David A. Weisblat

Abstract

The small glossiphoniid leech *Helobdella robusta* is among the best-studied representatives of the super-phylum Lophotrochozoa in terms of early development. The *Helobdella* embryo undergoes a modified version of spiral cleavage, characterized by stereotyped cell lineages comprising multiple examples of equal, and unequal divisions, many of which are well-conserved with respect to those of other clitellate annelids, such as the oligochaete *Tubifex*. Here, we review the early development of *Helobdella,* focusing on the variety of unequal cell divisions. We then summarize an experimental analysis of the mechanisms underlying the unequal first cleavage in *Helobdella*, concluding that the unequal first cleavages in *Helobdella* and *Tubifex* proceed by different mechanisms. This result demonstrates the evolvability of the basic cell biological mechanisms underlying well-conserved developmental processes. Finally, we propose a model in which the unequal *second* cleavage in *Helobdella* may be regulated by the polarized distribution of PAR protein homologs, convergent with the unequal *first* cleavage of the nematode *Caenorhabditis elegans* (super-phylum Ecdysozoa).

1 Introduction

This chapter summarizes our current understanding of unequal cell divisions in the development of the leech, *Helobdella robusta*, within the larger context of comparative studies of development and evolution. The general rationale for studying *Helobdella* is as follows.

To understand the evolutionary changes in developmental processes that have given rise to the diverse body plans of modern animals, we must compare the development of extant species, interpreting similarities and differences with respect to the phylogenetic tree by which their ancestors diverged. Similarities represent either ~~conservation~~ or the conservation of developmental processes present in the last common ancestor of the species being compared. Differences yield insights into the divergence of

University of California, Dept. of Molecular and Cell Biology, 385 LSA, Berkeley, CA 94720-3200, USA. E-mail: weisblat@berkeley.edu

Progress in Molecular and Subcellular Biology
Alvaro Macieira-Coelho (Ed.)
Asymmetric Cell Division

developmental mechanisms along different branches of the phylogenetic tree.

To avoid a self-defeating circularity in this undertaking, it is imperative ~~that we avoid using~~ developmentally-derived traits to construct the phylogenetic trees ~~that are used to interpret developmental comparisons~~. Constructing phylogenies on the basis of molecular sequence comparisons is not without severe problems of its own, but it does offer an escape from the circularity of ~~classical~~ phylogenies based on morphological traits.

Molecular phylogenies have converged on grouping bilaterally symmetric animals into three major clades, ~~a significantly reduced~~ Deuterostomia, ~~and, from the former Protostomia, two new super-phyla called~~ Ecdysozoa and Lophotrochozoa (Aguinaldo et al. 1997; Ruiz-Trillo et al. 1999). Combining this consensus phylogeny with paleontological evidence, one proposition is that the last common ancestor of these three groups was an unsegmented animal that relied on mucociliary locomotion and even lacked a true coelom (Valentine and Collins 2000). If so, many of the features we associate with modern bilaterian animals may have arisen largely independently within these three lines. On the other hand, others have proposed that the urbilaterian was a segmented eucoelomate with well-developed sensory structures and limbs (Holland 2000; Panganiban and Rubenstein 2002). In either case, but especially in the former, we anticipate that studies of taxa phylogenetically distant from the commonly used models may reveal novel combinations and applications of ancestral cellular and molecular processes, associated with the formation of diverse body plans over ~600 MY of separate evolution.

The model organisms on which most modern studies of development are carried out fall into either Deuterostomia (i.e., vertebrates) or Ecdysozoa (i.e., fly and nematode). In contrast, *Helobdella* is among the best studied and experimentally tractable representatives of Lophotrochozoa, home to at least one half of the present day phyla. Thus, developmental studies of *Helobdella* should be informative for deducing the features of the "ur-bilaterian", the "ur-protostome" and especially, when taken together with studies of molluscs, flatworms and other annelids, for understanding the divergence of developmental mechanisms involved in the evolution of the "spiral cleavers ", a diverse group of animals that now seem likely to form a monophyletic group within Lophotrochozoa.

The annelids, or segmented worms, make up one of the major spiralian taxa. The annelids were traditionally regarded as being composed of three monophyletic classes, polychaetes, oligochaetes and leeches. More recent molecular analyses indicate that the leeches are in fact a monophyletic group arising *within* the oligochaetes (Erseus and Kallersjo 2004). Collectively, leeches and oligochaetes are designated as the class Clitellata, arising within the polychaetes. Moreover, the polychaetes themselves may be polyphyletic with respect to other spiralian groups that were traditionally accorded phylum status, such as echiurans, pogonophorans and sipunculans.

The defining feature of spiralian development is the obliquely oriented and unequal cell divisions (spiral cleavage) by which quartets of smaller cells (micromeres) arise near the animal pole by successive rounds of divisions from larger vegetal cells (macromeres) beginning at third cleavage. The oblique divisions mean that each quartet of micromeres is displaced from the animal-vegetal (A-V) axis with respect to the macromeres, usually first in the clockwise direction and then in the counterclockwise direction with respect to the macromeres (Collier 1997).

The stereotypic cleavage patterns seen in spiralian development are often accompanied by highly determinate cell fates (Wilson 1892; Zackson 1984; Weisblat and Shankland 1985; Huang et al. 2002). However, among species known as "equal cleavers", the specific fates of the blastomeres in each quartet are interchangeable until the embryos reach roughly the 32-cell stage, depending on the species, at which point, inductive interactions break the initial 4-fold symmetry to establish the second embryonic axis (Collier 1997). This symmetry breaking process is known as "D quadrant specification"; in standard spiralian nomenclature, the four quadrants of the embryo are designated A–D, with D being defined as the quadrant that produces the bilaterally symmetric mesendoderm and post-trochal ectoderm. In "unequal cleavers", determinate cell fates are evident from the start, because the second embryonic axis is established by unequal cleavages that segregate cell fate determinants present in the zygote, first to blastomere CD at the two-cell stage, and thence to the cell defined as macromere D at the four-cell stage.

Presently it is accepted that equal cleavage is ancestral for spiralians and that unequal cleavage has arisen multiple times independently, at least among molluscs (Freeman and Lundelius 1992). The situation is less clear for annelids in this regard, however. So far, no embryological experiments have been published that demonstrate the developmental equipotency of the early quadrants of any of the putative equal cleavers. Evidence in favor of equal cleavage in annelids comes from a recent study of the polychaete *Hydroides* (Arenas-Mena, in press). In this putative equal cleaver, the early expression of a *forkhead*-related gene (for which the non-uniform expression around the blastopore is believed to be important in gastrulation) is expressed uniformly in all four quadrants of the early embryo. On the other hand, it has been suggested on the basis of lineage studies that unequal cleavage arose very early within the annelids and may even be ancestral to the polychaetes; in any case, unequal cleavage is clearly ancestral to the clitellate annelids such as *Helobdella* (Dohle 1999).

2 Summary of *H. robusta* Development

Hermaphroditic like all clitellate annelids, *Helobdella* is capable of both cross- and self-fertilization. It breeds year round in laboratory culture, feeding on small freshwater snails. Fertilization is internal but development

arrests in metaphase I of meiosis, and resumes upon zygote deposition. The zygotes are ~400 microns in diameter and are deposited in clutches of 10–100 in transparent cocoons, from which they can easily be removed and cultured to maturity in a simple salt solution. Development to the juvenile has been divided into 11 stages extending over approximately 10 days, but for more precise analyses, embryos may be timed relative to their passage through any easily observed transition (e.g. the initiation of first cleavage) and this is then translated into the time (at 23 °C) after zygote deposition (AZD) (Fernandez et al. 1987; Weisblat and Huang 2001) (Fig. 1).

In brief, the first and second polar bodies form at 50 and 105 min AZD, respectively, after which the male and female pronuclei migrate to the center of the zygote and fuse (karyogamy). During this period (105–180 min AZD), cytoplasmic rearrangements generate animal and vegetal domains of yolk-deficient cytoplasm (teloplasm), enriched for mitochondria and maternal mRNAs (Astrow et al. 1989; Fernandez et al. 1990; Holton et al. 1994). The first cleavage is unequal. The cleavage plane runs parallel to the animal-vegetal axis, thereby yielding a smaller blastomere AB and a larger blastomere CD, which inherits both pools of teloplasm.

Subsequent cleavages are asynchronous and mostly unequal. CD enters cytokinesis at ~375 min AZD, signaling the transition from the two-cell to the three-cell stage. This cleavage segregates teloplasm to cell D at the four-cell stage, then vegetal teloplasm migrates to the animal pole and mixes with that teloplasm, as the third, unequal division forms quartets of vegetal macromeres (A′–D′) and animal micromeres (a′–d′) (Holton et al. 1989). At fourth cleavage (stage 4b) macromere D′ divides along an obliquely equatorial plane. Both daughter cells inherit some of the teloplasm: the animal daughter cell, DNOPQ (2d in classical spiralian terminology), is the precursor of 8 ectodermal stem cells (N, O/P, O/P and Q teloblasts) plus ~~additional 13~~ micromeres; the vegetal daughter, DM (2D in classical spiralian terminology), is the precursor of 2 mesodermal stem cells (M teloblasts) plus 2 micromeres (Bissen and Weisblat 1989; Sandig and Dohle 1988). Macromeres A′–C′ undergo two more rounds of unequal divisions, yielding two sets of micromere trios (a″–c″ and a‴–c‴). The residual macromeres A‴–C‴ are classically regarded as the endodermal precursors, but the gut actually has a more complicated origin (see below).

The teloblasts are segmentation stem cells. Each teloblast undergoes repeated divisions to generate a column (bandlet) of segmental founder cells (m, n, o/p, o/p and q blast cells; 18–122 h AZD). On each side, the five bandlets come together in a parallel array (germinal band). The left and right germinal bands and the space between them are covered by an epithelium derived from micromeres that arise during cleavage. The germinal bands move over the surface of the embryo, eventually coalescing along the midline (79–135 h AZD) to form the germinal plate, from which segmental tissues arise.

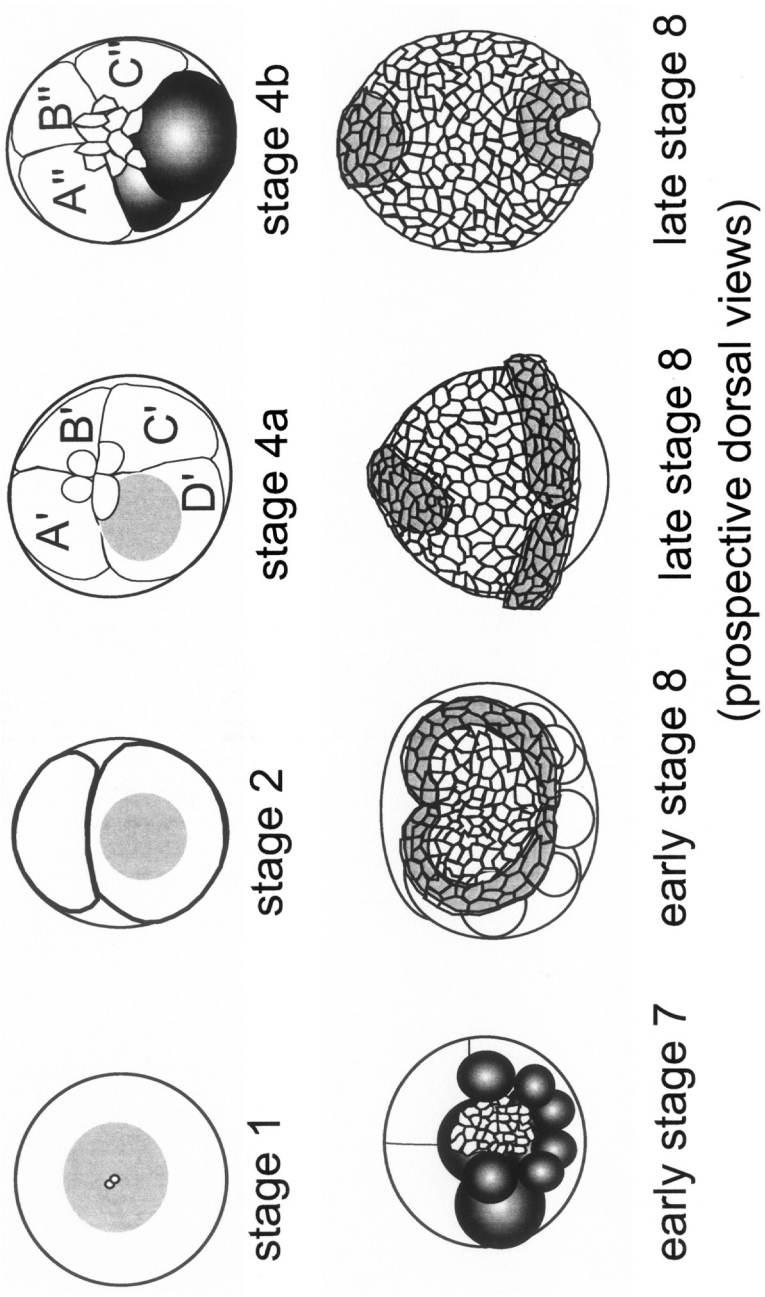

Fig. 1. Overview of *Helobdella* development. *Circles* at stage 1 denote polar bodies at animal pole; *shading* in stages 1–4a denotes teloplasm; *shading* in stages 4b and 7 highlights the origins of teloblasts from cells DM and DNOPQ; *shading* in stage 8 denotes the germinal bands and germinal plate; *expanding meshwork* in stages 4b–8 denotes micromeres and the epiboliziing epithelium to which they give rise

Coincident with the movements of the germinal bands, the epithelium spreads over the surface of the embryo. Within the germinal bands and germinal plate, blast cells undergo lineage-specific patterns of cell proliferation, migration and differentiation. The germinal plate expands dorsolaterally around the yolk and eventually coalesces along the dorsal midline, forming the body tube.

The gut forms by cellularization of a syncytial yolk cell (Nardelli-Haefliger and Shankland 1993), which forms by stepwise fusion, first among the macromeres and later still with the teloblast remnants and supernumerary blast cells (~120–160 h AZD) (Desjeux and Price 1999; Liu et al. 1998).The foregut (proboscis, proboscis sheath and esophagus) arises from specific micromere lineages (Huang et al. 2002).

Unequal cell divisions in the embryo of *Helobdella* fall into two categories (Scott Settle, unpublished observations) (Fig. 2). *Slightly unequal divisions*, defined as those that are clearly unequal but in which the ratio of sister cell diameters is less than 3, are seen among the large yolk-rich blastomeres beginning with first cleavage, and also in the stereotyped cell lineages leading from micromeres and blast cells to their definitive prostomial and segmental progeny, respectively. *Highly unequal divisions*, defined as those in which the ratio of sister cell volumes is greater than 3, consist of the micromere-forming divisions scattered throughout cleavage, and the production of blast cells by the repeated stem-cell divisions of the teloblasts. As will be illustrated below, the categorization of two different cell division as slightly unequal for example does not mean that they employ the same mechanism for regulating the position of the spindle apparatus, but nonetheless it's a starting point for addressing the problem. This chapter focuses on the mechanisms at work in slightly unequal cell divisions of the first two rounds of cell division.

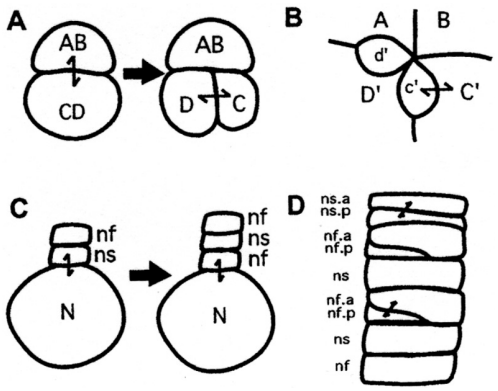

Fig. 2. A–D Unequal cell division in *Helobdella* development. In each panel, recent divisions are indicated by *double-headed arrows*: **A** animal view of the intact embryo at stages 2 and 3 depicts the first two, slightly unequal divisions leading

3
Unequal Cell Division at First Cleavage

In *Helobdella* as in other unequally cleaving spiralians, the chain of events initiated by the unequal first cleavage is critical to the normal development of the body plan. Centrifugation experiments showed that factors permitting the expression of the D quadrant fates are associated with the teloplasm. For instance, when zygotes are compressed to re-orient the mitotic apparatus and both daughters inherit teloplasm at first cleavage, they both make teloblasts (Nelson and Weisblat 1992). And when mild centrifugation is used to distribute teloplasm uniformly to the nominal C and D blastomeres at second cleavage, both these cells may form a full complement of teloblasts (Astrow et al. 1987). Thus, the segregation of teloplasm to cell CD by the unequal first cleavage, and thence to cell D by the unequal second cleavage, is critical for normal development. How are these unequal cell divisions achieved?

The nematode *Caenorhabditis elegans* also undergoes an unequal first cleavage such that, apart from the size difference, the two-cell stage appears very similar to that of *Helobdella*. An elegant body of work is emerging to provide a detailed mechanism for this unequal cleavage . Reviewing that work is beyond the scope of this chapter (Pellettieri and Seydoux 2002) but, in brief, the polarity of the zygote is set by the point of sperm entry, which defines the posterior end, and this initial cue is interpreted to establish posterior and anterior cortical domains in the zygote, marked by PAR1 and a widely conserved complex of proteins including PAR3, PAR6, atypical protein kinase C (aPKC) and one of the Rho family GTPases (CDC42),

Fig. 2. (*Cont'd*) from the zygote to cells AB and CD, and from cell CD to macromeres C and D; AB divides after CD; **B** depiction of the animal pole region, showing the production of micromeres by highly unequal cell divisions at third cleavage (stage 4a). The D quadrant divides first (yielding macromere D′ and micromere d′), then the C quadrant and then A and B divide synchronously; **C** teloblasts are bilaterally paired segmentation stem cells, which produce columns of segmental founder cells (blast cells) by iterated, highly unequal divisions at the rate of about one per hour (stages 6–8). Here, one of the primary neurogenic (N) teloblasts is depicted, which gives rise to two distinct classes of blast cells (nf and ns) in exact alternation; **D** an isolated column of blast cells derived from an N teloblast, showing the first mitoses of the nf and ns blast cells (stage 7–8). The nf and ns blast cells give rise to distinct, segmentally iterated sets of about 70 identifiable neurons, by lineages characterized by unequal cell divisions that are stereotyped according to the timing, orientation and degree of asymmetry. For example, each nf cell divides about 24 h after its birth from the N teloblast, and the anterior daughter (nf.a) is markedly larger than the posterior daughter (nf.p). In contrast, each ns cell divides only about 28 h after its birth, and the anterior daughter (ns.a) is only slightly larger than the posterior daughter (ns.p)

respectively (Nance 2005). Thus, astral microtubules emanating from the anterior spindle pole experience a different biochemical environment at the cell cortex than do those emanating from the posterior spindle pole (Labbe et al. 2003). The associated difference in astral microtubule dynamics results in displacement of the mitotic apparatus toward the posterior of the embryo and leads to the unequal first cleavage (Fig. 3). The posterior localization of a PAR1 homolog is also important in establishing the anterior-posterior polarity in *Drosophila* (Doerflinger et al. 2006), despite the vast differences in the early development of these two ecdysozoan models. Therefore, to ask if this mechanism for establishing zygotic polarity is also used in *Helobdella*, homologs of *par-1* and *par-6* (*Hro-par1* and *Hro-par6*) were cloned and antibodies were raised against them (Ren 2005).

No asymmetric immunostaining was detected in the zygote, suggesting that the mechanisms by which unequal first cleavage is achieved in *Helobdella* differs from that used in *Caenorhabditis elegans*. But by the two-cell stage and beyond, HRO-PAR1 and HRO-PAR6 showed complementary localization patterns, suggesting that the antibodies were recognizing their intended targets and that the proteins in leech are behaving in a biochemically similar manner to their homologs in other organisms. HRO-PAR1 is seen primarily at basolateral membranes, especially in the macromere-macromere junctions. In contrast, HRO-PAR6 is seen at the membrane abutting an intercellular space designated as the blastocoel at the two-cell stage and on both apical and basolateral junctions between micromeres in later stages.

The failure to detect a pre-established polarity in the *Helobdella* zygote was consistent with the results of previous embryological studies (Nelson and Weisblat 1992). Compressing the zygote so as to re-orient the mitotic apparatus at first cleavage does not disrupt development as long as both pools of teloplasm end up in the same blastomere at first cleavage, suggesting that there was no inherent polarization of the embryo along a prospective second axis prior to first cleavage.

In another approach to the question of the unequal first cleavage, carefully staged embryos were fixed at different time points during mitosis and immunostained for alpha-tubulin (to assess the morphology of the spindle) and gamma-tubulin (as a marker for the centrosomes) (Ren and Weisblat 2006). We found that the paternal centrosome duplicates prior to centration of the pronuclei and gives rise to a symmetric, diastral spindle in prophase and early metaphase (220–245 min AZD). Surprisingly, one centrosome then loses its gamma-tubulin immunoreactivity. Shortly after that, the associated aster becomes greatly reduced in size and the spindle shifts in the direction of the down-regulated aster, setting up the unequal cleavage. Gamma-tubulin immunoreactivity returns to the down-regulated centrosome during telophase, but the spindle remains asymmetric, setting up the unequal cleavage, with the larger aster corresponding to the future CD cell (Fig. 3).

These observations stand in contrast to those obtained previously for a different clitellate annelid, the oligochaete *Tubifex*. Ishii and Shimizu (Ishii and Shimizu 1997), using the same experimental approach, found that the centrosome of the mitotic spindle is maternal in origin and does not duplicate during the first cell cycle. The spindle pole associated with the (gamma-tubulin-positive) centrosome forms a large aster, while the spindle pole that lacks a centrosome (as judged by the absence of gamma-tubulin immunoreactivity) fails to generate an appreciable aster. As a result the first mitotic spindle is essentially monastral and strikingly asymmetric from prophase onwards. The spindle is displaced toward the anastral side of the zygote, resulting in an unequal first cleavage, with the astral half-spindle corresponding to blastomere CD (Fig. 3).

As described in the introduction, the clitellate annelids form a robust clade. The patterns of cell division during cleavage are highly conserved in this clade (Dohle 1999) and no equal cleaving clitellates have been described. So it seems beyond doubt that teloplasm formation and its segregation to the prospective D quadrant by unequal cleavage are unquestionably ancestral traits among clitellates. Thus, the strikingly different mechanisms operating during the unequal first cleavage in *Helobdella* and *Tubifex* must represent changes in the mechanism regulating the unequal first cleavage along one or both branches leading to these species from the ancestral clitellate, despite the fact that the inequality of that cleavage was conserved all along the way. Intriguingly, previous studies have also revealed differences in the cytoskeletal mechanisms underlying teloplasm formation, which is microfilament-dependent in *Tubifex* and microtubule-dependent in *Helobdella* (Astrow et al. 1989; Fernandez et al. 1998; Shimizu 1982).

A priori, it might be postulated that the more derived condition of leeches relative to oligochaetes in terms of adult morphology (e.g. loss of regenerative capabilities and formation of a posterior sucker) predicts that the mechanism governing the unequal first cleavage in *Helobdella* would also be derived with respect to that in *Tubifex*, i.e., that the monastral spindle mechanism is more likely to represent the mechanism of unequal cleavage in the ancestral clitellate. In fact, this conclusion is far from certain, and the relationship between morphological evolution and changes in developmental mechanisms is one of the key issues to be addressed by such comparative studies. Remember that *Tubifex* and *Helobdella* are both "modern" animals, equally far removed from the ancestral clitellate (for a further discussion of this critical issue, see Crisp and Cook 2005). And since we have just seen evidence that ~~the~~ a macroscopic process (e.g., unequal first cleavage) can be conserved while the underlying mechanisms evolve, it is impossible to conclude anything about the ancestral process by noting the differences between ~~these~~ two species. Fortunately, *Helobdella robusta* and *Tubifex tubifex* are but two among thousands of clitellate species. Examining additional judiciously chosen representatives should allow us to determine the variety of mechanisms regulating the unequal first cleavage and their phylogenetic origin(s) within this clade.

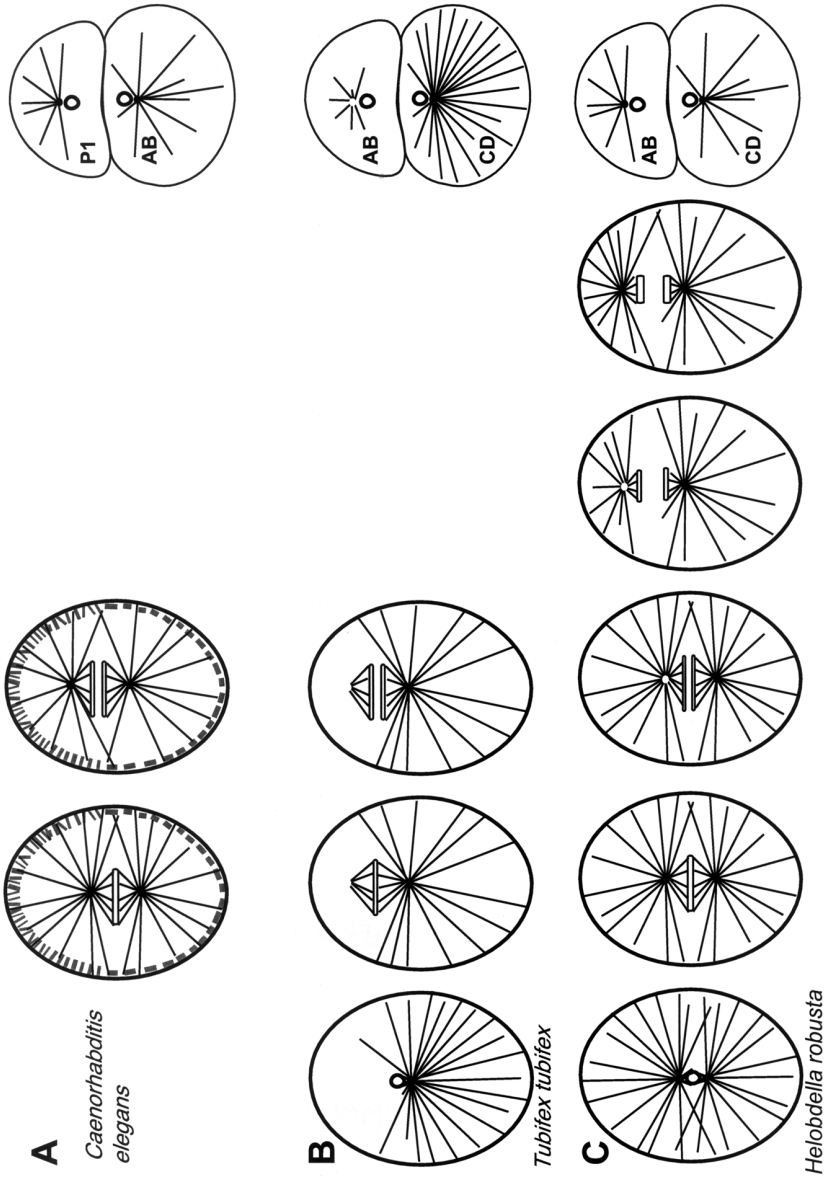

Fig. 3. **A–C** Comparison of the unequal first cleavage in nematode (*C. elegans*), earthworm (*T. tubifex*) and leech (*H. robusta*). Key steps in mitosis are indicated *from left to right* for each species: **A** in *C. elegans* (superphylum Ecdysozoa), events initiated at the point of sperm entry lead to the formation of distinct anterior and posterior domains at the cell cortex, marked by mutually exclusive localization of proteins such as PAR-3 and PAR-6 (*dashes*), and PAR-1 and PAR-2 (*bristles*), respectively. The diastral mitotic apparatus has two gamma-tubulin-positive centrosomes and is positioned symmetrically at the beginning of metaphase. Subsequently it shifts toward the PAR-1/PAR-2 domain, leading to an unequal cell division that yields a larger, anterior AB cell and a smaller, posterior P1 cell; **B** in *T. tubifex* (a clitellate annelid; superphylum Lophotrochozoa) the (maternally derived) centrosome fails to duplicate, so the mitotic apparatus is highly asymmetric from the start of mitosis; **C** *H. robusta* exhibits yet a third mechanism for this unequal first cleavage, despite the fact that it is clearly homologous to that in *Tubifex*. The (paternally derived) centrosome duplicates and the mitotic apparatus is symmetric through early metaphase. Then one centrosome is transiently down-regulated (indicated by the white center in the upper aster) and this is followed by a decrease in the size of the associated aster and the shift of the mitotic apparatus toward that side. Note that there is no biological significance to the AB cell designation in nematode and annelid

4
Unequal Cell Divisions at Second Cleavage

At second cleavage, blastomere CD also divides in a slightly unequal manner, so that teloplasm is further segregated to cell D at the four-cell stage. There has been no analysis of centrosome dynamics during this division for *Helobdella*, but double staining for tubulin and DNA revealed that the spindle was already positioned eccentrically by metaphase and that the asters associated with the prospective C and D blastomeres remained ~~uniformly~~ large, in contrast to the situation during first cleavage.

Other results, from blastomere isolation experiments, provide further evidence that the mechanism regulating the unequal second cleavage are different from those operating at first cleavage (Symes and Weisblat 1992). Specifically, when the fertilization envelope is removed and the AB and CD blastomeres are separated at first cleavage and cultured on an agarose bed, the cells assume more rounded shapes and cell CD tends to divide more equally than in the intact embryo; teloplasm is often inherited by both daughter cells and both may form teloblasts. In other experiments, the isolated CD cells were cultured in agarose wells in the presence of small sephadex beads, which deformed the CD blastomeres in a manner similar to that achieved by cell AB in the normal embryo. The mechanical deformation induced by the bead was sufficient to substantially restore the normal inequality of the CD division and teloplasm segregation. These results suggest that the unequal second cleavage is regulated in part by mechanical cues present in the two-cell stage that are not available to the more symmetrical zygote.

These observations on *Helobdella* complement previous work on *Tubifex*, suggesting that these species may be more similar during second cleavage than during first cleavage. Shimizu (Shimizu 1996) showed that the mitotic apparatus in blastomere CD has two asters, each associated with a gamma-tubulin positive centrosome. Asymmetry becomes evident just after metaphase, when the aster associated with the prospective C blastomere moves toward the membrane adjacent to the AB blastomere. Granted that appearances can be deceiving, but it appears as if the astral microtubules on that side of the prospective C aster have become attached to the cortex on that side of the cell and are undergoing a depolymerization-coupled traction toward the zone of AB/CD apposition, similar to those thought to be operating on kinetochore microtubules during anaphase (Westermann et al. 2006).

Separating the AB and CD blastomeres in *Tubifex* also causes the CD cell to undergo an equal division, as does moving the CD nucleus away from the membrane adjacent to blastomere AB by centrifugation (Takahashi and Shimizu 1997). From these results, it appears that cortical factors induced locally by contact with blastomere AB are required to asymmetrize what is otherwise a symmetric mitotic apparatus in cell CD.

At first, this seems to contradict the conclusion that the deformation of CD is sufficient to asymmetrize the cleavage in *Helobdella*. However, we suggest that these apparently disparate results may in fact just be two different aspects of the same process. On the one hand, the mild mechanical deformation induced by culturing CD blastomeres in the presence of a bead must somehow have biochemical consequences on the mitotic apparatus in order to affect the placement of the cleavage furrow. And, conversely, it may be assumed that co-culturing isolated CD blastomeres in the presence of other "inducing" blastomeres may lead to mechanical deformation of the CD cell as it adheres to the inducing cell. Thus biochemical and mechanical effects may reinforce one another in establishing cortical factors that asymmetrize the mitotic apparatus of cell CD in late metaphase/anaphase.

Why does CD cleave asymmetrically and not AB? A combination of seemingly disparate observations made in *Tubifex* and *Helobdella* suggest the outline of a possible answer.

The first observation is that during the two-cell stage in these embryos an extracellular cavity forms between the separating the apposing faces of the AB and CD blastomeres. This cavity is called the blastocoel, although it should not be concluded from this that it is necessarily homologous to the space of the same name in vertebrate embryos. This blastocoel develops midway through the two-cell stage and is initially surrounded entirely by the apposed AB and CD membranes. Later the blastocoel appears as an extracellular space between the micromeres and macromeres. The presence of the blastocoel at the two-cell stage means that we can distinguish three distinct spatial domains of cell membranes and cortical cytoplasm in the two-cell embryo: domain 1 consists of those membranes making up the outer surface of the embryo; domain 2 consists of the membranes in the region where the AB and CD cells are closely apposed; and domain 3 consists of those membranes making up the walls of the blastocoel itself (Fig. 4).

The second observation, made by Shimizu et al. (Shimizu et al. 1998) is that, during second cleavage, the microtubules of the smaller, essentially anastral mitotic apparatus present in the AB cell seem to have formed extensive contacts with the third domain, i.e. the blastocoel wall. In the larger mitotic apparatus of cell CD, by contrast, the astral microtubules of the aster associated with the prospective C macromere come into close apposition with domain 2 membranes in the region of cell apposition, and avoid the blastocoel wall (Fig. 4).

Finally, the third relevant observation is that, as alluded to in Sect. 2, the complementary localization of homologs of PAR1 (to the region of cell apposition) and PAR6 (to the blastocoel wall) (Ren 2005) suggests a biochemical mechanism by which the astral microtubules can distinguish between these different domains at least in *Helobdella*. Note that if the prospective C aster is attracted preferentially toward the PAR1-positive membrane domain, the presence of the PAR1-deficient blastocoel wall in

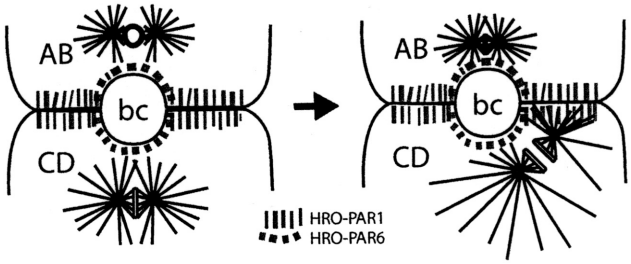

Fig. 4. Proposed mechanism for the unequal second cleavage, of blastomere CD, in *Helobdella*, based on a synthesis of observations made in *Helobdella* and *Tubifex*. ~~by~~ By the onset of mitosis in cell CD, a blastocoel (bc) has arisen at the interface of cells AB and CD. Cell membranes surrounding the blastocoel are enriched for HRO-PAR6, while those in direct apposition are enriched for HRO-PAR1. Greater affinity between one aster and the adjacent HRO-PAR1 would result in the displacement of the mitotic apparatus to that side. Why this displacement is invariably toward the right (as viewed from the animal pole) remains to be determined

the center of the embryo ensures that the mitotic apparatus shifts to one side or the other relative to the centrally located blastocoel, thereby setting up the unequal cleavage. Despite the differences in geometry and the fact that this is occurring at second cleavage rather than first, this situation is strikingly similar to that in the *C. elegans* zygote in terms of the movement of the mitotic apparatus relative to the polarized distributions of PAR1 and PAR6 and is presumably similarly governed by the differential effects of the PAR domains on microtubule dynamics (Labbe et al. 2003). We speculate that this represents an independent recruitment of the PAR-mediated cell polarity machinery to regulate an unequal cell division in the leech.

Whether or not this proposed mechanism ~~governing~~ the unequal second cleavage will prove true remains to be determined of course. In any event, several questions related to these observations remain such as: how does the clitellate blastocoel form and how are the PAR domains established? What accounts for the different cortical domain preferences of the mitotic apparatus in cell AB vs cell CD? And how is the chirality of the spirally cleaving embryo established?

In considering these questions, the speculations are almost entirely unbounded by any relevant factual observations. Blastocoel formation must involve differential localization of adhesion molecules including tight junctions, and also secretory apparatus and/or ion pumps, assuming that the fluid-filled cavity is inflated by an osmotic imbalance. However the blastocoel is formed, it is interesting to note that it provides an additional mechanical deformation of the membrane. Harking back to the bead experiments, might this be a factor in initiating or maintaining the discrete domains of PAR1 and PAR6 localization?

For *Tubifex*, an obvious difference between the mitotic apparatus in AB and CD cells is that the latter has gamma-tubulin reactive centrosomes and normal asters, while the former has neither of these (Shimizu, 1996). It is tempting to think that this may be important in determining the properties of the microtubules, but from the work in *Helobdella*, it seems that the AB mitotic apparatus has nice asters (Scott Settle, unpublished observations) and presumably centrosomes as well, since it inherits one from the first mitosis (Ren 2005). Other possibilities would be that the PAR1 immunoreactivity we see in the domain of cell-cell apposition is actually confined to cell CD, something that cannot be distinguished by current immunofluorescence observations, or that some other factor, possibly related to the teloplasm and thus present only in cell CD, is required for the mitotic apparatus to respond to the PAR1 and PAR6 domains. A related possibility is that the delay in mitosis of cell AB relative to CD is somehow responsible for the differential response of their mitotic apparatuses to the cortical factors.

Regarding the chirality of second cleavage, this handedness is manifested by the fact that cell CD cleaves so that cell C invariably lies at the counterclockwise side of cell D, when the embryo is viewed from the animal pole. In *Helobdella*, this corresponds to the prospective C aster being the one that shifts toward the PAR1 domain of cell-cell apposition. In principle of course, the elaboration of distinct animal-vegetal and AB-CD axes by the end of first cleavage provides sufficient information to reliably cue the orientation of the handedness of the following cleavages. However, like so many of the other questions in spiralian development, the molecular underpinnings of this process remain to be determined.

Acknowledgements Work described here from the author's laboratory has been supported by grants from the US National Science Foundation. I thank Deirdre Lyons and other members of the laboratory for helpful discussions.

References

Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA (1997) Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 387:489–493

Arenas-Mena C (in press) Dev Genes Evol

Astrow S, Holton B, Weisblat D (1987) Centrifugation redistributes factors determining cleavage patterns in leech embryos. Dev Biol 120:270–283

Astrow SH, Holton B, Weisblat DA (1989) Teloplasm formation in a leech, Helobdella triserialis, is a microtubule-dependent process. Dev Biol 135:306–319

Bissen ST, Weisblat DA (1989) The durations and compositions of cell cycles in embryos of the leech, Helobdella triserialis. Development (Cambridge) 106:105–118

Collier JR (1997) Gastropods, the snails. In: Gilbert SF, Raunio AM (eds) Embryology, constructing the organism. Sinauer, Sunderland, pp 189–217

Crisp MD, Cook LG (2005) Do early branching lineages signify ancestral traits? Trends Ecol Evol 20:122–128

Desjeux I, Price DJ (1999) The production and elimination of supernumerary blast cells in the leech embryo. Dev Genes Evol 209:284–293

Doerflinger H, Benton R, Torres IL, Zwart MF, St Johnston D (2006) Drosophila anterior-posterior polarity requires actin-dependent PAR-1 recruitment to the oocyte posterior. Curr Biol 16:1090–1095

Dohle W (1999) The ancestral cleavage pattern of the clitellates and its phylogenetic deviations. Hydrobiologia 402:267–283

Erseus C, Kallersjo M (2004) 18S rDNA phylogeny of Clitellata (Annelida). Zool Scr 33:187–196

Fernandez J, Olea N, Matte C (1987) Structure and development of the egg of the glossiphoniid leech Theromyzon rude: characterization of developmental stages and structure of the early uncleaved egg. Development (Cambridge) 100:211–226

Fernandez J, Olea N, Tellez V, Matte C (1990) Structure and development of the egg of the glossiphoniid leech Theromyzon rude: reorganization of the fertilized egg during completion of the first meiotic division. Dev Biol 137:142–154

Fernandez J, Roegiers F, Cantillana V, Sardet C (1998) Formation and localization of cytoplasmic domains in leech and ascidian zygotes. Int J Dev Biol 42:1075–1084

Freeman G, Lundelius JW (1992) Evolutionary implications of the mode of D quadrant specification in coelomates with spiral cleavage. J Evol Biol 5:205–247

Holland LZ (2000) Body-plan evolution in the Bilateria: early antero-posterior patterning and the deuterostome-protostome dichotomy. Curr Opin Genet Dev 10:434–442

Holton B, Astrow SH, Weisblat DA (1989) Animal and vegetal teloplasms mix in the early embryo of the leech, Helobdella triserialis. Dev Biol 131:182–188

Holton B, Wedeen CJ, Astrow SH, Weisblat DA (1994) Localization of polyadenylated RNAs during teloplasm formation and cleavage in leech embryos. Roux's Arch Dev Biol 204:46–53

Huang FZ, Kang D, Ramirez-Weber FA, Bissen ST, Weisblat DA (2002) Micromere lineages in the glossiphoniid leech Helobdella. Development 129:719–732

Ishii R, Shimizu T (1997) Equalization of unequal first cleavage in the Tubifex egg by introduction of an additional centrosome: implications for the absence of cortical mechanisms for mitotic spindle asymmetry. Dev Biol 189:49–56

Labbe JC, Maddox PS, Salmon ED, Goldstein B (2003) PAR proteins regulate microtubule dynamics at the cell cortex in C. elegans. Curr Biol 13:707–714

Liu NL, Isaksen DE, Smith CM, Weisblat DA (1998) Movements and stepwise fusion of endodermal precursor cells in leech. Dev Genes Evol 208:117–127

Nance J (2005) PAR proteins and the establishment of cell polarity during C. elegans development. Bioessays 27:126–135

Nardelli-Haefliger D, Shankland M (1993) *Lox10*, a member of the *NK-2* homebox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella*. Development 118:877–892

Nelson BH, Weisblat DA (1992) Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo. Development 115:103–115

Panganiban G, Rubenstein JL (2002) Developmental functions of the Distal-less/Dlx homeobox genes. Development 129:4371–4386

Pellettieri J, Seydoux G (2002) Anterior-posterior polarity in C. elegans and Drosophila–PARallels and differences. Science 298:1946–1950

Ren X (2005) Isolation and characterization of PAR-1 and PAR-6 homologs in Helobdella robusta. PhD thesis, Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, California

Ren X, Weisblat DA (2006) Asymmetrization of first cleavage by transient disassembly of one spindle pole aster in the leech Helobdella robusta. Dev Biol 292:103–115

Ruiz-Trillo I, Riutort M, Littlewood DT, Herniou EA, Baguna J (1999) Acoel flatworms: earliest extant bilaterian Metazoans, not members of Platyhelminthes. Science 283:1919–1923

Sandig M, Dohle W (1988) The cleavage pattern in the leech Theromyzon tessulatum (Hirudinea, Glossiphoniidae). J Morphol 196:217–252

Shimizu T (1982) Ooplasmic segregation in the Tubifex egg: mode of pole plasm accumulation and possible involvement of microfilaments. Wilhelm Roux's Arch 191:246–256

Shimizu T (1996) Behavior of centrosomes in early Tubifex embryos: asymmetic segregation and mitotic cycle-dependent duplication. Roux's Arch Dev Biol 205:290–299

Shimizu T, Ishii R, Takahashi H (1998) Unequal cleavage in the early Tubifex embryo. Dev Growth Differ 40:257–266

Symes K, Weisblat DA (1992) An investigation of the specification of unequal cleavages in leech embryos. Dev Biol 150:203–218

Takahashi H, Shimizu T (1997) Role of intercellular contacts in generating an asymmetric mitotic apparatus in the *Tubifex* embryo. Dev Growth Differ 39:351–362

Valentine JW, Collins AG (2000) The significance of moulting in Ecdysozoan evolution. Evol Dev 2:152–156

Weisblat DA, Huang FZ (2001) An overview of glossiphoniid leech development. Can J Zool Rev Can Zool 79:218–232

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

Westermann S, Wang HW, Avila-Sakar A, Drubin DG, Nogales E, Barnes G (2006) The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. Nature 440:565–569

Wilson E (1892) The cell lineage of Nereis. J Morphol 6:361–480

Zackson S (1984) Cell lineage, cel-cell interaction, and egment formation in the ectoderm of a glossiphoniid leech embryo. Dev Biol 104:143–160