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# Asymmetrization of first cleavage by transient disassembly of one spindle pole aster in the leech *Helobdella robusta*

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**Abstract**

Unequal first cleavage is characteristic of a diverse group of protostome animals. In the nematode *Caenorhabditis elegans*, unequal first cleavage is achieved through the interaction of an apparently symmetric mitotic spindle apparatus with a clearly polarized cell cortex. In the clitellate annelid *Tubifex tubifex*, by contrast, the spindle is monastral and contains only one gamma-tubulin-reactive centrosome; this monastral spindle is inherently asymmetric throughout mitosis. Here, we have used immunostaining for beta- and gamma-tubulin to follow spindle dynamics during the unequal first cleavage in another clitellate annelid, the leech *Helobdella robusta*. We find that the mitotic spindle is diastral and symmetric through early metaphase, then becomes asymmetric following the transient down-regulation of one centrosome, as judged by gamma-tubulin immunofluorescence. Low levels of drugs that affect microtubule dynamics can symmetrize the first cleavage without affecting the gamma-tubulin dynamics. Our results provide a striking example of the evolvability of cellular mechanisms underlying an unambiguously homologous developmental process.


*Keywords:* Asymmetric cell division; *Helobdella robusta*; Centrosome; Mitotic spindle dynamics

## Introduction

It is often assumed that developmental processes that are homologous between two organisms should proceed by similar mechanisms at the cell and molecular levels. Conversely, similarities in developmental mechanisms are taken as evidence for evolutionary homology. Whether or not these are reasonable starting assumptions, there are many examples where they break down. Here, we examine the former assumption as it applies to unequal first cleavage in clitellate annelids, and find evidence for a diversity of mechanisms in what are quite clearly homologous processes.

Mitotic sister cells that follow different fates are an essential feature of normal development for all higher metazoans. A priori, such fate differences may result either from unequal mitoses (i.e., those that form two inherently different cells) or from equal divisions followed by some sort of signaling process that causes equipotent cells to follow different fates.

Taxa that develop via spiral cleavage provide a rich source of experimental materials for analyzing and comparing mechanisms of equal and unequal divisions at the first zygotic mitosis. Examining the first cleavage is advantageous experimentally because it is generally more accessible experimentally and simpler geometrically than are later stages of development.

Comparisons among spiralian taxa should also be evolutionarily informative since recent molecular phylogenies are consistent with the hypothesis that spirally cleaving taxa form a monophyletic group (Aguinaldo et al., 1997). According to this hypothesis, the early cleavages giving rise to A–D "quadrants" of clonally related macromeres and micromeres are homologous among molluscs, annelids, non-acoel flatworms and their allies. As in most animals, the primary embryonic axis coincides with the animal–vegetal axis of the oocyte among spiralians. Establishment of the secondary axis, which conveys bilateral symmetry, is achieved in this group by specifying one of the four quadrants to be different from the other three; by convention, this is referred to as the D quadrant. In unequally cleaving spiralians, the prospective D quadrant and hence the second embryonic axis can be reliably predicted once the first

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cleavage has generated two non-equivalent blastomeres, designated AB and CD.

At present, wide scale comparisons among spiralians are hampered by uncertainties concerning the underlying phylogenetic relationships, especially within and among the *Annelida* (McHugh, 2000). This is now an area of active investigation. At present, it seems clear that polychaetes are a polyphyletic group encompassing taxa such as *Echiura* and *Pogonophora* that were previously categorized as separate phyla (McHugh, 1997). The two other classically defined classes of annelids, oligochaetes and leeches, combine to form a group, *Clitellata*, that is accepted on both molecular and morphological grounds as being monophyletic within the polychaetes (Kojima, 1998; McHugh, 1997).

Within *Clitellata*, the oligochaetes are paraphyletic and leeches are monophyletic (Erséus and Källersjö, 2004). Dohle (1999) showed that a particular pattern of asymmetric cleavages is very well conserved throughout the clitellates (and arguably throughout the annelids). Early steps in the clitellate cleavage pattern include a cytoplasmic rearrangement prior to first cleavage that yields animal and vegetal domains of yolk-free cytoplasm (teloplasm) followed by an unequal first cleavage that segregates both pools of teloplasm to cell CD and thence to macromere D at second cleavage. Macromere D is usually larger than A, B and C; during subsequent cleavages, it gives rise to progeny that make up the posterior growth zone and thus establishes the second axis of the clitellate embryo.

These processes have been best studied in glossiphoniid leeches (*Helobdella* spp. and *Theromyzon* spp.) and in the tubificid oligochaetes (*Tubifex* spp.). In general, the leech zygote is fertilized internally but arrested at first meiosis until deposition (Fig. 1A). Meiosis resumes after the egg is laid, and two polar bodies are extruded at the animal pole. After meiosis, the female pronucleus migrates toward the male pronucleus at the center of the zygote, on a vertical bundle of microtubules extending vegetally from the animal pole. Also during this period, the zygote undergoes cytoplasmic rearrangements to form pools of yolk-free cytoplasm at the animal and vegetal poles. The two pronuclei fuse close to or after the completion of teloplasm formation (Fernandez et al., 1990).

Before cleavage, the zygote shortens along its A–V axis and lengthens along the future AB–CD axis. This shape change is

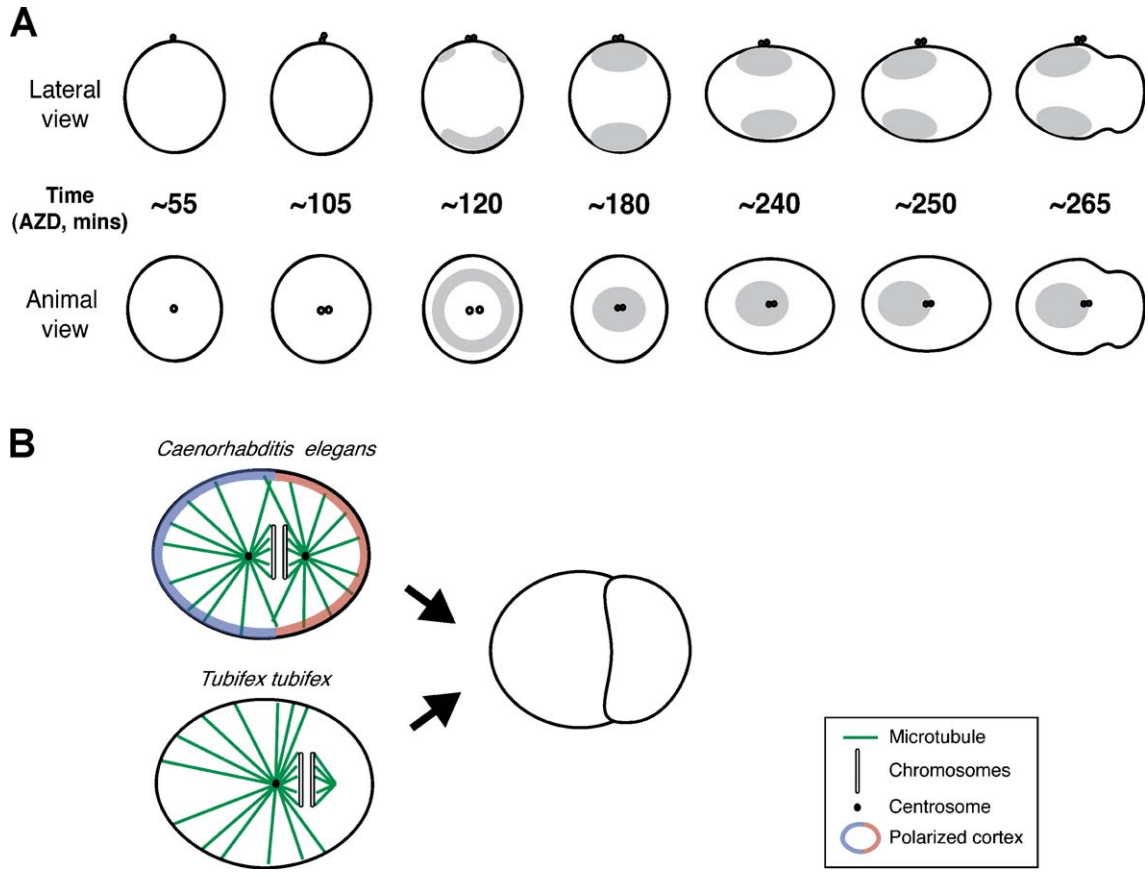



Fig. 1. (A) Timeline of events in the first cell cycle of *H. robusta* (Austin strain; first cleavage takes slightly longer than in the Sacramento strain). Fertilization is internal, but zygotes arrest in meiosis until deposited into cocoons. Thus, developmental events are indicated as occurring at a given time after zygote deposition (AZD). For the experiments presented here, small groups of embryos were synchronized by grouping those that formed a given polar body (small circles) within a 5 min interval. Teloplasm (gray shading) forms at the animal and vegetal poles after polar bodies have been produced. Polar bodies are indicated for reference throughout first cleavage, but, in reality, they are often difficult to detect once teloplasm formation is underway. (B) Mechanistic differences between unequal first cleavage in *C. elegans* and *Tubifex tubifex*. In *C. elegans*, the spindle is clearly biastral and roughly symmetric. Both centrosomes are retained throughout mitosis; the unequal cleavage is thought to result from asymmetric forces acting on the two asters, as a result of a spatially polarized cortex. In *T. tubifex*, the monastral spindle contains just one centrosome and so is highly asymmetric from the time it forms.

accompanied by movements of animal and vegetal teloplasm toward one side of the embryo along the AB–CD axis. Thus, at this point, it is possible to tell which side of the zygote is the prospective CD cell. The cytokinetic furrow forms displaced toward the other end of the zygote, so that the larger CD cell inherits the teloplasm and the smaller AB cell does not. Cells AB and CD differ not only in size and fate in normal development (Whitman, 1878), but also in developmental potential (Symes and Weisblat, 1992). Teloplasm is rich in maternal mRNAs (Holton et al., 1994) including one encoding a *nanos*-related gene (Kang et al., 2002; Pilon and Weisblat, 1997), and has been shown to contain the unknown D quadrant determining factor(s) (Astrow et al., 1987; Nelson and Weisblat, 1992).

One mechanism for producing unequally sized sister cells, best studied in the *Caenorhabditis elegans* zygote, is via a polarized cortex, which might then interact with the astral microtubules to generate asymmetric pulling forces on an otherwise symmetric mitotic spindle (reviewed by Schneider and Bowerman, 2003; Fig. 1B). Alternatively, unequal cleavage can result from an inherently asymmetric spindle. In the *Tubifex* zygote, for example, the paternal centrosome is inactivated, and the maternal centrosome fails to duplicate during first cleavage. As a result, a monastral spindle is formed, and the cleavage plane becomes asymmetrically situated (Ishii and Shimizu, 1997; Fig. 1B). Previous studies involving compression of the *Helobdella* prior to first cleavage provided no evidence for the operation of cortical mechanisms (Nelson and Weisblat, 1992), and the homology of unequal cleavage among clitellate annelids made it seem likely that *Helobdella* would use the same mechanism as in *Tubifex*.

Here, we present the results of a study of spindle dynamics during the first cleavage of *Helobdella*, showing what seems to be a third mechanism for generating an asymmetric mitosis. In contrast to the situation in either *Tubifex* or *Caenorhabditis*, the mitotic spindle in *Helobdella* is initially symmetric and contains two centrosomes. It becomes asymmetric only during metaphase, through a process involving transient down-regulation of gamma-tubulin at the AB spindle pole. We also show that this polarized spindle is necessary for the asymmetry of the first cleavage.

## Materials and methods

### *Animal culture and embryo manipulations*

Laboratory colonies of the leech *Helobdella robusta* were cultured in spring water (1% Instant Ocean) and fed with fresh water snails. Two strains of *H. robusta* were maintained, one originating from Sacramento, CA and the other from Austin, TX. Zygotes of the Austin strain (used for the descriptive studies presented in Figs. 1–5) undergo first cleavage ~10 min later than those of the Sacramento strain (used for the drug studies presented in Figs. 6–11). Embryos were cultured in Htr medium (4.8 mM NaCl, 1.2 mM KCl, 2 mM $MgCl_2$, 8.0 mM $CaCl_2$ 1 mM malic acid, adjusted to pH 6.6 with 1 N KOH) at 23°C. To synchronize stage 1 embryos, zygotes were collected shortly after egg laying and grouped according to the time of emission of the first or second polar body (Fig. 1A). A general description and staging system for *Helobdella* general have been provided previously (reviewed by Weisblat and Huang, 2001). For greater precision in timing developmental events, they are designated as occurring at a particular time (at 23°C) after zygote deposition (AZD), at which point the embryos are released from meiotic arrest.

### *Histochemistry*

Embryos were immersed in a biphasic mixture of MES buffer (200 mM MES, 4 mM $MgCl_2$, 4 mM EDTA, 4 mM EGTA, pH 6.1), heptane and 37% formaldehyde (4:5:1) for 15 min at room temperature with gentle rotation (Mitchison and Sedat, 1983). Fixed embryos were washed briefly in TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4), during which time the vitelline membrane was dissected away with fine pins. The washed devitellinized embryos were then incubated in blocking buffer (10% normal donkey serum, 3% BSA and 1% Triton X100 in TNE) overnight at 4°C and then in primary antibody diluted in block at 4°C for 7 to 10 days. Mouse monoclonal antibody against beta-tubulin (Sigma, T-0198 clone number D66) was used at 1:500; rabbit polyclonal antibody against gamma-tubulin (Sigma, T-3559) was used at 1:2000; mouse monoclonal antibody against histone (Chemicon, MAB052) was used at 1:1000.

Following primary antibody incubation, embryos were washed in block for 6 h at room temperature with frequent solution changes then incubated in fluorescent dye-conjugated secondary antibody, diluted in block at 4°C, again

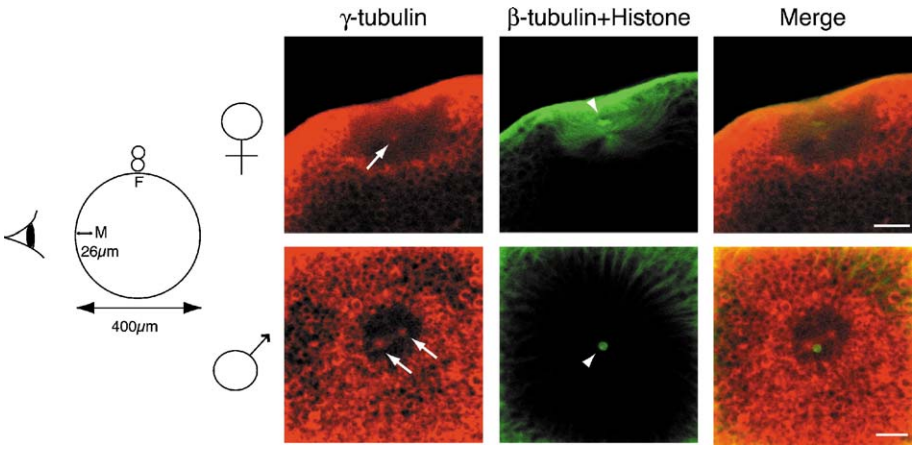



Fig. 2. Pronucleus migration and centrosome duplication. Stacks of 4 confocal images (1 μm steps) showing the vicinity of the female (top row; F in schematic) and male (bottom row; M in schematic) pronuclei of an embryo fixed at 108 min AZD then immunostained for gamma-tubulin (pseudocolored red) to reveal centrosomes (arrows) and histone to reveal nuclei (arrowheads) + beta-tubulin (pseudocolored green). Only one faintly staining centrosome is seen in the vicinity of the female pronucleus, while the paternal centrosomes have already duplicated, though the male has just begun its migration to the center of the zygote (see schematic). Panels at the right show a merge of red and green images. Scale bar, 20 μm.

for 7 to 10 days. $F_{ab}$ fragments of secondary antibodies were used to facilitate penetration. Alexa-dye-conjugated secondary antibodies were used at 1:800 (Molecular probes); Cy3- and Cy5-conjugated antibodies were used at 1:800 (Jackson Immunosciences). After secondary antibody incubation, embryos were washed in TNE-T (1× TNE, 1% Triton X100) buffer for 6 h at RT with frequent solution changes. Embryos were dehydrated through an ethanol series and cleared in 3:2 benzyl benzoate:benzyl alcohol (BBBA) prior to viewing. Immunostained embryos were examined under a laser scanning confocal microscope (Zeiss 510 Axioplan META confocal microscope) and analyzed using the Zeiss LSM software. Measurements were made using Zeiss LSM software.

In some embryos, teloplasm was visualized by acridine orange staining as described previously (Astrow et al., 1987).

### *Drug treatments*

Stock solutions of taxol (Paclitaxel® from *Taxus yannanensis* or *T. brevifolia*; Sigma; 10 mM in DMSO) and nocodazole (10 μM in DMSO) were stored at −20°C. Drugs were diluted in Htr medium immediately before use. Stage 1 embryos were cultured in Htr medium until just after formation of the second polar body, then the medium was replaced with drug-containing solutions. For most experiments, 15 μM taxol and 15 nM nocodazole were used. To ensure that the nocodazole had not degraded, a few embryos in each nocodazole experiment were treated in parallel with 50 nM nocodazole, which inhibits teloplasm formation in *Helobdella* embryos (Astrow et al., 1989). In each experiment, control embryos were treated with 0.2% DMSO in Htr medium.

## Results

### *Spindle dynamics during first cleavage*

The mitotic spindle of the living *Helobdella* zygote is obscured by yolk, so time lapse imaging is not feasible. Therefore, to reconstruct the dynamics of the mitotic spindle during first cleavage, we selected small groups of embryos, synchronized to within 5 min of one another with respect to polar body formation (Fig. 1A). We fixed these synchronized zygotes at selected time points and analyzed the microtubule cytoskeleton immunohistochemically, using a cross-reactive monoclonal antibody raised against mouse beta-tubulin. Similarly, a cross-reactive polyclonal antibody against gamma-tubulin was used to assess the status of the centrosomes during first cleavage (see Materials and methods for details).

Detailed descriptions of cytoskeletal dynamics during the zygote stage of glossiphoniid leeches have been provided for a different glossiphoniid leech genus, *Theromyzon* sp., by Fernandez and Olea (1995) Fernandez et al. (1994, 2004). Events in *Helobdella* follow a similar sequence, albeit with a different time course. Fertilization is internal, but the zygotes arrest in meiosis I until they are deposited into the external cocoons (0 min AZD). By the time the first polar body has formed (55 min AZD), the male pronucleus has begun moving toward the animal–vegetal axis. After the second polar body has formed (105 min AZD), the female pronucleus descends along the animal–vegetal axis, joining the male pronucleus. Immunostaining for gamma-tubulin reveals that the paternal centrosome has duplicated by the time it begins migrating (Fig. 2). At this time, the maternal centrosome has not duplicated and stains only faintly for gamma-tubulin (Fig. 2). At later time points during centration, we were unable to observe the maternal centrosome (data not shown). From these data, we conclude that the monastral array of microtubules observed during the first interphase and prophase (next paragraph) is organized around the duplicated paternal centrosomes, as in most species where this has been examined (Shatten, 1994).

Following polar body formation, cytoplasmic rearrangements lead to teloplasm formation at the animal and vegetal poles of the embryo (120–180 min AZD; Fig. 1A). In parallel, the female pronucleus descends from the animal pole to join the male pronucleus at the center of the zygote. Associated with these processes, and presumably underlying them (Fernandez et al., 1998), is the elaboration of a large monastral structure that extends microtubules throughout the zygote in three domains (Fig. 3; 220 min AZD). One domain consists of radial microtubules extending to the cortex in the equatorial regions of the zygote. The other two consist of polar microtubules extending into the animal and vegetal teloplasm, respectively.

The bipolar mitotic spindle arises during the interval of about 220–240 min AZD by the reorganization of the monaster (Fig. 3), presumably using the paternal centrosomes (Fig. 4). During the early stages of mitosis, through early metaphase (230–240 min AZD), the spindle grows in size but remains symmetric, as judged by the form and intensity of the microtubule immunofluorescence signal and by two quantitative measures, the spindle pole-to-DNA distance and the spindle pole-to-cortex distance (Fig. 5). The centrosomes are also symmetric during this early phase of mitosis, as judged by gamma-tubulin staining (Fig. 4).

Asymmetry in the mitotic spindle becomes evident during metaphase (~240 min AZD). One spindle pole continues to enlarge, extending prominent arrays of astral microtubules to the cortex in equatorial regions and into the teloplasm at the poles (Fig. 3, 250 min AZD). This larger prospective CD aster develops an apparently microtubule-deficient pericentrosomal zone that creates the impression of an aster with a hollow center. During this period, the other spindle pole becomes condensed, losing most of its astral microtubules and its association with teloplasm (Fig. 3, 250 min AZD). The spindle pole not associated with the teloplasm is the prospective AB spindle pole. It does not develop the hollow centered appearance of the prospective CD spindle pole (Fig. 3, 250 min AZD).

The compaction of the AB spindle pole is associated with a reduction in gamma-tubulin staining relative to the CD spindle pole (Fig. 4). Moreover, the disappearance of gamma-tubulin staining precedes the overt polarization of the spindle (Fig. 4), suggesting that the destruction of the centrosome is responsible for the compaction of the future AB spindle pole. Shortly after the compaction of the AB spindle pole, as the cell enters anaphase, gamma-tubulin staining is again observed at the AB spindle pole (Fig. 4, 250 min AZD) and the AB spindle pole aster regrows (Fig. 3, 260 min AZD). The spindle remains asymmetric, however (Fig. 3). The mitotic spindle remains perpendicular to the A–V axis of the zygote throughout mitosis.

The asymmetrization of the mitotic spindle during metaphase is evidenced by the appearance of differences in the spindle pole-to-DNA and spindle pole-to-cortex distances at the two ends of the spindle (Fig. 5). The shift in the position of the

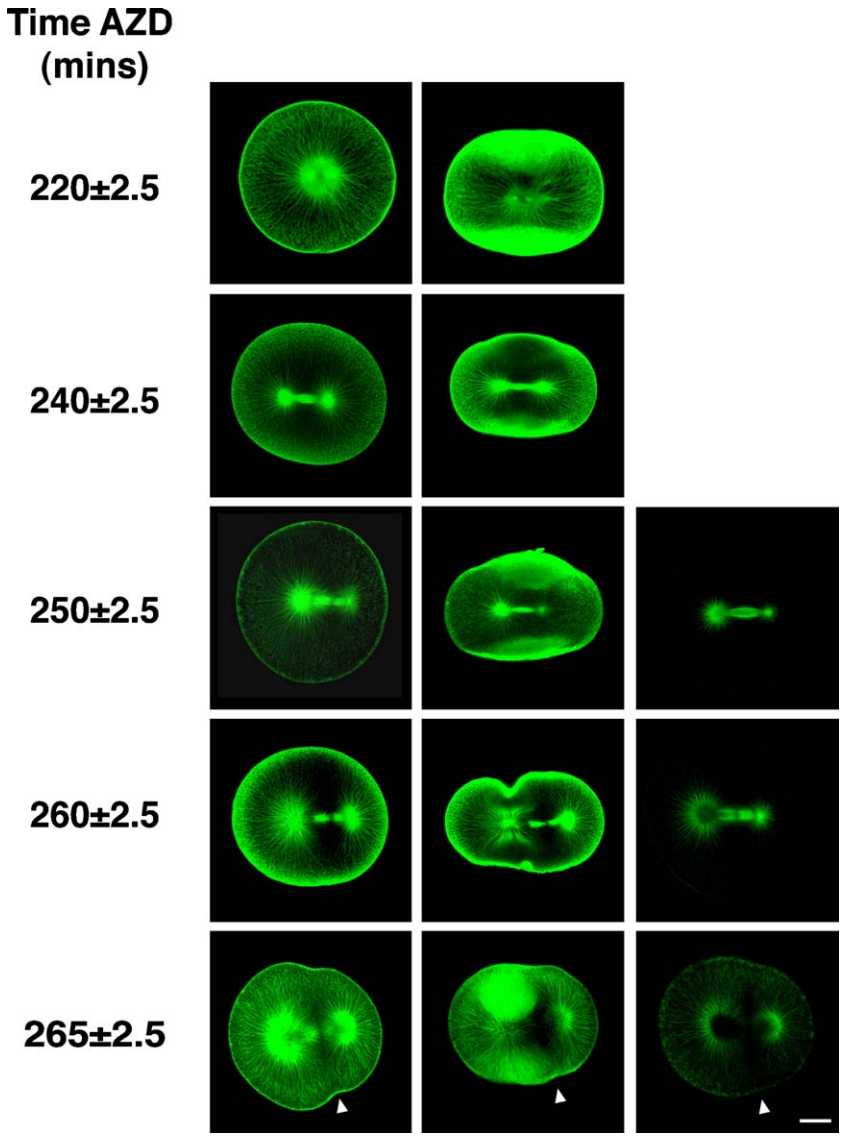



Fig. 3. Spindle aster dynamics during first cleavage. Stacks of 16–20 confocal images (2 μm steps), centered on the aster(s) of embryos fixed and immunostained for beta-tubulin at the indicated time points during first cleavage. Animal views in leftmost column. Lateral views (animal pole, up) in center column (teloplasm at the animal and vegetal poles appears bright through combined tubulin immunoreactivity and autofluorescence). When it is discernable (beginning at 250 min AZD), the prospective AB pole is to the right. Note that the spindle is initially symmetric (240 min AZD) and that the onset of asymmetrization (250 min AZD) is accompanied by a transient partial disassembly of the prospective AB spindle aster, which later reverses (260 min AZD). Brightness of the images in the right and center columns is adjusted for optimum visibility of the astral microtubules. For images in the rightmost column, the brightness is adjusted to show that, by 260 min AZD, the prospective CD spindle pole contains a microtubule-deficient pericentrosomal zone that creates the impression of an aster with a hollow center. The deformation visible in the lateral view of the embryos fixed at 260 min is a processing artifact and not the cleavage furrow, which is evident by 265 min (arrowheads). Scale bar, 100 μm.

spindle relative to the cell cortices is most obvious, but there is also a significant shift in the position of the spindle midbody relative to the poles. Measurements of five metaphase spindles revealed that the kinetochore microtubules emanating from the prospective CD spindle pole are 1.3 times longer than those from the prospective AB spindle pole ($n = 5$; two-tailed $t$ test $P \leq 0.01$).

*Disrupting microtubule dynamics symmetrizes cleavage and the mitotic spindle: taxol*

From the preceding descriptive analysis, it seemed likely that the asymmetrization of the mitotic spindle is required to establish the normal asymmetries of cell size and teloplasm distribution in the first cleavage of *Helobdella*. Moreover, since one aster grows and the other shrinks during asymmetrization, it seemed that disrupting the dynamic instability of the microtubules in either direction might disrupt this process.

To test these ideas, we treated various sets of zygotes with either taxol which stabilizes microtubules (Schiff and Horwitz, 1980) or nocodazole, which binds to free tubulin dimers, thereby favoring microtubule depolymerization (Lee et al., 1980). Either of these drugs blocks mitosis at sufficiently high concentration (Jordan et al., 1992; Jordan et al., 1993), and teloplasm formation in *Helobdella* is also sensitive to nocodazole (Astrow et al., 1989). We tested both drugs at a

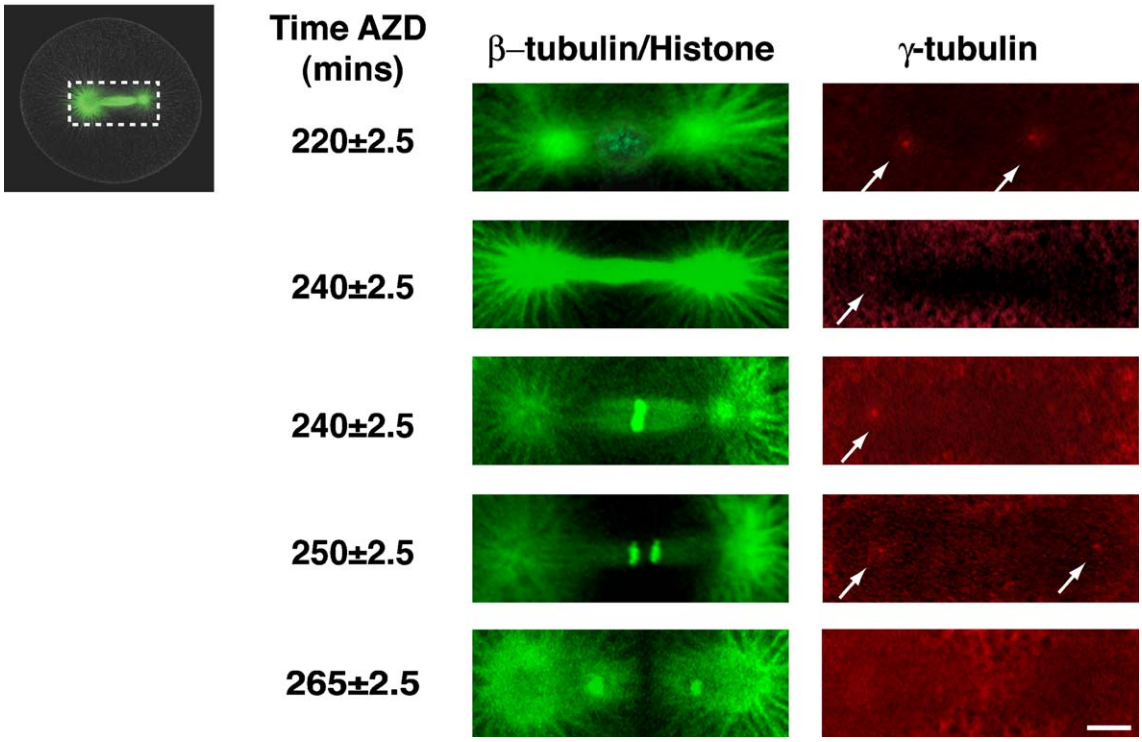



Fig. 4. Centrosome dynamics during first cleavage. Stacks of 3 to 4 confocal images (1 μm steps) from embryos fixed and immunostained for beta-tubulin + histone (pseudocolored green) and gamma-tubulin (pseudocolored red). The images are cropped to the center on the spindle poles, as indicated by the dotted contour in the image at left. Gamma-tubulin-reactive centrosomes (white arrows) are present at both poles when the symmetric spindle first forms (220 min AZD), but, shortly thereafter, one centrosome has lost its gamma-tubulin immunoreactivity, even in zygotes where the spindle is still symmetric (240 min AZD, upper). Due to the 5 min time spread present in each sample, other embryos at this time point have begun to exhibit overall spindle asymmetry (240 min AZD, lower), indicating that down-regulation of one centrosome is quickly followed by down-regulation of the associated aster, corresponding to the prospective AB pole. As anaphase is underway (250 min AZD), two centrosomes are again visible. At 265 min AZD, the centrosomes are no longer detected, presumably because the cell is in telophase. Scale bar, 20 μm.

range of concentrations to find those that would not block mitosis or teloplasm formation, then examined the effects of those concentrations on the symmetry of the first cleavage and the first mitotic spindle.

As expected, the effect of taxol on first cleavage was concentration-dependent. At low concentrations (0.1 to 1.0 μM), cleavage was normal and asymmetric for all embryos. At high concentrations (20 μM), cleavage was meroblastic and slow or arrested; many zygotes formed multiple cytokinetic furrows at this concentration (data not shown).

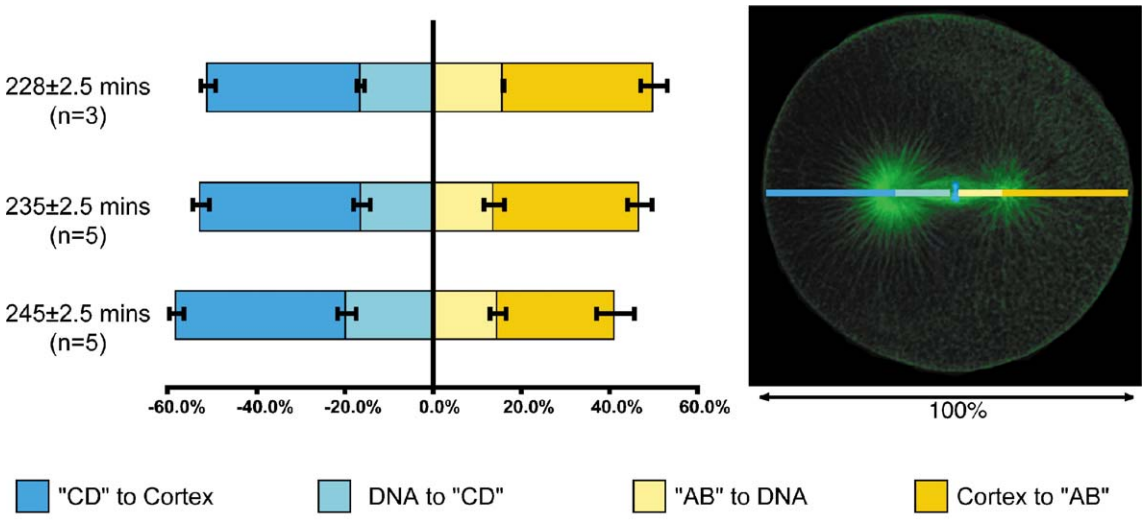
At an intermediate taxol concentration (15 μM), teloplasm formed normally, and all the zygotes cleaved at about the normal time, but 58% (15/26) divided symmetrically (Figs. 6A, B). Moreover, in the equally dividing embryos, teloplasm often failed to segregate and was partitioned into both cells. Embryos treated with DMSO only all divided normally (24/24) (Figs. 6A, B).

Interestingly, taxol treatment did not inhibit teloplasm formation even at the highest concentration tested (20 μM). Teloplasm components are thought be transported on a microtubule network under the cortex of the zygote (Fernandez et al., 2002). The failure of taxol to prevent teloplasm formation suggests that this part of the microtubule network is highly stable and therefore not dependent on the dynamic instability of the microtubule network.

To examine how taxol treatment affected the microtubule cytoskeleton, we visualized the mitotic spindles in treated and control embryos during anaphase. As expected, spindles of DMSO-treated controls were invariably asymmetric at this stage (Fig. 6C), and only one pole of each spindle had its aster embedded in the teloplasm (Fig. 7). But in 63% (14/22) of the taxol-treated embryos, the mitotic spindle at mid-anaphase was much more symmetric, including the “hollowing out” of the pericentrosomal region (Fig. 6C). The spindle pole asters were equal in size and morphology, and both sets of astral microtubules were in contact with the teloplasm (Fig. 7).

During metaphase, the kinetochore microtubules of the two spindle poles exhibited no significant length differences ($n = 5$, two-tailed $t$ test $P \leq 0.26$) in taxol-treated embryos (Fig. 8A). Near the end of cytokinesis, the spindle poles of equally dividing taxol-treated embryos were equal in size (data not shown). In all these respects, both ends of the spindles of taxol-treated zygotes resembled the prospective CD end of the spindle in normal zygotes.

Because fixed embryos were used in these experiments, it cannot be known with certainty whether any of these embryos would have later divided equally. However, the percentage of symmetric mitotic spindles observed in taxol-treated embryos (14/22, 63%) is similar to the percentage of such embryos that underwent equal divisions (15/26, 58%) when allowed to complete mitosis. These results suggest that the polarized spindle is necessary for the asymmetric first cleavage in the leech.




| | Cortex to "CD" | "CD" to DNA | "AB" to DNA | Cortex to "AB" | Cortex to Cortex |
|---|---|---|---|---|---|
| 228±2.5 mins | 100.9±9.4 | 48.9±4.7 | 45.3±1.3 | 99.1±4.8 | 291.0±12.5 |
| 235±2.5 mins | 106.4±2.4 | 48.4±8.6 | 39.6±8.3 | 96.5±6.5 | 292.1±12.9 |
| 245±2.5 mins | 114.8±9.4 | 60.1±14.3 | 41.6±4.9 | 79.4±7.3 | 297.1±18.8 (μm) |

Fig. 5. Spatial dynamics of the first mitotic spindle. Embryos were fixed at selected time points during metaphase, and immunostained for beta-tubulin and histone, then viewed using confocal microscopy. Distance measurements were made as indicated in the color-coded image at right, with the vertical blue bar marking the chromatin. For early metaphase (228 min AZD) when the spindle was symmetric and it was not possible to distinguish the prospective CD pole of the symmetric spindle, the side with the longer kinetochore–spindle distance was treated as the CD pole for each specimen. Even with this bias, the spindle is indeed symmetric in early metaphase, as judged by either the kinetochore–spindle distances or the spindle–cortex distance. By late metaphase (245 min AZD), the prospective AB pole was closer to the cortex and the chromatin had also shifted closer to the AB spindle pole, thereby setting up the zygote for an unequal first cleavage.

*Disrupting microtubule dynamics symmetrizes cleavage and the mitotic spindle: nocodazole*

At high concentrations (greater than 30 nM), nocodazole inhibited teloplasm formation and cleavage, as reported previously for *H. triserialis* (Astrow et al., 1989). However, at low concentrations (15 nM), teloplasm formation and first cleavage occurred, albeit delayed by about 20 min relative to DMSO-treated controls. As with taxol treatment, a substantial fraction of the zygotes cleaved equally when treated with nocodazole (14/22,64%; Figs. 9A, B). Embryos treated with DMSO alone divided normally (30/30, 100%; Figs. 9A, B).

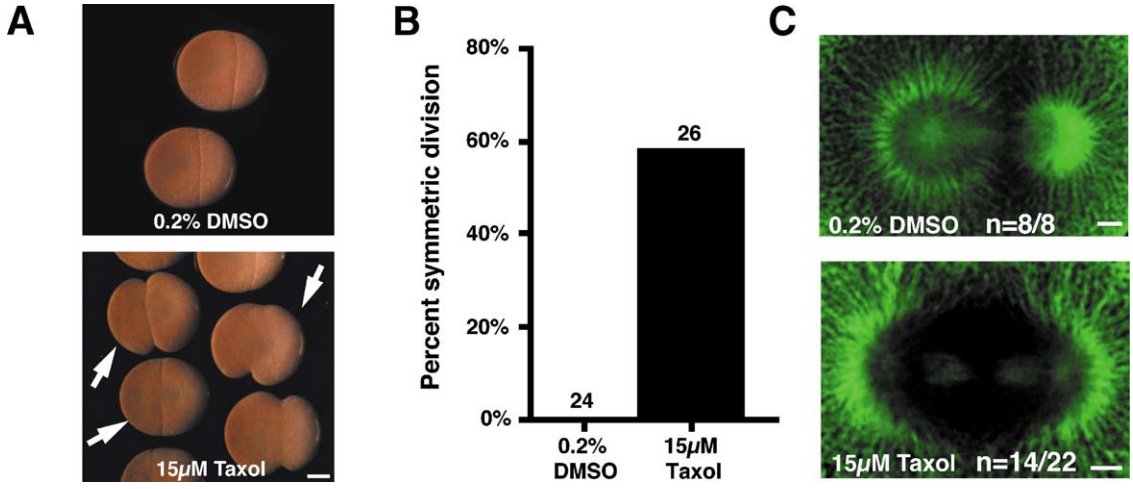



Fig. 6. Low concentrations of taxol symmetrize the spindle and first cleavage. (A) Examples of 2-cell embryos arising from zygotes treated with either 15 μM taxol or solvent (0.2% DMSO). Several embryos underwent equal cleavage (arrows), including partitioning of teloplasm to both daughters. (B) Pooled results from 5 such experiments showed that taxol treatment symmetrized first cleavage in 58% of the embryos. (C) To observe the effects of taxol on the spindle, embryos treated with either taxol or DMSO alone were fixed during anaphase and immunostained for beta-tubulin. While all DMSO-treated specimens showed the expected asymmetry (upper confocal image), more than half of the taxol-treated specimens showed a symmetric spindle (lower confocal image) in which both asters resembled the prospective CD aster of controls. Scale bar, 20 μm.

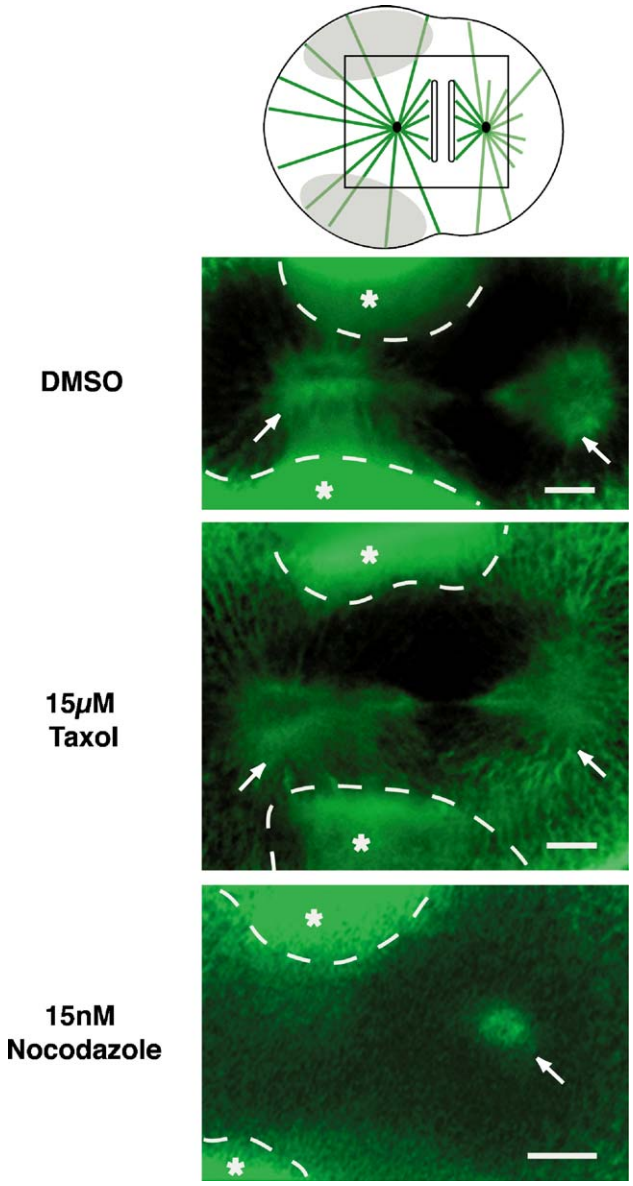



Fig. 7. Interactions between astral microtubules and teloplasm in normal and drug-treated embryos. Control and drug-treated embryos were fixed at anaphase and immunostained for beta-tubulin. Stack of 5 confocal images (2 μm steps) showing a lateral view, as indicated by the boxed area of the schematic. Dotted lines mark the margins of the teloplasm (asterisk). Spindle poles are indicated by arrows. In control embryos (top), the prospective CD aster (at left) made extensive contact with both pools of teloplasm, and the prospective AB aster is displaced from between the pools of teloplasm. In taxol-treated embryos (center), the teloplasm was located more centrally between the two asters and was contacted by both of them. In nocodazole-treated embryos (bottom), astral microtubules are not evident. Moreover, both the teloplasm and the spindle are mis-oriented, so that an image plane containing both pools of teloplasm includes only one eccentrically placed spindle pole. Scale bar, 20 μm.

To examine spindle morphology in nocodazole-treated embryos, we compared them with control embryos fixed 20 min earlier to compensate for the developmental delay resulting from nocodazole treatment. The mitotic spindles of the nocodazole-treated embryos (11/18, 61%) were small, and their astral microtubules did not extend into the teloplasm (Fig. 7), resembling the prospective AB spindle pole aster in control embryos. In nocodazole-treated embryos, the mitotic spindle at mid-anaphase was more symmetrical and smaller than in control embryos (Fig. 9C). At metaphase, the kinetochore microtubule lengths of the two spindle poles were not significantly different in nocodazole-treated embryos (Fig. 8A; $n = 7$, two-tailed $t$ test $P \leq 0.16$).

The percentage of symmetric mitotic spindles observed in nocodazole-treated embryos (11/18, 61%) is similar to the percentage of equal cleavages observed when treated embryos were allowed to complete mitosis (13/22, 60%; Fig. 9B). Near the end of cytokinesis, the spindle asters of equally dividing nocodazole-treated embryos were equal in size (Fig. 9C).

Although both taxol and nocodazole could symmetrize cleavage and the mitotic spindle in the *Helobdella* zygote, there were significant differences in embryos treated with microtubule stabilizing versus destabilizing drugs. First, as described above, the asters of the symmetric spindles in taxol-treated embryos were larger and the centrosome–kinetochore distances

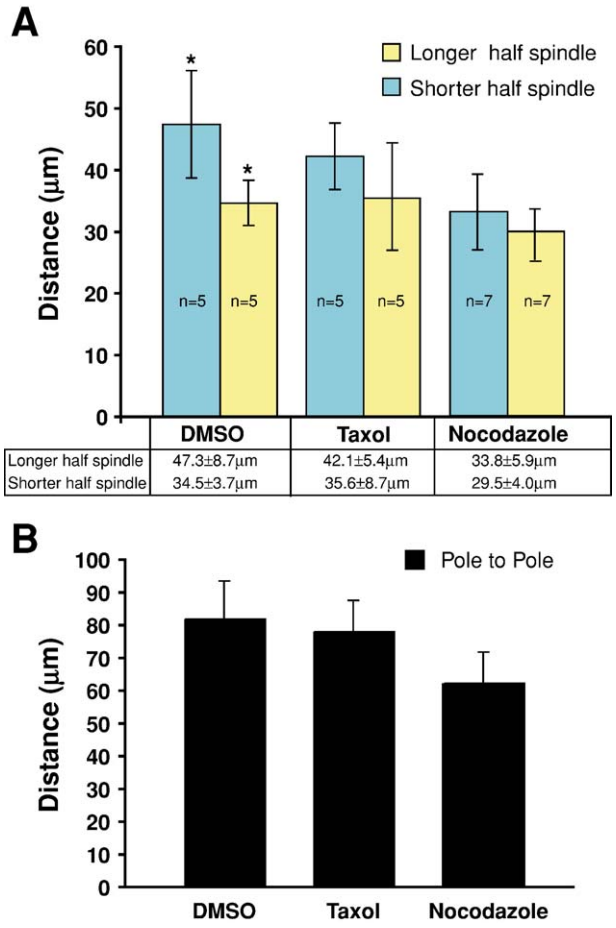



Fig. 8. Low doses of taxol and nocodazole affect metaphase spindle dimensions. Control and drug-treated embryos were fixed at metaphase then immunostained for beta-tubulin + histone. Distances from the spindle pole to chromatin (A) and from spindle pole to spindle pole (B) were measured from confocal images. For the former, the longer distances were pooled for each data set, but only the control (DMSO-treated) embryos showed a statistically significant asymmetry. Nocodazole treatment was also correlated with a decrease in the overall spindle dimensions relative to taxol-treated and control embryos, but this difference was not statistically significant by either measurement.

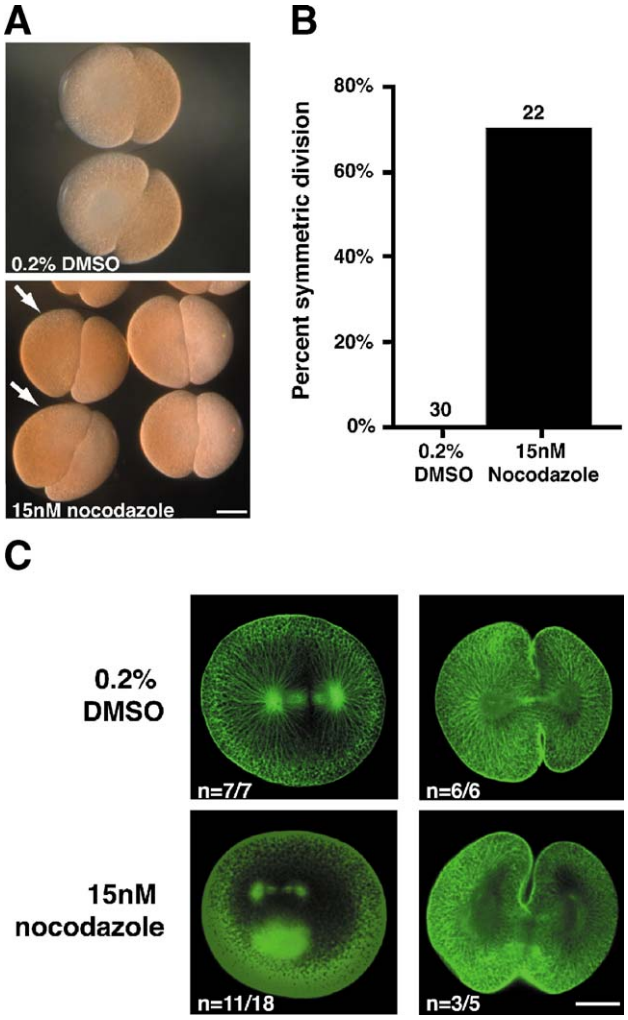



Fig. 9. Low concentrations of nocodazole symmetrize first cleavage. (A) Examples of 2-cell embryos arising from zygotes treated with either 15 nM nocodazole or solvent (0.2% DMSO) during first cleavage. Arrows indicate embryos that had undergone equal cleavage. (B) Pooled results from 6 such experiments showed that taxol treatment symmetrized first cleavage in more than 60% of the embryos. (C) To observe the effects of nocodazole on the spindle, embryos treated either with nocodazole (lower images) or DMSO alone (upper images) were fixed during anaphase (left) or cytokinesis (right). Note the marked reduction in the prominence of microtubules in the nocodazole-treated embryo at anaphase. But, the spindle poles still differ in size and teloplasm is abnormally positioned relative to the spindle. Another feature of nocodazole-treated zygotes is that cytokinesis often proceeds irregularly compared with controls. Scale bar, 100 μm in panels A and C.

were longer than those in nocodazole-treated embryos (Fig. 8B). Correlated with this, when the late mitotic spindle was examined, it was often observed that both spindle poles were in contact with the pools of teloplasm (Fig. 7). [In control embryos, only one spindle pole is associated with the teloplasm (Fig. 7).] Presumably as a result of this, teloplasm in equal cleaving taxol-treated zygotes tended to distribute equally into the two daughter blastomeres (9/16, 62%; Fig. 10).

In contrast, teloplasm tended to segregate more randomly in the 2-cell embryos resulting from nocodazole treatment (Fig. 10). The random distribution of teloplasm after cleavage correlated with variability in the placement of mitotic spindle with respect to the animal and vegetal pools of teloplasm. While in control and taxol-treated embryos, the mitotic spindle axis was invariably perpendicular to the axis passing through the two pools of teloplasm, the mitotic spindle axis in nocodazole-treated embryos was often displaced from the teloplasm axis (i.e., not intersecting it and not necessarily at a right angle to it; Fig. 7). These data suggested that the astral microtubules normally anchor the spindle to the teloplasm within the zygote.

### *Gamma-tubulin dynamics in the first cleavage are not affected by drug treatment*

Given that the mitotic spindle in the *Helobdella* zygote is initially symmetric and then becomes asymmetric during metaphase, how is it determined that one aster grows and the other shrinks? A priori, the differential stability of the prospective AB and CD spindle pole asters could reflect inherent differences, interactions with external factors or stochastic interactions between the spindle poles themselves.

Regarding possible external factors that might affect spindle asymmetry in the zygote, polarization of the cell cortex along the A–P axis has been described in the zygote of *C. elegans* zygote (reviewed in Schneider and Bowerman, 2003). But, previous experiments, involving compression to constrain the orientation of the first division in *Helobdella*, provided no evidence of a pre-determined AB–CD axis (Nelson and Weisblat, 1992). Moreover, no polarization of PAR protein homologs has been observed for the *Helobdella* zygote (Ren, 2005).

In the experiments presented here, we observed that during prophase and early metaphase, both spindle pole asters are associated with teloplasm, and the collapse of the spindle pole aster associated with the prospective AB cell in normally developing embryos was preceded by the loss of gamma-tubulin staining from its centrosome. These observations led us to consider a model for spindle asymmetrization in which factors required to maintain the centrosome are located in the teloplasm and transported to the centrosome along microtubules. According to this model, as the spindle grows in size during prophase and metaphase, one spindle pole aster could be "captured" by the teloplasm, so that its centrosome receives the centrosome stabilizing factor from the teloplasm, while the centrosome of the spindle pole aster that receives less of the teloplasm factor is destabilized, leading to disappearance of the centrosome and collapse of the associated aster. According to this hypothesis, we predicted that stabilizing the teloplasm–microtubule connections by taxol treatment would result in symmetrized spindles in which both centrosomes remain intact. Conversely, depolymerizing microtubules with nocodazole should block the transport of any teloplasm factors and result in symmetrized spindles in which both centrosomes disappear.

To test this hypothesis, we examined gamma-tubulin staining during late metaphase of drug-treated embryos. Taxol and nocodazole do not directly affect gamma-tubulin stability. They might affect gamma-tubulin distribution indirectly by stabilizing or destabilizing microtubules after long treatments (Vorobjev et al., 2000), but given the short incubation times used in

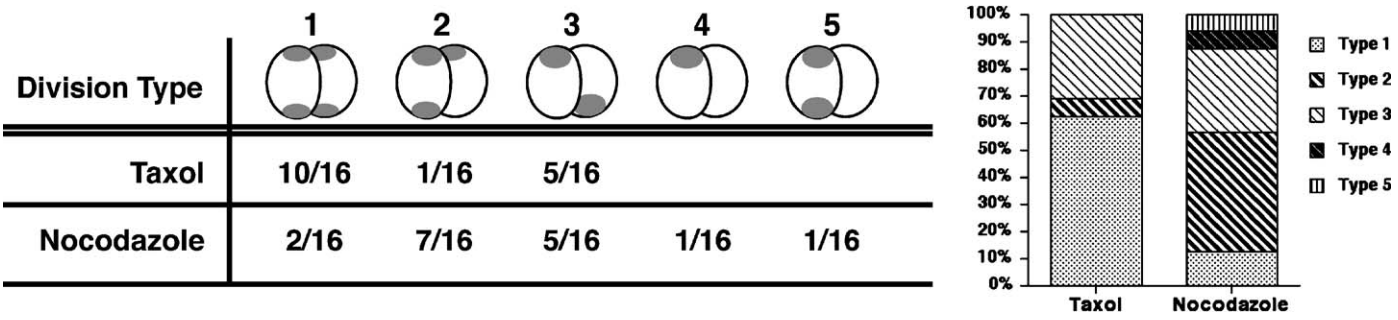



Fig. 10. Low concentrations of taxol and nocodazole affect teloplasm distribution differently at first cleavage. In normal development, both animal and vegetal pools of teloplasm are invariably segregated to the larger CD blastomere at first cleavage (type 5). In contrast, embryos treated with taxol or nocodazole exhibited a variety of aberrant inheritance patterns ranging from equal division of both pools of teloplasm (type 1) to segregation of animal and vegetal pools to different cells (type 3). Most taxol-treated embryos underwent the type 1 pattern, but nocodazole-treated embryos showed a more random distribution of teloplasm inheritance patterns including one case where the two pools of teloplasm appeared to have merged (type 4). This result is consistent with the notion that microtubules are important in maintaining the position of teloplasm relative to the mitotic spindle.

our experiments, no significant changes in gamma-tubulin distribution are expected by this mechanism. However, if microtubule-mediated interactions with teloplasm are required for the stabilization of the centrosomes, then we expected that gamma-tubulin staining of the two centrosomes in nocodazole-treated embryos would be uniform and faint, whereas in taxol-treated embryos the gamma-tubulin staining of centrosomes would be uniform and bright.

No such results were obtained. At late metaphase, when normally the spindle is at its most asymmetric state and only one centrosome is immunoreactive for gamma-tubulin, as expected, only one centrosome was detected in 8 control embryos. Similar results were obtained in drug-treated embryos. Of the symmetric metaphase spindles examined (Fig. 11), 8 nocodazole- and 5 taxol-treated embryos showed only one gamma-tubulin-positive centrosome at the spindle poles. These results suggest that interactions with teloplasm are not necessary for either the maintenance of the CD spindle pole centrosome or the down-regulation of the AB spindle pole centrosome.

## Discussion

### *Spindle dynamics associated with the unequal first cleavage in H. robusta*

Carefully regulated asymmetric cell divisions are a prominent feature of development in various taxa, including spiralians (Whitman, 1878; Wilson, 1892). Classic experiments with sand dollar eggs by Rappaport (1961) showed the cleavage furrow forms where two half spindles interact. More recently, assembly of the central spindle has been shown to be critical for positioning the furrow (Giansanti et al., 2001). Thus, asymmetric positioning of the astral microtubules and/or the asymmetric positioning of the central spindle within the mitotic spindle could contribute to eccentric placement of the cleavage plane (reviewed in Kaltschmidt and Brand, 2002; Kusch et al., 2003).

In the experiments presented here, we have analyzed the dynamics and regulation of the mitotic spindle during the

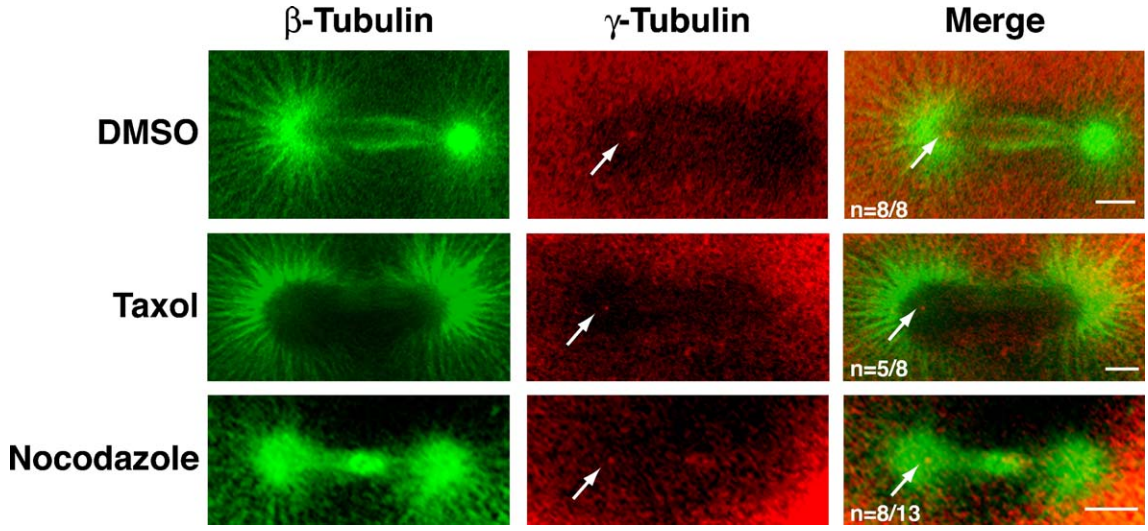



Fig. 11. Down-regulation of the centrosome is not affected by low levels of taxol or nocodazole. Embryos treated with DMSO alone, taxol or nocodazole were fixed in late metaphase then immunostained for beta- and gamma-tubulin. Just one gamma-tubulin-reactive centrosome was detected in each sample (arrows), despite the fact that taxol and nocodazole symmetrized the spindle. Scale bar, 20 μm.

unequal first cleavage of a clitellate annelid, the glossiphoniid leech *H. robusta*. We found that the unequal first cleavage in *Helobdella* is correlated with the asymmetrization, during metaphase, of an initially symmetric mitotic spindle.

There are two aspects to the asymmetrization of this mitotic spindle. One is the down-regulation of the prospective AB centrosome, as judged by gamma-tubulin immunoreactivity, and the subsequent compaction of the AB spindle pole aster. During anaphase, the centrosome at the AB spindle pole recovers. The associated aster regrows but remains smaller than the prospective CD spindle pole aster. The second aspect of the asymmetrization is the eccentric positioning of the spindle midbody, as reflected by a difference in the kinetochore microtubule lengths of the two spindle poles.

From these observations, it appears that, in normal development, the compaction of the prospective AB aster is driven by the down-regulation of the centrosome. This is consistent with results of UV micro-radiation experiments carried out on mammalian cells in culture; when one centrosome was destroyed, the astral microtubules in the associated half-spindle disassembled within 10 min of the destruction (Uzbekov et al., 1995).

This normal chain of events can be circumvented. Low doses of drugs affecting microtubule stability in either direction (taxol and nocodazole) prevent the normal process of spindle asymmetrization and equalize the first cleavage, without affecting the centrosome dynamics. The drug treatments also block the asymmetrization of the kinetochore microtubule lengths.

The results with taxol and nocodazole suggest that a proper balance between microtubule stability and instability is necessary for the normal asymmetrization of the first mitotic spindle and the resultant unequal cleavage. Moreover, the fact that these drugs do not affect the normal centrosome dynamics suggests that the transient down-regulation of one centrosome is not dependent on microtubule-mediated interactions with the teloplasm or the cortex.

How it is that just one of the centrosomes is down-regulated in each zygote remains to be determined. Our results suggest that the centrosomes in the first mitotic spindle in *Helobdella* arise by duplication of the paternal centrosome prior to centration, in contrast to the situation in *Tubifex*, where the maternal centrosome contributes to the monaster (Shimizu, 1996a). Details of centrosome structure and duplication remain unclear (reviewed by Shatten, 1994; Ou and Rattner, 2004). We find no results from other systems suggesting a possible mechanism for asymmetrizing the mitotic centrosomes in the *Helobdella* zygote with no apparent cortical polarization. Nonetheless, it is intriguing to note that, among their various roles in cell cycle regulation, centrosomes are now also thought to play a role in regulating the metaphase-to-anaphase transition (reviewed Doxsey et al., 2005).

In another spiralian, the mollusc *Ilyanassa obsoleta*, Lambert and Nagy (2002) found that intrinsic differences between centrosomes play a role in asymmetric segregation of mRNAs during cleavage; those differences might reflect the positioning of centrosomes relative to the cortex. But, our experiments in which the normal microtubule dynamics were disrupted with taxol or nocodazole suggest that this selection is not mediated exclusively by microtubule contacts with teloplasm or cortex in *Helobdella*. It is also tempting to speculate that transient down-regulation of a paternally derived centrosome in *Helobdella* represents an evolutionary transition from the situation in *Tubifex*, in which the paternal centrosome is absent altogether.

So far as we know, regulation of asymmetric first cleavage by means of the absence (in *Tubifex*) or transient down-regulation (in *Helobdella*) of one centrosome during first cleavage has not been observed outside *Clitellata*. Differences in the structure of centrosome pairs, as judged by gamma-tubulin or centrin immunoreactivity, have been observed in association with various instances of asymmetric cell divisions in other systems but have not been implicated in regulating the asymmetry of those divisions. For example, in *Drosophila* neuroblasts, which undergo asymmetric stem-cell-like divisions, the spindle aster associated with the (larger) neuroblast is more elaborate than the spindle aster associated with the (smaller) ganglion mother cell (Giansanti et al., 2001). Correlated with this difference, the spindle pole of the prospective neuroblast exhibits a brighter gamma-tubulin staining (Giansanti et al., 2001). However, it is unlikely that unequal centrosomes are necessary for this asymmetric division because neuroblasts still divide asymmetrically in a mutant that lacks centrosomes (Giansanti et al., 2001). In the sea urchin embryo, during the asymmetric cleavage that leads to the generation of vegetal micromeres at fourth cleavage, the micromere centrosome contains less centrosomal material than the macromere centrosome and the spindle morphology is also asymmetric (Holy and Schatten, 1991), but there is no evidence for a causal link between them.

### *Divergent mechanisms of unequal cleavage among clitellate annelids*

Our results indicate that the unequal first cleavages of the *Helobdella* and *Tubifex* zygotes are achieved by different mechanisms, since, as described in the Introduction, the first mitotic spindle in *Tubifex* is monastral, with just one maternally derived, gamma-tubulin-positive centrosome and a highly asymmetric mitotic apparatus from the beginning of mitosis (Ishii and Shimizu, 1995; Shimizu, 1996a). Thus, our results taken with Shimizu's elegant studies provide a clear example of a process being evolutionarily conserved at the operational level, despite changes in the underlying mechanism.

The dramatic differences in the asymmetrization processes of *Helobdella* and *Tubifex* are surprising because both are clitellate annelids. *Clitellata* is a monophyletic taxon by both molecular and morphological criteria (Erséus and Källersjö, 2004; Martin, 2001). Teloplasm formation prior to first cleavage and its segregation to the prospective D quadrant by unequal cleavages is clearly ancestral to *Clitellata* (Dohle, 1999). Both these processes are well conserved at the operational level between *Helobdella* and *Tubifex* (reviewed by Shimizu et al., 1998; Weisblat and Huang, 2001). Thus, it might be expected that the sub-cellular mechanisms by which these processes proceed also

would be conserved. But, on the contrary, it has been known for some time that teloplasm formation proceeds by an actin-dependent mechanism in *Tubifex* (Shimizu, 1982) and by a microtubule-dependent mechanism in *Helobdella* (Astrow et al., 1989). Our present results show that these early differences extend to the process by which the unequal first cleavage is achieved.

Given their unambiguous phylogenetic relationship, it is clear that the asymmetrization mechanisms seen in *Tubifex* and *Helobdella* derive from the process operating in an ancestral clitellate. Several questions arising from these findings are as follows.

Do the changes represent evolutionary drift (i.e., the cumulative effects of many neutral changes) or positive selection? Both process generate healthy adults, so it is unlikely that selection could work at that level, but positive selection could be involved in giving a more reliable first cleavage under different ecological conditions in which they live or perhaps in response to differences in the proportion of self- versus cross-fertilization. (All clitellate annelids are hermaphroditic.)

Did the ancestral clitellate generate its unequal first cleavage as in *Helobdella*, as in *Tubifex*, by some combination of the two, or by yet another mechanism? This question can never be answered with absolute certainty, given that the ancestor no longer exists. But, an important related question is that of how frequently the asymmetrization mechanisms have changed within *Clitellata* and where within the clitellate tree those changes have occurred, and the answer to this question could help with the earlier ones as well.

For example, if all groups branching earlier than leeches employ the *Tubifex* mechanism, we might be fairly confident that that is the ancestral mode. Or if asymmetrization mechanisms vary widely and frequently in *Clitellata*, even among species occupying similar ecological and reproductive niches, it would constitute evidence for neutral changes. Obviously, these questions will only be resolved by examining cleavage mechanisms more densely within the clitellate annelids.

### *Comparison with non-clitellate species*

Despite the differences discussed above, the clitellate asymmetrization processes, as exemplified by *Tubifex* and *Helobdella*, are similar in that both make an unequal first cleavage using asymmetric spindles with no evidence for involvement of a polarized cortex (Ishii and Shimizu, 1995; Nelson and Weisblat, 1992; Ren, 2005; Shimizu, 1996b).

In contrast to these results stands what is arguably the best understood example of unequal first cleavage, that of the nematode *C. elegans*. In *C. elegans*, the spindle remains essentially symmetric throughout mitosis, and unequal division involves a positional shift of the spindle within the cell. In *C. elegans*, and presumably in other systems with symmetric spindles, this is accomplished by the reciprocally polarized localization of cortical proteins (Rose and Kemphues, 1998), which leads to differences in the pulling forces between the cortex and the two spindle poles (Grill et al., 2001; Labbe et al., 2004).

Among the spiralians, it has been demonstrated experimentally that the molluscan phylum includes both unequal and equal cleaving (van den Biggelaar, 1996; van den Biggelaar and Guerrier, 1979). Freeman and Lundelius (1992) concluded that equal cleavage is ancestral (but this question should probably be re-examined in the light of possible revisions to the underlying phylogeny based on molecular data). Two unequally cleaving species that have been examined are the surf clam *Spisula solidissima* (Inoue and Dan, 1987) and the zebra mussel *Dreissena polymorpha* (Luetjens and Dorresteijn, 1998). In both these bivalve species, the asymmetrization process resembles that in *C. elegans*, the mitotic spindle remains symmetric but shifts dramatically toward the cortex at one side, apparently due to asymmetric pulling forces acting on microtubules at the cortex.

Among the annelids, various species have been described as equal cleavers on the basis of cell size (Freeman and Lundelius, 1992). However, it is important to note that none of these has been shown by experimental manipulation to contain developmentally equipotent cells at the 2- or 4-cell stage, and Dohle (1999) argued that unequal cleavage is ancestral, at least among annelids. In summary, while no firm conclusion can yet be drawn, current information is consistent with the possibility that the mechanism of polarizing the mitotic spindle by regulating centrosomal stability might be unique to the annelids, having arisen at some point prior to the separation of *Clitellata* from other annelids.

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