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Review

Formation and localization of cytoplasmic domains in leech and ascidian zygotes

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Introduction

Most eggs display an heterogeneous distribution of organelles and cytoskeletal elements established during oogenesis and/or after fertilization and subsequent cell cycles (see Capco, 1995). Heterogeneities are easily visualized in eggs that have microscopically discernible cytoplasmic domains due to the accumulation of organelles and sometimes pigmented vesicles or granules (Fernández, 1980; Sardet *et al.*, 1994). These distinct cytoplasmic domains were first perceived a century ago in eggs of protostomes and deuterostomes, including those of leeches, sea urchins, ascidians, molluscs and insects (Whitman, 1878; Boveri, 1895; Conklin, 1897,1905; Wilson, 1925).

The presence of naturally-marked cytoplasmic regions has allowed cell lineage analysis and the demonstration that cell fate depended on the presence or absence of particular domains. Early observations formed the basis of a theory of "cytoplasmic localization" (Wilson, 1925). It was gradually recognized that visible as well as invisible cytoplasmic regions might contain 'determinants' that caused the cells that inherited these domains to follow different differentiation programs. In the last 2 decades evidence, principally coming from *Drosophila* embryos, has shown that determinants can be heterogeneously distributed macromolecules whose location may be or may not be necessarily coincident with microscopically identifiable structures (Davidson, 1986; St Johnston and Nüsslein-Volhard, 1992; Nüsslein-Volhard, 1996). The best example of a determinant associated with a structure is that of the oskar mRNA in *Drosophila*. As a result of cytoskeletal reorganizations, oskar mRNA is normally present in a distinct cytoplasmic domain known as "germ plasm". Ectopic localization of this mRNA promotes the appearance of germ plasm and functional pole cells in ectopic regions of the blastula (Ephrussi and Lehmann, 1992). Except for germ plasms, which exhibit common structural and biochemical features in egg

Abbreviations used in this paper: AT, animal teloplasm; CAB, centrosome-attacting body; CH, chorlon; CR, contraction ring; CY, cytaster; ER, endoplasmic reticulum; FK, female karyomeres; GS, gray spot; Ins P3, inositol triphosphate; KM, karyomeres; MB, meridional bands of contraction; MF, monaster fibers; MI, first metaphase of meiosis; MO, monaster; MP, male pronucleus; MS, melotic spindle; PB, first polar body; PP, perinuclear plasm domain; PR, polar ring; SC, sperm centrosome; SN, sperm nucleus; VT, vegetal teloplasm; ZN, zygote nucleus.

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from a wide variety of species (electron dense RNA-rich granules immersed in a "nuage" of mitochondria, see Rongo and Lehman, 1996), other recognizable cytoplasmic domains exhibit a widely different structure, composition and localization (Sardet *et al.*, 1994; Capco, 1995). This is reflected in the various names given to them (myoplasm, pole plasm, teloplasm). Yet common mechanisms must be at work in the formation, positioning and maintenance of these domains. Cytoskeletal elements (microtubules, microfilaments and possibly intermediate filaments) have been implicated in the progressive formation, positioning and relocation of these domains with respect to the embryonic axes (Sardet *et al.*, 1994; Fernández and Olea, 1995; Etkin, 1997; Gard *et al.*, 1997). There is also increasing evidence that cytoskeleton-associated motors, and their membrane receptors, are a major factor in the transport, anchorage and possibly in the function of mRNAs and other determinants (Glotzer and Ephrussi, 1996; Bassell and Singer, 1997; Gavis, 1997; Goodson *et al.*, 1997).

Leech and ascidian eggs were among the first cells in which formation of microscopically visible domains were associated with cell fate determination (Whitman, 1878; Conklin, 1905). In this review, we compare the nature of the cytoplasmic domains and the mechanisms responsible for their localizations in leech and ascidian zygotes. We also consider their importance for development. Recent reviews, books and papers have appeared that deal with these and other species (Sardet *et al.*, 1994; Capco, 1995; King, 1996; Pilon and Weisblat, 1997).

Leech and ascidian oocytes, zygotes and embryos

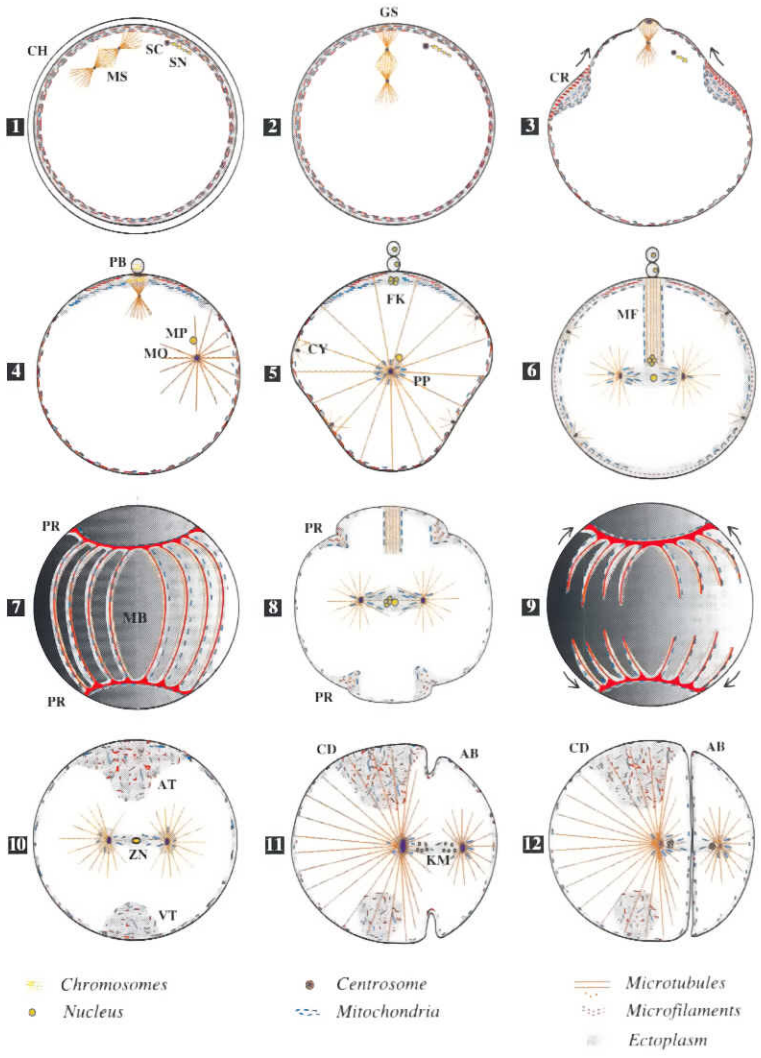
Leeches are protandric annelids that lay cocoons containing from a few to several dozen eggs fertilized in the ovaries. Diameter of laid zygotes ranges from about 100 μm (Hirudinid leeches) to several mm (Glossiphoniid leeches). Some species breed several times a year *(Helobdella)* while others *(Theromyzon)* breed only once in their life and then die. The hirudinid leech *Hirudo medicinalis* and the glossiphoniid leeches *Helobdella robusta*, *Helobdella triserialis* and *Theromyzon rude* have been used for developmental studies at cellular and molecular level.

At the beginning of the breeding season, when leeches are males, the animals copulate. The transmitted sperm is retained inside the body for periods of weeks to months. The animals then become female and produce mature oocytes that are fertilized internally before proceeding to first metaphase (MI) of meiosis. As soon as the zygotes are laid, development is initiated. In some leeches *(Theromyzon)* development is direct while in others *(Hirudo)* it is indirect, giving rise to a cryptolarva. Development to sexual maturity can take weeks or months (Fernández *et al.*, 1992). In the laid zygote one can clearly distinguish an acellular chorion, a thin layer of peripheral cytoplasm deficient in yolk (ectoplasm) and a core of yolk platelet-rich cytoplasm (endoplasm) (see Panel A, Fig. 1). Except for the animal pole region which contains a clear area occupied by the meiotic spindle, and the presence of larger yolk platelets in the vegetal hemisphere, the newly-laid zygote lacks distinct identifiable cytoplasmic domains. A network of microfilaments is present across the entire ectoplasm, whereas microtubules seem to be restricted to the meiotic spindle (Fernández *et al.*, 1987). When the zygote is laid, the nucleus derived from the fertilizing sperm lies at the periphery of the animal hemisphere, often near the animal pole. The sperm-derived centrosome has not yet grown astral microtubules and the screw-shaped nucleus contains condensed chromatin. The naked acrosome and flagellum as well as the single sperm mitochondrion are found nearby (Fernández *et al.*, 1994). In rare cases polyspermy occurs, and sperm nuclei are found at the periphery of the egg in both hemispheres.

Study of the embryology of leeches goes back to Whitman (1878,1887), who was the first investigator to attempt cell lineage analysis using glossiphoniid leech embryos. Embryonic development in leeches was first considered to be essentially determinate and to rely on invariant and independent cell lineages (Mori, 1932). More recently it was shown that development of some cell lines also depends on cell interactions (Blair and Weisblat, 1984; Shankland, 1987) and that indeterminacy and transfating take place under certain experimental conditions (Shankland and Weisblat, 1984; Weisblat and Blair, 1984). Like many protostome embryos, the leech embryo undergoes spiral cleavage. A series of unequal stereotyped divisions results in blastomeres of different size, position, structure and fate. These give rise to precursor cells whose descendants can be unambiguously identified (Fernández and Olea, 1982; Weisblat *et al.*, 1987). A particular feature of leech development is that the ectoderm and mesoderm are produced by a discrete number of stem cells (teloblasts), located at the caudal end of the embryo, that inherit particular cytoplasmic domains (teloplasms) (Fernández, 1980; Fernández and Stent, 1980). These domains, originally located at both the animal and vegetal poles of the zygote, are produced through a series of cytoplasmic rearrangements that will be described and discussed in this review. Another interesting feature of leech embryonic development deals with segmentation. Leeches have a constant number of segments (32) with an additional non-segmented prostomium. The leech embryo is a particularly convenient model for the study of the cellular and molecular events involved in segment determination and organization (Fernández and Stent, 1980; Weisblat *et al.*, 1988,1994; Shankland *et al.*, 1991).

Ascidians are hermaphroditic marine invertebrates (urochordates, tunicates) with a small genome. Most species exhibit indirect embryonic development but a few species develop directly (Satoh, 1994). Most ascidians develop rapidly into simple swimming tadpoles whose organization is typical of the chordate embryo. Within days of hatching, tadpole embryos attach to the substrate and metamorphose into a reproductive sessile adult. Ascidians are deuterostome organisms characterized by stereotypic bilaterally symmetrical cleavage divisions that produce a blastula with a relatively small number of cells in a few hours. The fate of these blastomeres, up to the tadpole and juvenile stages, has been partly or completely traced in *Ciona intestinalis, Phallusia mammillata, Styela partita* and *Halocynthia roretzi* (Zalokar and Sardet, 1984; Venuti and Jeffery, 1989; Nishida, 1992,1996,1997). In addition, study of closely related interhybridizing ascidian species that develop into tadpole larvae with or without tails has yielded novel information about differentiation of embryonic tissues (Swalla and Jeffery, 1990; Swalla *et al.*, 1993).

Ascidian oocytes can be fertilized internally or externally depending on the species. The sperm undergoes an acrosomal reaction as it goes through an acellular chorion lined with follicle cells. This layer can be chemically removed to study fertilization and embryonic development (Zalokar and Sardet, 1984). Oocytes, ranging in diameter from about 150 μm *(Ciona, Phallusia, Styela)* to 300 μm *(Halocynthia)*, can be obtained in large quantities in appropriate seasons or throughout the year. They are usually arrested in MI of meiosis ready to be fertilized.



Panel A: Leech. Figs. 1-12.

Cytoplasmic reorganizations in the leech *Theromyzon rude* from oviposition to first cleavage. *With the exception of Figures 7 and 9, each figure represents a meridional section of a zygote. In all figures the animal pole of the zygote is up. Figures 1-3 show zygotes in the first meiotic cell cycle, figures 4 and 5 zygotes in the second meiotic cell cycle, figure 6 a zygote terminating early first interphase, figures 7 and 8 zygotes in mid-interphase, figures 9 and 10 zygotes in late first interphase and figures 11 and 12 cleaving zygotes. Times of development at room temperature for these different stages are indicated below.* **1.** *Structure of the zygote at the time of laying. The layer of peripheral cytoplasm (ectoplasm) contains numerous microfilaments as well as organelles (represented here by mitochondria). CH, chorion; MS, meiotic spindle; SC, sperm centrosome; SN, sperm nucleus.* **2.** *Formation of the gray spot (about 0.5 h of development). The developing spindle has rotated about 90° and its peripheral pole, tethered to the future animal pole, forms the gray spot (GS).* **3.** *Contraction ring during meiosis I (about 1.5 h of development). The contraction ring (CR), moving towards the animal pole (arrows), is composed of numerous actin filaments as well as organelle-rich ectoplasm.* **4.** *Monaster formation and establishment of an animal pole cytoplasmic domain (about 2 h of development). The first polar body (PB) has been released and the sperm centrosome originated the nascent monaster (MO), that appears associated with the male pronucleus (MP). The ectoplasm formed a cup-shaped cytoplasmic domain in the animal pole region.* **5.** *Monaster centration, formation of the perinuclear plasm domain and release of the second polar body (about 2.5 h of development). The sperm centrosome and male pronucleus have reached the center of the zygote and the monaster microtubule bundles now extend throughout the internal cytoplasm (endoplasm). CY, cytasters; FK, female karyomeres; PP, perinuclear plasm domain.* **6.** *Formation of the amphidiaster, centration of the female karyomeres and thickening of the ectoplasmic layer (about 3 h of development). The perinuclear plasm domain, that encloses the amphidiaster, has grown into a disk-shaped structure in the center of the zygote. The decondensing female karyomeres, together with organelle-rich cytoplasm, have migrated from the animal pole along a subset of monaster fibers (MF). Note that the thickened ectoplasm includes numerous organelles and cytasters.* **7.** *Surface view of a zygote at the peak of furrowing (about 3.5 h of development). The zygote adopts a pumpkin-like appearance due to formation of annular furrows (polar rings, PR) and meridional bands of contraction (MB). This is the most common furrowing pattern detected during mid interphase. Organelle-rich ectoplasm, actin microfilaments and microtubules appear concentrated at the wall of the furrows. The discontinuous line marks the animal/vegetal axis and corresponds to the plane of the meridional section displayed in Figure 8.* **8.** *Accumulation of ectoplasm at the wall of the polar rings and meridional bands of contraction. This drawing illustrates the internal structure of the zygote in Figure 7 viewed in a meridional section not passing through the tips of the furrows. Notice that the female karyomeres have completed their centration and lie adjacent to the male pronucleus at the center of the perinuclear plasm domain. Meanwhile, the subset of microtubules associated with the migration of the female karyomeres have started shortening towards the animal pole. PR, polar rings.* **9.** *Surface view of a zygote showing poleward displacement of the polar rings and "shortening" of the meridional furrows (about 4 h of development). This modification of the furrowing pattern prior to mitosis (arrows indicate the poleward displacement of the rings and bands of contraction) is accompanied by bipolar displacement of organelle-rich ectoplasm, actin microfilaments and microtubules.* **10.** *Completion of teloplasm formation (about 4.5 h of development). Furrowing has ceased and cytoplasm enriched in organelles and cytoskeletal elements accumulate at the poles of the zygote to form the animal (AT) and vegetal (VT) teloplasms. The zygote nucleus (ZN) has formed at the center of the dumbbell-shaped perinuclear plasm domain.* **11.** *First cleavage division (about 5 h of development). The mitotic spindle has a large aster located in the CD sector and a small aster located in the AB sector of the zygote. Astral microtubules penetrate into the substance of the teloplasms and reach the zygote cortex. Karyomeres (KM) formed at the end of the first cleavage division appear close to the poles of the cleavage spindle.* **12.** *The first two blastomeres (about 6 h of development). The cleavage furrow has divided the zygote unequally and the large CD blastomere has inherited most of the animal and vegetal teloplasms and the large aster. The enlarged perinuclear plasm (compare with Fig. 5), however, has divided symmetrically and thus the two blastomeres inherit similar amounts of this cytoplasmic domain.*

Experimental embryology of ascidians, dating back to the days of Chabry (1887) and Conklin (1905), showed that precursor blastomeres of muscle cell lineages contained cytoplasmic domains, or plasms (myoplasm), inherited from particular regions of the zygote. These domains are established before or after fertilization as a result of two main episodes of cytoplasmic and cortical reorganization (ooplasmic segregation), which are described and discussed in this review. In recent years these morphogenetic movements, and the developmental potential of parts of zygotes and embryos, have been analyzed rigorously (Bates and Jeffery, 1987; Nishida, 1994,1996,1997; Sardet *et al.*, 1994; Yamada and Nishida, 1996). These studies, complemented with the analysis of

early expression of certain genes (including homologs of vertebrate genes), have revealed that the differentiation of some cell types (such as primary muscle cells) is highly determinate while that of other cells (such as notochord cells) relies on cell interactions (Satoh *et al.*, 1996; Corbo *et al.*, 1997). The composition, cleavage pattern and lineage of all the cells in the early embryo are now known in detail, reinforcing the potential of the ascidian embryo as a model to understand early ontogeny of deuterostomes at a molecular, cellular and physiological level (Nishida, 1997).

Formation and localization of cytoplasmic domains

Fertilization and meiotic events

In the leech, meiosis is initiated in the ovary and is completed 150 to 180 min after egg laying (Panel A, Figs. 1-5). Although the sperm has already penetrated the egg when it is laid, the zygote stored in the leech body remains arrested in MI of meiosis. The zygote only resumes meiosis once out of the body (Fernández and Olea, 1982). The first detectable sign that meiosis has resumed is the reorientation of the meiotic spindle, such that its long axis becomes perpendicular to the surface of the animal pole. Meanwhile, the peripheral pole of the meiotic spindle together with its astral microtubules have approached the zygote surface where they form the gray spot (a lighter area that will constitute the animal pole) (Panel A, Figs. 1 and 2). This phenomenon is blocked by colchicine and hence depends on microtubules (Fernández *et al.*, 1990). The gray spot becomes detectable about 30 min after zygote laying (Fernández, 1980). An hour later a cell constriction, the contraction ring, appears at the equator and progresses toward the animal pole at a speed of about 30 μm/min (Panel A, Fig. 3). Concomitantly, the animal pole surface begins to bulge up in anticipation of first polar body formation. Formation and displacement of the contraction ring is inhibited by cytochalasin B, indicating that these processes involve actin filaments. Examination of the contraction ring reveals that it contains not only a high concentration of actin filaments, but also microvilli, granular material, vesicles and numerous mitochondria as well as other organelles, all of which move towards the animal pole. Hence, poleward translation of the meiotic contraction ring is coupled to both emission of the first polar body and segregation of plasmalemmal domains, the actin cytoskeleton and organelles. This is the first episode of ooplasmic segregation in the leech zygote (Fernández *et al.*, 1990). The cytoplasmic domain thus created appears as a distinct disk-shaped region beneath the animal pole (Panel A, Fig. 4).

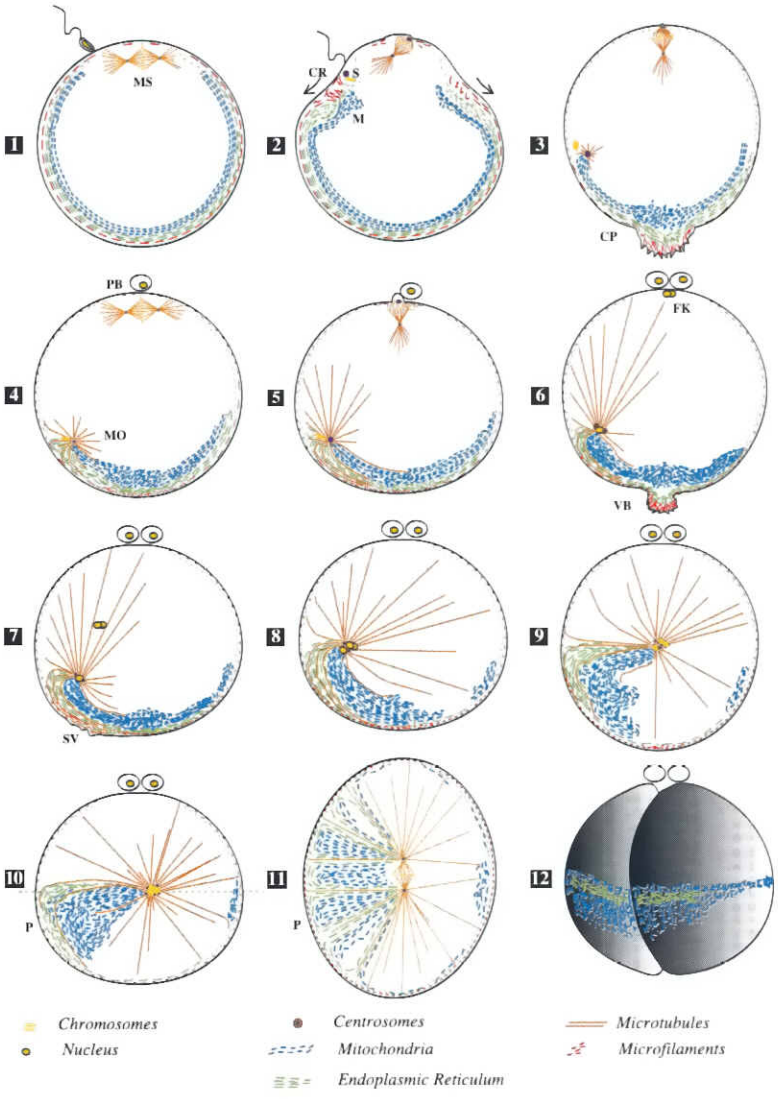
Ascidian eggs, which are also generally arrested in MI phase of meiosis at the time of fertilization *(Halocynthia, Styela, Ciona, Phallusia)*, also undergo a characteristic cortical contraction which is dependent on actin filaments (Jeffery and Meier, 1983,1984; Sawada and Osanai, 1985; Sardet *et al.*, 1989; Roegiers *et al.*, 1995). It is the fertilizing sperm, that preferentially penetrates the egg in the animal hemisphere, what triggers the cortical contraction (Panel B, Figs.1 and 2). This contraction starts close to the site of sperm entry and progresses towards the vegetal hemisphere at a speed of about 80 μm/min, forming a characteristic microfilament and microvilli-rich protrusion called the contraction pole (Panel B, Fig. 3). The progress of the cortical contraction in ascidian zygotes clearly depends on a calcium wave that traverses the cell at a speed of 5 μm/sec from the sperm entry site (Speksnijder *et al.*, 1990; McDougall and Sardet, 1995; Roegiers *et al.*, 1995). In ascidians, this first phase of ooplasmic segregation leads to the concentration of a subcortical mitochondria-rich region (the myoplasm) in the vegetal hemisphere. The myoplasm is a thin subcortical layer that forms during oocyte maturation (Swalla *et al.*, 1991). It is readily observed in living eggs of some species *(Styela)* because it also contains naturally pigmented (yellow/orange) vesicles (Conklin, 1905; Jeffery and Meier, 1983) and is best visualized using fluorescent dyes that accumulate in mitochondria (Sardet *et al.*, 1989). The cortical contraction moves the introduced sperm nucleus and centrosome vegetally (Speksnijder *et al.*, 1989). It also creates a new cytoplasmic domain sandwiched between the myoplasm and the microfilamentous cortex and microvilli-rich surface of the contraction pole (Speksnijder *et al.*, 1993). This new cytoplasmic domain consists of numerous sheets and tubes of endoplasmic reticulum (ER) (Panel B, Figs. 2 and 3). In both ascidian and leech zygotes the end result of these microfilament-driven cortical contractions is the redistribution of cell components such as mitochondria, cytoskeletal elements and probably many associated organelles with respect to the future position of the embryonic body axes. In leeches the contraction moves symmetrically, with respect to the animal/vegetal axis, towards the animal pole whereas in ascidians the contraction moves vegetally but may be asymmetrical with respect to the preexisting animal-vegetal axis (Sawada and Osanai, 1985; Sardet *et al.*, 1989; Jeffery, 1995; Roegiers *et al.*, 1995).

When the first polar body is emitted in the leech zygote (after about 90 min of development), small cytasters with short microtubules are already detected in the ectoplasm. Cytasters are small microtubule organizing centers also found in the cytoplasm of zygotes from other species (see Harris *et al.*, 1980; Maro *et al.*, 1988; Fernández *et al.*, 1994). Shortly after emission of the first polar body, bundles of long microtubules (astral fibers) grow from around the centrosome provided by the fertilizing sperm (Panel A, Fig. 4). As the astral fibers grow longer, the centrosome together with the sperm nucleus, acrosome, mitochondrion and flagellum move toward the center of the zygote (Panel A, Figs. 4 and 5). A large monaster develops while the spiral-shaped nucleus unwinds and its chromatin begins to decondense (Fernández *et al.*, 1994). After 150-180 min of development, a transient deformation of the vegetal pole accompanies the emission of the second polar body (Fernández *et al.*, 1987). This is followed by formation of karyomeres, enclosing the female post-meiotic chromosomes, in the thickened ectoplasm underlying the animal pole (Panel A, Fig. 5) (Fernández and Olea, 1995).

By comparison, completion of meiosis in ascidians is characterized by periodic contraction-relaxations of the zygote surface in synchrony with periodic calcium waves. These waves are initiated in the vegetal pole area from the ER-rich domain of the zygote and propagate cortically towards the animal pole (Speksnijder *et al.*, 1990; McDougall and Sardet, 1995). The monaster generated around the sperm-derived centrosome grows slowly in the zygote periphery between the first and second meiotic metaphase (Panel B, Figs. 4,5 and 6). The zygote then rounds up and the second polar body is emitted. Depending on the ascidian species being considered, this process is completed after 20 to 40 min of development (Sawada and Schatten, 1988; Sardet *et al.*, 1989). At the end of meiosis, a large subcortical monaster is found in the vegetal hemisphere of the zygote where the sperm centrosome has been dragged by the contraction wave (Sardet *et al.*, 1989; Roegiers *et al.*, 1995). As the astral rays reach the animal pole, the female chromosomes are enclosed in karyomeres and a nuclear envelope forms around the decondensing sperm chromatin. The female

Panel B: Ascidian. Figs. 1-12.

Cytoplasmic reorganizations between fertilization and first cleavage division in the zygote of the ascidian *Phallusia mammillata*. *All figures are meridional sections of the egg along the animal/vegetal axis except for Figure 11 (equatorial section of an egg in mitosis) and Figure 12 (oblique surface view of a cleaving egg seen from the posterior pole). Only cortical and subcortical cytoplasmic domains and large cytoplasmic structures (asters, spindles, nuclei) are represented.* **1.** *Egg at the time of fertilization. A cortical basket of actin microfilaments, sheets and tubes of ER is sandwiched between the plasma membrane and a mitochondria-rich domain called the myoplasm (this domain also contains vesicles and a low density of ER which are not indicated). The meiotic spindle (MS) of the egg arrested in metaphase of the first meiotic cell cycle, lies at the periphery and parallel to the egg surface. The basket of actin microfilaments opens towards the animal pole.* **2.** *Sperm penetration. The sperm penetrates preferentially in the animal hemisphere, introducing its centrosome and nucleus (S). The entering sperm triggers a calcium wave which causes the cortical microfilament basket to contract. The contraction ring (CR) propagates vegetally (arrows) dragging with it the mitochondria-rich myoplasm (M) as well as the sperm nucleus (S) and adjoining centrosome.* **3.** *Formation of the contraction pole (5 min after fertilization). A contraction pole (CP) forms in the vegetal hemisphere. It is characterized by numerous microvilli, actin microfilaments and abundant sheets and tubes of ER. The sperm chromosomes and centrosome have moved vegetally with the contraction wave.* **4.** *Completion of the first meiotic cell cycle (10 min after fertilization). The first polar body (PB) has been emitted. The contraction pole has resorbed but an accumulation of cortical ER persists in the vegetal hemisphere. A monaster (MO) develops around the introduced sperm centrosome.* **5.** *Oscillation period during meiosis (10-20 min after fertilization). The zygote exhibits periodical contractions and relaxations as 10 to 20 calcium waves propagate rhythmically from the site of the vegetally-localized contraction pole. Monaster microtubules grow in length .* **6.** *Completion of the second meiotic cell cycle (20-25 min after fertilization). Completion of meiosis is characterized by emission of the second polar body and formation of the female karyomeres (FK). The monaster begins to grow rapidly throughout the interior of the zygote and the centrosome duplicates. A protrusion called the vegetal button (VB) containing microvilli, microfilaments and ER arises in the region where the contraction pole formed previously.*



7. *Folding of myoplasm and female karyomere migration (30 min after fertilization). The vegetal button has resorbed and the myoplasm starts folding. The female karyomeres are seen migrating toward the center of the monaster. Surface vibrations (SV) occur near the ER-rich cortical domain.* **8.** *Meeting of the pronuclei and early migration of monaster (35 min after fertilization). The male and female pronuclei meet each other and together with the disc-shaped centrosome and aster start migrating toward the center of the zygote. This drags myoplasm and accumulated ER in the same direction. The cup-shaped myoplasm tears.* **9.** *Centration of the monaster and cytoplasmic domains (45 min after fertilization). The aster together with the accompanying cytoplasmic domains move towards the center of the zygote. Breakdown of the pronuclear envelope occurs during this movement.* **10.** *Syngamy and posterior displacement of cytoplasmic domains (45 min after fertilization). Cortical deformation movements, arising from the vegetal pole region, participate in the equatorial displacement of the bulk of the myoplasm and the ER-rich domain and in their localization at the future posterior pole (P) of the embryo. A small portion of myoplasm remains at the future anterior pole of the zygote.* **11.** *Mitosis and first cleavage division (Equatorial section of the zygote 45 min after fertilization). The plane of section corresponds to the dashed line of Figure 10. Cytoplasmic domains rich in mitochondria are enclosed in microtubule-rich/ER-rich domains. The cleavage spindle is symmetrical.* **12.** *Oblique surface view of the posterior sector of the zygote after completion of the first cleavage division (50 min after fertilization). The bulk of the myoplasm and ER-rich domains are represented. The cleavage furrow has partitioned these domains equally between the first two blastomeres.*

karyomeres then move along the astral rays towards the centrosome and sperm pronucleus (Panel B, Figs. 6 and 7).

In both leech and ascidian zygotes a large number of organelles gather around the sperm centrosome and derived monaster. In leeches, this accumulation constitutes the so-called "perinuclear plasm" domain (Panel A, Fig. 5). Drug studies have implicated microtubules in the centripetal accumulation of organelles around the leech centrosome to form this domain. Interestingly, active gathering and proliferation of mitochondria are both involved in the growth of the perinuclear plasm (Fernández *et al.*, 1994). In *ascidians* the sperm monaster gathers ER and a wedge-shaped domain of the mitochondria-rich myoplasm (see Panel B, Fig. 6).

It should be noted that in the ascidians *Phallusia* and *Ciona* a small microfilament-and-ER-rich protrusion, the "vegetal button", forms transiently at this time at the site where the contraction pole first formed. The vegetal button appears at the time the second polar body forms and subsides about 10 min later (Panel B, Fig. 6) (Sardet *et al.*, 1989; Roegiers *et al.*, 1995).

Pronuclear movements and interphase events

After the completion of meiosis in the leech, the female karyomeres coalesce and together with the surrounding organelle-rich cytoplasm start moving centripetally toward the zygote center (Panel A, Fig. 6). This movement occurs along a subset of monaster microtubules that link the animal pole to the central perinuclear plasm domain. These animal pole to center microtubules are more stable than the other astral microtubules and persist for a longer time. As the karyomeres move along these microtubules towards the zygote center, they start fusing and their chromatin decondenses. The fusing karyomeres enter the enlarged perinuclear plasm domain and reach the male pronucleus (Panel A, Fig. 8). Following completion of chromatin decondensation the female pronucleus forms besides the male pronucleus (Fernández and Olea, 1995).

In ascidians, the female karyomeres also migrate toward the center of the sperm monaster which in this case is situated in an asymmetric position. A wedge of the mitochondria-rich myoplasmic domain occupies about a quarter of the monaster near the zygote cortex and is closely apposed to the envelope of the male pronucleus (Panel B, Fig. 7). In both leech and ascidian zygotes, the paternally-derived centrosome duplicates at the time the female karyomeres start moving along the astral rays (early during the first interphase). In this manner, a disk-shaped perinuclear plasm domain houses the amphidiaster in the leech zygote (Panel A, Figs. 6 and 8). In ascidians, the amphidiaster with its disc-shaped centrosomes together with the male and female pronuclei, and the cytoplasmic domains embedded in the aster, migrate to the zygote center (Panel B, Figs. 7 and 8). The translocation of the mitochondria-rich myoplasm and adjoining ER-rich domain are coordinated. They move in 2 characteristic phases, first in a slow phase (6 μm/min) and then in a fast and smoother phase (25 μm/min) (Sardet *et al.*, 1989). These movements depend mostly on microtubules. They result in that the cup-shaped myoplasm first buckles and then ruptures (Panel B, Figs. 7 and 8). The bulk of the mitochondria-rich myoplasmic domain and the vegetally situated ER-rich domain move along the cortex towards the equatorial and central region of the zygote (Panel B, Fig. 9). They form the so-called "yellow crescent" in naturally pigmented species (Conklin, 1905; Jeffery and Meier, 1983). The ultimate position of this crescent depends on the location of the sperm aster at the end of meiosis and corresponds to the future posterior pole of the ascidian embryo (Sardet *et al.*, 1989; Speksnijder *et al.*, 1993; Jeffery, 1995).

In the leech zygote the ectoplasm thickens considerably during early first interphase (Panel A, Fig. 6). This process relies on the centrifugal recruitment of organelles, from the neighboring endoplasm, as well as on the proliferation of mitochondria in the ectoplasm itself. Drug treatment shows that translocation of organelles to the periphery of the egg is a microtubule-based process (Fernández *et al.*, 1998). Once the ectoplasm has thickened, intense furrowing activity leads to the formation of 2 contraction rings (polar rings) lying towards the poles of the zygote. A dozen meridional furrows linking the rings then appear as the zygote enters mid interphase (about 3 h of development) (Panel A, Figs. 7 and 8) (Fernández *et al.*, 1987). When meridional furrows form, organelles move in the plane of the ectoplasm and accumulate within the walls of the rings and bands of contraction. Actin filaments have been implicated in these processes (Fernández *et al.*, 1998). The poleward movement of the contraction rings and meridional bands of contraction, during late first interphase (about 4 h of development), is accompanied by a massive bipolar translocation of organelles and cytoskeletal elements (Panel A, Fig. 9). This leads to the accumulation of organelles, microtubules and actin filaments at both zygote poles and the establishment of the new cytoplasmic domains called teloplasms (Panel A, Fig. 10) (Fernández and Olea, 1995). Drug treatment indicates that these movements towards the poles depend on both microtubules and actin filaments. Teloplasm formation, like the previous expansion of the perinuclear plasm domain, involves both recruitment and proliferation of mitochondria (Fernández *et al.*, 1998). The teloplasms are cytoplasmic domains destined to be inherited by large caudally-situated embryonic stem cells called teloblasts. The teloplasm of these cells is utilized in the manufacture of the ectodermal and mesodermal founder cells that will give rise to the paired germinal bands of the embryo (Fernández, 1980; Fernández and Stent, 1980).

Mitotic events and the partitioning of cytoplasmic domains

In ascidians the first cleavage spindle is small and situated at the center of the zygote. Each spindle pole is associated with a large aster whose microtubules often reach the zygote surface. The cleavage plane bisects the zygote generating 2 identical blastomeres in which the cytoplasmic domains exhibit mirror image distributions (Panel B, Figs. 11 and 12). During the subsequent cleavage divisions, the domains are progressively allocated to different blastomeres with different cell fates (Venuti and Jeffery, 1989; Nishida, 1992,1997; Satoh, 1994 ; Satoh *et al.*, 1996).

In leeches the first cleavage spindle is huge and asymmetric, with one spindle pole (corresponding to the CD sector of the zygote) much larger than the other (corresponding to the AB sector of the zygote). The asymmetric positioning of the spindle is such that the cleavage furrow divides the zygote unequally (Panel A, Figs. 11 and 12). As a consequence, the larger CD blastomere frequently inherits the entire animal and vegetal teloplasms (Fernández *et al.*, 1987).

Mechanisms and perspectives

Fertilization and early meiotic events

In ascidians it is clear that a first phase of cytoplasmic reorganization, lasting about 5 min, is triggered by a cortical contraction. The zygote cortical microfilament basket, with its opening at the animal pole, contracts in response to the intracellular release of calcium caused by the fertilizing sperm.

The sequence of events leading to this first phase of cytoplasmic reorganization is likely to be the following: 1) transmission of an activation signal to the egg by a sperm factor and/or via activation of a G protein (Whitaker and Swann, 1993; Sette *et al.*, 1997), 2) release of intracellular calcium at the site of sperm entry. This event is probably mediated by the secondary messenger InsP3 (McDougall and Sardet, 1995; Albrieux *et al.* 1997); 3) initiation of a microfilament-based cortical contraction which drags surface proteins, cortical and subcortical organelles, as well as the nucleus and centrosome introduced by the sperm. If the sperm penetrates near the animal pole (the most common situation), the calcium

wave reaches simultaneously all sides of the opening of the microfilament basket and the cortical contraction proceeds symmetrically in the vegetal direction, forming a contraction pole exactly opposite the animal pole. If the sperm penetrates near the equator the calcium wave reaches one side of the microfilament basket first and this side contracts first. This side of the basket corresponds to the position of the sperm entry site. One consequence of this situation is the asymmetrical contraction of the microfilament basket. In such cases, the resulting contraction pole can be situated as much as 45 degrees away from the vegetal pole (Speksnijder *et al.*, 1990; Roegiers *et al.*, 1995). These events result in a large reorganization of the ascidian zygote cortex and cytoplasm into distinguishable domains (mitochondria-rich myoplasm/subcortical, ER-rich cytoplasm/microfilaments and microvilli-rich contraction pole region). These domains are radially organized around a new developmental axis that predicts the future site of gastrulation and dorso-ventral axis of the embryo. This axis is exactly coincident (if the contraction is symmetrical) or not coincident (if the contraction is asymmetrical) with the original animal/vegetal axis of the zygote (Speksnijder *et al.*, 1990; Roegiers *et al.*, 1995).

The mechanism by which the initial calcium signal provokes the contraction of the actin microfilament network remains to be established. We need to know if there is assembly of new actin filaments and how organelles are connected to the contracting microfilament network and concentrated in the various cytoplasmic domains. Other cytoskeletal elements (intermediate filaments) may also be involved. Finally, we would like to know how these large reorganizations influence the distribution and functions of membrane components.

One clear consequence of the creation of a contraction pole region highly enriched in microvilli, microfilaments and sheets and tubes of ER is the establishment of a calcium wave "pacemaker" capable of generating from 10 to 20 calcium waves during meiosis (see McDougall and Sardet, 1995). As in mammalian eggs, oscillatory calcium signals in ascidian eggs are involved in the regulation of the meiotic cell cycle (Speksnijder *et al.*, 1990; McDougall and Sardet, 1995; Russo *et al.*, 1996). These signals may also be necessary for proper embryonic development since ablation of a sizeable fraction of this region prevents a normal cleavage pattern and gastrulation (Bates and Jeffery, 1987; Nishida, 1996). It remains to be seen what domains and molecular and cellular functions are affected in such experiments.

Eggs of many other organisms also display cortical contractions triggered by the sperm as a consequence of transient elevations of intracellular calcium. The best documented case is that of the amphibian eggs in which the calcium wave initiated by the fertilizing sperm is followed by a symmetrical contraction (along the animal/vegetal axis), which drags subcortical organelles and the incorporated sperm nucleus towards the animal pole (Cheer *et al.*, 1987). This poleward motion brings the male and female pronuclei closer to each other (Elinson, 1983) but is not thought to have major consequences on the establishment of a developmental axis.

In leeches, the microfilament-driven meiotic contraction is clearly delayed with respect to fertilization and zygote laying (about 90 min). The poleward progression of the contraction ring terminates with the emission of the first polar body and the accumulation at the animal pole region of plasmalemma, organelles and actin filaments. At present we have not been able to detect calcium signals at the time of contraction (Fernández and Sardet, unpublished observations). Localized regulation of contractile components in the cortex by cell-cycle dependent phosphorylation may be involved in generating these contractions. A similar bout of microfilament-driven contractions has been reported in the zygotes of the phylogenetically related oligochaete *Tubifex* at the conclusion of the first meiotic division (Shimizu, 1982a). In this case, several meridionally-oriented equatorial grooves, rather than one contraction ring, are formed. Rearrangements of the preexisting actin filaments network have been documented (reviewed in Shimizu, 1995).

Late meiotic events

Once the early microfilament-driven reorganizations have taken place the next phase of cytoplasmic reorganization in leech and ascidian zygotes is linked to the growth of an aster around the centrosome introduced by the sperm (establishment of the first monaster). This microtubular structure is essential in the recruitment and relocalization of organelles and the establishment of new cytoplasmic domains.

In ascidians, the aster grows slowly and at first symmetrically, beneath the cortex in a period of 20 min following emission of the first polar body. After the second polar body is formed, the monaster grows in an asymmetric manner with its longest fibers reaching the animal region, where the female karyomeres have formed. The karyomeres then move toward the center of the monaster containing the male pronucleus, presumably using microtubule motors (see Houliston *et al.*, 1995; Vernos and Karsenti, 1996).

In leech zygotes the process is quite similar. The monaster is initially situated beneath the cortex and grows asymmetrically over a period of about 60 min. As it grows the monaster glides in a vegetal direction towards the center of the zygote following an arc-like path. Both microfilaments and microtubules are involved in monaster centration in the leech egg (Fernández *et al.*, 1994). The microtubules linking the centrosome to the animal pole region, where the newly-formed female karyomeres lie, are stabilized allowing movement of the karyomeres toward the egg center. Organelles recruited from the adjacent endoplasm form a perinuclear plasm domain that also encloses the sperm-derived centrosome and the developing male pronucleus.

In both ascidians and leeches growth of the sperm centrosome-derived monaster is clearly related to progression from MI to the MII stage of meiosis. This process is likely to depend on cell cycle regulation of microtubule dynamics and motor activity (Vernos and Karsenti, 1996). In both ascidians and leeches these microtubule-driven events during meiosis II are accompanied by transient microfilament-based deformation of the vegetal cortex. Microfilament-driven deformation of the vegetal hemisphere also takes place during meiosis of other invertebrate zygotes. In mollusc and annelid zygotes such deformations result in polar lobe formation (Dohmen, 1983). Meridional furrows also form at the equator of the *Tubifex* zygote during emission of the second polar body (Shimizu, 1982a). Recent experiments by Shimizu (1997) indicate that in *Tubifex* the reorganization of cortical actin network requires activation of Protein kinase C and an increase in intracellular calcium.

Interphase and mitotic events

In ascidians, duplication of the sperm-derived centrosome during first interphase modifies the shape of the centrosomal region, from a sphere to a disc. This starts a relocalization process that

involves the coordinated translocation of the aster and the pronuclei towards the center of the zygote and that of adjoining cytoplasmic domains towards the future posterior pole of the embryo. This process is mainly dependent on the integrity of microtubules and may be analogous to the "cortical rotation". In amphibian eggs, since microtubule-driven displacement of cytoplasmic regions likewise takes place in relation to the zygote cortex, it will be informative to examine the organization and polarity of cortical and subcortical microtubules during these movements and to assess the importance of microtubule polymerization and displacement, with respect to other microtubules and organelles, as well as the involvement of microtubule based motor molecules in this process (Houliston, 1994). The slow and fast phases of translocation that we have described during this second phase of ooplasmic segregation in ascidians probably correspond to the building up of tensions brought about by the sliding of microtubules against the cortex and then to the release of tensions when the cup-shaped myoplasm tears. In ascidians, these movements ultimately result in the relocalization of the bulk of cytoplasmic domains (mitochondria-rich and ER-rich domains). Because of the stereotyped cleavage pattern of the ascidian embryo these domains will be inherited by the 2 ventro-posterior blastomeres of the 8-cell embryo. These blastomeres will give rise autonomously to the primary muscle cells of the ascidian tadpole and even develop muscle cells in isolation (Satoh, 1994; Nishida, 1997). It is interesting to note that a particular cortical structure, called the CAB (Centrosome-Attracting Body), plays a key role in the unequal cleavage pattern of posterior blastomeres in 16-64 cell embryos (Hibino *et al.*, 1998). We may hypothesize that the CAB, or its precursor, is formed in the posterior cortex during translocation and centration of the aster and accompanying cytoplasmic domains.

Centrosome duplication in the leech zygote also takes place in the interphase period that follows completion of meiosis. The duplication, in this case, causes the perinuclear plasm domain to change its shape from spherical to ellipsoidal and finally into a dumbbell-shaped body oriented perpendicularly to the animal/vegetal axis. In such a large zygote, restriction of mitotic activity (meiosis) to the cytoplasm around female karyomeres, located in the animal pole region of the zygote, might contribute to the stability of some interphase microtubules, a situation that also occurs in the even larger eggs of *Beroe* and *Xenopus* (Houliston *et al.*, 1995; Pérez-Mongiovi *et al.*, 1998).

By mid interphase distinct circular and meridional furrows, whose formation is dependent on microfilaments, appear at the leech zygote surface (Fernández and Olea, 1995; Fernández *et al.*, 1998). It is not known what directs the formation of these furrows and whether furrowing relies on the recruitment and contraction of preexisting microfilaments and/or on the polymerization of new actin filaments. Microtubules and organelles accumulate at the walls of rings and bands of contraction. In this manner, organelles, microtubules and microfilaments are brought together to concentrate in regions of thickened ectoplasm. The origin of ectoplasmic microtubules has not yet been fully determined but they may derive from the numerous cytasters present in the ectoplasm during meiosis (see Panel A, Figs. 5 and 6). The bipolar displacement of organelles, microfilaments and microtubules associated with the poleward motion of the polar rings and the shortening of meridional furrows results in a large accumulation of ectoplasm at both zygote poles. In the zygote of the leech *T. rude* this process involves both microtubules and microfilaments (Fernández *et al.*, 1998). In the zygote of other leeches, it seems to be microtubule-dependent (Astrow *et al.*, 1989), whereas in *Tubifex* it appears to involve mainly microfilaments (Shimizu, 1982b,1995). Although the large zygote of glossiphoniid leeches and oligochaetes are favorable models to study the formation of cytoplasmic domains, much remains to be done to disclose the respective roles of different cytoskeletal elements and motors in vectorial translocation of organelles and cytoskeletal elements.

In the leech, the last and decisive event for the allocation of cytoplasmic domains to one of the first two blastomeres is the generation and positioning of an asymmetric mitotic apparatus (Panel A, Figs. 11 and 12). This allows unequal division of the zygote such that the larger cell contains all or most of the animal and vegetal teloplasms. At present we have no clue as to what determines the asymmetry of the mitotic spindle. It is possible that the teloplasms and the adjoining cortical regions, in which astral microtubules are embedded, affect the dynamic equilibrium of spindle pole microtubules in the CD sector of the egg (Fernández *et al.*, unpublished observations). In *Tubifex*, formation of an asymmetric cleavage spindle in the one cell embryo does not apparently depend on cortical mechanisms but rather on the inheritance of a single maternal centrosome during mitosis (Ishii and Shimizu, 1997). In contrast, formation of an asymmetric cleavage spindle in the two cell embryo of *Tubifex* relies on cortical differences dependent on cell contacts (Takahashi and Shimizu, 1997).

We have stressed similarities between leech and ascidian zygote reorganization during the mitotic and meiotic cell cycles. It is evident, however, that there are also important differences. For example, in ascidians it is clear that the point of sperm entry and consequent localization of the sperm aster play an essential role in defining the localization of cytoplasmic domains and thus the anterior/posterior axis of the embryo. In the leech zygote localization of cytoplasmic domains is dictated by the distribution of cytoskeletal elements along the animal/vegetal axis that is determined during oogenesis. Other axes in the leech, such as that running along cells AB and CD, do not appear to be strictly determined during oogenesis or by the site of sperm entry (Pilon and Weisblat, 1997). It is also worth noting that unequal partitioning of cytoplasmic domains (teloplasms) is rather precocious in leeches taking place during the first mitotic cycles, whereas in ascidians, unequal partitioning of myoplasm and ER rich domains takes place much later (at the 16-32 cell stage) when smaller posterior blastomeres are formed (Nishida, 1997).

Summary

Leech and ascidian embryos are well suited for the study of certain developmental processes. Although leeches and ascidians belong to different bilateralia groups (protostomes and deuterostomes, respectively) they share important developmental features and, in particular, the determinate character of their embryogenesis. In both types of embryos this property is related to the presence of specific cytoplasmic domains that are selectively allocated to different blastomeres during cleavage. In this review leech and ascidian eggs and zygotes are compared in terms of the structure of these cytoplasmic domains and of the cellular mechanisms involved in their formation and localization. During meiosis the zygote of leeches and ascidians undergo stereotypic actin-dependent contraction movements related to both the emission of

the polar bodies and the formation and relocalization of cytoplasmic domains. After completion of meiosis, during first interphase, monaster microtubules nucleated from the sperm-derived centrosome play a key role in pronuclear migration. In addition, these astral microtubules direct the relocalization of cytoplasmic domains and the translocation and accumulation of organelles in the interior of the zygote. Microtubules and microfilaments, on the other hand, are involved in cortical reorganizations and organelle translocations in both zygote species during interphase and cleavage divisions. In the case of leech zygotes, this process leads to formation of characteristic polar cytoplasmic domains called teloplasms. These domains are selectively inherited by teloblasts, precursor stem cells of ectodermal and mesodermal tissues in the leech embryo. In the ascidian zygote, the cytoplasmic movements observed during interphase and mitosis lead to relocalization of the bulk of a mitochondria-rich domain, called the myoplasm, along with an endoplasmic reticulum-rich domain towards the future posterior pole of the embryo. The myoplasm is inherited by a subset of posterior blastomeres committed to become the primary muscle cells of the ascidian tadpole.

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