

Chapter 12

Asymmetric and Unequal Cell Divisions in Ascidian Embryos

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Abstract Asymmetric cell division during embryogenesis contributes to cell diversity by generating daughter cells that adopt distinct developmental fates. In this chapter, we summarize current knowledge of three examples of asymmetric cell division occurring in ascidian early embryos: (1) Three successive cell divisions that are asymmetric in terms of cell fate and unequal in cell size in the germline lineage at the embryo posterior pole. A subcellular structure, the centrosome-attracting body (CAB), and maternal *PEM* mRNAs localized within it control both the positioning of the cell division planes and segregation of the germ cell fates. (2) Asymmetric cell divisions involving endoderm and mesoderm germ layer separation. Asymmetric partitioning of zygotically expressed mRNA for Not, a homeodomain transcription factor, promotes the mesoderm fate and suppresses the endoderm fate. This asymmetric partitioning is mediated by transient nuclear migration toward the mesodermal pole of the mother cell, where the mRNA is delivered. In this case, there is no special regulation of cleavage plane orientation. (3) Asymmetric cell divisions in the marginal region of the vegetal hemisphere. The directed extracellular FGF and ephrin signals polarize the mother cells, inducing distinct fates in a pair of daughter cells (nerve versus notochord and mesenchyme versus muscle). The directions of cell division are regulated and oriented but independently of FGF and ephrin signaling. In these examples, polarization of the mother cells is facilitated by localized maternal factors, by delivery of transcripts from the nucleus to one pole of each cell, and by directed extracellular signals. Two cellular processes— asymmetric fate allocation and orientation of the cell division plane—are coupled by a single factor in the first example, but these processes are regulated independently in the third example. Thus, various modes of asymmetric

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cell division operate even at the early developmental stages in this single type of organism.

12.1 Ascidians as Model Organisms for Studies of Asymmetric Cell Division

When one mother cell divides asymmetrically to generate two daughter cells with distinct developmental fates, the mother cell is polarized before the mother cell starts to divide in order to generate distinct daughters after the division. Then, the orientation of the division plane must be properly regulated, so that the division plane is perpendicular to the direction of the preestablished polarization of the mother cell. In many cases of asymmetric cell division, these two cellular processes (orienting cell polarization and spindle) are harmonized by common basic factors, although these processes may not necessarily be coupled, especially in early embryos. Thus, in this chapter, we use the term “asymmetric cell division” to refer to cell division that produces two different daughters from the time they are generated. Polarization of mother cells is directed by asymmetrically distributed cell-intrinsic factors or cell-extrinsic signaling molecules (Chen et al. 2016).

Ascidians are globally distributed marine invertebrates belonging to the subphylum Tunicata (Urochordata), constituting a sister group to vertebrates in the phylum Chordata (Delsuc et al. 2006). Ascidians spawn enormous numbers of eggs and exhibit an invariant embryonic cleavage pattern, which is also shared among diverse ascidian species, have long facilitated making them an ideal organism for investigations of embryogenesis up to the hatching tadpole larva stage (Fig. 12.1a) (Chabry 1887; Conklin 1905). Ascidian tadpole larvae possess the basic body plan of chordates, having an axial mesoderm including muscles and notochord, a dorsal central nerve system, and a brain. The embryonic cleavage pattern of the solitary ascidian, *Styela partita*, was documented for the first time in amazing detail by Conklin, and subsequent studies using modern techniques have reconfirmed Conklin’s descriptions and traced the embryonic cell lineages (Nishida and Satoh 1983, 1985; Nishida 1986, 1987; Stach and Anselmi 2015).

The cleavage pattern of ascidian embryo is bilaterally symmetrical but not simple (Figs. 12.1 and 12.2a, b). The cell fates of most blastomeres are restricted to give rise to a single type of tissue by the 110-cell stage (after seven rounds of initial cell divisions) (Fig. 12.2a) (Nishida 1987; Kumano and Nishida 2007). The ascidian fate map (Fig. 12.2b) shows similarity to that of the frog in terms of the geographical topology of the tissue precursor cell territories (Lemaire et al. 2008). Both the cleavage pattern and fate map are highly conserved among ascidian species, which have become phylogenetically diversified (Hudson and Yasuo 2008; Lemaire 2009; Tsagkogeorga et al. 2009). We have confirmed that species spanning seven genera [*Styela* (Conklin 1905), *Halocynthia* (Nishida 1987), *Ciona* (Conklin 1905), *Phallusia* (Zalokar and Sardet 1984), *Ascidia* (H. Nishida, unpublished observation), and *Boltenia* (http://gvondassow.com/Research_Site/

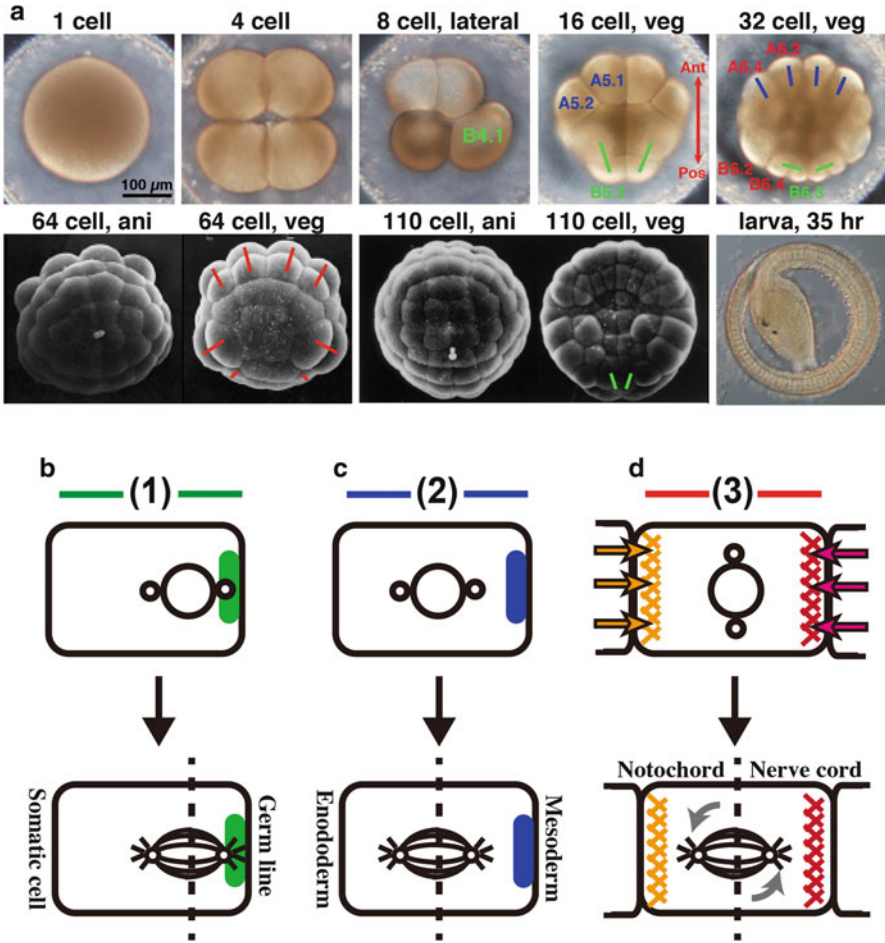


Fig. 12.1 Ascidian eggs develop into tadpole larvae through a relatively simple process of development involving a small number of constituent cells. (a) The photos show live and scanning electron microscopy images of *Halocynthia roretzi* embryos at various stages from the fertilized egg to the larva (35 h of development). The larvae exhibit the basic body plan of chordates, having a dorsal neural tube, a notochord flanked by bilateral muscle, and a brain with two sensory pigment cells. The notochord, a characteristic feature of chordates, is visible in the tail of the larva, consisting of exactly 40 disc-shaped cells arranged in a single line. The total number of cells in the hatched larva, 1.5 mm in length, is approximately 3000. The lines connecting the blastomeres show the directions of asymmetric cell division relevant to the three subheadings of this chapter. In 16- to 110-cell embryos, anterior is up. *Green bars* indicate three successive unequal and symmetric cell divisions resulting in generation of small primordial germ cells at the posterior pole. *Blue bars* indicate asymmetric divisions that segregate the endoderm and mesoderm cell fates at the 32-cell stage. *Red bars* correspond to asymmetric cell divisions that separate the notochord/nerve code fates in the anterior half and mesenchyme/muscle fates in the posterior half at the 64-cell stage. Names of some blastomeres are indicated. (b–d) Schematic representation of three types of asymmetric cell division that are described in this review. See details in text. (b) Asymmetric division in germ cell lineage. It corresponds to cell divisions designated by the *green*

[Picture_of_the_week/Entries/2010/5/9_Embryogenesis_in_the_ascidian_Boltenia.html](#)) and *Corella* (http://gvondassow.com/Research_Site/Video_-_Corella_early_cleavage.html)] share the common cleavage pattern. This implies that the cell division patterns in ascidian embryos are regulated through evolutionarily robust mechanisms.

In order to generate primordial tissue precursor cells during seven rounds of cell division after fertilization, many cell divisions take place to generate daughter cells with distinct prospective cell fates. These divisions are indicated by red bars in Fig. 12.2a. These red bars represent one out of a total of one at the 4-cell stage in the bilateral half, two out of two at the 8-cell stage, three out of four at the 16-cell stage, seven out of eight at the 32-cell stage, seven out of 16 at the 64-cell stage, and seven out of 23 at the 110-cell stage. However, these do not always represent asymmetric cell divisions because cell fates may be determined after the mother cell has divided. In asymmetric cell division, there should be evidence to indicate that the mother cell has already become polarized before division begins. In this chapter, we summarize current knowledge of three types of genuine asymmetric division occurring in ascidian early embryos as outlined below. Hereafter, we refer to the mitotic division responsible for production of daughter cells with different developmental potential as “asymmetric” cell division and that produce daughter cells differing in size through eccentric positioning of the division plane as “unequal” cell division.

1. Three successive asymmetric and unequal cell divisions at the posterior pole (green letters and bars in Fig. 12.1a, b). A subcellular structure, the centrosome-attracting body (CAB), and maternally localized mRNAs control both the positioning of the cell division planes and the segregation of cell fates.
2. Asymmetric cell divisions involving segregation of the endoderm and mesoderm germ layers (blue letters and bars in Fig. 12.1a, c). Asymmetric partitioning of zygotically expressed mRNA for Not, a homeodomain transcription factor, promotes the mesoderm fate and suppresses the endoderm fate. This asymmetric partitioning is mediated by nuclear migration toward the mesodermal pole within the mother cell. In this case, there is no special regulation of cleavage plane positioning, as the cell divides into daughters with approximately same size and the cleavage plane positioning simply follows Sach’s rule that the new division plane is formed perpendicularly to the previous one.

Fig. 12.1 (continued) bars in (a). *Large circle* is nucleus, and *small circle* is centrosome. *Green oval* represents the centrosome-attracting body (CAB). *Broken line* indicates the next cell division plane. (c) Separation of endoderm and mesoderm. It corresponds to the *blue bars* in (a). *Blue oval* represents the asymmetrically localized mRNA of Not transcription factor in *Halocynthia*. (d) Asymmetric cell divisions in the marginal region of the vegetal hemisphere. It corresponds to the *red bars* in (a). *Yellow arrows* and *crisscrosses* indicate FGF signaling on one side. *Red arrows* and *crisscrosses* indicate Ephrin antagonizing signaling on the opposite side. Veg, vegetal view. Ani, animal view. Ant, anterior. Pos, posterior. SEM images have been reproduced from Nishida (1986) with permission

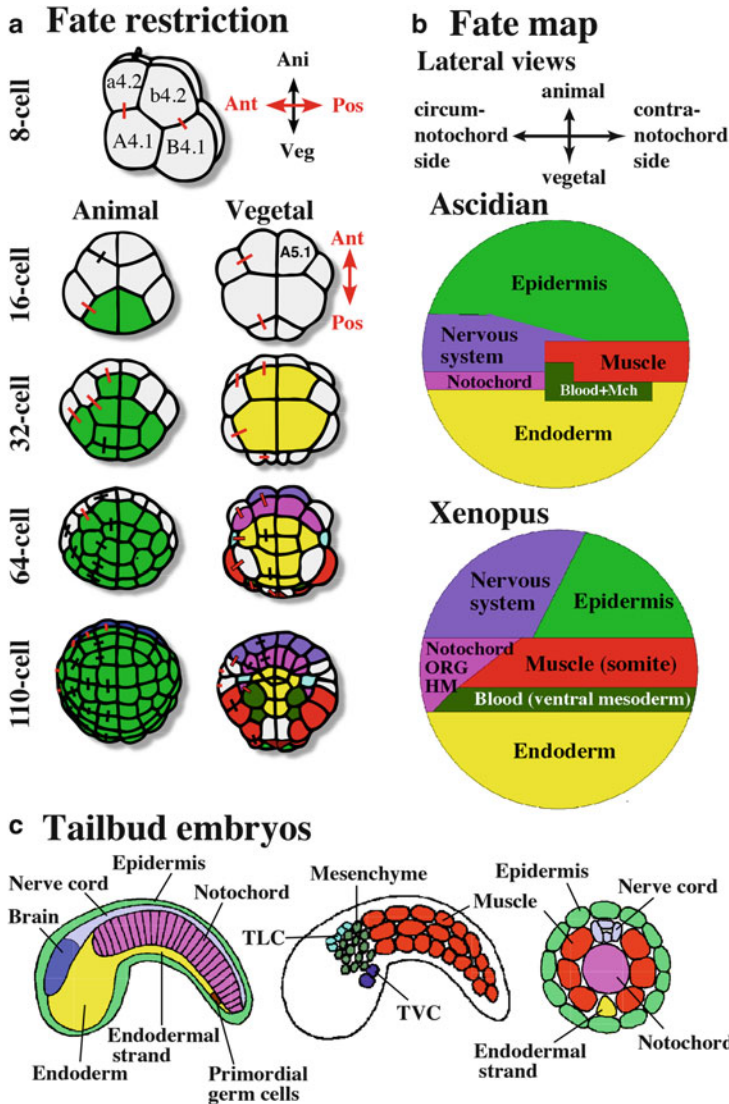


Fig. 12.2 Developmental fates of blastomeres of the ascidian embryo. Orientation of the each drawing is indicated. **(a)** Cell fate restriction during the cleavage stages. Blastomeres are colored when their fate is restricted to a single cell type. The colors correspond to those of the larval tissues indicated in **(c)**. Fate restriction in ascidian early embryos proceeds quickly. Sister blastomeres are connected by bars. *Red bars* indicate that the prospective cell fates of the two sister blastomeres are distinct. The fate map is bilaterally symmetric. **(b)** Schematic fate maps of ascidians and *Xenopus* blastulae. Lateral views. Circum-notochord side is to the *left*. Note the topographic similarity of the presumptive tissue territories in the two fate maps. ORG, organizer; HM, head mesoderm; Mch, mesenchyme (precursors of adult tunic cells). **(c)** Organization of tailbud embryos. Mid-sagittal planes, sagittal planes, and transverse sections of the tail. TLC, trunk lateral cells (precursors of adult blood and body wall muscle); TVC, trunk ventral cells (precursors of adult heart and body wall muscle). Drawings have been reproduced with permission from Nishida (2005) and Lemaire et al. (2008)

3. Asymmetric cell divisions in the marginal region of the vegetal hemisphere (red letters and bars in Fig. 12.1a, d). The directed extracellular FGF and antagonizing ephrin signals polarize the mother cells, inducing distinct fates in a pair of daughter blastomeres (nerve versus notochord and mesenchyme versus muscle). The direction of cell division is regulated and oriented but independently of FGF and ephrin signaling.

12.2 Segregation of Maternally Localized Transcripts and Cell Divisions Unequal in Size

12.2.1 *Localized Maternal Transcripts: Postplasmic/PEM mRNAs in Ascidians*

Studies using many animals have revealed that localized maternal factors initiate establishment of the embryonic axes (Gurdon 1992; Johnston and Nüsslein-Volhard 1992; Bowerman et al. 1993). This mechanism would be a likely candidate for the process involved in asymmetric cell divisions during the cleavage stages. In ascidians, Conklin (1905) described the segregation pattern of yellow-colored ooplasm in a region known as the posterior–vegetal cytoplasm/cortex (PVC; myoplasm in Conklin’s description) of fertilized eggs and embryos. About a hundred years after this original description, it was discovered that a genuine muscle determinant, maternal mRNA of *macho-1* transcription factor, is present in the myoplasm (Nishida and Sawada 2001). *macho-1* is a member of the so-called *postplasmic/PEM* mRNAs.

In 1996, Yoshida et al. first reported that in *C. savignyi* a maternal mRNA was localized to the PVC of the egg and the posterior pole of the embryo, and the gene was named “*posterior end mark*” (*pem*). *pem* is the most abundant maternally localized mRNA in the ascidian egg. Since the discovery of *pem*, approximately 50 mRNAs showing the same pattern of localization as *macho-1* and *pem* have been identified (Kawashima et al. 2000; Makabe et al. 2001; Nishida and Sawada 2001; Nakamura et al. 2003; Yamada 2006; Paix et al. 2009). These mRNAs are referred to as *postplasmic/PEM* mRNAs (*macho-1*, *pem*, *Wnt5*, *POPK-1*, etc.) (reviewed in Sardet et al. 2005; Prodon et al. 2007; the most recently updated list of *postplasmic/PEM* RNAs is available in Makabe and Nishida 2012). They are first concentrated at the vegetal pole and then move to the posterior pole after fertilization (Fig. 12.3, 1st and 2nd phases), later becoming further concentrated into the centrosome-attracting body (CAB) at the posterior pole during the cleavage stages, as mentioned later (Fig. 12.3, the 2- to 110-cell embryos). The results of various types of screening, including large-scale *in situ* hybridization and microarray, suggest that the localization pattern shared by *postplasmic/PEM* RNAs is the sole pattern of localization of maternal mRNAs (Makabe et al. 2001; Yamada et al. 2005). There

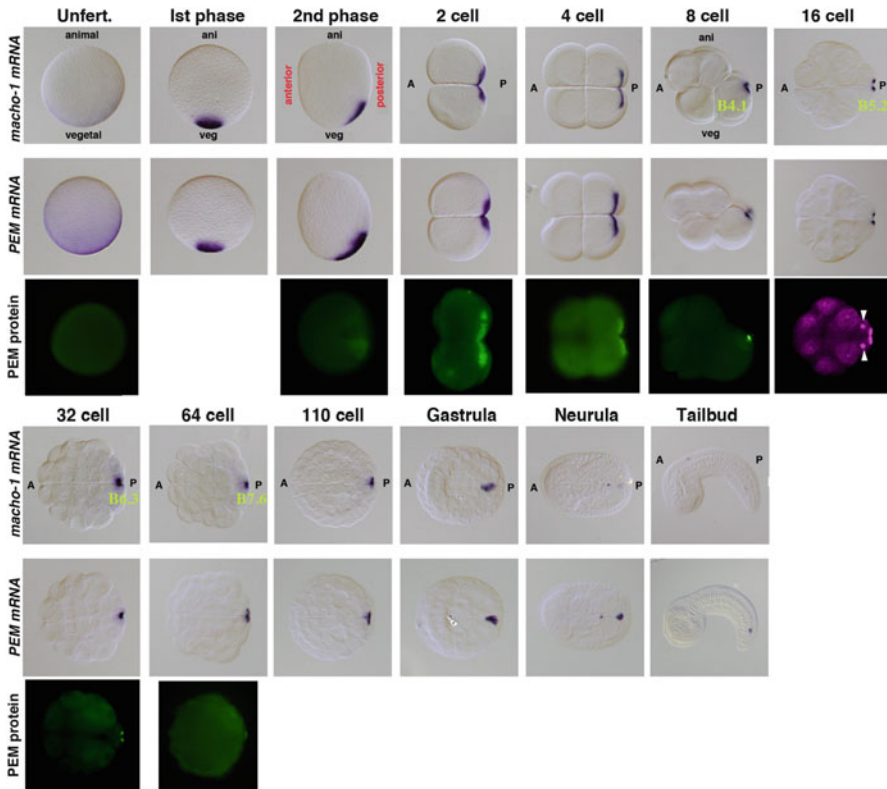


Fig. 12.3 Localization of two representative *postplasmic/PEM* mRNAs, *PEM*, and *macho-1*, during early embryogenesis. *PEM* protein localization is also shown. In the 16-cell-stage embryos, *PEM* protein is detected with higher sensitivity, revealing the presence of *PEM* protein in nuclei of the posterior blastomeres in addition to localization of the CAB (arrowheads). Egg and 8-cell embryos are lateral views. The 2-, 4-, 32- to neurula-stage embryos are vegetal views. Names of posterior-most blastomeres which inherit the *postplasmic/PEM* RNAs are indicated. Unfert., unfertilized egg. Ani, animal pole. Veg, vegetal pole. A, anterior. P, posterior. Images have been reproduced from Negishi et al. (2007) and Kumano et al. (2011) with permission

have been no reports of maternal RNA localized to the animal and anterior regions of the egg.

12.2.2 Successive Unequal Cell Divisions

Up to the 8-cell stage, the fertilized egg is cleaved equally in accordance with Sach's rule that the new division plane is formed perpendicularly to the previous one (Strome 1993). At the end of the four-cell stage, the third cleavage planes intersect the animal-vegetal axis, although the posterior cleavage planes are

slightly tilted. As a result, the ascidian 8-cell-stage embryo shows a unique shape with the posterior–vegetal blastomeres (B4.1¹) protruding posteriorly (Figs. 12.1a, 12.2a and 12.4a). Subsequently, three successive unequal cell divisions take place at the posterior pole of the vegetal hemisphere (Figs. 12.1, green bars, and 12.4a). These unequal divisions always generate smaller daughter cells at the posterior pole. The resulting posterior most and smallest cells of the 64-cell embryo are the precursors of the germline (Shirae-Kurabayashi et al. 2006). In contrast to the posterior–vegetal region, the blastomeres in the animal half and the anterior–vegetal region divide equally according to Sach’s rule, except for a certain case at the sixth cleavage, as will be described later.

One strategy for achieving unequal cell division is eccentric placement of the mitotic spindle (Siller and Doe 2009). In the one-cell zygote of *Caenorhabditis elegans*, such unequal cell division is driven by the attachment of microtubules emanating from the centrosome to cortical dynein motors (Grill et al. 2001). Investigation of astral microtubules in ascidians has revealed a bundle of microtubules linking the centrosome to the posterior pole (Fig. 12.4b, c). The nucleus/centrosome complex approaches the posterior pole as the bundle is shortened, and then the mitotic apparatus forms close to the posterior pole (Hibino et al. 1998). This is repeated during three rounds of unequal cell divisions. Treatment with microtubule dissociation agent, nocodazole, prevents the nuclei from approaching to the posterior pole (Nishikata et al. 1999). Detailed behavior of nucleus, centrosome, and spindle during the B5.2 unequal cell division has been reported in Prodon et al. (2010).

In cytoplasm-extracted embryos, a brilliant structure has been observed at the posterior pole under light microscope. This structure has been designated the centrosome-attracting body (CAB) (Fig. 12.4b, d) (Hibino et al. 1998). The CAB at the posterior cortex is always inherited by the smaller blastomere at each division. The CAB is not present in the egg, and it is first recognized as a group of small dots with high refraction index at the late two-cell stage (Hibino et al. 1998). The location of this subcellular structure corresponds exactly to that of the *postplasmic/PEM* mRNAs (Yoshida et al. 1996; Hibino et al. 1998; Nakamura et al. 2003). Microsurgical removal and transplantation of the PVC of eggs have shown that the PVC is responsible for formation of the CAB and unequal cleavages (Nishikata et al. 1999).

Observations of the CAB using transmission electron microscopy (TEM) and isolation of the cell cortex have revealed that the CAB is characterized by an electron-dense matrix/granule (EDM) that resembles the germ plasm of other animals (Fig. 12.4d) and a network of cortical rough endoplasmic reticulum (cER) (Iseto and Nishida 1999; Sardet et al. 2003). Some *postplasmic/PEM*

¹Nomenclature of ascidian blastomeres. Each blastomere is assigned its name such as B4.1, A5.2, and b5.6. Capital letters are for the cells of the vegetal hemisphere. Lower case letters are for the animal hemisphere cells. A and a for the anterior blastomere. B and b for the posterior blastomeres (see Fig. 12.2a, 8-cell embryo). First digit represents the generation. Second digit is a unique number based on its relative position after cell division.

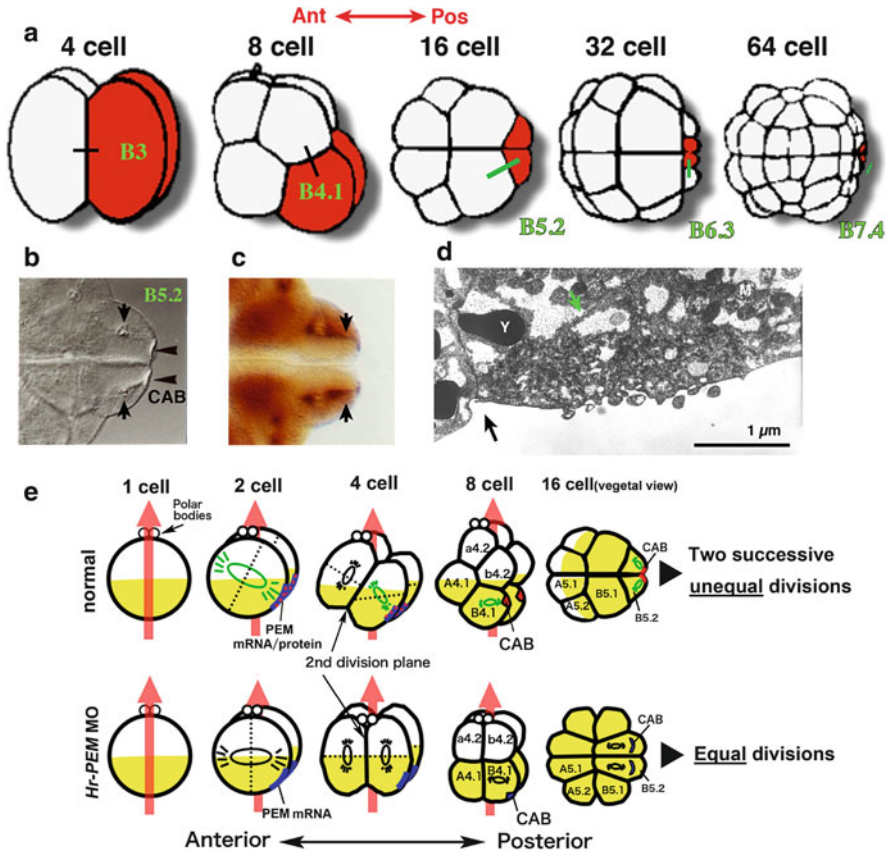


Fig. 12.4 Unequal cell divisions taking place at the posterior pole. (a) Red blastomeres divide unequally in size from the 8-cell to 64-cell stages, always generating smaller cells at the posterior pole. Green bars indicate three successive unequal cell divisions resulting in generation of small primordial germ cells at the posterior pole. The 4- and 8-cell embryos are lateral views. The 16- to 64-stage embryos are vegetal views. Name of relevant cells are indicated in green letters. Ant, anterior. Pos, posterior. (b) Posterior part of the extracted 16-cell embryo. These cells have divided unequally and are about to divide unequally again. The CAB is present at the posterior pole (arrowheads). Positions of the nuclei are indicated by arrows. (c) A bundle of microtubules (arrows) connects the posterior centrosome to the CAB. The bundle is shortening to pull the nucleus toward the CAB. (d) Ultrastructure of the CAB. The CAB is characterized by an electron-dense matrix, which is considered to be the putative germ plasm. Black arrow indicates the midline of the embryo. Green arrow indicates a microtubule. Y, yolk granule. M, mitochondrion. (e) Model for control of orientation and positioning of the cell division plane by the PEM. (Upper) In a normal embryo at the two-cell stage, the centrosome axis rotates as the posterior centrosome is attracted toward the posterior-vegetal cortex (blue cortex), where PEM protein is localized (red dots on blue cortex), and a tilted spindle (green) forms. The second division plane is oblique to the A-V axis (large red vertical arrow). Broken lines indicate the planes of the next division. At the 4-cell stage, similar events occur again, only in the posterior blastomeres. Therefore, the B4.1 blastomere protrudes posteriorly at the 8-cell stage. Yellow color shows the vegetal half of the cytoplasm. In the B4.1 blastomeres, the centrosome/nucleus complex approaches the CAB, and the blastomere divides unequally. At the 16-cell stage, a smaller B5.2 blastomere forms at the posterior pole. This posterior-most blastomere undergoes two further unequal cleavages. (Lower) Without PEM protein, the cleavage pattern is quite regular as there is no centrosome-attracting

mRNAs are known to be anchored to the cER and others to granules in the CAB (Paix et al. 2009). Their protein products, including *pem* and *vasa*, are mostly localized to the CAB (Fig. 12.3) (Shirae-Kurabayashi et al. 2006; Negishi et al. 2007; Paix et al. 2009; Kumano et al. 2010). Some additional proteins translated from “non”-*postplasmic/PEM* mRNAs also show localization to the CAB; these include the aPKC–Par3–Par6 complex, β -catenin, Dishevelled, the regulators of translation initiation (MnK-4EBP-S6 K), and Polo-like kinase1 (Patalano et al. 2006; Kawai et al. 2007; Negishi et al. 2011; Paix et al. 2011). Thus, the CAB is a multifunctional subcellular structure that attracts the centrosome, provides a structural scaffold for the localization of *postplasmic/PEM* mRNA and several proteins, and contains the putative germ plasm. The CAB plays a robust role in these asymmetric and unequal cell divisions by organizing the orientation of cleavage planes and asymmetric partitioning of the *postplasmic/PEM* mRNAs. In our knowledge, the CAB is unique to ascidians because in cell-autonomous unequal cell divisions in other animals such as *C. elegans*, *Drosophila*, and sea urchin, there has been no report on visible macroscopic structure like the CAB. In these examples, it seems that microtubules emanating from the centrosome interact with wide area of the cortex of the cell (Grill et al. 2001).

12.2.3 Control of Cell Division Planes and Germ Cell Formation by Localized PEM Protein

What are the molecules involved in the orientation of cell division planes and germ cell specification? *PEM* was the first *postplasmic/PEM* mRNA to be identified and is the most abundant, as mentioned previously. *PEM* protein is translated and localized in the CAB (Fig. 12.3). When the function of *PEM* is inhibited with the antisense morpholino oligo (MO), unequal cell divisions are converted to equal ones, and the posterior cleavage pattern becomes similar to the anterior pattern. In *PEM*-deficient embryos, the astral microtubules do not focus on the CAB, and consequently the cells divide equally (Fig. 12.4e) (Negishi et al. 2007). In addition, *PEM*-deficient eight-cell embryos show no protrusion of the posterior–vegetal blastomere (Fig. 12.4e). *PEM* localization also determines the orientation of mitotic planes at the second and third cleavages before the start of successive unequal cell divisions. Observations of the mitotic spindle by immunostaining and live imaging have supported the idea that from the 2- to the 32-cell stages the closest centrosomes are always attracted and pulled toward the cortex where *PEM* protein is localized (Negishi et al. 2007).

Fig. 12.4 (continued) activity. No specific regulation of the cleavage pattern occurs. Ant, anterior. Post, posterior. Drawings and images have been reproduced from Hibino et al. (1998), Iseto and Nishida (1999), and Negishi et al. (2007) with permission

Similar localization and function of the PEM have been observed in three ascidians, *Halocynthia*, *Phallusia*, and *Ciona*, and are thought to underlie the common cleavage pattern of ascidians (Prodon et al. 2010; Shirae-Kurabayashi et al. 2011). However, ascidian PEM has no known domain except the C-terminal WPRW sequence that is a binding site for groucho, a transcriptional corepressor, and there is no apparent homolog in other deuterostomes. It has shown that aa 258–341 of the PEM of *Halocynthia*, where some conserved amino acid stretches are shared among ascidian species, are required for this function, although the molecular mechanism responsible for pulling of the centrosome toward the CAB is still unclear (Kumano et al. 2011).

Interestingly, a certain amount of PEM protein is translocated into the nuclei of posterior-most cells, which are of germ cell lineage, and mediates transcriptional quiescence in the germline precursor (Kumano et al. 2011; Shirae-Kurabayashi et al. 2011). In the *Halocynthia* embryo, aa 342–426 in the C-terminal region of the PEM, where some conserved amino acid stretches are shared among ascidian species, are crucial for transcriptional quiescence. This region interacts with the P-TEFb complex, resulting in suppression of zygotic transcription (Kumano et al. 2011). Suppression of P-TEFb is a conserved mechanism for germline quiescence by localized germ plasm factors in ascidians, *Drosophila*, and *C. elegans* (Nakamura and Seydoux 2008). Having two distinct domains that mediate cell division and germ cell development, PEM protein ensures that proper asymmetric cell divisions occur by harmonizing both orientation of the cell division planes and germ cell fate specification.

12.3 Asymmetric Cell Divisions to Separate Endoderm and Mesoderm Cell Fates

In most bilaterians, one of the most important steps during early embryogenesis is the formation of three germ layers (Kimelman and Griffin 2000; Rodaway and Patient 2001; Davidson et al. 2008). Since fate restriction in ascidian embryos is accomplished with a small number of constituent cells, some studies have investigated the molecular mechanism responsible for separation of the mesoderm and endoderm lineages at the single-cell level (Takatori et al. 2010, 2015; Hudson et al. 2013). In the 16-cell ascidian embryo, three pairs of vegetal cells (A5.1, A5.2, and B5.1 of the 16-cell embryos in Fig. 12.1) divide asymmetrically into mesoderm daughter cells (A6.2, A6.4, and B6.2) that are located in the marginal zone and endoderm daughter cells (A6.1, A6.3, and B6.1) that are located in the vegetal pole region (Conklin 1905; Nishida 1987) (Fig. 12.1, blue bars in the 32-cell embryos).

In *Halocynthia*, asymmetrically distributed mRNA for *Not*, which encodes a conserved homeodomain transcription factor (Von Dassow et al. 1993; Utsumi et al. 2004), is preferentially partitioned into the daughter cell on the marginal side, where *Not* promotes the mesodermal fate and suppresses the endoderm fate

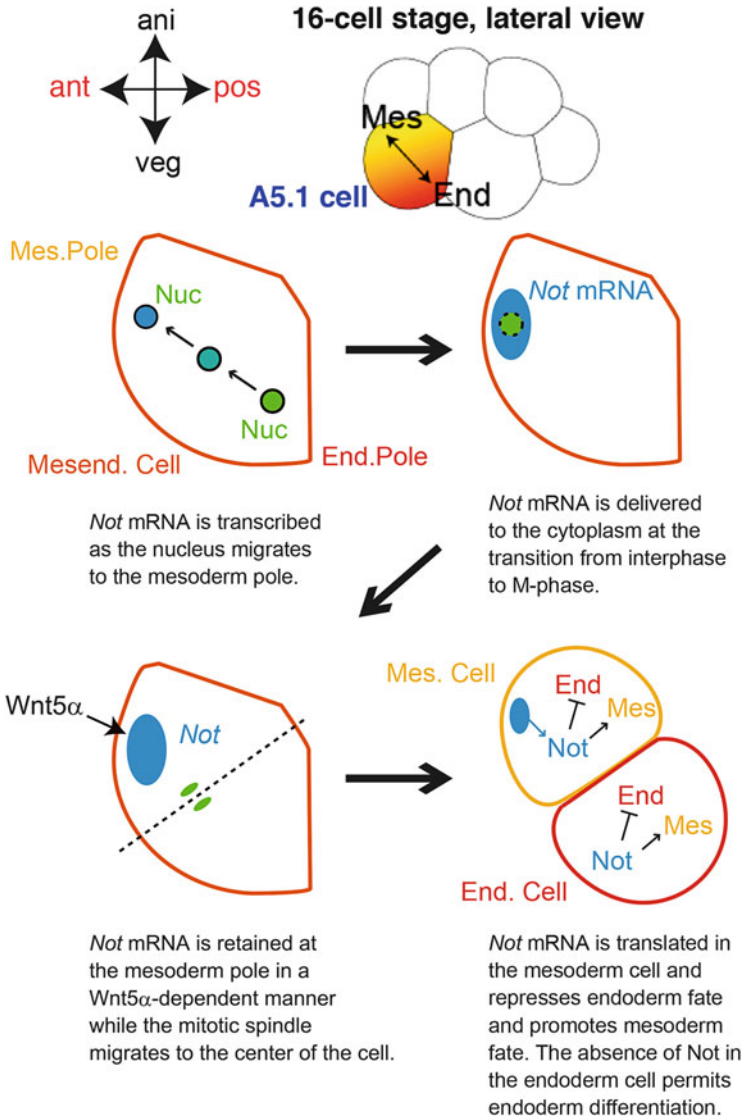


Fig. 12.5 Schematic diagram of *Not* mRNA asymmetric partitioning and mesendoderm fate segregation. Lateral view of the mesendoderm cell (A5.1 cell) from the 16- to 32-cell stage. Animal pole is up. *Not* mRNA (blue) is transcribed in the nucleus (green) as it migrates to the future mesoderm-forming side of the mesendoderm cell. *Not* mRNA is then delivered from the nucleus to the future mesoderm cell cytoplasm at the transition from interphase to M-phase. Wnt5 α -dependent mechanism retains *Not* mRNA at the mesoderm pole as the mitotic spindle repositions to the center of the cell (green) and subsequent cell division partitions *Not* mRNA to the mesoderm precursor cell. Dotted line indicates the plane of the next cell division. After the cell division, Not protein is translated in the mesoderm cell (yellow), inhibiting the endoderm fate and promoting the mesoderm fate. Absence of Not in the endoderm (red) permits endoderm differentiation. Nuc, nucleus; ani, animal; veg, vegetal; ant, anterior; pos, posterior; Mesend, mesendoderm; Mes, mesoderm; End, endoderm. Drawings have been reproduced with permission from Takatori et al. (2010, 2015)

(Fig. 12.5) (Takatori et al. 2010). *Not* mRNA segregation is coupled to transient migration of the nucleus, which is zygotically transcribing *Not* mRNA, to the future mesodermal region (mesoderm pole) of the mother cell via a microtubule network (Fig. 12.5) (Takatori et al. 2010). After delivering *Not* mRNA to the cytoplasm of the mesoderm pole, the mitotic apparatus returns to the center, and consequently the mRNA is preferentially partitioned into a mesoderm daughter cell. It has been revealed that, subsequently, the direction of displacement of the nucleus within the mother cell is determined by the localized distribution of PtdIns(3,4,5)P₃ (PIP3), a plasma membrane-bound lipid. The region of PIP3 localization is determined by localization of the PIP3-producing enzyme, phosphatidylinositol 3-kinase (PI3K). It is also intriguing that the gradient of PI3K along the animal–vegetal axis is established by ooplasmic movement within 5 min after fertilization (Takatori et al. 2015). These results indicate that, in the ascidian embryo, some processes important for germ layer segregation have already been initiated in the early fertilized egg and that the mesoderm and endoderm fates are separated into two distinct cells following a series of early cleavages.

While the cleavage pattern and fate map are well conserved among the divergent ascidian species, the molecular pathways responsible for specifying cell fates occasionally vary (Hudson and Yasuo 2008; Lemaire 2009). A study using *Ciona* and *Phallusia* has shown that the mechanism responsible for mesoderm versus endoderm fate separation differs from that in *Halocynthia*. Differential nuclear β -catenin activity coupled with two rounds of mitotic divisions along the animal–vegetal axis separates the mesoderm and endoderm fates. The ON-to-OFF sequence of nuclear β -catenin accumulation during the 16- to 32-cell stage is sufficient to induce the mesoderm fate without the involvement of *Not* asymmetric segregation (Hudson et al. 2013), although it is still unclear how nuclear accumulation of β -catenin is lost only in the mesoderm precursor cell at the 32-cell stage. The difference in molecular mechanisms of mesoderm specification between *Halocynthia* and *Ciona/Phallusia* provides a good example of the developmental system drift (DSD) (True and Haag 2001; Burgess 2011). DSD is the theory that, despite the strong conservation of developmental processes across closely related species at the morphological level, the underlying molecular mechanisms may be diverse. In this case as well as secondary muscle and secondary notochord fate induction in ascidian embryos (Hudson and Yasuo 2008; Lemaire 2009), homologous cell fates involving homologous cells can nonetheless be specified by completely different molecular mechanisms in *Halocynthia* and *Ciona*. Thus, the constraints on embryo anatomy appear stronger than those on the choice of underlying regulatory molecules. In both cases of *Halocynthia* and *Ciona/Phallusia*, there is no special regulation of cleavage plane positioning, as the cleavage plane simply follows Sach's rule and forms perpendicularly to the previous one. Accordingly, depletion of key molecules for fate specification (*Not*, PI3K, or β -catenin) does not alter the pattern of cell division (Takatori et al. 2010, 2015; Hudson et al. 2013).

12.4 Asymmetric Cell Divisions in the Marginal Zone of the Vegetal Hemisphere

12.4.1 *Localized FGF Signaling Polarizes Mother Cells that Divide into Daughter Cells with Distinct Fates*

After separation of the mesoderm and endoderm lineages, subsequent asymmetric cell divisions along the animal–vegetal axis occur radially in the mesodermal blastomeres located in the marginal region (Fig. 12.1, red bars in the 64-cell embryo) (Nishida 2005; Kumano and Nishida 2007). In these cell divisions from the 32- to 64-cell stages, the posterior marginal cells (B6.2 and B6.4 cells of the 32-cell embryo in Fig. 12.1, namely, MM cells: mesenchyme/muscle mother blastomeres, shown as red stripes on a white background in Fig. 12.6a, c) divide into the marginal side muscle precursors (colored red in Fig. 12.6b, c) and the vegetal pole side mesenchyme precursors (colored green). Likewise, in the anterior region, marginal cells (A6.2 and B6.4 cells of the 32-cell embryo in Fig. 12.1, namely, NN cells: notochord/nerve cord mother blastomeres, white in Fig. 12.6a, c) divide into the marginal side nerve cord precursors (light purple) and the vegetal pole side notochord precursors (pink). Notochord and mesenchyme are the induced fates, while nerve cord and muscle are the default uninduced fates. In this system, the inducer blastomeres, stage of induction, signaling molecules and intracellular signal transduction, spatial and temporal regulation of cellular competence, and the identities of competence factors have been extensively revealed in the last two decades.

All of these asymmetric cell divisions in the marginal region are mediated by FGF signaling. The mother cells respond to the FGF signal, but the daughter cells are no longer able to respond (Nakatani et al. 1996; Hashimoto et al. 2011). Therefore, the mother cells are polarized by localized FGF signaling (Fig. 12.6, blue arrows) and then divide asymmetrically. The same FGF signal induces notochord in the anterior region and mesenchyme in the posterior region. The difference in response is regulated by the intracellular competence factors, zygotic FoxA and Zic within the anterior mother cells and maternal macho-1 within the posterior mother cells (Fig. 12.6c) (Nishida 2005). Extracellular FGF signaling is transduced through Ras, ERK/MAPK, and ETS transcription factor (Nakatani and Nishida 1997; Kim and Nishida 2001).

In the posterior region, when FGF is applied from both sides of the mother cell, both of the daughter cells assume a mesenchyme fate, whereas abrogation of the FGF signal results in formation of two muscle cells (Fig. 12.6d). Transplantation of ectodermal cells that artificially express FGF and simultaneous suppression of endogenous FGF production can reverse the direction of asymmetric cell division (Kim et al. 2007). This clearly demonstrates that polarity of the muscle/mesenchyme mother cell is determined solely by the direction from which the FGF9/16/20 signal is presented.

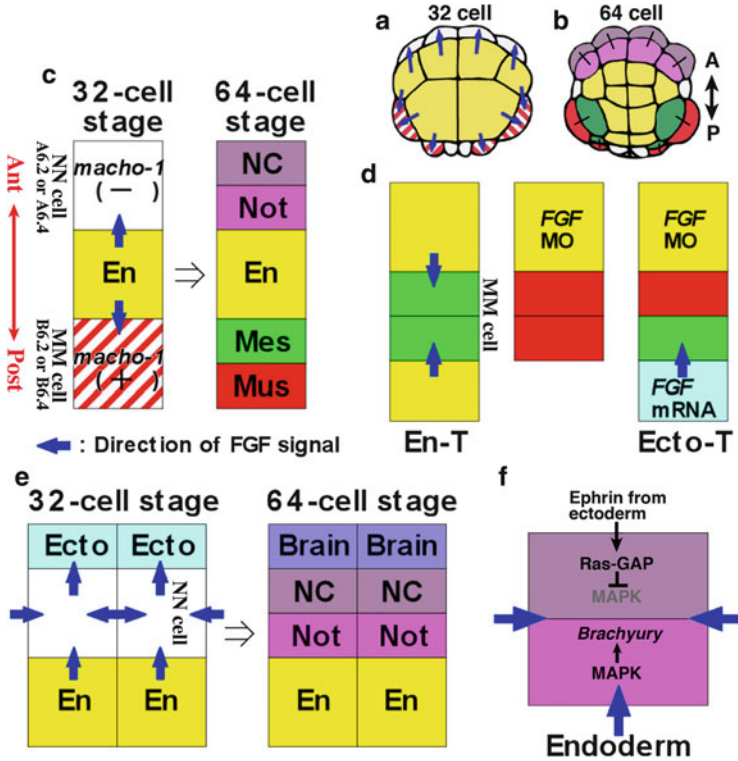


Fig. 12.6 Directed FGF signaling and asymmetric cell divisions during mesenchyme and notochord induction. (a) Position of each cell at the 32-cell stage. FGF signaling (blue arrows) takes place in the anterior and posterior margins of the vegetal hemisphere. (b) Then, asymmetric cell divisions occur to generate sister cells that assume induced and default cell fates. The bars connecting the cells show the directions of asymmetric cell divisions in the anterior and posterior regions. (c) Patterning in normal embryos shown as schematic diagrams of the arrangement of cells along the anterior–posterior axis. Cell types are highlighted by the same color code as in (b). NC, nerve cord. Not, notochord. En, endoderm. Mes, mesenchyme. Mus, muscle. At the division to the 64-cell stage, FGF-mediated asymmetric divisions take place in the anterior (NC vs. Not) and posterior (Mes vs. Mus) marginal zones. *macho-1* is a maternal and intrinsic competence factor for mesenchyme induction. (d) Experimental results confirming that the direction of asymmetry for segregation of the muscle and mesenchyme fates is determined by the direction from which the FGF signal comes. FGF MO, FGF morpholino antisense oligo-injected endoderm cell. En-T, transplanted endoderm cell. Ecto-T transplanted ectoderm cell that has been injected with *FGF* mRNA. (e) In normal embryos, FGF is also expressed in the nerve cord/notochord precursor to induce brain in the ectoderm (Ect) marginal zone. (f) Polarity of asymmetric division that produces nerve cord and notochord precursors is determined by the direction from which the inhibitory ephrin signal comes. The ephrin signal from ectoderm inhibits activation of MAPK by FGF signaling via activation of Ras-GAP on the nerve cord side and consequently results in the inhibition of the notochord-specific *Brachyury* gene. Drawings have been reproduced with permission from Nishida (2002) and Kim et al. (2007)

Similarly in the anterior marginal region, the daughter cell in which ERK/MAPK is activated adopts a notochord fate, whereas the other adopts a nerve cord fate. However, in this system, the mechanism is more complex because the notochord/nerve cord mother cells themselves express FGF in order to induce a brain fate (dark purple in Fig. 12.6e) in the adjacent ectoderm region. Polarization of the mother cells requires not only the FGF signal but also an antagonizing signal from the adjacent ectodermal cell (Kim et al. 2007). This antagonizing action is mediated by ephrin–Eph, which is better known for its roles in axon guidance. The ephrin–Eph signal attenuates ERK/MAPK activation in the nerve cord-fated daughter cell via activation of p120 Ras GAP (GTPase-activating protein) (Fig. 12.6f) (Picco et al. 2007; Haupaix et al. 2013). In combination with a directional antagonizing signal from the ectoderm, the nondirectional FGF signal polarizes the mother cell. In this case, the polarity of the notochord/nerve cord mother cell is determined by the direction from which the ephrin signal is presented. The unique feature of this system is that an ectodermal GPI membrane-bound form of ephrin ligand (Arvanitis and Davy 2008) ensures one-way signaling from the ectoderm cell to the notochord/nerve cord cells, so that ephrin does not interfere in an autocrine manner within the ectoderm with brain induction by FGF secreted from the notochord/nerve cord cells.

12.4.2 Distinct Modes of Mitotic Spindle Orientation in a Sister Cell Pair for Proper Allocation of Blastomeres

The abovementioned studies of signaling for asymmetric cell fate allocation have suggested that the polarity of asymmetric cell division is determined by the direction from which the inducing or antagonizing signal is presented. Because the division plane of the notochord/nerve cord mother cell is parallel to the previous one, this cell division does not follow Sach's rule, suggesting the presence of some form of division plane control (Figs. 12.2a, 12.6a, b and 12.7a) (Conklin 1905; Kumano and Nishida 2007). In the asymmetric division of the EMS cell of the *C. elegans* 4-cell embryo, the Wnt signal as an extrinsic cue from the adjacent posterior cells controls both the specification of cell fate and orientation of the mitotic spindle (Goldstein 1995; Rocheleau et al. 1997; Thorpe et al. 1997). In ascidians, however, depletion of FGF and ephrin signaling does not alter the direction of cell division (Kim and Nishida 2001; Kumano et al. 2006; Kim et al. 2007; Picco et al. 2007). Thus, cell fates and the division plane are regulated independently in the notochord/nerve mother cell. The mechanism of spindle orientation has been investigated using *Phallusia*, which has a transparent egg and embryo, and is able to efficiently translate mRNA injected into unfertilized eggs, making it a suitable organism for live imaging (Prodon et al. 2010). This approach has revealed that the mitotic spindle in the notochord/nerve mother cell

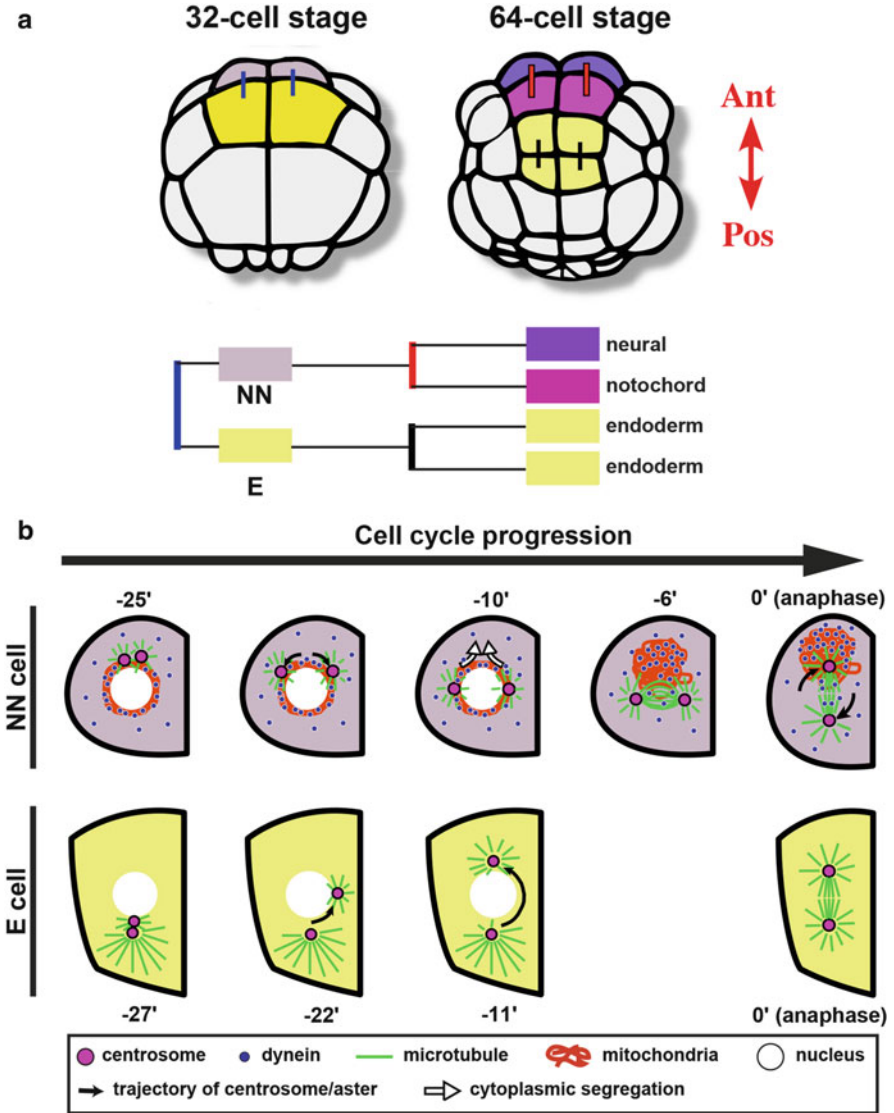


Fig. 12.7 Oriented cell division of the medial NN and E cells. **(a)** Drawings of the 32- and 64-cell-stage embryos highlighting the cell lineage of the sister cells. Embryos are in vegetal pole view with the anterior side up. Asymmetric cell division produces notochord/nerve cord mother cells (NN cell, in gray) and endodermal precursors (E cell in yellow) at the 32-cell stage. The first division axis is parallel to the anterior–posterior axis. These two daughter cells then divide again along the anterior–posterior axis to generate one nerve cord precursor (purple), one notochord precursor (magenta), and two endoderm precursors (yellow) at the 64-cell stage. Green bars indicate a pair of sister blastomeres. **(b)** Model of the mechanisms of spindle orientation in NN and E cells. Time is indicated with 0 min corresponding to the beginning of anaphase. In the NN cell, the centrosome is duplicated on the anterior side of the nucleus. The centrosomes migrate 90° in opposite directions and result in formation of the mitotic spindle orthogonal to the A–P axis. In

(NN cell in Fig. 12.7a) rotates 90° to align along the animal–vegetal axis (Fig. 12.7b) (Negishi and Yasuo 2015). This spindle rotation is coupled with segregation of the mitochondria toward the marginal end (Zalokar and Sardet 1984; Takatori et al. 2010; Kobayashi et al. 2013). It is likely that the dynein motor protein accumulated in the mitochondria-rich region exerts pulling force on both poles of the spindle via microtubules (Fig. 12.7b, arrows in NN cell) (Negishi and Yasuo 2015). This idea is supported by a series of inhibitor treatments: (1) low doses of the microtubule inhibitor (nocodazole) impairs the rotation of spindle, while allowing normal segregation of mitochondria; and (2) treatment with the two dynein inhibitors (erythro-9-[3-(2-hydroxy-nonyl)]adenine and Ciliobrevin A) after mitochondrial segregation blocks the spindle rotation (Negishi and Yasuo 2015). The eventual selection of which of the two spindle poles is actually pulled is merely random. In addition, the spindle orientation is cell-autonomous process because segregation of mitochondria and the spindle rotation occur in the isolated cells from embryos (Negishi and Yasuo 2015).

The anterior midline endoderm precursor cell of the 32-cell embryo (E cell in Fig. 12.7a), which is a sister cell of the notochord/nerve mother cell, also divides contrary to Sach's rule. During this cell division, the duplicated centrosomes show asymmetric migration in interphase and then the mitotic spindle forms along the animal–vegetal axis from the beginning of M phase (Fig. 12.7b) (Negishi and Yasuo 2015). After duplication, one centrosome stays on the vegetal side and the other migrates to the animal side of the nucleus. This contrasts with the notochord/nerve cord mother cell, where the duplicated centrosomes migrate symmetrically and the spindle rotates to align along the animal–vegetal axis. This asymmetric centrosome behavior is reminiscent of that in male germline stem cells and in neuroblasts of *Drosophila* (Yamashita et al. 2003; Rebollo et al. 2007; Yamashita and Fuller 2008), and indeed, in the ascidian anterior midline endoderm precursor cell, the centrosome with a larger aster becomes immotile, similar to these examples in *Drosophila*. Interestingly, ablation of the neighboring cells of ascidian embryos suggests that cell shape may direct asymmetric centrosome migration (Negishi and Yasuo 2015). Although both daughter cells of the anterior midline endoderm precursor cell assume the same endodermal fate, proper spatial arrangement of endodermal cells may be important for gastrulation when these cells play a major role in morphogenesis (Sherrard et al. 2010).

Fig. 12.7 (continued) this cell, cytoplasmic dynein and mitochondria, which are initially distributed around the nucleus, segregate toward the anterior side in an actomyosin-dependent manner. This enrichment of cytoplasmic dynein exerts the pulling force for rotation of the spindle in the NN cell. In the E cell, the duplicated centrosomes are first positioned at the posterior side of the nucleus. After duplication, the centrosome having a larger aster shows less movement, and the other migrates to the opposite side of the nucleus. The spindle then forms along the A–P axis from the beginning of M phase. Images have been reproduced with permission from Negishi and Yasuo (2015)

12.5 Perspectives

Ascidian early embryogenesis involves several unequal and asymmetric cell divisions, with or without regulation of division plane control. In some cases, prelocalized maternal factors are involved. In endoderm and mesoderm segregation, nuclear migration and asymmetric partitioning of the transcript play a role. These are controlled by the intrinsic and cell-autonomous factors. In other cases, directed extracellular signals induce asymmetric allocation of cell fates. In unequal divisions of germline lineage cells, cell fate specification and division plane orientation are coupled by the CAB. In endoderm and mesoderm segregation, there is no specific regulation of the division plane. In other cases, cell fate specification and division plane orientation are regulated independently. Thus, various modes of asymmetric cell division may operate, even in the early developmental stages of any single organism. A specific series of the stereotypic asymmetric cell divisions allows ascidian embryos to establish reproducible cell fates from a small number of constituent cells.

In ascidian embryos, the functions of the two major protein complexes involved in asymmetric cell division and orientation of the division axis in a wide range of animals have not been elucidated. Although the aPKC–PAR3–PAR6 complex (Kemphues et al. 1988; Tabuse et al. 1998; Kemphues 2000; Ohno 2001; Macara 2004) is conserved in bilaterians and is localized to the CAB in ascidians (Patalano et al. 2006), its function is still unclear. It has only been confirmed recently that this complex has a role in the tubulogenesis of ascidian notochord cells (Denker et al. 2013). Another major complex, LGN–NuMA–G α i, has also been shown to have a highly conserved role in asymmetric cell division and spindle orientation in many animals (Schaefer et al. 2000; Du and Macara 2004; Couwenbergs et al. 2007; Nguyen-Ngoc et al. 2007). In ascidians, however, its role has not yet been elucidated. Although LGN and G α i are apparently present in ascidian genomes, a simple BLAST search fails to identify NuMA ortholog because of low degree of conservation in the sequences of NuMA proteins across taxa.

In addition to the three kinds of asymmetric cell divisions described in this chapter, there are two other examples that have been investigated in ascidian embryos. Both of them involved unequal cell divisions and the two daughter cells assume distinct cell fate. First, precursors of the secondary notochord cell are generated at the 110-cell stage. The precursor is smaller than its sister cell that assumes mesenchyme fate. FGF and BMP sequentially induce the secondary notochord in *Halocynthia*, while Nodal and Notch are players in the relay in *Ciona* (Darras and Nishida 2001; Hudson and Yasuo 2006). However, it is not known whether these extracellular signals polarize the mother cell prior to cell division and promote genuine asymmetric cell division. Unlike notochord and mesenchyme induction described in the fourth section in this chapter, the system is not a simple binary cell fate switch because the notochord progenitor does not adopt its sister fate (mesenchyme) in Nodal or Notch signaling-inhibited embryos in *Ciona* (Hudson and Yasuo 2006). It is currently not known whether the unequal

cleavage is affected in those embryos. Second, asymmetric cell divisions that generate heart precursor cells take place at late neurula stage. Each of four cardiac founder cells divides into a smaller daughter (heart precursor) and a large daughter (tail muscle precursor). The mother cells are exposed uniformly to FGF, which is required for heart precursor fate, but matrix adhesion on ventral side of the cell polarizes the cell. In free plasma membrane, FGF receptors are internalized and degraded, while these processes are suppressed in adherent membrane, resulting in elevated levels of FGF signaling. In addition, membrane remodeling mediated by Caveolin enhances the receptor enrichment at the adherent membrane. The mechanism mediates both asymmetric fate specification and positioning of cell division plane (Davidson et al. 2006; Cooley et al. 2011; Cota and Davidson 2015). On the other hand, detailed quantification of the volume of every blastomere at each stage of *Ciona* embryo development has revealed other cases of unequal cell division, which do not involve the CAB (Tassy et al. 2006). Further investigation of the mechanisms and functions of these newly found unequal cell divisions would be intriguing.

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