Chapter 4
Vertebrate Embryonic Cleavage Pattern Determination

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Abstract The pattern of the earliest cell divisions in a vertebrate embryo lays the groundwork for later developmental events such as gastrulation, organogenesis, and overall body plan establishment. Understanding these early cleavage patterns and the mechanisms that create them is thus crucial for the study of vertebrate development. This chapter describes the early cleavage stages for species representing ray-finned fish, amphibians, birds, reptiles, mammals, and proto-vertebrate ascidians and summarizes current understanding of the mechanisms that govern these patterns. The nearly universal influence of cell shape on orientation and positioning of spindles and cleavage furrows and the mechanisms that mediate this influence are discussed. We discuss in particular models of aster and spindle centering and orientation in large embryonic blastomeres that rely on asymmetric internal pulling forces generated by the cleavage furrow for the previous cell cycle. Also explored are mechanisms that integrate cell division given the limited supply of cellular building blocks in the egg and several-fold changes of cell size during early development, as well as cytoskeletal specializations specific to early blastomeres

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including processes leading to blastomere cohesion. Finally, we discuss evolutionary conclusions beginning to emerge from the contemporary analysis of the phylogenetic distributions of cleavage patterns. In sum, this chapter seeks to summarize our current understanding of vertebrate early embryonic cleavage patterns and their control and evolution.

Keywords Blastomere • Spindle orientation • Cleavage plane determination • Aster centering • Scaling • Cytoskeleton • Compaction • Cell cleavage type • Evolution

4.1 Introduction

The pattern of early cell divisions in vertebrate embryos varies widely. It is important to understand this patterning and its origin, since, in most organisms, the arrangement of cells resulting from the early cleavages is responsible for generating the earliest features of the embryo's body plan. The cleavage pattern of the early embryo, and the arrangement of cells that results, generates the early embryonic anatomy. During activation of zygotic gene expression at the midblastula transition (MBT), new gene expression initiates new patterns of cell behavior, generating morphogenetic movements that further modify the embryo. However, early cleavage pattern constrains subsequent developmental processes. Moreover, many cellular decisions, including axis induction, germ layer specification, and germ cell formation, occur prior to MBT. The early embryonic anatomy, which originates from the interaction between the initial egg structure and dynamic processes driven by maternally inherited components, must facilitate, or at least be compatible with, such inductive processes.

Various factors, such as embryo size, patterns of yolk deposition (in nonmammalian vertebrates), the symmetry of yolk deposition with respect to oocyte polarity, localization of molecular cues, and cell shape, influence patterns of cell division. The resulting cellular assembly in combination with inductive processes lays the foundation for embryonic morphogenesis. This chapter addresses these cellular and molecular processes, which generate this late blastula architecture, and their relation to the early pattern of the embryo. We explore some of the variety in vertebrate embryonic cleavage patterns and discuss processes involved in their creation. We first examine the two main classes of embryonic cleavage in vertebrates (meroblastic and holoblastic cleavage). Later, we describe cellular mechanisms required for furrow placement during early embryonic development, an essential factor in generating the early embryonic cleavage pattern. We also address additional cellular and developmental mechanisms underlying morphological landmarks of the embryo, such as the formation of specialized cytoskeletal structures in large embryonic cells and cellular compaction, as well as the regulated use of maternal building blocks in cleaving embryonic cells. We also summarize current knowledge on patterns and underlying molecular cues in other vertebrates, including mammals, and in a well-studied proto-vertebrate system.

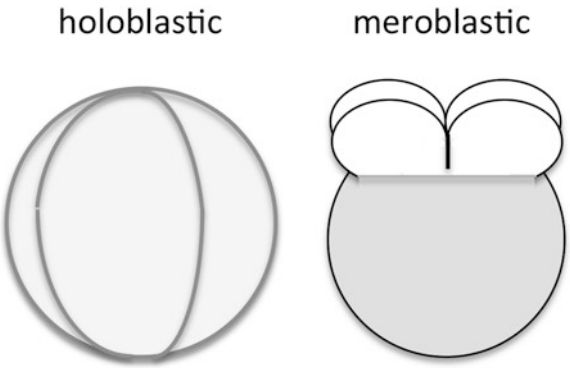

Fig. 4.1 Types of embryonic cleavage. Holoblastic cleavage encompasses the entirety of the embryo, involving meridional planes that cleave through the animal and vegetal poles of the embryo. Meroblastic cleavage involves an early separation between cells at the animal pole and yolk at the vegetal pole of the egg, and cell furrows encompass only the animal-most region of the embryo, leaving the yolk intact

4.2 Main Patterns of Embryonic Cleavage in Vertebrates: Holoblastic Versus Meroblastic

Early embryonic cell division patterns in vertebrates can be broken into two broad categories, holoblastic cleavage (e.g., most amphibians and mammals) and meroblastic cleavage (e.g., birds, reptiles, and teleost fishes) (Fig. 4.1). In holoblastic cleavage, the entire egg undergoes cellularization, and yolk platelets are either absent (e.g., in mammals) or present as cytoplasmic inclusions that partition among cells (e.g., in amphibians). Cleavage furrows encompass the embryo completely, from the animal pole (corresponding to the region containing the meiotic spindle in the oocyte) to the vegetal pole. In meroblastic cleavage, in contrast, cell division does not divide the embryo in its entirety. Instead, embryonic cells divide in the animal pole independently of the vegetally located yolk, with cells typically remaining syncytial to the yolk cell for a period of time that varies among species. We would like to point out that pure holoblastic or meroblastic cleavage patterns are only idealized extremes. We will discuss that many embryos cleave with an intermediate geometry, in which the entire embryo cleaves but the strong asymmetry of yolk leads to a bias of cleavage planes. In any of these cases, dividing cells are called blastomeres regardless of whether they contain yolk.

In all vertebrates, determination of the animal and vegetal poles of the egg develops during oogenesis, with the oocyte typically containing a pre-existing animal region with distinct properties (see Chap. 5). This animal region will contain the nuclear DNA after fertilization and, in embryos with meroblastic cleavage, accumulates yolk-free ooplasm after fertilization. The vegetal region of the egg, in addition to acting as the site for yolk storage in meroblastic species, is also an essential player in early embryogenesis, containing signals involved in early axial patterning

and cell specification that are transmitted to blastomeres (see Chaps. 6–8). In the following sections, we explore the factors that govern holoblastic and meroblastic cleavage patterns, especially the substantial variation observed within these two broad categories.

4.3 Cellular Mechanisms Underlying Cell Cleavage Pattern Determination

The pattern of early cell division in an embryo influences parameters of the forming blastula, such as the number of cells, depth of stacking within a cell layer, and relationship to extraembryonic spaces and structures. The cleavage pattern depends on cell division planes in individual blastomeres. Here, we address cellular mechanisms that influence the furrow plane and which lead to the cellular arrangement of the early embryo.

4.3.1 Induction of Cell Cleavage Plane by Chromatin and Amphiasters

Typically, the cell divides perpendicular to its longest axis into two equal daughter cells. At the core of this process is the spindle apparatus (Fig. 4.2) with the two microtubule asters (amphiasters) emanating from its centrosomes. The spindle apparatus is an arrangement of microtubules that organize around the microtubule-organizing centers (MTOCs) and the DNA during M phase of the cell cycle and is responsible for chromosome segregation during cell division. Kinetochore microtubules connect the spindle pole to chromosomes. The microtubules between the poles that are not connected to the kinetochore are called interpolar microtubules. While we currently know little about microtubule length distribution in the mitotic spindles of early development, in the closely related meiosis II spindles, interpolar microtubules are very short compared to spindle length (Needleman et al. 2010). Another set of spindle-associated microtubules are astral microtubules, which emanate outward from each of the MTOCs in a radial fashion. Typically, microtubule themselves are arranged with their minus ends pointing toward the closest MTOC in the spindle pole. As described below, signals both from chromatin at the spindle midzone and from astral microtubules are involved in furrow induction and therefore cleavage plane determination.

Early observations indicated a correlation between the appearance of the furrow plane and the location of the spindle apparatus, with the furrow forming perpendicular to the spindle at a location equidistant from the spindle poles (Hertwig 1893). This plane normally corresponds to that of aligned chromosomes during the preceding metaphase, as well as the region where astral microtubules overlap.

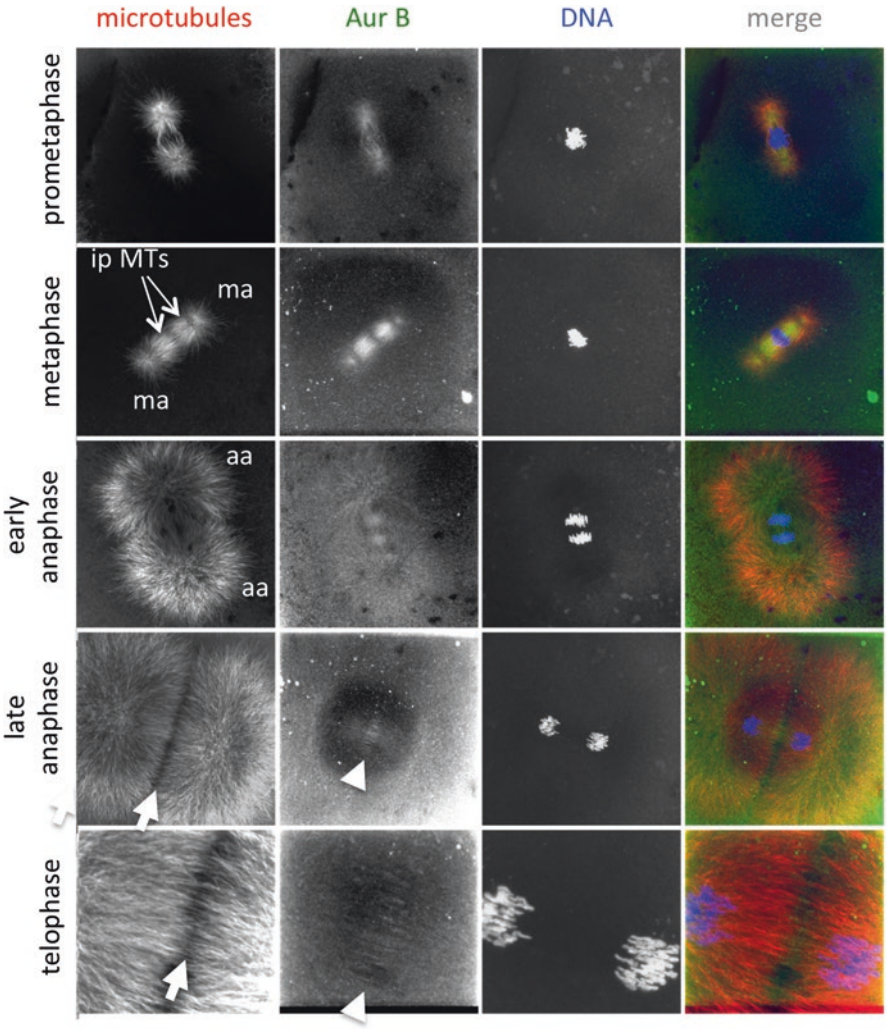

Fig. 4.2 Dynamic changes in spindle morphology during the cell cycle. The spindle apparatus begins to form before metaphase. During metaphase, interpolar microtubules (ipMTs) link the spindle poles, and short metaphase asters (ma) form that are too short to reach the cortex (Wühr et al. 2010). Asters during anaphase appear to be much more developed, reaching a wider radius. A zone of microtubule exclusion, the microtubule interaction zone, forms in the region in which asters meet and which presages the furrow (arrows). AurB, a CPC protein, shows recruitment of this complex to the spindle midzone (arrowhead at late anaphase) and at the tip of FMA tubules in more distal regions of the furrow (arrowhead in telophase; see also Yabe et al. 2009). A zone of microtubule exclusion, the microtubule interaction zone, forms in the region in which asters meet and which presages the furrow (arrows). Microtubules in red, AurB in green, DNA in blue. Figure adapted from Yabe et al. (2009)

Chromosomes therefore can typically be separated equally between the two daughter cells. Analyses of various cell types have shown furrow-inducing activity in midzone microtubules of the spindle (Martineau et al. 1995; Cao and Wang 1996). Studies in amphibians show that neighboring asters induce a cleavage furrow, but only if chromatin is located between them (Brachet 1910), showing a key role for a chromatin-derived factor in furrow induction. However, experimental manipulations of sand dollar embryos by Rappaport, which created a barrier between the poles of a forming spindle to force overlap of normally nonadjacent asters in other regions of the blastomere, showed the induction of an ectopic furrow in the region of overlap in the absence of nearby chromatin, confirming a role for astral microtubules in furrow induction (Rappaport 1961, 1996). These experiments indicate that signals present in both the midzone and astral microtubules contribute to furrow induction (Rappaport 1996; Mishima 2016). The degree to which one of these two mechanisms alone is able to induce a cleavage furrow varies between different species (Brachet 1910; Rappaport 1961; Rappaport and Rappaport 1974; Su et al. 2014; Field et al. 2015). Because the location of the midzone and that of astral microtubule overlap typically occur in the same position, both of these structures act together to establish a robust positioning mechanism to place the furrow at a plane halfway between spindle poles.

The large cells of some embryos like fish or amphibians require some special adaptations related to spindle structure and function. A major adaptation involves aster morphology. In smaller cells, nearly all microtubules' minus ends are believed to be close to the centrosome. However, such an arrangement, due to its radial nature within the volume of the blastomere, would lead to severely reduced microtubule densities near the cortex in very large cells. Instead, large cells such as the zebrafish and *Xenopus* embryonic blastomeres implement an alternative strategy, in which sites of microtubule nucleation are evenly distributed throughout these large asters (Figs. 4.2 and 4.4). The implementation of these internal microtubule nucleation sites results in microtubule density remaining near constant, independent of distance from the aster center (Wühr et al. 2009). The induction of microtubule nucleation sites within the aster can be explained with a chemical trigger wave that relies on microtubule-dependent nucleation (Ishihara et al. 2014).

Another major adaptation of asters which is particularly apparent in very large embryos is the formation of an aster–aster interaction zone, a region depleted of microtubules at the site of overlap between adjacent asters (Wühr et al. 2010; Nguyen et al. 2014; Figs. 4.2 and 4.4). As described below, this interaction zone seems to enable to communicate the proper plane for cell division from the mitotic spindle apparatus to the cell cortex, which can be separated by several hundred micrometers. Furthermore, the interaction zone preempts the barrier of the future cleavage plane allowing the aster to center and orient along the longest axis of the future daughter cell before it actually exists.

Analysis of the function of the Chromosomal Passenger Complex (CPC) component Aurora kinase B (Aur B) in zebrafish embryos provides additional insight into this redundancy as it applies to the large embryonic cells (Yabe et al. 2009). Embryos from females homozygous for a maternal-effect mutant allele in the gene *cellular*

island, which encodes Aur B, exhibit defects in furrow formation. However, in these embryos, cytoskeletal structures associated with the furrow (see below) appear to be induced relatively normally in the center of the blastomere, whereas they are entirely absent in more distal regions of the furrow. As expected, Aur B protein in these embryos is found both at the spindle midzone and the tips of astral microtubules that contact the forming furrow (Fig. 4.2). Genetic analysis of the *Aur B* maternal-effect *cellular island* mutant allele indicates that it retains some functional activity, as homozygotes for complete loss of function alleles are zygotic lethal and do not survive to adulthood, in contrast to homozygotes for the maternal-effect mutant allele. A comparison of the maternal-effect *cellular atoll* phenotype, which allows formation in the medial furrow region, with the effects of a specific Aur B inhibitor, which cause furrow inhibition throughout the furrow, suggests that the partial activity in the maternal-effect *cellular island* allele is provided by Aur B function present in the spindle. Such spindle-provided Aur B may be at a higher concentration or have a higher functional activity than that present in astral microtubule ends. Consistent with this interpretation, embryos maternally mutant for both *futile cycle*, which fails to form spindle structures, and *cellular island* lack furrow-associated structures throughout the length of the furrow, in both medial and distal regions. Altogether, these observations suggest that, in large embryonic blastomeres, CPC activity and potentially other signals from astral microtubules are essential for furrow induction in distal regions of the cell, which are presumably too distant to be influenced by inducing signals from chromatin present at the spindle midzone (Figs. 4.2 and 4.4). Indeed, in several amphibians, early cleavage furrow advance may depend on signals propagated solely through the cortex via the furrow's distal ends (Sawai 1974, 1980; Mabuchi et al. 1988; Sawai and Yomota 1990). These spatial functional specializations of furrow-inducing activity may be a necessary adaptation to the small coverage of the spindle relative to the much larger embryonic blastomeres.

Furrow-inducing activity from the spindle midzone and astral microtubules is concentrated at the point halfway between the spindle poles, and in a plane perpendicular to that of the spindle, thus functionally linking cleavage patterning to the mechanistic determinants of spindle orientation within dividing blastomeres. This linkage will be discussed in the next section.

4.3.2 Centering and Orienting Asters and Spindles

For over 125 years, scientists have known that mitotic spindles tend to align along the longest axis of a dividing cell and that cleavage furrows tend to form perpendicular to the mitotic spindle (Hertwig 1893). The tendency holds even when cell shape is deliberately manipulated to change the orientation of a cell's longest axis. In the original manipulations, artificially elongating frog embryos by compression generated a reorientation of the cleavage plane consistent with realignment of the spindle (Pflüger 1884; Hertwig 1893; Black and Vincent 1988). This phenomenon,

named Hertwig's rule, has been consistently observed in numerous cell types, including both embryonic and somatic cells, in a variety of organisms including vertebrates, invertebrates, and unicellular organisms. The sensitivity of spindle orientation to cell shape underlying Hertwig's rule has been explained by interactions between astral microtubules and the cell cortex, thought to generate forces that become balanced and energetically favorable when the spindles become aligned to the long axis of the cell (Bjerkness 1986; Grill and Hyman 2005; see below).

An important question to understand cleavage plane determination is how astral structures sense cellular geometry. Spindles may sense cell shape through their asters, which extend outward in a radially symmetric manner and are therefore ideally suited to sense intracellular surroundings and the cortex. However, in some large embryos, astral microtubules of the spindle are too short to reach the cortex. In these cells, the task of sensing cellular geometry is performed by the cell-spanning anaphase asters of the preceding cell cycle. For the first cell cycle, the task is performed by the sperm-aster, a mono-aster that forms in the zygote immediately after fertilization (Chambers 1939).

4.3.2.1 The Role of the Sperm-Aster

In most vertebrate lineages after fertilization, centrioles are inherited through the sperm, having been lost during oogenesis. This arrangement is thought to be essential to maintain a constant number of centrioles from one generation to the next (reviewed in Delattre and Gönczy 2004). Surprisingly, however, there are numerous variations on this general theme. Sperm may bring a pair of centrioles, a pair with an incomplete centriole, or a single centriole. In the latter two cases, biogenesis of the second centriole is completed in the zygote after fertilization. Sperm-derived centriolar pairs are thought to act as a template to mediate the reconstitution of a centrosome by nucleating maternally derived centrosome components (Lessman 2012). During interphase of the first cell cycle, this centrosome acts as an MTOC to generate the structure with a single aster, termed the sperm-aster.

The primary function of the sperm-aster is to mediate pronuclear fusion. During fertilization, the maternal pronucleus is formed after reinitiation of meiosis II triggered by egg activation, whereas the paternal pronucleus is introduced by the sperm. After fertilization, the paternal pronucleus remains in close proximity to the centrosome, and the sperm-aster is required for the movement of the maternal pronucleus toward the MTOC and closely associated paternal pronucleus. This movement occurs through the movement of the maternal pronucleus toward the microtubule minus end at the MTOC at the center of the sperm-aster. This movement has been shown in a number of vertebrate species (Chambers 1939; Navara et al. 1994), to be mediated by transport via the minus-directed microtubule-based motor dynein (Reinsch and Karsenti 1997).

In most vertebrates, multiple layers of regulation are in place to inhibit fertilization by more than one sperm (Just 1919). If polyspermy is induced artificially, each sperm produces its own sperm-aster (Brachet 1910). The asters space each other out

in the embryo and multiple mitotic spindles each induce their own cleavage furrow. This condition is lethal for the embryo. However, in most urodeles (newts and salamanders), polyspermy is the natural mode of fertilization (Fankhauser 1932). Although each sperm gives rise to its own sperm-aster, only the one sperm-aster that reaches the female pronucleus becomes dominant to span large parts of the cell. The other sperm-asters disintegrate. It seems necessary that the DNA of the supernumerary asters must also somehow be destroyed later on to prevent polyploidy of subsections of the embryo. The molecular mechanisms underlying this fascinating phenomenon are not understood.

In the zebrafish, the sperm-aster has also been shown to facilitate the multimerization of maternally inherited ribonucleoparticles (RNPs) that confer the germ cell fate, termed the germplasm. This appears to be achieved by the action of sperm-aster ends on an actin-based network associated with these RNPs, where the radial growth of sperm-aster microtubules generates a wave of RNP aggregation (Theusch et al. 2006; Nair et al. 2013), in anticipation of their accumulation at the furrows produced during the early cell divisions (Eno and Pelegri 2013; reviewed in Eno and Pelegri 2016). A similar function for the sperm-aster in germplasm RNP multimerization in other model systems has not yet been shown, although in some systems, such as in frogs and chicken, germplasm RNPs exhibit patterns of accumulation at the furrows that are similar to those occurring in the zebrafish (see Chap. 8).

4.3.2.2 Nuclear–Cytoskeletal Attachment During Nuclear Fusion

In the zebrafish, transport of the paternal pronucleus along the sperm-aster has also been shown to require the function of another maternal-effect gene, *futile cycle/lrmp* (Lindeman and Pelegri 2012). This gene encodes a KASH-domain protein with gene products localized to the nuclear envelope of maternal and paternal pronuclei. This protein is thought to mediate a link between the outer nuclear membrane and the microtubule cytoskeleton. Interestingly, the *futile cycle/lrmp*-dependent nuclear–microtubule connection is also required to maintain the close attachment of the paternal pronucleus to the centrosome at the sperm-aster MTOC (Lindeman and Pelegri 2012). Thus, attachment of the outer nuclear membrane to the cytoskeleton is essential for both long-range transport of the maternal pronucleus toward the MTOC and local attachment of the paternal pronucleus to the MTOC. This coordinated set of processes drives the movement of the maternal pronucleus toward the MTOC while keeping the paternal pronucleus close to this structure, thus mediating the encounter of both pronuclei leading toward their fusion.

Nuclear envelope–cytoskeletal interaction likely continues throughout early embryonic blastomere divisions, as suggested by the highly localized pattern for futile cycle/lrmp proteins at the nuclear membrane–centrosome interphase (Lindeman and Pelegri 2012). This nuclear–centrosomal linkage, coupled to the centering of the (aster-containing) spindle apparatus, guarantees the even distribution of chromatin content among the newly formed blastomeres.

The process of pronuclear fusion is additionally integrated with aster centering. In amphibians, sperm entry occurs at random locations in the animal hemisphere, generating an immediate need for aster centering. Even in zebrafish, where the mature egg contains a sperm entry site at the approximate center of the animal pole that will constitute the future blastodisc area, the aster still likely centers itself along the depth of the forming blastodisc (see below). Below, we describe how aster centering is achieved in a large embryonic cell.

4.3.2.3 Tug-of-War Forces and Aster Centering

Early studies showed growing microtubules can generate pushing forces against an object (Hill and Kirschner 1982), which suggested a mechanism for aster (and bipolar spindle) centering in small cells such as yeast (Tran et al. 2001). However, the larger size of most metazoan cell types necessitates longer astral microtubules in order to contact the cortex, which, due to their greater length and greater tendency to undergo buckling, limit the force that might be transmitted through a pushing mechanism (Dogterom et al. 2005). Instead, in these cells, aster centering has been hypothesized to depend on pulling forces. Indeed, experiments in fertilized sand dollar eggs showed that generating a zone of microtubule polymerization (by localized inactivation of the microtubule inhibitor colcemid) generates movement toward the zone of polymerization (Hamaguchi and Hiramoto 1986), not away, as would have been predicted by a pushing model for aster centering (Fig. 4.3a). This led to a model in which astral microtubules are centered by pulling, rather than pushing forces, a mechanism that was subsequently supported by experiments in *Caenorhabditis elegans* and yeast (Bukarov et al. 2003; Grill et al. 2003; Grill and Hyman 2005). Consistent with the colcemid-inhibition experiments in sand dollar embryos (Hamaguchi and Hiramoto 1986) and a microtubule pulling model, the converse experiment, involving partial aster depolymerization via UV-mediated uncaging of the microtubule inhibitor combretastatin 4A in early zebrafish embryos, results in spindle movement away from the site of depolymerization (Wühr et al. 2010; Fig. 4.3b).

These pulling interactions were assumed to occur between microtubules and the cell cortex (Dogterom et al. 2005; Grill and Hyman 2005; Kunda and Baum 2009). Due to astral microtubule orientation, with minus ends at the aster center and plus ends facing away, such pulling forces could be mediated by the minus end-directed microtubule-based motor dynein (reviewed in Kotak and Gönczy 2013). However, pulling from the cortex under most circumstances will not lead to aster centering but rather MTOC pulling close to the cortex. Grill and Hyman suggested a scenario in which a limited concentration of cortical dynein compared to the number of plus-end microtubules reaching the cortex could lead to stable aster centering (Grill and Hyman 2005).

Nevertheless, studies have suggested that, at least in large cells such as those in early zebrafish and *Xenopus* embryos, dynein is not exerting a force by pulling from the cortex but rather by anchoring of astral microtubules to internal elements of the

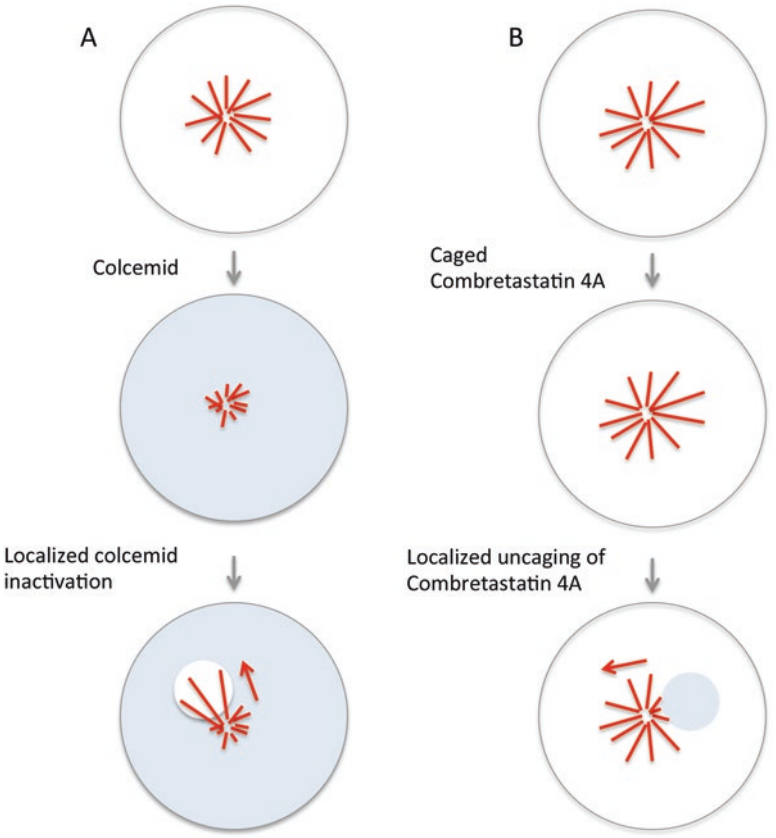

Fig. 4.3 Demonstration that asters are centered by pulling, not pushing, forces. (**a**) Treatment of embryos with colcemid leads to inhibition of microtubule polymerization, and localized inactivation of colcemid with UV light results in the movement of the aster toward the site of microtubule growth (Hamaguchi and Hiramoto 1986). (**b**) Local uncaging of the microtubule inhibiting drug combretastatin 4A results in the movement of the aster away from the site of microtubule inhibition (Wühr et al. 2010). A model in which asters become centered by microtubule pushing forces predicts the opposite effects on aster movement and is ruled out by observations

cytoplasm. This was initially suggested by the observation that both the sperm-aster after fertilization and bipolar spindles during cleavage become centered within the cell before astral microtubules reach the cortex. Similarly, in the abovementioned microtubule inhibitor uncaging experiment (Wühr et al. 2010), spindle movement away from the site of local microtubule depolymerization occurred before the aster reached the cortex. As in cultured cells, interference with dynein function in zebrafish blastomeres (in these experiments through injection of p150-CC1, a dominant-negative form of the dynein partner dynactin) results in spindles that are uncentered as well as misoriented (Wühr et al. 2010). A similar requirement for dynein function was demonstrated for the centering of the sperm-aster immediately after fertiliza-

tion in *Xenopus* eggs (Wühr et al. 2010). These studies suggest that, in zebrafish and *Xenopus*, dynein-dependent pulling forces are required for the centering of astral microtubules during pronuclear fusion and blastomere divisions. Because in these species the centering and experimentally induced movements occur prior to astral microtubules contact with the cortex, the pulling force is unlikely to be generated at the cortex, but is instead generated along presently unknown internal elements of the cytoplasm. Knockdown experiments in *C. elegans* suggest that, in this system, vesicles are likely the anchor for cytoplasmic dynein to generate pulling forces on microtubules (Kimura and Kimura 2011). Distribution of such internal anchors through the cytoplasm could result in a length-dependent force, such that longer microtubules contribute larger pulling forces than shorter ones, resulting in net aster movement. A similar model for microtubule length-dependent pulling depending on cell volume (i.e., internal stores) as opposed to cell surface has been derived through cell shape manipulations and mathematical modeling (Minc et al. 2011). Thus, although pulling from cortical anchors has been documented to orient the spindle in smaller differentiated cells (McNally 2013), large embryonic blastomeres appear to use a tug-of-war pulling mechanism from internal sites, whose consequences for embryonic cleavage patterning are described below.

4.3.3 Mechanisms Underlying Spindle Orientation in Large Embryonic Cells in Fish and Amphibians

Two model systems, zebrafish and *Xenopus*, provide insight on the mechanisms that drive cleavage patterning in early vertebrate embryos with large blastomeres. Embryos from these species exhibit similar behaviors with regard to the spatial arrangement of the spindle and associated DNA in relation to the blastomere center. Due to the small size of the spindle relative to the large size of the blastomere, each of the resulting nuclear masses is at the end of anaphase in a location relatively close to the previous furrow, off-center with respect to the newly formed daughter blastomeres. Thus, in preparation for the next cell division, an important initial requirement is the centering of the forming spindle within the daughter blastomeres. Additionally, the cell cleavage plane in these embryos is known to alternate with each cell division, with each cleavage plane at a 90° angle relative to that of the previous one.

4.3.3.1 Spindle Orientation Based on Cell Geometry Can Be Overridden by Molecular Cues

As described above, aster-mediated forces influence the position of both sperm-asters and amphiasters emanating from bipolar spindles. As will be discussed shortly, in the case of bipolar spindles, stress forces and/or coordinate centering of

astral microtubules at each of the spindle poles is thought to additionally contribute to the alignment of the spindle within the cell. In smaller cells, such alignment is clearly influenced by interactions of the cell boundaries and the metaphase spindle, according to Hertwig's rule of alignment with the longer cell axis. The idea that astral centering occurs by internal pulling forces can be reinterpreted in this context, since forces on MTOCs and the spindle structure will be dependent on the length of astral microtubules emanating from these structures. Since the cortex acts as a barrier to microtubule growth, the long axis of the cell allows for longer microtubule growth and consequently stronger internal pulling forces, which contribute to spindle alignment along this axis. In this manner, cell shape can influence the orientation of the spindle.

The general principle of cells forming a cleavage furrow perpendicular to their longest axis can be overridden by molecular cues, where mechanisms of spindle orientation are influenced by asymmetrically distributed factors (reviewed in Sousa-Nunes and Somers 2013; Williams and Fuchs 2013; Rose and Gönczy 2014; Schweisguth 2015). This mechanism tends to be most common during the division of polarized cells, such as those found in tissue epithelia and cells that are beginning to differentiate into specific lineages. This phenomenon has been observed in some cell fate determination systems that depend on asymmetric cell division mediated by the orientation and/or position of the spindle with regard to intracellular polarity factors, such as in *C. elegans* embryonic development, *Drosophila* neuroblast formation, micromere formation in echinoid embryos, and neural precursor divisions in the vertebrate nervous system. As exemplified in *C. elegans*, off-center positioning of the spindle in addition to its orientation also mediates the formation of different cell sizes of daughter cells. Controlled orientation of spindle and cell cleavage plane has also been observed during extension of the vertebrate axis, with cleavage plane orientation mediating axis elongation.

However, in many vertebrate embryos such as zebrafish and *Xenopus*, early blastomeres are generated through a relatively uniform process in the absence of apparent signals that generate cell asymmetry. Under these conditions, how is the spindle orientation of a sequence of daughter cells determined, and how can this explain the overall cleavage pattern of an early embryo?

4.3.3.2 Transmission of Spindle Orientation Cues During Rapid Cycling

While a cortex-sensing mechanism can explain spindle alignment in smaller cells, studies in zebrafish and *Xenopus* have shown that it cannot explain spindle orientation in the large embryonic blastomeres in those species. In such embryos, spindles become aligned immediately prior to metaphase even before microtubule asters are long enough to contact the cortex (Fig. 4.4). It is only during anaphase, which in these cells coincides with interphase for the following cell cycle, when astral growth becomes extensive enough to reach the cortex. The early commitment of the spindle orientation by the metaphase spindle and refinement of orientation by the telophase astral microtubules has been demonstrated by following the effects of induced cell

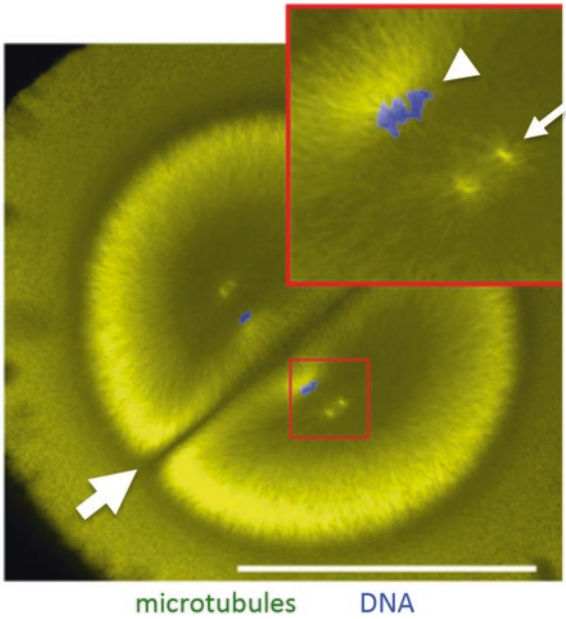

Fig. 4.4 Spindle alignment occurs early in the cell cycle and conforms to an alternating perpendicular pattern. Shown are microtubules (green) and DNA (blue, arrowhead in insert) in a frog embryo shortly before the first cleavage. Alignment of the axis of the incipient spindle is concurrent with telophase for the previous (in this embryo, first) mitotic spindle. Arrow in insert points to the nascent second mitotic spindle indicating the spindle axis. The forming spindle is parallel to the microtubule interaction zone, which indicates the location of the first cleavage furrow (thick arrow in the main panel; Wühr et al. 2010). The second spindle is therefore oriented perpendicular to the spindle of the previous cell cycle. Scale bar corresponds to 500 μm. Figure adapted from Wühr et al. (2010)

shape changes at different time points (Wühr et al. 2010). By applying pressure to *Xenopus* blastomeres to artificially impose a cleavage plane bisecting the elongated cell axis, the authors found that in prophase, prior to nuclear breakdown, the forming spindle (as determined by the inter-centrosomal axis) was already aligned with the long axis of the cell, within about 5° (compared to 45° if randomly oriented). Between anaphase and cytokinesis, when the telophase aster would be expected to have an effect, this alignment improved slightly yet significantly, to 1–2°. These experiments clearly show that (1) the metaphase spindle largely acquires its final orientation at a time when astral microtubules do not reach the cortex and (2) anaphase asters contribute to the fine-tuning of spindle alignment.

The second of these structures—anaphase astral microtubules—appears to provide two functions to the *ongoing* cell cycle. As in the case for the sperm-aster (see above), anaphase astral microtubules experience dramatic growth due to continuous nucleation at internal sites, resulting in microtubules becoming nucleated as they expand outward toward the cortex (Ishihara et al. 2014). This internal priming mechanism may allow astral tips to reach the cortex in spite of the large size of the

blastomeres (see above). For the ongoing cell cycle, a primary function of the anaphase aster appears to be furrow induction at the site of overlap of microtubules from each spindle pole, presumably by providing furrow induction signals such as CPC factors as discussed above. A second function, indicated by the slight improvement in spindle alignment observed in anaphase, seems to be to add precision late in the cell cycle to the orientation of the alignment of the spindle acquired earlier in the cell cycle. The precise mechanistic nature of this late alignment has not been studied, but presumably involves length-dependent internal pulling forces and/or (because anaphase microtubules do reach the cortex) cortex-sensing mechanisms.

The second function, fine-tuning of orientation, can be explained by a cortex-sensing mechanism. But, how does the spindle largely acquire its future orientation early in the cell cycle, even prior to its ability to sense the cortex and possibly cell shape? A key concept for understanding this mechanism of early spindle alignment is that, in the rapidly cycling blastomeres of the early embryo, cytoskeletal structures involved in cell division exhibit a degree of overlap between cell cycles. Thus, the spindle for a given cell cycle is starting to form at a time in which telophase astral microtubules of the previous cell cycle are still carrying out essential cell division functions. In particular, telophase astral microtubules, in addition to executing the above-described functions on furrow induction and spindle alignment fine-tuning for an *ongoing* cell cycle, provide cues for the early orientation of the metaphase spindle for the *following* cell cycle (Wühr et al. 2010).

This influence of furrow orientation from one cycle to the next appears to depend on a zone of microtubule exclusion that forms at the site of anaphase astral microtubule overlap, which develops in the plane in which the future cytokinetic furrow will cleave (Figs. 4.2, 4.4, and 4.5). As mentioned above, metaphase asters are relatively small and do not reach the cortex. On the other hand, anaphase asters grow dramatically to fill in the entire space of blastomeres and contact the cortex, where they provide signals to induce furrowing during cytokinesis. As telophase asters from opposite sides of the spindle reach the midzone, a microtubule-free (microtubule interaction) zone appears which clearly delineates the site of the forming furrow. This zone of microtubule exclusion generates a “dome”-shaped aster, where the sides of the dome correspond to the new spindle’s long axis, aligned parallel to the plane of the forming furrow (Wühr et al. 2010). This dome shape, with microtubules being longer on the side of the spindle opposite the microtubule interaction zone at the furrow (Figs. 4.4 and 4.5), generates an asymmetric force that moves the MTOCs toward the center of the future daughter cells and aligns the nascent spindle along the axis parallel to the forming furrow. The mechanism underlying the formation of the microtubule interaction zone is poorly understood. Embryos mutant for the zebrafish maternal-effect gene *motley*, which encodes an isoform of the CPC component survivin, do not exhibit a microtubule interaction zone at the furrow. In these mutants, anaphase astral microtubules instead cross the furrow boundary from opposite directions to generate a diffuse region of overlap (Nair et al. 2013).

The series of cellular events that result in early spindle alignment in zebrafish and *Xenopus* embryos (and possibly other vertebrates with large blastomeres) can

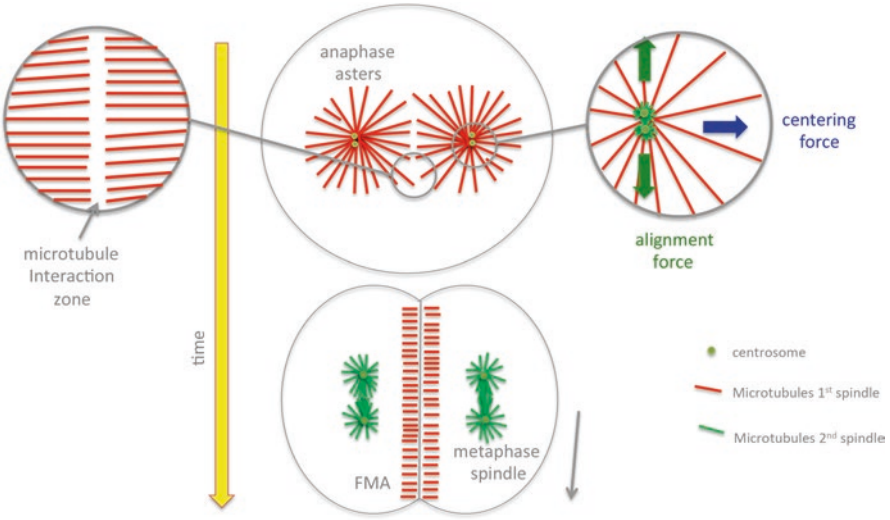

Fig. 4.5 Mechanism of spindle centering and alignment in large embryonic cells. Rapid cell cycling results in temporal overlap between processes corresponding to different cell cycles (see also Fig. 4.4). Anaphase astral microtubules for a given cycle (depicted in red for the first spindle in the top panel) expand all the way to the cortex. The asters form a microtubule exclusion region, the microtubule interaction zone, at the site of aster overlap (left insert). Formation of an incipient spindle for the following cell cycle (depicted in green for the second spindle in the bottom panel and right insert) occurs simultaneous with events associated with the previous cell cycle, including microtubule furrow array (FMA) formation and reorganization (see also Fig. 4.10). The influence of the microtubule interaction zone on the forming spindle for the following cycle results in asymmetric microtubule growth. Coupled to pulling from internal sources, the resulting astral microtubule length asymmetry generates a centering force (blue arrow) for the forming spindle apparatus within the forming blastomere. This same asymmetric influence, coupled to spindle elongation and a hypothesized stress force within the spindle, results in spindle alignment in an orientation parallel to the furrow (and perpendicular to the spindle) for the previous cell cycle (green arrows)

be described as follows (Fig. 4.5). During anaphase, at a time when nuclear envelope membranes are being reformed, a microtubule interaction zone forms at the plane along which the furrow will cleave. Because of the rapid cycling of early blastomeres, this time period also coincides with the separation of centrosomes and start of formation of the spindle for the following cell cycle. The nascent spindle is located in relatively close proximity to the microtubule interaction zone at the furrow corresponding to the previous cell cycle. The close proximity of astral microtubules to this aster interaction zone limits the length of astral microtubules directed toward the furrow. Under these circumstances, pulling forces on these shorter astral microtubules (extending between the interaction zone and the aster center) are weaker than forces on the opposite side of the aster center (extending toward the central and larger portion of the blastomere). This pulling force differential results in a net force on the spindle that moves it away from the furrow plane toward the center of the blastomere, resulting in spindle centering. Asymmetric pulling forces on the radially structured metaphase astral microtubules can also

explain the alignment of the spindle in the same direction as the furrow from the previous cycle, if the sister centrosomes are connected and exert a stress force on each other. Because aster microtubules oriented in the same orientation as the aster interaction zone (for the previous cell cycle) do not experience a microtubule length limit, internal pulling along the same in the direction of the previous furrow would not be restricted. Together, these forces result in alignment of the spindle for the new cell cycle along the same axis as the furrow plane for the previous cell cycle. This in turn results in the orientation of the new furrow, for any given cell cycle, in a plane perpendicular to that of the previous cell cycle. In zebrafish, where this sequence of events has been studied in more detail, the process is repeated to generate the embryonic cleavage pattern at least until about the sixth cell cycle, when the blastomeres become small enough to be contacted by the metaphase asters. As described in the following sections, this mechanism has been proposed to mediate the stereotypical cleavage patterns in zebrafish and *Xenopus* early embryos.

4.3.3.3 Cleavage Pattern Determination in Zebrafish

In the zebrafish, embryonic cleavage is known to be not only highly synchronous, a characteristic of pre-MBT cleavage patterns (see Chap. 9), but also stereotypical in terms of the orientation of the furrow plane during each cell cycle (Kimmel et al. 1995). As mentioned above, zebrafish embryos have meroblastic cleavage. This cleavage pattern is presaged by the structure of the oocyte, which has two primary regions, a wedge-shaped region at the animal pole of the egg where the oocyte nucleus is arrested in metaphase II of meiosis and the remaining region that contains a mixture of ooplasm and yolk granules (Selman et al. 1993). In zebrafish, the sperm site of entry is found at a specific location within the oocyte animal pole region which corresponds to the approximate center of the blastodisc that forms after egg activation (Hart and Donovan 1983; Hart et al. 1992). This likely facilitates sperm-aster centering with respect to a plane parallel to that of the blastodisc itself (which we refer to as the *x*–*y* plane). Although not yet shown, centering in a perpendicular dimension (in the *z*-direction, along the height of the forming blastodisc and the animal–vegetal axis of the embryo) may still occur. Similar to sperm-aster centering in *Xenopus* (Wühr et al. 2010), sperm-aster centering along the *z*-axis in zebrafish could occur through asymmetric forces on the sperm-aster due to microtubule length restriction on the side of the cortex (see below).

The earliest embryonic cleavage cycles exhibit a largely stereotypic pattern (Kimmel et al. 1995; Fig. 4.6a). During the first five cycles, blastomeres divide along the *x*–*y* plane (with *x* being the dimension along the first cell division and *y* along the second cell division) and in an orientation that alternates 90° every cell cycle. This generates, in subsequent cell cycles, a pattern of one-tiered blastomere arrays of 2 × 1 (two-cell embryo), 2 × 2 (four-cell), 4 × 2 (eight-cell), 4 × 4 (16-cell), and 8 × 4 (32-cell). Furrow positioning for the sixth cell cycle bisects the blastomeres along a *z*-plane, generating a two-tiered 8 × 4 blastomere array (64-cell embryo). This cleavage pattern is remarkably constant, although detailed live imag-

ing has found variations in the cell cleavage pattern during the earliest cell cycles (Olivier et al. 2010). Later cell cycles continue to be temporally synchronized but exhibit a pattern of furrow placement of increased variability. Because furrow induction and positioning are determined by the spindle midzone and its orientation, the observed pattern is generated by changes in spindle alignment in each successive cleavage.

The mechanistic model described above is consistent with spindle orientation changes in the early zebrafish and therefore its largely invariant cleavage pattern. According to observations and the model, metaphase asters in the early cell cycles are too small to sense the shape of the cell via microtubule–cortex interactions. However, aster alignment depends on internal pulling forces that are asymmetric due to the microtubule interaction zone at the cleavage plane for the previous cell cycle. As described above, these asymmetric forces result in alignment of the spindle in an orientation parallel to the furrow (and perpendicular to the spindle from the previous cell cycle). During each cell cycle, this asymmetric force results in both spindle orientation and spindle centering and, importantly for the overall cleavage pattern, cell furrows forming at alternating perpendicular angles.

This regular cleavage pattern explains the alternating furrow orientation pattern for the first cell divisions, but why do blastomeres stay in a single plane, forming a one-tiered structure, and why does the pattern change during the sixth cell cycle to generate a two-tiered structure? In terms of the spindles, why do spindles lie in the *x–y* plane during the first five cell cycles, whereas during the sixth cell cycle, spindles reorient vertically along the z-axis? The first question, of why spindles remain along a single x–y plane, may be related to a cell shape-sensing mechanism. The cell cortex gradually becomes close enough to the spindle to allow astral microtubules to increasingly contact the cortex and respond according to shape-sensing forces (Wühr et al. 2010; Xiong et al. 2014). Blastomeres are initially relatively elongated along the x–y plane compared to the z-dimension, i.e., they are longer and wider rather than taller, which would tend to align spindles along the x–y plane. The answer to the second question, of why spindles tend to reorient along the z-axis during the sixth cell cycle, may be related to the same mechanism if, as blastomeres divide and acquire a smaller size, their dimensions along the x and y axes become smaller, relative to the z-axis, which has remained relatively unchanged (e.g., blastomeres become taller, in the z-axis, than wider, along the x–y dimensions). Thus, a shape-sensing mechanism may cause spindles to realign from the x–y plane to the z-axis when new blastomere dimensions promote this realignment. An effect of changing cell dimensions on cleavage plane orientation has also been implicated in the regulation of the thickness of epithelial layers at later stages of development (Da Silva-Buttkus et al. 2008; Luxenburg et al. 2011; Lázaro-Diéguez et al. 2015). The stereotypic pattern of division orientation in zebrafish embryos ceases to be apparent at the seventh cycle and beyond (Kimmel et al. 1995; Hoh et al. 2013).

The emerging picture is that blastomeres of the early embryo may utilize a mechanism in which cell shape is sensed by the combined action of the mitotic spindle, oriented by an asymmetry defined by the zone of microtubule exclusion, and interphase asters allowing cell shape sensing. Together with changes in blastomere

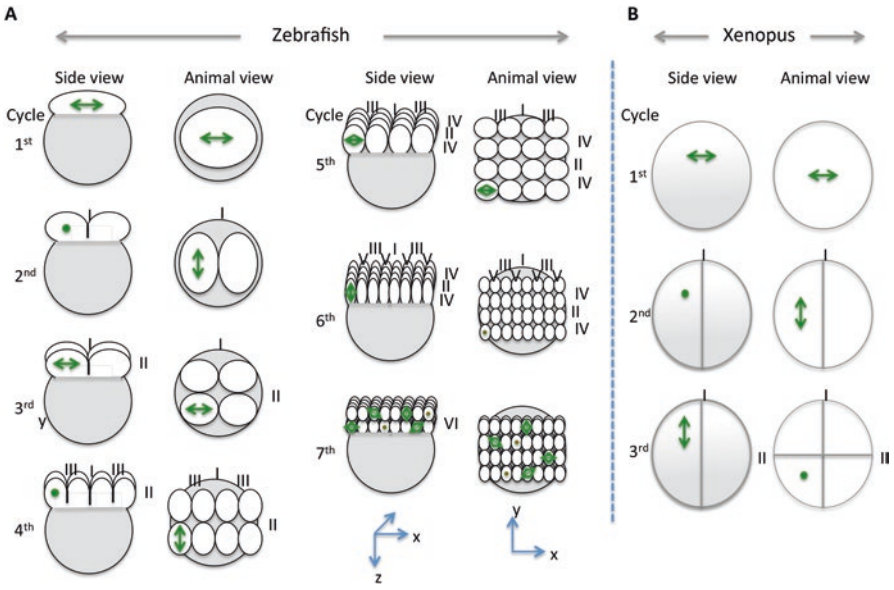

Fig. 4.6 Cleavage pattern orientation in early zebrafish and *Xenopus* embryos. (**a**) Stereotypic cleavage pattern in zebrafish. During the first five cell cycles, spindles (double arrows) remain aligned to a single, horizontal (x–y) plane parallel to the blastodisc plane, alternating 90° every cell cycle. During the sixth cell cycle, spindles tend to align in the vertical (z-axis) orientation, generating two tiers of blastula cells. Spindle orientation becomes random during the seventh cell cycle. (**b**) Canonical cleavage pattern in *Xenopus*. The first two cell cycles have spindles aligned to the x–y plane, also exhibiting an alternating 90° pattern, resulting in meridional cleavages. Spindle tends to reorient along the z-axis during the third cell cycle to generate a cleavage plane parallel to the equator. During the following cell cycles, the spindle aligns with the longest axis of the cell (Strauss et al. 2006; Wühr et al. 2010). Graded shading represents asymmetric yolk distribution, which is enriched in vegetal regions. Asymmetrically distributed yolk is hypothesized to interfere with microtubule length and/or pulling forces to generate a force that places the spindle in an eccentric position, biased toward the animal pole. In both (**a**) and (**b**), spindle orientation is indicated with green double arrows (which appear as a dot when entering the plane of the page). The order of furrow formation (corresponding to the cell cycle in which those furrows form) is indicated by Roman numerals

dimensions, this combined mechanism results in changes in spindle orientation that begin to transform a two-dimensional single-cell layer into a three-dimensional blastula.

4.3.3.4 Cleavage Pattern Determination in *Xenopus*

The mechanistic model described above, which explains the early zebrafish cleavage pattern, is also consistent with the generation of cell cleavage pattern in *Xenopus laevis*, another embryo with large blastomeres (Wühr et al. 2010; Fig. 4.6b). As in zebrafish, blastomere cleavage planes exhibit an alternating 90° pattern (Nieuwkoop

and Faber 1967; Sawai and Yomota 1990). Also similar to zebrafish, spindles acquire their final orientation early in the mitotic cycle, before astral microtubules are long enough to reach the cortex (Wühr et al. 2010).

In contrast to zebrafish, fertilization of anuran amphibian eggs, such as *Xenopus*, does not occur through a spatially defined sperm entry point, but instead occurs at random locations in the animal hemisphere (Elinson 1975; Schatten 2012). This generates in the *Xenopus* embryo an immediate need for sperm-aster centering along the x–y plane. This centering likely occurs by internal pulling forces acting on the sperm-aster, formed immediately after fertilization and generated by reconstitution of a centrosome around the sperm-derived centrioles, which act as an MTOC for the sperm-aster. The location of the sperm centrosome immediately below the surface will automatically result in centering: the membrane generates a restriction on the astral microtubule lengths, and pulling forces from the opposite (internal) side generate an overall asymmetric force that centers the sperm-aster (Wühr et al. 2010). The aster's closeness to the cortex induces an asymmetry generating a long sperm-aster axis that is roughly parallel to the tangent of the cortex at the sperm entry point. The aster seems to sense this long axis and transfer it to the nascent first mitotic spindle (see below). This might explain the old observation that the first cleavage plane typically cuts through the sperm entry point (Roux 1903).

During the first division cycles, *Xenopus* MTOCs tend to move toward the animal-most third of the embryo (Wühr et al. 2008). This movement in the animal direction may also be explained if, as in zebrafish, yolk granules present in more vegetal regions limit astral microtubule attachments. More yolk could inhibit microtubule growth or result in fewer cytoplasmic structures for dynein to pull on, thus again providing a force differential that pulls the spindles animally until reaching an equilibrium point at the observed location. However, the proposed mechanism by which asters and yolk interact is not understood. Induction of furrows in *Xenopus* initiates at the animal region and only gradually moves vegetally (Danilchik et al. 1998), consistent with asymmetric location of the spindles, which can act to induce a furrow first on the more closely located animal cortex. The first two divisions occur with spindles aligned along an x–y plane parallel to the equator, alternating 90 °C to generate four cells whose furrows span meridians along the animal–vegetal axis. This arrangement is similar to the cleavage pattern in zebrafish and, even with the obvious contrast that cleavage in *Xenopus* is holoblastic, is easily explained by the microtubule exclusion model described above. As in zebrafish, maintenance of the spindles in the x–y plane may rely on a differential force that precludes z-axis tilting during these cycles. The frog egg is not perfectly spherical but slightly oblate, and vegetal yolk likely weakens pulling forces on asters in the z-direction, two conditions that would promote spindle orientation along the x–y plane.

Xenopus embryos, also like zebrafish, exhibit a transition of spindle alignment from the x–y plane to a z-axis orientation (as in zebrafish, the latter also corresponds to the animal–vegetal axis of the embryo). However, this transition normally occurs in the third cycle in *Xenopus*, compared to the sixth cycle in zebrafish. This earlier transition in *Xenopus* may reflect a differing balance between microtubule pulling lengths along the x–y axis (limited by membranes laid out between blastomeres)

and vegetally located yolk. The result of this z-axis shift in spindle orientation is cell furrowing along a plane parallel to the equator (above the equator due to the spindles being located closer to the animal pole), generating four smaller animal blastomeres and four larger vegetal blastomeres. Subsequent cleavage planes generally follow a rule for alternating shifts in spindle orientation albeit showing increasing variation: cleavage in cycle 4 again tends to generate longitudinal furrows, reflecting a realignment of the spindle with the x–y plane, whereas cleavage in cycle 5 tends to generate furrows parallel to the equator, reflecting a second z-axis reorientation. The alternating alignment of the spindle along the x–y plane and the z-axis may reflect, as in zebrafish, a cell shape-sensing mechanism influenced by changing dimensions of the blastomeres as they undergo cell division (Strauss et al. 2006; Wühr et al. 2010).

Thus, and in spite of exhibiting an entirely different type of cleavage pattern (meroblastic compared to holoblastic), the global pattern of cleavage orientation in zebrafish and *Xenopus* embryos can be explained spatially and temporally through the cleavage stages using the same simple mechanistic model (Wühr et al. 2010). This model initially relies on microtubule length-dependent forces and the influence of the furrow from the previous cell cycle, together with additional intracellular modulation, such as the distribution of yolk. Over time cells acquire a smaller size and the patterning system may transition to the spindle being able to directly sense the cortex (Strauss et al. 2006; Wühr et al. 2010; Xiong et al. 2014). Together, these influences generate a three-dimensional blastula.

The fact that species as phylogenetically distant as teleosts and amphibians appear to obey a conserved set of cell-biological mechanisms, which generate manifestly different cleavage patterns from different initial starting conditions, suggests that a common set of rules may provide the basis for the various cell arrangements observed in many early vertebrate embryos. During evolution, such unifying rules may be all that is necessary to accommodate limited changes in starting blastodisc dimensions and/or the influence of modifying factors (i.e., embryo size, affinity of internal anchors, amount or nature of yolk particles) to generate cleavage pattern variation. It will be interesting to test this hypothesis through further studies in additional species.

4.3.4 Cell Cleavage Orientation in Other Vertebrate and Proto-vertebrate Systems

Studies in amphibians and teleosts have, until now, contributed the most to our mechanistic understanding of cleavage plane determination in vertebrate embryos. However, a full comparative picture will involve patterns in other vertebrate systems such as Aves, reptiles, and mammals. The chordates include invertebrates such as tunicates in addition to vertebrates, and mechanisms involved in cell cleavage pattern have been well described in ascidian (sea squirt) tunicate embryos. We summarize our current knowledge in these systems below.

4.3.4.1 Meroblastic Cleavage in Aves and Reptiles

Most knowledge concerning the cleavage stages of avian embryos comes from studies in the domestic chicken (*Gallus gallus*). However, understanding of cellular and molecular processes during cleavage stages is limited even in this well-known developmental model organism (Lee et al. 2013; Sheng 2014; Nagai et al. 2015). This is because, by the time the egg is laid, the embryo is long past the blastula stage. Thus, studying cleavage-stage embryos in the chick requires the use of methods for obtaining eggs still developing in utero (Lee et al. 2013).

The chick embryo undergoes meroblastic cleavage, with blastomeres dividing atop a large yolky mass (reviewed in Sheng 2014; Fig. 4.7). Fertilization is notable because, while only a single female and single male pronucleus will give rise to the zygotic genome, polyspermy is not uncommon (Lee et al. 2013). These supernumerary sperm, which can be few or many, are found in both yolk and blastoderm. Their function, if any, is unclear, but they are capable of producing pseudo-furrows that do not fully ingress.

Another notable feature of these embryos is that the point at which the first two cleavage furrows meet is not centered at the middle of the embryo (Sheng 2014; Nagai et al. 2015). There is a known correlation between asymmetric inheritance of maternally deposited factors and establishment of PGCs, but whether this is associated with off-center early cleavages is unclear. It has also been proposed that the off-center cleavages cause blastomeres to inherit heterogeneously deposited maternal factors asymmetrically as they cellularize, leading to symmetry breaking and axis induction. However, the ability to experimentally change the dorsal–ventral axis after egg laying challenges this early-establishment hypothesis (reviewed in Sheng 2014).

The first two cleavages in the chick are stereotypical, with the second forming perpendicular to the first in the plane of the blastoderm. The third furrow follows this pattern, though evidence suggests that divisions at this point become asymmetric, resulting in smaller cells in the interior and larger cells at the periphery (Lee et al. 2013). Cell division becomes asynchronous at the fourth cleavage, and presumably some asynchronous division continues (Sheng 2014).

It is also roughly around this time that the embryo begins to form two layers of blastomeres. As cleavage progresses, the embryo will reach 5–6 layers of cells in thickness before thinning again during and after gastrulation (Sheng 2014). A study found that approximately 75 % of surface cells (i.e., those in the uppermost layer) divided in a direction parallel to the blastoderm plane, yielding two daughters in the same layer, while deeper cells show more variation, with approximately 56 % dividing in a direction 30–90° from that of the blastoderm plane (Nagai et al. 2015; see below). These data suggest that cleavage orientation only partly explains increasing layer number, but other mechanisms, such as rearrangement of already cellularized blastomeres, may also be involved. The increase in layers also roughly corresponds to the onset of formation of the subgerminal cavity, a space that separates part of the blastoderm from the yolk. This structure may also influence blastoderm thickness.

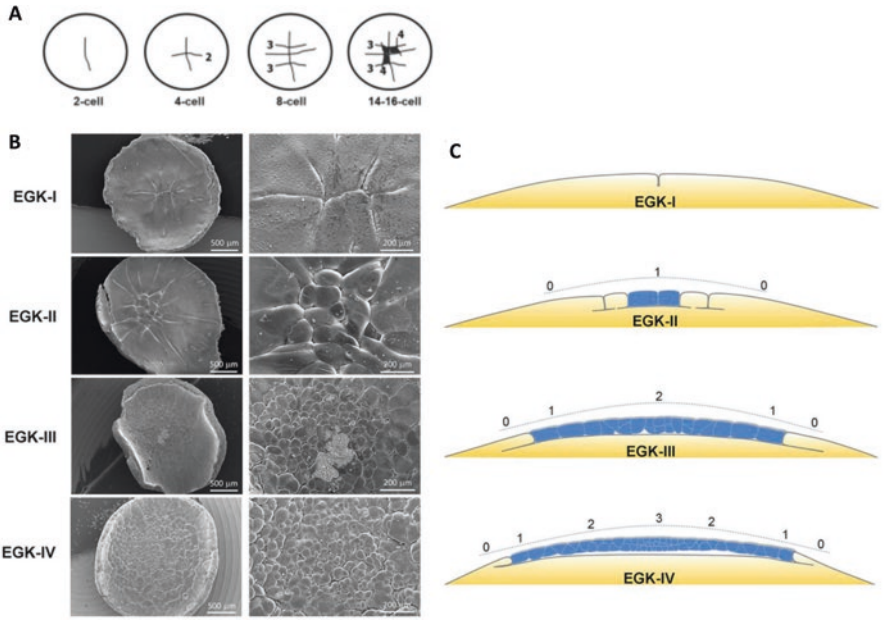

Fig. 4.7 Cleavage pattern in the early chick embryo. Early cleavage in chick embryos during the early to mid-cleavage stages (EGKI–EGKIV) tends to exhibit a perpendicularly alternating pattern, diagrammed in (**a**) and visualized through scanning electron micrographs in (**b**). (**c**) Diagram of a side view of the animal pole of the embryo, depicting meroblastic cleavage, with cellularized cells in blue and the number of layers indicated by numbers. Reproduced from Lee et al. (2013) (a) and Nagai et al. (2015) (**b**, **c**), with permission

Study of chick cleavage stages reveals some striking similarities to groups outside amniotes. For example, evidence suggests that zygotic gene activation in the chick occurs around the seventh or eighth cell division (precision is difficult due to cell cycle asynchrony). This places the midblastula transition somewhere between cycles 7 and 9, quite similar to the timing observed in zebrafish and *Xenopus* (Nagai et al. 2015). These researchers also find evidence of a yolk syncytial layer in the chick, though it is unclear if it plays a regulatory role similar to the YSL in zebrafish (see Chap. 7). The division of a blastomere with the cleavage plane parallel to the blastoderm, resulting in an inner cell and an outer cell that will go on to different fates, is reminiscent of a similar process in *Xenopus* (Sheng 2014). This evidence, combined with overall morphological similarity of cleavage-stage embryos in birds, reptiles, and teleost fish, suggests deep ancestry or convergent evolution of many characteristics of cleavage-stage embryonic development in vertebrates (Nagai et al. 2015).

Reptiles warrant a mention here. They are an important group to study to understand evolution of embryonic cleavage patterning in vertebrates and are known to undergo meroblastic cleavage. Unfortunately, data on their earliest cleavage stages

are scarce (Wise et al. 2009; Matsubara et al. 2014). Reasons for this can include difficulty of establishing breeding colonies in the lab, small clutch sizes, fertilization time uncertainty for wild-caught pregnant females, difficulty in culturing embryos due to extreme temperature and humidity sensitivity, and difficulty isolating embryos because of embryo adhesion to the egg's inner surface. However, recent work has begun to overcome these obstacles (reviewed in Wise et al. 2009; Matsubara et al. 2014). A method for culturing embryos of the Japanese striped snake (*Elaphe quadrivirgata*) (Matsubara et al. 2014) is a major step forward. While the earliest stage depicted in the study was a gastrula-stage embryo, it appeared quite similar to a chick embryo at the same stage. The data focused on somitogenesis, but this method has promise for examining early cleavage stages in snakes.

Other studies have suggested various lizards, such as the leopard gecko (*Eublepharis macularius*), as a model for development in that group (reviewed in Wise et al. 2009) and presented staging series. So far though, the majority of these focus on embryos in eggs that have already been laid, which is too late for characterization of cleavage stages. However, easy husbandry of these animals, combined perhaps with methods similar to those mentioned above for snakes and chicks, has potential to further the study of cleavage patterning in this phylogenetically important group of reptiles.

4.3.4.2 Early Cleavage Divisions in Mammals

Analogous to fish, amphibians, birds, and reptiles, mammalian zygotes initially undergo a series of cleavage divisions following fertilization to produce an increasing number of progressively smaller cells without changing the overall size of the embryo. However, the introduction and optimization of in vitro fertilization (IVF) and embryo culture techniques have revealed several notable differences in how these early divisions occur between mammals and other vertebrate animals. First, mammalian species exhibit rotational cleavage, whereby meridional division is observed along the animal–vegetal axis in the first cleavage, but during the second cleavage, the daughter cells can divide either meridionally or equatorially by dividing perpendicular to the animal–vegetal axis (Gulyas 1975; Fig. 4.8). As a consequence, each blastomere inherits equivalent cytoplasmic material from both the animal and vegetal region at the two-cell stage and potentially differentially allocated animal and vegetal portions when the embryo divides from two cells to four cells (Gardner 2002). The type of second division each daughter cell undergoes determines which of the four distinct classes (meridional–meridional, meridional–equatorial, equatorial–meridional, or equatorial–equatorial) a four-cell embryo will become, and this may impact both cell fate and developmental potential as previously suggested (Piotrowska-Nitsche and Zernicka-Goetz 2005). More specifically, it has been shown that four-cell mouse embryos containing at least two blastomeres with both animal and vegetal material are much more likely to develop to term than embryos where all blastomeres have either only animal or only vegetal cytoplasmic inheritance (i.e., the equatorial–equatorial class; Piotrowska-Nitsche et al. 2005).

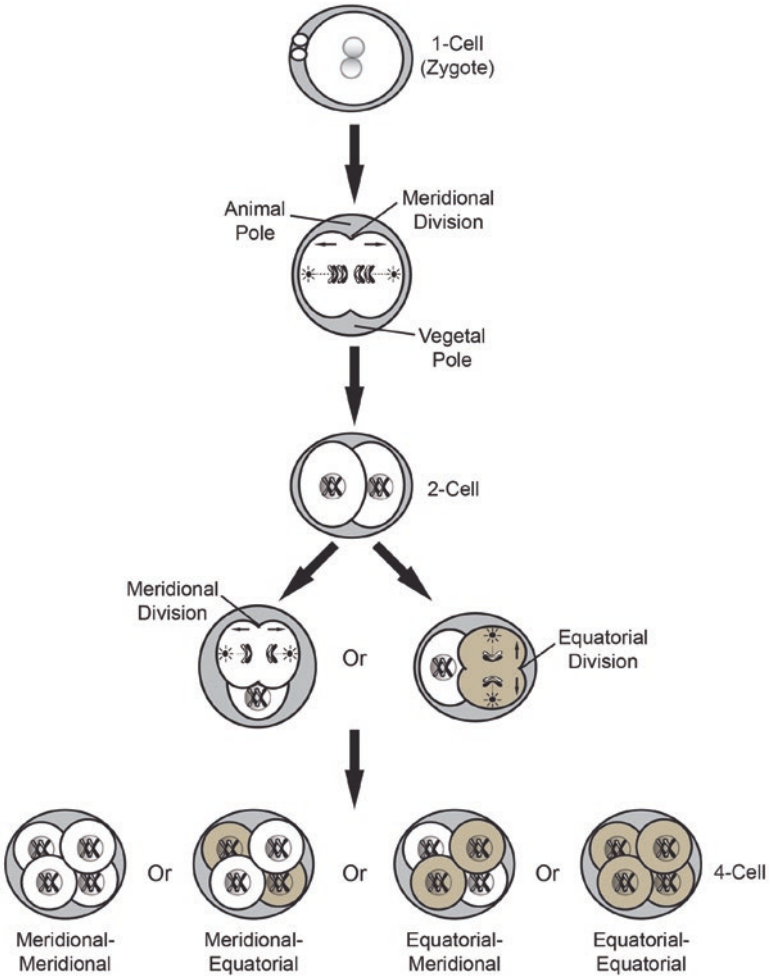

Fig. 4.8 Cleavage pattern in the early mammalian embryo. The first division is meridional, along the animal and vegetal poles, and the second division can be either meridional or equatorial. This generates four possible cellular arrangements at the four-cell stage with meridional–equatorial and equatorial–meridional divisions distinguishable by virtue of one of the blastomeres in the two-cell embryo dividing prior to the other. In embryos that exhibit the equatorial–equatorial pattern, putative factors localized to the animal and vegetal poles segregate to different daughter cells. See text for details

Based on their differential distribution between the animal and vegetal poles in the zygote, several candidates have been proposed to mediate this cleavage-related asymmetry (reviewed in Ajduk and Zernicka-Goetz 2015). Apart from findings of asymmetric localization, however, none of these candidate proteins have been shown to function in the determination of whether a meridional or equatorial division occurs at the two-cell stage. Thus, the exact molecular mechanism(s)

mediating the developmental fate of each blastomere and distinction between the four classes of four-cell mammalian embryos remains to be determined (Ajduk and Zernicka-Goetz 2015).

In addition to unique cell orientation, the time between cleavage divisions in mammalian embryos is more prolonged, typically between 8 and 24 h apart depending on the species, compared to that in many nonmammalian vertebrates. For instance, the time between the first and second mitosis in mice is approximately 20 h and for the majority of other mammals, including humans, 8–12 h (O'Farrell et al. 2004; Wong et al. 2010; Weinerman et al. 2016). Besides longer time intervals, blastomeres in early cleavage-stage mammalian embryos also undergo asynchronous cell division rather than simultaneously dividing like in other vertebrates. Therefore, mammalian preimplantation embryos do not increase exponentially in cell number from the two- to four- or four- to eight-cell stage but can contain an odd number of blastomeres at certain times in development (Gilbert 2000). Lastly, in contrast to other vertebrate animals, the mammalian genome becomes activated much earlier, and many of the mRNA transcripts and protein products produced from embryonic genome activation (EGA) are required for subsequent cell divisions. Because of this requirement, the stage of embryo development that coincides with EGA is most susceptible to cleavage arrest or "block" in several mammalian species (Ko et al. 2000). The mouse embryo exhibits EGA earliest, at the two-cell stage; however, minor transcription of certain mRNAs also occurs in mouse embryos at the one-cell stage and is often referred to as zygotic gene activation (ZGA) (Flach et al. 1982; Ko et al. 2000; Hamatani et al. 2004; Wang et al. 2004; Zeng et al. 2004). Similar to the mouse, human embryos have also been shown to undergo minor transcriptional activity of preferential mRNAs prior to the major wave of EGA, and some of the transcripts include cell cycle regulators (Dobson et al. 2004; Zhang et al. 2009; Galán et al. 2010; Vassena et al. 2011). Thus, it is likely that preimplantation embryos from other mammalian species also exhibit "waves" of gene expression during the transition from maternal to embryonic transcriptional control, which may impact embryo behavior and cell division timing if these cell cycle-related genes are aberrantly expressed. Regardless of which wave it occurs under, the production of embryo-derived transcripts is clearly established by day 3 of human preimplantation development even in embryos that arrested prior to the eight-cell stage (Dobson et al. 2004; Zhang et al. 2009; Galán et al. 2010; Vassena et al. 2011), suggesting that EGA is a function of time rather than cell number per se.

Several studies have shown that male mammalian preimplantation embryos may actually cleave faster than female embryos cultured in vitro (Xu et al. 1992; Pergament et al. 1994; Peippo and Bredbacka 1995), although other studies detected no difference in the sex ratios between early- or late-cleaving human embryos (Ng et al. 1995; Lundin et al. 2001). This suggests that potential sex-related differences in the cleavage rate of male versus female human embryos do not occur until later during post-implantation development or that, alternatively, this phenomenon is restricted to only certain mammalian species. Nevertheless, early cleavage in general is a strong indicator of embryo viability since human embryos that undergo the first mitotic division sooner appear to have greater potential for successful implanta-

tion and positive pregnancy outcome (Lundin et al. 2001). More recent findings, however, suggest that it is the duration of the first cleavage division rather than its onset, together with the time intervals between the first three mitotic divisions, that is highly predictive of which human embryos will reach the blastocyst stage (Wong et al. 2010). Since this initial report, other studies have confirmed the importance of early cleavage divisions and identified additional cell cycle or morphological parameters predictive of developmental success (Cruz et al. 2011, 2012; Meseguer et al. 2011, 2012; Azzarello et al. 2012; Dal Canto et al. 2012; Hashimoto et al. 2012; Hlinka et al. 2012; Rubio et al. 2012; Liu et al. 2014, 2015; Stensen et al. 2015) as well as underlying chromosomal composition (Chavez et al. 2012, 2014; Campbell et al. 2013; Basile et al. 2014; Yang et al. 2014). Whether the first three mitotic divisions are similarly predictive of embryo viability and/or chromosomal status for other mammalian species is still under investigation, but an examination of early mitotic timing in murine, bovine, and rhesus monkeys has suggested that this is likely the case (Pribenszky et al. 2010; Sugimura et al. 2012; Burruel et al. 2014). As mentioned above, however, the precise timing between the first cell divisions can vary between different mammalian species (O'Farrell et al. 2004; Wong et al. 2010; Weinerman et al. 2016), and the underlying cause(s) remains largely unknown. Besides a later EGA onset in comparison to the mouse, human embryos have also been shown to express diminished levels of cell cycle checkpoints and robust expression of cell cycle drivers at the cleavage stage (Harrison et al. 2000; Los et al. 2004). This can impact not only embryo chromosomal stability, as shown by the high incidence of whole chromosomal abnormalities (aneuploidy) in cleavage-stage human embryos (Vanneste et al. 2009; Johnson et al. 2010; Chavez et al. 2012; Chow et al. 2014), but may also produce preimplantation embryos that cleave at a faster rate over other mammals. Moreover, time-lapse monitoring of early embryonic development has demonstrated that human embryos also frequently undergo multipolar divisions, whereby zygotes or blastomeres divide into three or more daughter cells rather than the typical two. Indeed, it has been estimated that approximately 12 % of human zygotes cultured in vitro are characterized by multipolar divisions (Chamayou et al. 2013), and this phenomenon could further explain differences in mitotic timing between mammalian species. While the potential impact of higher-order divisions at the two-cell stage and beyond may not be as detrimental and is still being investigated, embryos that exhibit multipolar divisions at the zygote stage are much less likely to form blastocysts and implant than zygotes that undergo a bipolar division (Hlinka et al. 2012).

4.3.4.3 Proto-vertebrates

Studies in ascidians have provided important insights into patterns of cell cleavage in a lineage basal to vertebrates, which may reflect ancestral developmental mechanisms. Cleavage pattern in ascidian species is holoblastic, invariant, and characterized by bilateral symmetry (Conklin 1905; Nishida and Satoh 1983; Nishida 1987). The pattern of cell cleavage orientation in ascidians provides insights into our

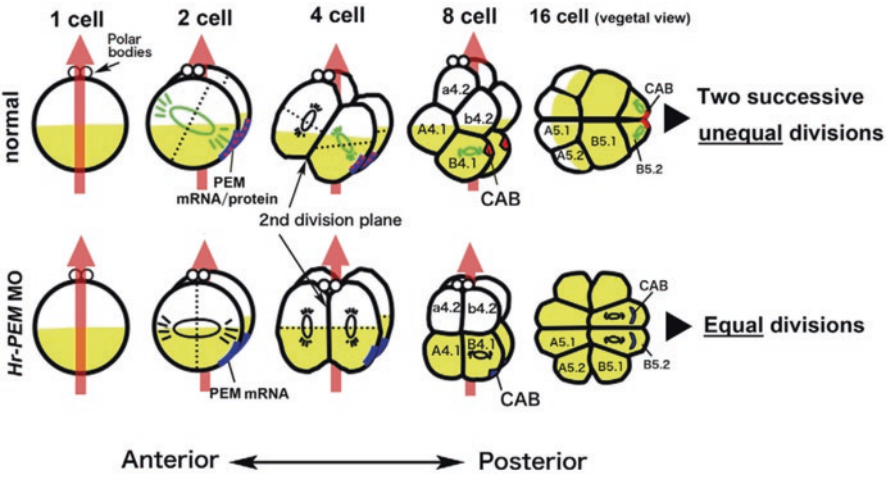

Fig. 4.9 Early embryonic cleavage pattern in ascidians. Early cleavages in general follow an alternating perpendicular pattern, consistent with a dependence on cell shape. Spindle orientation in the posterior-vegetal cell is influenced by the attraction of one of the spindle poles to the CAB (blue), which becomes condensed and attached to the posterior-vegetal cortex in the eight-cell and 16-cell embryo. This causes the eccentric placement of the spindle at the posterior end of the posterior-vegetal blastomere during these stages, resulting in consecutive asymmetric cell divisions that generate a larger blastomere anteriorly and a smaller blastomere posteriorly. Spindle orientation is also influenced by the condensing CAB during formation of the two-cell embryo, resulting in a shift of the cleavage plane axis with respect to the polar bodies at the embryo animal pole and animal–vegetal axis (orange arrow) of the embryo, as well as during formation of the four-cell embryo, resulting in the observed protrusion of posterior-vegetal blastomeres (B4.1) at this stage. Spindle centering in anterior- and posterior-animal blastomeres, which do not contain the CAB, occurs normally resulting in symmetric cell division. Attraction of the spindle pole by the CAB is dependent on the function of PEM, a maternal product localized to the CAB. Reproduced, with permission, from Negishi et al. (2007)

general understanding of cell cleavage patterning in early embryos. The mechanism appears to involve symmetric cleavages in alternating orientations, consistent with shape-sensing spindle orientation mechanisms acting in large embryonic cells. This underlying cell division pattern is further modified in a subset of cells by a specialized structure capable of influencing the orientation and position of the spindle through multiple cell cycles (at least cycles 3–6), adding asymmetric details to the blastula, which begin to sculpt the embryo. This modified structure, capable of influencing spindle placement and cell pattern arrangement, also contains factors that function in cell shape specification. Thus, basal mechanisms of cell division interact with a specialized structure to coordinate cell division cues and cell fate signals to generate the basic body plan (Fig. 4.9).

The first two cleavage planes in ascidian embryos are meridional, passing through the animal and vegetal poles, with the second cleavage oriented perpendicular to the first to generate four equal-sized blastomeres. The third cleavage plane is also perpendicular to the first and second cleavage but equatorial, divid-

ing each blastomere to generate an eight-cell blastula with four cells in each of the animal and vegetal halves of the embryo. These first three cleavages are nearly symmetric, with the exception of an outward tilt of the division axis for the two posterior blastomeres during the third cell division, resulting in a slight outward protrusion of the resulting posterior-vegetal blastomeres. The next three cell cycles continue a cell division pattern that is symmetric in animal and anterior-vegetal blastomeres but is asymmetric in posterior-vegetal blastomeres. These posterior-vegetal blastomeres instead divide asymmetrically, each cell cycle generating a small cell posteriorly and a larger cell anteriorly. The resulting cells in the blastula exhibit unique lineages and cell fates (Conklin 1905; Nishida 1987).

The ascidian cleavage pattern thus exhibits some similarities to those observed in vertebrate systems, especially vertebrates such as teleosts and amphibians. In particular, the holoblastic, mutually orthogonal divisions of ascidians during the first three cycles are aligned in a pattern that is at least superficially identical to that in the canonical cleavage pattern in *Xenopus*: two divisions with spindles oriented along the x–y plane (to generate blastomeres along a single plane) and then a third along the z-axis (to generate two tiers of blastomeres). Although not yet directly tested, this symmetric, alternating pattern may result from the same mechanisms described above that govern spindle orientation in other vertebrates, namely, a combination of asymmetric forces generated by the furrow for the previous cell cycle and cortex sensing mediated by astral microtubules (see Sect. 4.3.3 and below).

With regard to cell cleavage pattern, two differences stand out in ascidians compared to amphibians. The first difference is the bilateral symmetry of the embryo. Subcellular mechanisms maintaining bilateral symmetry in cell arrangement have not yet been studied. It is possible that this feature involves no more than the absence of cell mixing between the two embryonic halves prior to differentiation, itself possibly reflecting strong intercellular adhesive forces along the earliest cleavage planes, with cell division tightly coordinated with cell-autonomous fate commitment.

A second unique characteristic of ascidian cleavage pattern is the asymmetric cell division of posterior-vegetal cells. A posteriorly localized cytoplasmic structure, termed the centrosome-attracting body (CAB), has been shown to be involved in both the posterior tilting of the cleavage axis during the third cell cycle, generating the posterior-vegetal protrusion of the blastula, and the asymmetric cell division of the posterior-vegetal cells during the next three cell cycles (Hibino et al. 1998; Nishikata et al. 1999). The CAB is derived from cytoplasm associated with the posterior-vegetal cortical region that is enriched in cortical ER (cER) and associated factors. The cER becomes enriched at the vegetal pole through cytoplasmic reorganization during the first cell cycle (Roegiers et al. 1995, 1999; Sardet et al. 2003; Prodon et al. 2005) and subsequently undergoes a posterior displacement to coalesce by the third cell cycle into a tight mass at the posterior cortex, constituting the CAB (Hibino et al. 1998; Iseto and Nishida 1999; Nishikata et al. 1999). Interestingly, observed patterns of cER reorganization are similar in three evolutionarily distant ascidian species, *Halocynthia roretzi*, *Ciona*

intestinalis, and *Phallusia mammillata*, indicating a high degree of conservation for cytoplasmic mechanisms involved in cell cleavage in ascidian lineages (Sardet et al. 2003; Prodon et al. 2005). Ablation of the posterior-vegetal cytoplasm from which the CAB forms results in the absence of spindle tilting and asymmetric cell division at subsequent cell cycles (Nishida 1994, 1996). The CAB has an electron-dense appearance at the ultrastructural level (Iseto and Nishida 1999) and is known to contain a number of localized mRNAs, the so-called type I postplasmic/PEM RNAs, some of which are known to have cell-determining functions (Nishida 2002; Sardet et al. 2006).

The CAB is also able to associate with the cytoskeleton. Indeed, the posteriorly located CAB appears to direct the rearrangement of microtubules between the CAB and the interphase nucleus, forming a bundled array of microtubules which undergo shortening. This results in the posterior movement of the nucleus, which is followed by the attachment of one of the centrosomes of the metaphase spindle to the CAB. The combined process results in a posteriorly displaced spindle apparatus, which is attached through one centrosome to the posterior cortex. This eccentric placement of the spindle results in the asymmetric cell division that occurs in these cells. Segregation of the CAB to the posterior-most membrane insures that the posterior-most blastomere inherits this structure, which continues to promote the eccentric spindle location in the following cell cycle. The electron-dense nature of the CAB as well as its ability to localize mRNAs is similar to that of nuage or germplasm, the specialized cytoplasm that specifies primordial germ cells (Wylie 2000; see Chap. 8), suggesting that these may be related structures. Consistent with this interpretation, the smallest, most posterior blastomeres in the 64-cell blastula are fated to become primordial germ cells (Shirae-Kurabayashi et al. 2006). Although a short microtubule bundle is not observed at the third cell cycle stage, the posterior tilting of the spindle that occurs during this earlier stage in posterior-vegetal blastomeres is thought to have the same underlying cause as spindle eccentric movement, namely, the attraction of one centrosome toward the CAB (Negishi et al. 2007). The influence of CAB precursor components at the posterior-vegetal cortex has been proposed to influence spindle orientation as early as the second cell cycle, resulting in an observed shift of the second division cleavage plane with respect to polar bodies at the animal pole of the embryo (Negishi et al. 2007).

One of these CAB-localized mRNAs that code for the novel protein posterior end mark (PEM) has been shown to be directly involved in CAB-induced microtubule reorganization. In embryos with PEM functional knockdown, the CAB appears to form normally, but the microtubule bundle linking the centrosome to the posterior cortex does not form (Negishi et al. 2007). Embryos with PEM functional knockdown also lack the tilting of the spindle characteristic of the third cell cycle as well as the cleavage plane shift at the second cell cycle (Negishi et al. 2007). Thus, PEM mRNA is localized to the CAP, and its protein product has a function essential for the association of the CAP at the posterior cortex with the spindle centrosome, involved in both the cell division tilting and the eccentric placement of the spindle leading to asymmetric cell division.

4.4 Cell Division Machinery During the Early Cleavage Stage

Cell division in the early embryo is influenced by features characteristic of the egg-to-embryo transition, such as the shift from meiotic to mitotic cycles, the inheritance of a limited supply of cellular building blocks, and specializations for large cellular size and unique embryo architecture. We address these topics in this section.

4.4.1 Maternal Loads and Scaling of Spindle Size During Early Cell Divisions

The early embryo develops with unique restrictions since, prior to zygotic gene activation at the midblastula transition (see Chap. 9), all embryonic processes by necessity are driven solely by maternal products. Thus, while cells at later stages of the embryo and the adult produce on their own new products essential for cell growth and division, cells in the early embryo are limited to the supply of cellular building blocks originally stored in the mature egg. Species have evolved specialized systems for the storage in the egg and controlled use of maternal products during early embryonic development. In addition to energy and essential building blocks, the embryo must generate subcellular structures as it becomes multicellular. It has been shown that the overall protein composition from the fertilized egg to the midblastula transition only changes minimally (Lee et al. 1984; Peshkin et al. 2015). Hence, the embryo must generate vital subcellular structures that can fulfill their tasks under drastically different dimensional scales, while the cell size changes by orders of magnitude from the fertilized egg to the midblastula transition. In particular, the cellular machinery must adapt to use a finite initial supply of building blocks for vital subcellular structures as they are being used by the embryo and in the context of a several-fold change in blastomere size. Interestingly, the limited supply of histones and replication factors and their utilization by the exponentially increasing DNA amount have been shown to trigger the onset of the midblastula transition (Newport and Kirschner 1982a, b; Collart et al. 2013; Amodeo et al. 2015). In this section, we use the scaling of spindle-associated structures as an example of maternal product inheritance and adaptation to different length scales in the early cleaving vertebrate embryo.

An important advance in the analysis of intracellular processes involved in early embryonic cell division was the ability to reconstitute asters and bipolar spindles in *Xenopus* extracts, first from oocytes to generate structures analogous to meiotic spindles (Lohka and Maller 1985; Sawin and Mitchison 1991; Mitchison et al. 2013) and later from early embryos to generate sperm-asters and mitotic spindles (Wühr et al. 2008). In both situations, remarkably normal spindles formed, but, in spite of the absence of any cell boundaries in this in vitro system, the spindles exhib-

ited similar sizes to the in vivo equivalent. Furthermore, an upper limit to spindle size was also shown to occur in *Xenopus* embryos: during the first four cell cycles, the spindle size is relatively constant at an upper limit similar to that observed in spindles formed in vitro using an early mitotic or meiotic extract (Wühr et al. 2008; Good et al. 2013; Hazel et al. 2013). The small size of the mitotic spindle compared to the cell size requires some special adaptation for proper DNA segregation. While the mitotic spindle still is responsible for the initial separation of sister chromatids, the majority of the DNA movement into the center of the future daughter cell is executed by cell-spanning anaphase/telophase asters (see Sect. 4.3.2.3).

Starting at the fifth cell cycle, spindle length begins to scale with blastomere length, exhibiting an approximately linear relationship. These observations showed a transition between spindle length control mechanism, with very early and late blastomeres exhibiting different control mechanisms. The observations that the upper spindle length limit is observed in in vitro-reconstituted spindles (Wühr et al. 2008) as well as in embryos where cells are too large to be contacted by metaphase spindle asters (Wühr et al. 2010; see above) suggest the presence of a length-determining mechanism intrinsic to the spindle. The precise nature of this mechanism remains unknown, but it is thought to depend on a balance between microtubule nucleation dynamics and the function of microtubule-associated motors, as proposed for the meiotic spindle (Burbank et al. 2007; Cai et al. 2009; Dumont and Mitchinson 2009; Reber et al. 2013). Subsequently in smaller cells, spindle length does become reduced coordinately with blastomere cell size.

As stated above, spindle size does scale with blastomere size during the later blastomere cycles (Wühr et al. 2008). A simple model by which subcellular structures may scale to the decreasing size of embryonic blastomeres invokes a limiting-component mechanism, developed through studies in the nematode *C. elegans* embryo (Decker et al. 2011). Under this mechanistic model, the size of subcellular structures, in this case centrosomes, scales according to cell volume due to the inheritance during cell division of a limiting amount of structure precursor material that necessarily decrements with each cell division.

The limiting-component mechanism appears to also apply to spindle formation in *Xenopus laevis*, as shown by the analysis of spindles forming in cytoplasmic compartments produced by microfluidic technology (Good et al. 2013; Hazel et al. 2013). In these compartments, spindle size correlates with cell volume. By deforming the compartments to maintain cell volume while changing droplet diameter, the authors showed that spindle length depends on a volume-sensing mechanism as opposed to a boundary-sensing mechanism. Direct measurements shows a decrease in free cytoplasmic tubulin in smaller blastomeres, consistent with spindle scaling being dependent on the concentration of a limiting spindle factor precursor (Good et al. 2013). Interestingly, encapsulated cytoplasm from early stages shows an upper limit in spindle length in vivo as well as in within large droplets (Good et al. 2013; Hazel et al. 2013). Together, these findings indicate that spindle size is regulated by two separate yet interacting systems, one dependent on cytoplasmic composition which imparts an upper limit to spindle length in large cells and a second which

depends on cytoplasmic volume and a limiting subunit such as free tubulin and results in spindle scaling in smaller cells.

A limiting-component mechanism may be a simple and widely used property of animal embryos, including those of vertebrates. Spindle size in early mammalian early embryos is also consistent with a limiting cytoplasmic factor (Courtois et al. 2012), and centrosome size in the early zebrafish embryo is significantly larger in early blastomeres than in later ones (Lindeman and Pelegri 2012). Various components may also interact. For example, centrosome size is known to influence spindle length (Greenan et al. 2010), and it will be interesting in the future to assess the role of centrosome apportioning to spindle scaling. Mechanisms similar to those proposed to regulate spindle and centrosome size, dependent on limiting components inherited in the egg, likely act in the regulated generation of other subcellular organelles in the early embryo.

4.4.2 Specialization of the Cytoskeleton in the Early Embryo

The morphogenetic forces that produce each species' unique embryonic cleavage pattern must integrate with several other cellular activities during cell division to construct the pregastrular embryo. These activities include the establishment of a specialized basolateral membrane domain in each cleavage plane, the zippering together of apical–basolateral margins along advancing furrows to produce a tight-junctional osmotic barrier, and, ultimately, the osmotically driven inflation of interstitial spaces such as the blastocoel. Where the egg begins with only a single outer (apical) surface, the blastula must develop a functional, polarized epithelium to physiologically isolate the interstitial space and/or blastocoel from the outside world. During cleavage, blastomeres become adherent and distinct apical and basolateral membrane domains develop, separated by apical tight junctions (Muller and Hausen 1995; Merzdorf et al. 1998; Fesenko et al. 2000). This process, referred to as compaction, occurs at different developmental times in different organisms. For example, as discussed below (Sect. 4.4.4), in mammalian embryos, compaction is a distinct phase beginning at about the eight-cell stage or later (Ducibella and Anderson 1975; Fleming et al. 2000), while in holoblastically cleaving eggs of amphibians and sturgeon (Zotin 1964; Bluemink 1970; Kalt 1971a, b; Bluemink and deLaat 1973), it occurs contemporaneously with the earliest cleavages.

4.4.2.1 Basolateral Membrane Formation in Xenopus Cleavage

Amphibian early embryos are distinctive among dividing cells for the comparatively large amount of membrane added continuously during the cleavage process (Bluemink and deLaat 1973). Cleavage furrowing is said to be unipolar because it begins at one pole of the egg, and the contractile band then assembles and travels as an arc extending progressively around the egg surface, eventually forming a complete ring which then constricts inward while large amounts of new surface area are

added near the base of the advancing furrow. The new plasma membrane therefore has a composition different from that of the original egg surface (Kalt 1971a, b; Sanders and Singal 1975; Byers and Armstrong 1986; Aimar 1997). The main source of the new basolateral membrane appears to be a pool of post-Golgi vesicles produced during oogenesis which contributes membrane lipids, glycoproteins, and extracellular matrix components to the growing surface (Kalt 1971a, b; Servetnick et al. 1990). Roberts et al. (1992) provided direct evidence that Golgi-derived vesicles generated during late oogenesis can fuse specifically with the new membrane.

4.4.2.2 Microtubule-Dependent Exocytosis of Basolateral Membrane in the Cleavage Plane

The massive localized delivery of new basolateral membrane to the advancing cleavage furrow is known to be microtubule dependent in both Xenopus and zebrafish. A distinctive array of antiparallel microtubule bundles, referred to as a furrow microtubule array, develops along the base of advancing furrows in both *Xenopus* (Danilchik et al. 1998) and zebrafish (Jesuthasan 1998; Fig. 4.10). Their appearance and general geometry distinguish furrow microtubule arrays from other

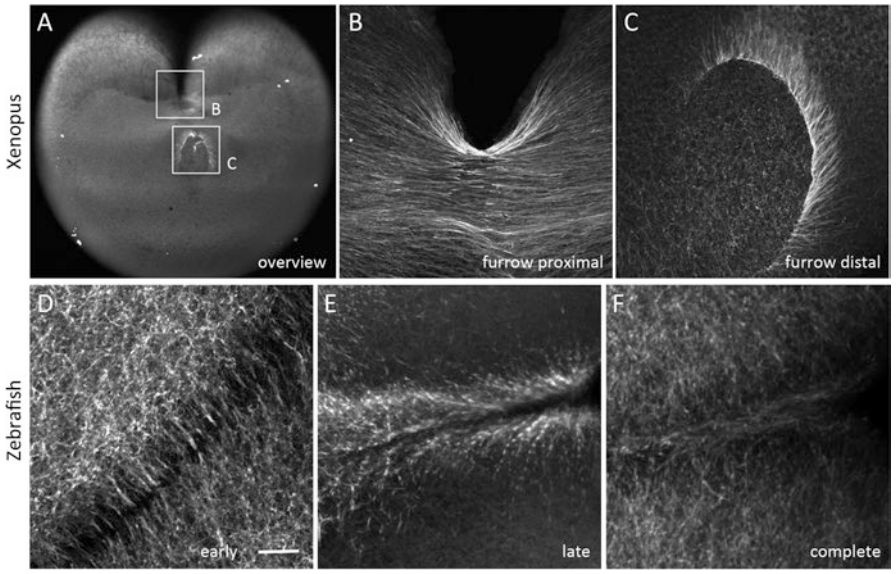

Fig. 4.10 FMA reorganization in *Xenopus* and zebrafish. (**a**–**c**) FMA in *Xenopus*: overview (**a**) and magnified views at the base of the furrow (**b**) and in more vegetal regions of the advancing furrow (**c**). (**d**–**f**) FMA in zebrafish furrows: early furrow shows a parallel arrangement of FMA tubules, oriented perpendicular to the plane of the furrow (**d**), maturing furrow shows distally accumulating FMA tubules in a tilted, V-shaped arrangement (**e**), and complete furrows show FMA disassembly (**f**). Scale bar in (**d**) corresponds to 10 μm for panels (**d**–**f**). Panels (**d**)–(**f**) courtesy of Celeste Eno

microtubule-containing structures in the cleavage plane, such as interzonal spindle microtubules. The rapid expansion of new membrane sustained by the furrow microtubule array is sometimes regarded as an embryonic amplification of the mid-body-dependent localized exocytosis required for abscission of dividing cells of organisms ranging from yeasts to plants and animals (Straight and Field 2000; Glotzer 2001).

4.4.2.3 Protrusive Activity During Cleavage Furrow Closure

Various kinds of membrane protrusions develop along cleavage furrow margins (Danilchik and Brown 2008; Danilchik et al. 2013). Long microvilli are seen associated with stress folds at the cortex, and lamellipodia and filopodia extend near the furrow base itself. These protrusions are actively motile and can make relatively robust contacts across the extracellular space separating the furrow margins. Abrogating normal actin assembly by microinjection of constitutively active rho and cdc42 disrupts normal furrow margin protrusive activity and cleavage furrowing, suggesting that the protrusions play a role in normal furrow closure and blastomere adhesion (Danilchik and Brown 2008).

4.4.2.4 Blastocoel Formation

In amphibian embryos, the initiation of blastocoel formation is evident well before the first contractile ring has finished closing (Kalt 1971a, b). During advance of the cleavage furrow, a new domain of plasma membrane becomes inserted in the plane of the plasma membrane on either side of the contractile ring, resulting in two expanding basolateral surfaces facing each other between separating blastomeres (Bluemink 1970; Kalt 1971a, b). A similar phenomenon likely occurs in the zebrafish (Feng et al. 2002; Urven et al. 2006; Eno et al. 2016). As discussed above, this rapid expansion of new membrane depends on localized exocytosis of maternally derived vesicles immediately behind the advancing contractile ring. The new cleavage planes express maternally encoded C- and EP-cadherins which facilitate adhesion between sister blastomeres (Heasman et al. 1994a, b; Kühl and Wedlich 1996) and integrins. Each successive cleavage event inserts more basolateral surface between dividing daughter cells; the blastocoel, resting at the intersection of all the early cleavage planes, thus is entirely lined by basolateral surface and, with the development of apical–basolateral tight junctions, becomes osmotically isolated from the outside world. Although definitive extracellular matrix fibers, including fibronectin, do not develop until mid- to late-blastula stage (Boucaut et al. 1984; Davidson et al. 2004, 2008), the volume of the blastocoel is nevertheless entirely filled with extracellular matrix at all stages (Keller 1986, Danilchik, unpublished). The blastocoel undergoes continuous expansion and change in shape throughout the cleavage stage, via osmotic uptake of water (Slack and Warner 1973; Han et al. 1991; Uochi et al. 1997), as well as the progressive epibolic thinning of the

blastocoel wall (Longo et al. 2004). Other forces involved with blastular morphogenesis remain obscure but certainly include localized regulation of cell–cell interactions, as illustrated by the effective obliteration of the blastocoel following interference with cadherin-dependent adhesion or Eph–ephrin-mediated cell–cell repulsion (Heasman et al. 1994a, b; Winning et al. 1996).

4.4.3 Transition of Cell Division Factors from Oocytes to Embryos

Egg activation, typically coincident with fertilization, is regarded as a natural boundary between egg and zygote. Indeed, egg activation involves many processes that turn a quiescent cell into an actively dividing one. A dramatic example of this sharp transition is the cortical calcium wave associated with egg activation and/or sperm entry, which triggers the exocytosis of cortical granules and remodeling of surrounding egg membranes to act as a block against polyspermy (see Chap. 1). Another dramatic example is the initiation of maternal transcript polyadenylation upon egg activation, which results in the translation of their cognate protein products for use during embryonic development (see Chap. 2).

Besides the abrupt physiological transition brought about by egg activation and the associated process of fertilization, several other profound alterations in cellular processes are known to take place between the end of oogenesis and early embryonic development. This is exemplified by the transition between two different mechanisms for spindle formation in mouse embryos (Courtois et al. 2012). Vertebrates exhibit degeneration of centrioles during oogenesis; centrioles are provided solely by the sperm, an arrangement that ensures a constant centriole number through generations and is an obstacle to parthenogenetic development (Symerly et al. 1995; Delattre and Gönczy 2004). Thus, in vertebrates, spindle formation during meiosis utilizes a centriole-independent pathway in which microtubules self-organize into a pair of microtubule foci which, although wider than those organized by centriole pairs, can nevertheless direct the formation of a barrel-shaped bipolar spindle (Heald et al. 1996, 1997; Gaglio et al. 1997; Walczak et al. 1998; Schuh and Ellenberg 2007). During cleavage stages, embryonic cells typically use bipolar spindles whose formation relies on centrioles inherited through the sperm. However, in rodent lineages, not only oocyte centrioles but also sperm centrioles degenerate (Woolley and Fawcett 1973; Schatten 1994; Manandhar et al. 1998), and the early rodent embryo has to rely on a centriole-independent mechanism to generate MTOCs and spindles. Courtois et al. (2012) found that, unlike most organisms, the mechanism for MTOC formation in the early mouse embryo fails to undergo a sharp transition at fertilization. Instead, early mouse embryos form MTOCs and spindles that have morphological properties similar to those in oocytes during meiosis. During the first eight embryonic cleavages, MTOC and spindle morphology change gradually from the meiotic pattern to one that is typical of later embryonic stages,

e.g., a centriole-dependent MTOC nucleation and the presence of conventional centriole markers. These studies further revealed a similar requirement for the centriole-independent formation of meiotic and early mitotic spindles on microtubule-dependent motors, such as dynein and kinesin-5 (Schuh and Ellenberg 2007; Courtois et al. 2012), showing not only a continuing reliance on meiotic factors during the early mitotic divisions but also the deployment of these factors in similar cellular programs.

Another example of gradual transitioning from oocytes to embryos is the finding in zebrafish of copies of housekeeping genes, which are specialized for maternal expression and which function during both meiosis and early mitosis. This phenomenon has been observed in a maternally expressed form of the protein survivin, which, as mentioned above, is a component of the CPC complex involved in furrow induction and maturation. A mutation in the gene motley was found to affect one of two *survivin* (a.k.a. *birc*) genes in the zebrafish genome, *birc5b* (Nair et al. 2013). This gene exhibits predominantly maternal expression, whereas the related gene *birc5a* is expressed both maternally and throughout zygotic development. Mutations in *motley/birc5b* as well as Birc5b protein localization indicate a specialized role for this gene copy in cytokinesis during both meiotic divisions and early zygotic mitotic divisions. A similar dual function in meiotic and early embryonic mitotic divisions is observed in the case of another maternal zebrafish gene, *tmi* (Nair and Pelegri, unpublished). These examples highlight the continuation of cellular programs across the generational boundary occurring at fertilization, possibly because, in the absence of ongoing transcription, reutilization of programs involved in oocyte formation is an effective way to implement processes subsequently required for early embryonic development. Further studies will be required to understand the prevalence of such cellular program reutilization across the fertilization boundary as well as the role of gene duplication in the generation of genes acting in such processes.

4.4.4 Mammalian Embryo Compaction

In mammals, the cleavage-stage embryo undergoes several mitotic divisions until compaction, or intracellular adhesion, occurs to form a morula. Initially described by Mulnard and Huygens (1978) in mouse embryos and further characterized by others (Ziomek and Johnson 1980; Batten et al. 1987; Natale and Watson 2002), the formation of a morula represents the first morphological disruption in embryo radial symmetry (Fig. 4.11). It is thought that compaction is required for subsequent morphogenetic events such as lineage specification, but how this process is regulated is generally not well understood (Kidder and McLachlin 1985; Levy et al. 1986). In the mouse, compaction is mediated by the formation of adherens junctions, or protein complexes between cells, of which epithelial cadherin (E-cadherin) is a major component (Vestweber et al. 1987). As a type-1 transmembrane protein, E-cadherin relies on calcium ions (Ca^{2+}) and its intracellular binding partners, alpha-catenin (α-catenin) and beta-catenin (β-catenin), to function. However, since E-cadherin

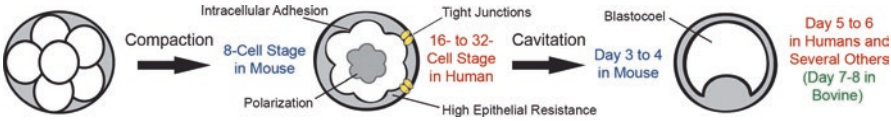

Fig. 4.11 Mammalian embryo compaction and blastulation. The processes of compaction, intracellular adhesion, and polarization result in the formation of a morula at the eight-cell and 16- to 32-cell stage in mouse and human embryos, respectively. In mammals, compaction is mediated by the development of adherens junctions and results in the development of the first developmental asymmetry in the embryo. Once compaction is complete, the assembly of tight junctions in the embryo initiates cavitation and the formation of a fluid-filled cavity called the blastocoel. Most mammalian species, including humans, form blastocysts between days 5 and 6, whereas mouse embryos begin blastulation earlier between days 3 and 4 and bovine embryos later between days 7 and 8

and α-/β-catenin are expressed in mouse embryos as early as the zygote stage, it is unclear how the process of compaction is initiated (Vestweber et al. 1987; Ohsugi et al. 1996). Of note, compaction still occurs even if transcription is inhibited beginning at the four-cell stage (Kidder and McLachlin 1985) and in fact can be prematurely induced by incubating four-cell embryos with protein synthesis inhibitors (Levy et al. 1986). This suggests that all the components required for embryo compaction have been synthesized by the four-cell stage, and, given that premature compaction is observed in the presence of protein kinase activation as well, it also indicates that compaction is under the control of posttranslational regulation via phosphorylation (reviewed in Cockburn and Rossant 2010). Indeed, both E-cadherin and β-catenin become phosphorylated at the time of compaction (Pauken and Capco 1999), and a recent report demonstrated that E-cadherin-dependent filopodia are responsible for the cell shape changes necessary for compaction in mouse embryos (Fierro-González et al. 2013). Using live-cell imaging and laser ablation, this study determined that filopodia extension is tightly coordinated with blastomere elongation and that the inhibition of filopodia components, E-cadherin, α-/β-catenin, F-actin, and myosin-X, prevented cellular elongation and mouse embryo compaction. Whether other mammalian embryos establish and/or maintain cell elongation by similarly extending filopodia is not known, but an earlier time for the initiation of compaction correlates with implantation success in human IVF embryos (Landry et al. 2006; Skiadas et al. 2006).

Along with cell elongation, intracellular polarization also occurs during compaction, whereby the outward-facing, or apical, region of each blastomere becomes distinct from the inward-facing (basolateral) regions at least in mouse embryos. In particular, the blastomere nuclei move basolaterally, whereas both actin and microtubules concentrate apically concomitant with differential localization of membrane and polarity protein complexes (Reeve and Kelly 1983; Johnson and Maro 1984; Houliston and Maro 1989). Cell–cell contact appears to be essential for establishing the orientation of polarity, since blastomeres polarize along the axis perpendicular to cell contact and apical poles form farthest from the contact point. However, additional mechanisms are also likely involved (Ziomek and Johnson 1980; Johnson

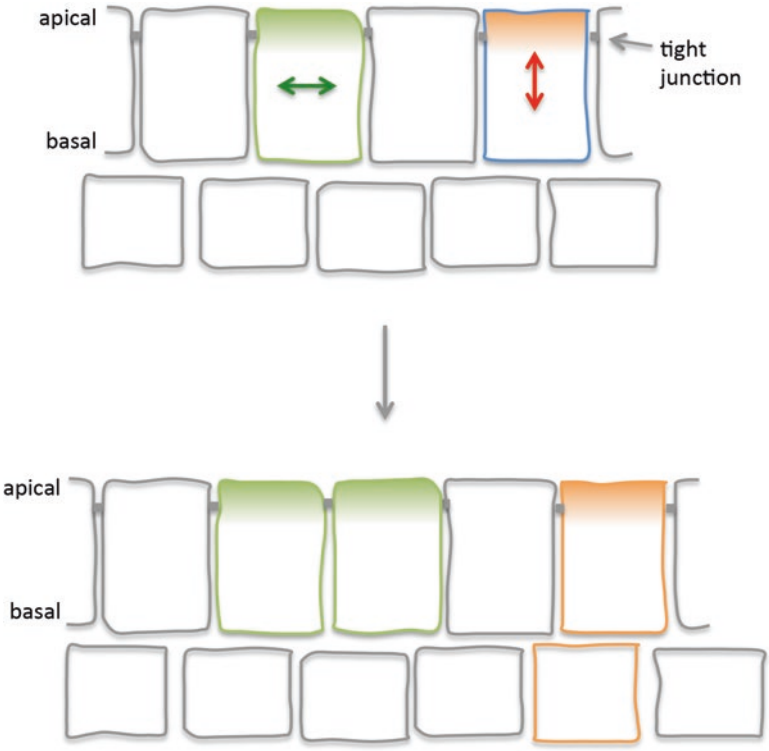

Fig. 4.12 Cell division in a polarized epithelium results in different patterns depending on spindle orientation. Spindle orientation perpendicular to the direction of cell apical–basal polarization (green) results in a cleavage plane that maintains polarization in both daughter cells. Spindle orientation parallel to the direction of cell polarization (red) results in one daughter cell that is polarized and another that is not and which exits the epithelium. Spindle orientation is indicated by a double arrow

and Ziomek 1981a, b). Once the embryo has compacted and polarized, subsequent cell divisions are influenced by the orientation of the cleavage plane so that the established polarity is inherited in the daughter cells (Fig. 4.12). If a blastomere divides at an angle parallel to its axis of polarity, both daughter cells will be polar and remain on the outside of the embryo. However, if a blastomere undergoes mitosis perpendicular to its axis of polarity, one daughter cell will be polarized and contribute to the outside of the embryo, whereas the other daughter cell will be apical and become a part of the inside of the embryo (Johnson and Ziomek 1981a, b; Sutherland et al. 1990). These symmetric versus asymmetric cleavage divisions eventually result in the generation of two distinct cell populations; the cells on the inside will become a part of the inner cell mass (ICM), and the cells on the outside will contribute to the trophectoderm (TE) layer of the blastocyst.

As expected, much of what we know about embryo compaction and blastocyst formation has been derived from studies in mice, and therefore relatively little is

known about these processes in other mammalian species, including humans. Notably, compaction occurs much earlier in mouse embryos, at the eight-cell stage, than in human embryos, where it begins at the 16-cell stage and, even later, at the 32-cell stage, in bovine embryos (Steptoe et al. 1971; Edwards et al. 1981; Nikas et al. 1996; Van Soom et al. 1997). Almost immediately following compaction, the formation of a fluid-filled cavity called the blastocoel is initiated by the assembly of tight junctions, which includes occludin, cingulin, as well as other components, and the establishment of high epithelial resistance in TE cells until the 32-cell stage (Fleming et al. 1993; Sheth et al. 1997, 2000). Once the blastocoel is formed, human embryos are likely to undergo at least one additional round of cell division to form a ~256-cell blastocyst, whereas mouse blastocysts typically comprise ~164 cells (Niakan and Eggan 2013). Until recently, it was unknown how these differences in the timing of compaction or number of cells may affect polarization and asymmetric cell divisions in the human embryo. In contrast to the mouse, wherein TE and ICM fates are established in a positional and cell polarization-dependent manner at the morula stage as described above, human embryos appear to establish the TE as well as the epiblast and primitive endoderm lineages concurrently at the blastocyst stage (Petropoulos et al. 2016). This study also noted that human embryo compaction is not as prominent as in the mouse, with only partial compaction occurring in a certain number of blastomeres on embryonic day 4. Consequently, it is not until embryonic day 5 and upon blastocyst formation that distinct inner and outer compartments are observed in human embryos. Whether other mammals undergo compaction via a cell polarization-dependent mechanism at the morula stage for lineage specification or establish the first lineages concomitant with blastocyst formation is unclear, but partial compaction has been observed in bovine, porcine, and rabbit embryos (Reima et al. 1993; Koyama et al. 1994). Taken together, this suggests that although mammalian embryos morphologically resemble each other during preimplantation development, there are several notable species-specific differences that may limit extrapolation between mammals.

4.5 Evolutionary Relationship Between Holoblastic and Meroblastic Cleavage Types

The phylogenetic distribution of holoblastic and meroblastic cleavage indicates that the latter has evolved independently five times in craniates (a phylogenetic group containing the vertebrates and hagfish (Myxini)) (Collazo et al. 1994; Collazo 1996; Fig. 4.13). Several closely related groups outside of craniates, such as ascidians, tunicates, and echinoderms, exhibit holoblastic cleavage, suggesting that this type of cleavage is the ancestral mode. Within craniates, meroblastic cleavage appears to have evolved independently in Myxini (hagfish), Chondrichthyes (sharks, skates, and rays), Teleostei (largest infraclass of ray-finned fishes), Actinistia (coelacanths), and Amniota (certain non-eutherian mammals (e.g., egg-laying monotremes), birds,

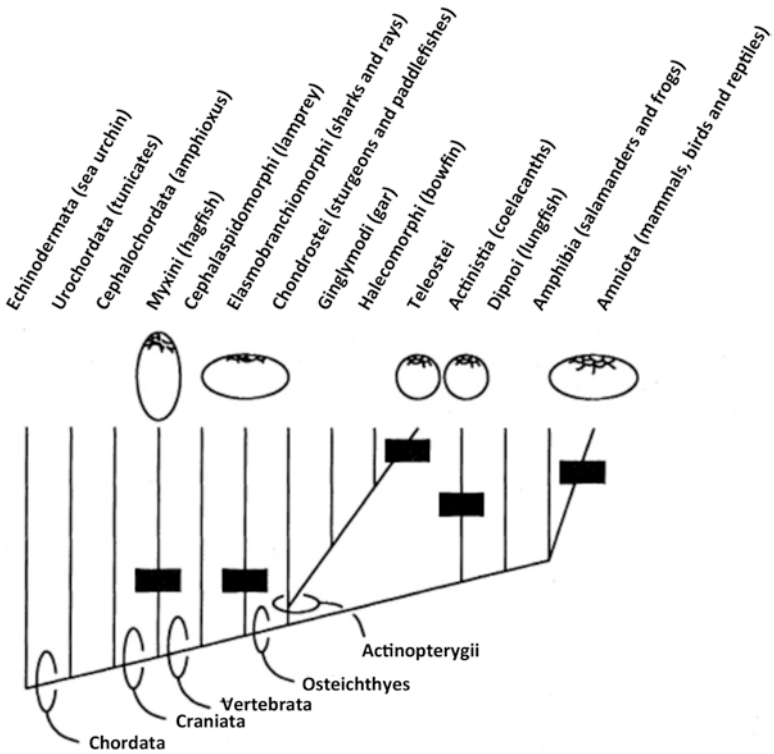

Fig. 4.13 Independent appearance of meroblastic cleavage in various vertebrate phylogenetic lineages. Phylogenetic tree of lineages from sea urchin to vertebrates, showing that meroblastic cleavage (black rectangles) arose multiple times and independently within these lineages. Diagram reproduced from Collazo et al. (1994), with permission

and reptiles). Convergent evolution of meroblastic cleavage is further supported by differences in early development between the various lineages that undergo meroblastic cleavage (Collazo 1996). Independent evolution of meroblastic cleavage appears to reflect a selective advantage. Once arisen within a lineage, meroblastic cleavage is typically not lost, again consistent with an evolutionary advantage. A notable exception to this pattern is the inferred reversion of cleavage pattern within Amniota, from meroblastic to holoblastic as found in eutherian mammals and marsupials.

In amphibian holoblastic cleavage, like that observed in the model vertebrate *Xenopus*, all blastomeres eventually contribute to one of the three germ layers. The ancestral nature of holoblastic cleavage is largely responsible for the widely held assumption that amphibian-like cleavage represents the ancestral form of holoblastic cleavage in vertebrates. However, evidence from bichir (*Polypterus*), a basal actinopterygian (ray-finned fish), and lamprey (*Lampetra japonica*), a basal vertebrate, challenges this view (Takeuchi et al. 2009). Vegetal cells in embryos of these organisms do not express mesodermal or endodermal markers as in the case in amphibi-

ans. Analyses of such embryos suggest that some vegetal cells do not contribute to any of the three germ layers and are instead nutritive yolk cells only. This fact, combined with the evolutionary position of basal fish like bichirs and lampreys, points to the conclusion that, while holoblastic cleavage is ancestral in vertebrates, the particular form observed in amphibians, with all blastomeres contributing to embryonic tissues, is derived. This conclusion is further bolstered by the observation that a maternally expressed homologue of vegT, which is crucial for early amphibian endoderm development, appears to only be found in amphibians and not other vertebrate species, such as mice, bichirs, lampreys, and teleosts (Takeuchi et al. 2009).

The ancestral trait of having explicitly nutritive yolk cells in vertebrates may have provided early embryos an evolutionarily advantageous ability to implement cell division in the absence of yolk granules while nevertheless maintaining embryonic nutritive stores, an advantage that may have been maintained in meroblastic cleaving embryos. Developing embryos are known to rely on exquisitely precise cellular processes, such as cytoskeletal reorganization and the recycling of membrane particles during cell division, and it is easy to imagine that the presence of yolk particles may interfere with, or add variability to, this process. Selection against such interference could be one cause of a transition to a meroblastic cleavage system in an animal's lineage.

The inference of explicitly nutritive yolk cells in ancestral vertebrates may also make it easier to understand precisely how meroblastic cleavage might evolve from holoblastic cleavage. For example, fusion of yolky blastomeres into a single nondividing mass is proposed to be the second of two changes that occurred leading to evolution of the teleost embryo, the first being loss of bottle cells that are still present near the beginning of gastrulation in more basal taxa (Collazo et al. 1994). Given this, groups such as bowfins (*Amia*) and gars (*Lepisosteus*) (Ballard 1986a, b; Long and Ballard 2001), which exhibit cleavage patterns that appear to be partially meroblastic (Fig. 4.14a), may be representative of ancestral transitional states along a continuum from holoblastic to meroblastic cleavage.

An important correlation that has long been observed in the study of cleavage pattern evolution is that meroblastic cleavage often correlates with large egg size (Collazo 1996). Egg size correlation can be most clearly seen in amniotes where large eggs and meroblastic cleavage are predominant, with the small embryos of eutherian mammals and marsupials having returned to essentially holoblastic cleavage. Furthermore, as discussed above, the generally small eggs of amphibians exhibit a likely derived form of holoblastic cleavage. In such cases, selected traits such as differing degrees of reliance on egg nutritive stores may underlie the correlation between cleavage type and egg size, although other explanations have been proposed (Collazo 1996).

Teleosts constitute a major exception to this correlation, as there appears to be a sharp decrease in egg size in their stem phylogenetic lineage (Collazo 1996). Conversely, the Puerto Rican tree frog, *Eleutherodactylus coqui*, has an egg approximately 20 times the size of *Xenopus laevis* but in spite of this enormous size maintains a holoblastic cleavage pattern. How these particular species may escape the

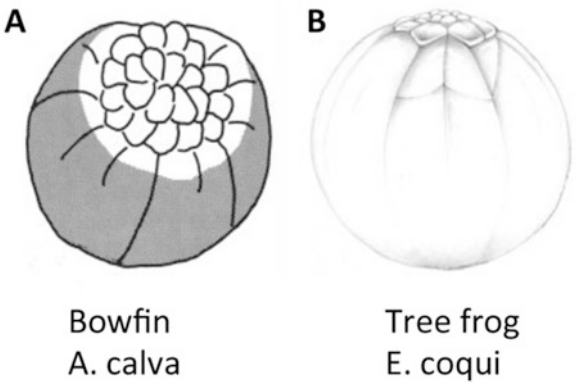

Fig. 4.14 Intermediates between holoblastic and meroblastic cleavage patterns. (**a**) Partially meroblastic cleavage in bowfin fish (*A. calva*). The yolk-rich vegetal region ceases to cleave after the formation of 16 cleavage furrows. (**b**) Formation of nutritional endoderm in the Costa Rican tree frog (*E. coqui*). Yolk-rich vegetal cells divide although do not eventually become part of the embryo proper (Buchholz et al. 2007). Diagrams reproduced from Ballard (1986a, b), in panel (**a**) and Buchholz et al. (2007) in panel (**b**), with permission

general rule of egg size and type of cleavage is unknown. However, blastula embryos for *E. coqui* exhibit a distinct population of nutritional vegetal cells that are not destined to become endodermal tissue (or any other embryonic cell type) (Buchholz et al. 2007; Fig. 4.14b), and, as mentioned above, the presence of such nutritional cells may represent an important step in the evolutionary transition from holoblastic to meroblastic cleavage (Buchholz et al. 2007; Elinson 2009).

Thus, it appears that holoblastic cleavage is the ancestral pattern of cleavage in craniates. Meroblastic cleavage, in which only a portion of the embryo is made up of dividing blastomeres, has evolved independently at least five times within this group. This evolution is sometimes, though not always, correlated with increased egg size. Meroblastic cleavage evolution in teleosts is a significant exception and has been shown to involve two evolutionary changes, loss of bottle cells and fusion of yolk into a single mass. The presence of a population of nutritive cells, whether ancestral or derived in a lineage, may be a key innovation on the way toward meroblastic cleavage. These innovations likely conferred a selective advantage, possibly an increased ability to implement subcellular programs required for early embryogenesis.

4.6 Conclusions

In this chapter, we have described mechanisms underlying patterns of cell cleavage arrangement in early vertebrate embryos. Much of our knowledge on this topic stems from studies in tractable developmental systems such as amphibians and teleosts.

These studies highlight major challenges that the cleavage-stage embryo, in these and likely other vertebrate species, has to overcome. These include structures such as spindle asters that are too small relative to the large early blastomeres, a limited supply of cellular building blocks within a changing landscape of cell size and organization, and the requirement for cytoskeletal specializations adapted to very large cells. We discuss how the vertebrate embryo appears to use simple rules to drive development even under these limitations, such as the use of interphase astral microtubules from a given cycle to orient the spindle for the following cell cycle or the use of limiting inherited reagents, such as tubulin, to scale spindles according to cell size in later-stage embryos. Such simple rules provide elegant solutions to overcome constraints associated with the transition from an egg into a three-dimensional embryonic blastula. Notably, we examine how a combination of cell shape-sensing cues, including those from a microtubule exclusion zone at the furrow for the previous cell cycle to orient the spindle, explain the sequence of blastomeric divisions leading to the basic cell arrangement in both zebrafish and *Xenopus* embryos, and possibly other vertebrates as well, though this remains to be determined. Interestingly, dynamic changes in the embryonic developmental landscape contribute to developmental decisions as they occur. For example, changes in cell dimensions in teleost blastomeres likely result in the eventual shift of the spindle plane from a horizontal (x–y) axis to a vertical (z) axis, generating a two-tiered blastula.

Although not as well studied, similar rules may exist across the range of vertebrate species, for example, mechanisms for spindle scaling in mammals and mechanisms of cleavage plane positioning in proto-vertebrates such as ascidians, suggesting that cellular mechanisms involved are highly conserved. This hypothesis is bolstered by examination of the phylogenetic distribution of major cleavage patterns across vertebrates and their close relatives.

It is possible that a wide variety of cleavage patterns can be explained with the same simple rules but with different initial parameters or conditions. For example, the presence of yolk enriched in the vegetal pole in Xenopus likely creates a pulling force bias on the spindle, resulting in the animal movement of the spindle and eventually an asymmetric cell division leading to a smaller animal pole cell and a larger vegetal pole cell. As another example, a change in shape in the initial blastodisc may lead to changes in the relative proportions of blastomere allocated to different dimensions in the resulting blastula. Basic cell shape-sensing mechanisms also interact with specialized cytoplasmic structures, such as the CAB in ascidians, resulting in the generation of added cell cleavage pattern variation. The observed embryonic patterns thus appear to be the outcome of a temporal sequence based on initial embryonic conditions (starting shape, amount, and type of relevant factors) as they are modified by ongoing cycles of cell division by the factors inherited by the egg itself. Future studies will continue to address detailed mechanistic aspects that drive these early embryonic processes and will also allow us to understand how changes in various conditions and parameters lead to diversity in blastomere arrangements encountered in different species. Cell cleavage pattern is a backdrop on which cell fate decisions are overlain, and it will also be important to better understand the interconnection between these two types of processes. Future studies

will surely continue to provide us with a view of the elegant mechanisms embryos use to solve the unique problem of transforming an egg into a multicellular blastula.

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