Zebra Fish: cell Movement and early development, patterning

Early Zebrafish Development

In recent years, the teleost (bony) fish *Danio rerio*, commonly known as the zebrafish, has joined *Xenopus* as a widely studied model of vertebrate development (see Figure 11.1B). Despite differences in their cleavage patterns (*Xenopus* eggs are holoblastic, dividing the entire egg, whereas the yolky zebrafish egg is meroblastic, wherein only a small portion of the yolky cytoplasm forms cells), *Xenopus* and *Danio* form their body axes and specify their cells in very similar ways.

Zebrafish have large broods, breed all year, are easily maintained, have transparent embryos that develop outside the mother (an important feature for microscopy), and can be raised so that mutants can be readily discovered and propagated in the laboratory. In addition, these fish develop rapidly. By 24 hours after fertilization, the embryo has already formed most of its organ primordia and displays a characteristic tadpole-like form (**FIGURE 11.32**; see Granato and Nüsslein-Volhard 1996; Langeland and Kimmel 1997). Furthermore, the ability to microinject fluorescent dyes into single blastomeres and to generate transgenes driving cell-type-specific fluorescent protein expression has allowed scientists to follow individual living cells as an organ develops.

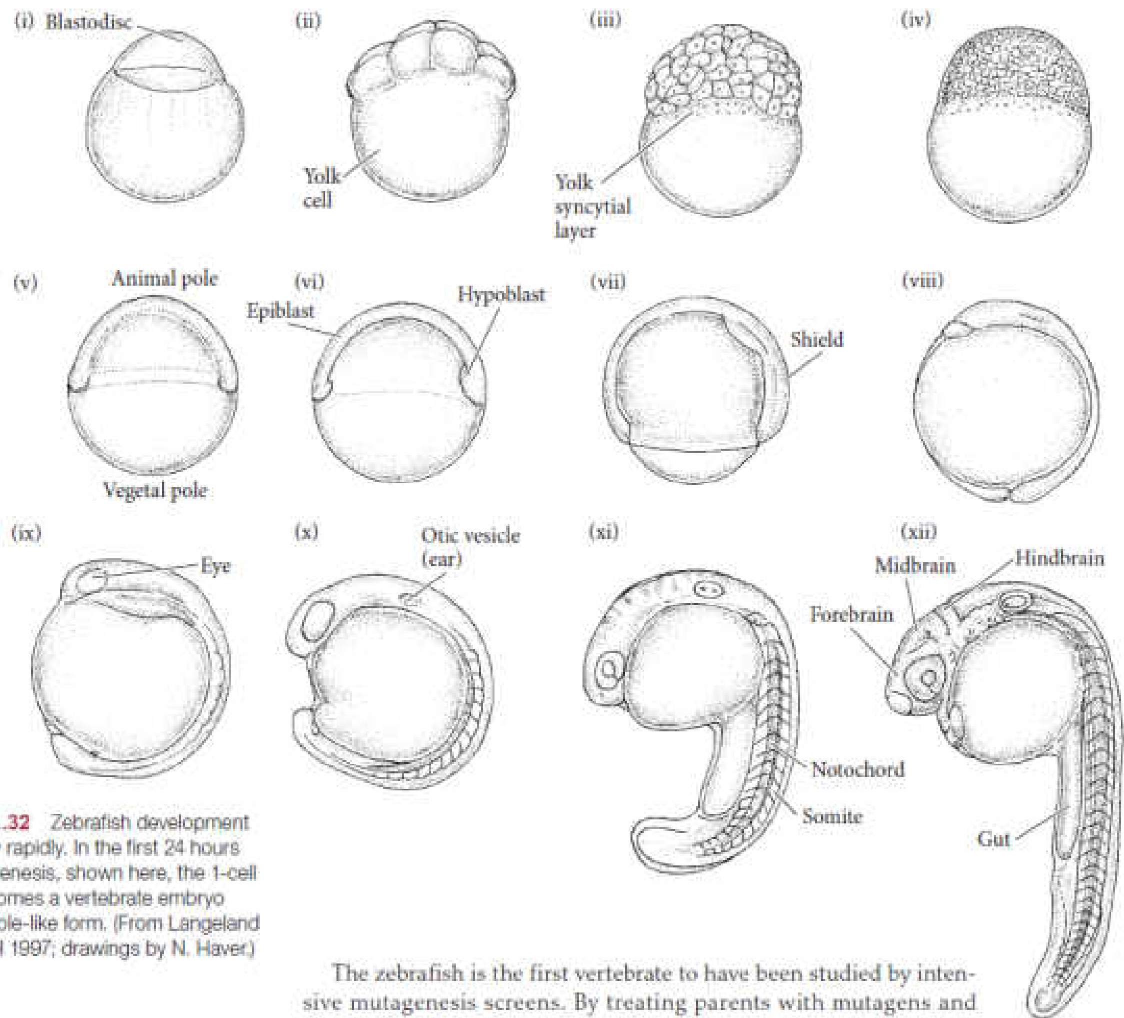

FIGURE 11.32 Zebrafish development occurs very rapidly. In the first 24 hours of embryogenesis, shown here, the 1-cell zygote becomes a vertebrate embryo with a tadpole-like form. (From Langeland and Kimmel 1997; drawings by N. Haver.)

The zebrafish is the first vertebrate to have been studied by intensive mutagenesis screens. By treating parents with mutagens and selectively breeding the progeny, scientists have found thousands of mutations whose normally functioning genes are critical for development. The traditional method of genetic screening (modeled after large-scale screens in *Drosophila*) begins when the male parental fish are treated with a chemical mutagen that will cause random mutations in their germ cells. Each mutagenized male is then mated with a wild-type female fish to generate F_1 fish. Individuals in the F_1 generation carry the mutations inherited from their father. If the mutation is dominant, it will be expressed in the F_1 generation. If the mutation is recessive, the F_1 fish will not show a mutant phenotype, since the wild-type dominant allele will mask the mutation. The F_1 fish are then mated with wild-type fish to produce an F_2 generation that includes both males and females that carry the mutant allele. When two F_2 parents carry the same recessive mutation, there is a 25% chance that their offspring will show the mutant phenotype (**FIGURE 11.33**). Since zebrafish development occurs in the open (as opposed to within an opaque shell or inside the mother's body), abnormal developmental stages can be readily observed, and the defects in development can often be traced to changes in a particular group of cells (Driever et al. 1996; Haffter et al. 1996). Recently, high-throughput methods of gene analysis and the CRISPR genome editing system have propelled the analysis of zebrafish development, enabling mutations in particular genes to be rapidly generated, identified, and bred (see Gonzales and Yeh 2014; Vashney et al. 2015).

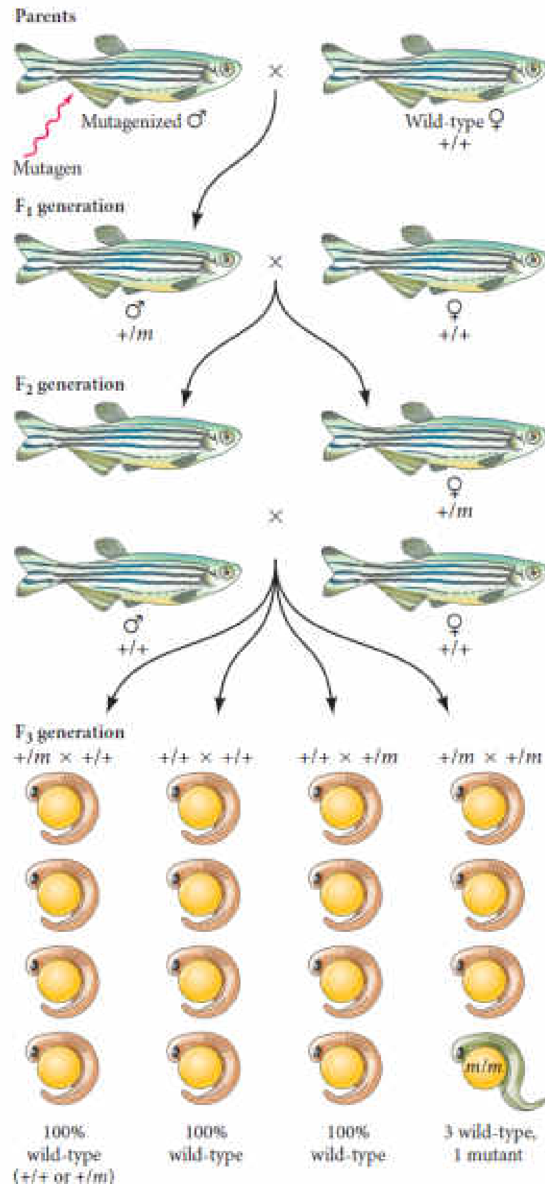

FIGURE 11.33 Screening protocol for identifying mutations of zebrafish development. The male parent is mutagenized and mated with a wild-type (+/+) female. If some of the male's sperm carry a recessive mutant allele (*m*), then some of the F_1 progeny of the mating will inherit that allele. F_1 individuals (here shown as a male carrying the mutant allele (*m*) are then mated with wild-type partners. This creates an F_2 generation wherein some males and some females carry the recessive mutant allele. When the F_2 fish are mated, approximately 25% of their progeny will show the mutant phenotype. (After Haffter et al. 1996.)

Like *Xenopus* embryos, zebrafish embryos are susceptible to morpholino antisense molecules (Zhong et al. 2001), and researchers can use this method to test whether a particular gene is required for a particular function. Furthermore, the green fluorescent protein reporter gene can be fused with specific zebrafish promoters and enhancers and inserted into the fish embryos. The resulting transgenic fish express GFP at the same times and places as the endogenous proteins controlled by these regulatory sequences. The amazing thing is that one can observe the reporter protein in living transparent embryos (**FIGURE 11.34**).

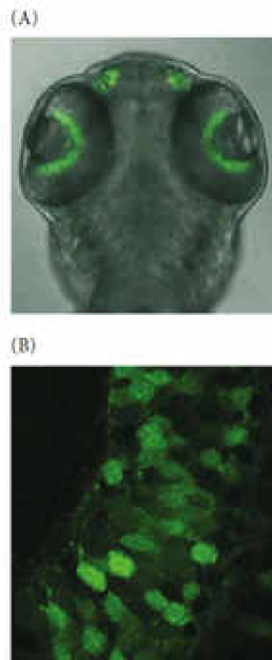

FIGURE 11.34 The gene for green fluorescent protein (GFP) was fused to the regulatory region of a zebrafish *sonic hedgehog* gene. As a result, GFP was synthesized wherever Hedgehog protein is normally expressed in the fish embryo. (A) In the head, GFP is seen in the developing retina and nasal placodes. (B) Because GFP is expressed by individual cells, scientists can see precisely which cells make GFP, and thus which cells normally transcribe the gene of interest (in this case, *sonic hedgehog* in the retina). (Photographs courtesy of U. Strähle and C. Neumann.)

The similarity of developmental mechanisms among all vertebrates and the ability of *Danio rerio* to be genetically manipulated has given this small fish an important role in investigating the genes that operate during human development (Mudbhary and Sadler 2011). When developmental biologists screened zebrafish mutants for cystic kidney disease, they found 12 different genes. Two of these genes were already known to cause human cystic kidney disease, but the other 10 were as-yet unknown genes that were found to interact with the first two in a common pathway. Moreover, that pathway, which involves the synthesis of cilia, was not what had been expected. Thus, the zebrafish studies disclosed an important and previously unknown pathway to explore human birth defects (Sun et al. 2004).

Zebrafish embryos are also permeable to small molecules placed in the water—a property that allows us to test drugs that may be deleterious to vertebrate development. For instance, zebrafish development can be altered by the addition of ethanol or retinoic acid, both of which produce malformations in the fish that resemble human developmental syndromes known to be caused by these molecules (Blader and Strähle 1998). As one zebrafish researcher joked, "Fish really are just little people with fins" (Bradbury 2004).

Cleavage

The eggs of most bony fish are **telolecithal**, meaning that most of the cytoplasm is occupied by yolk. Cleavage can take place only in the **blastodisc**, a thin region of yolk-free cytoplasm at the animal pole. The cell divisions do not completely divide the egg, so this type of cleavage is called **meroblastic** (Greek *meros*, "part"). Since only the blastodisc becomes the embryo, this type of meroblastic cleavage is referred to as **discoidal**.

Scanning electron micrographs show beautifully the incomplete nature of discoidal meroblastic cleavage in fish eggs (**FIGURE 11.35**). The calcium waves initiated at fertilization stimulate the contraction of the actin cytoskeleton to squeeze non-yolky cytoplasm into the animal pole of the egg. This process converts the spherical egg into a pear-shaped structure with an apical blastodisc (Leung et al. 1998, 2000). In fish, there are many waves of calcium release, and they orchestrate the processes of cell division. The calcium ions are critical for coordinating mitosis. They integrate the movements of the mitotic spindle with those of the actin cytoskeleton, deepen the cleavage furrow, and heal the membrane after the separation of the blastomeres (Lee et al. 2003).

The first cell divisions follow a highly reproducible pattern of meridional and equatorial cleavages. These divisions are rapid, taking only about 15 minutes each. The first 10 divisions occur synchronously, forming a mound of cells that sits at the animal pole of a large **yolk cell**. This mound of cells constitutes the **blastoderm**. Initially, all the cells maintain some open connection with one another and with the underlying yolk cell, so that moderately sized (17 kDa) molecules can pass freely from one blastomere to the next (Kane and Kimmel 1993; Kimmel and Law 1985). Remarkably, as the daughter cells migrate away from one another, they often retain these bridges through long tunnels connecting the cells (Caneparo et al. 2011).

Maternal effect mutations have shown the importance of oocyte proteins and mRNAs in embryonic polarity, cell division, and axis formation (Dosch et al. 2004; Langdon and Mullins 2011). As in frogs, the microtubules are important roads along which morphogenetic determinants travel, and maternal mutants affecting the formation of the microtubule cytoskeleton prevent the normal positioning of the cleavage furrow and of mRNAs in the early embryo (Kishimoto et al. 2004).

Fish embryos, like many other embryos, undergo a mid-blastula transition (seen around the tenth cell division in zebrafish) when zygotic gene transcription begins, cell divisions slow, and cell movements become evident (Kane and Kimmel 1993). At this time, three distinct cell populations can be distinguished. The first of these is the **yolk syncytial layer**, or **YSL** (Agassiz and Whitman 1884; Carvalho and Heisenberg

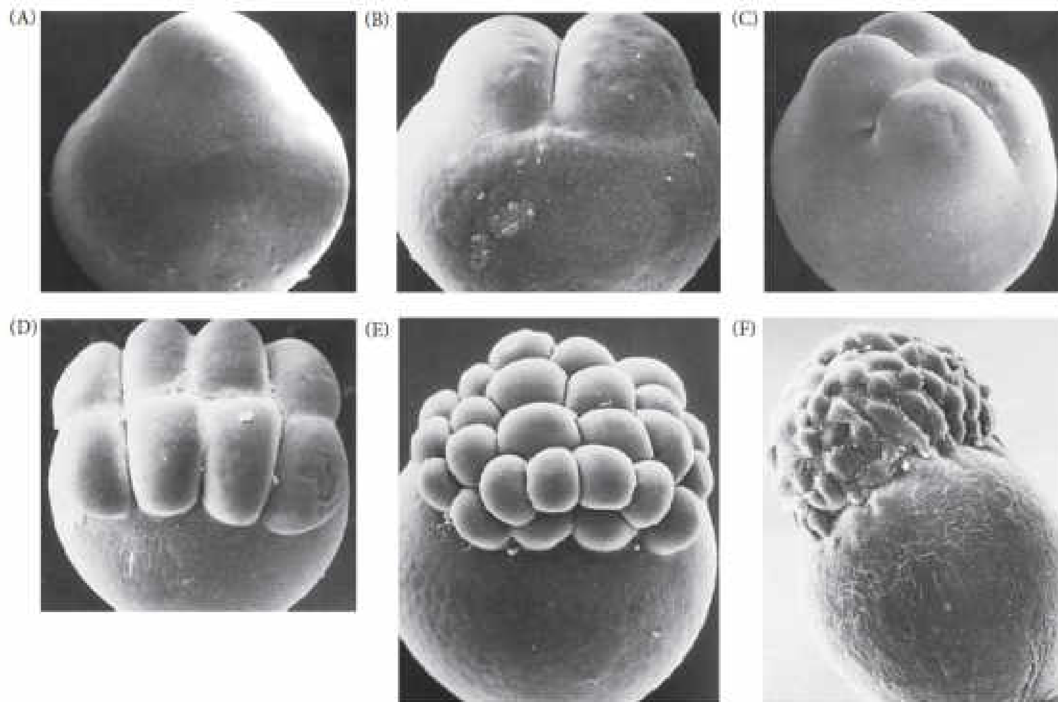

FIGURE 11.35 Discoidal meroblastic cleavage in a zebrafish egg. (A) 1-Cell embryo. The mound atop the cytoplasm is the blastodisc. (B) 2-Cell embryo. (C) 4-Cell embryo. (D) 8-Cell embryo, wherein two rows of four cells are formed. (E) 32-Cell embryo. (F) 64-Cell embryo, wherein the blastodisc can be seen atop the yolk cell. (From Beams and Kessel 1976, courtesy of the authors.)

2010). The YSL will not contribute cells or nuclei to the embryo, but it is critical for generating the fish organizer, patterning the mesoderm, and leading the epiboly of the ectoderm over the embryo (Chu et al. 2012). The YSL is formed at the tenth cell cycle, when the cells at the vegetal edge of the blastoderm fuse with the underlying yolk cell. This fusion produces a ring of nuclei in the part of the yolk cell cytoplasm that sits just beneath the blastoderm. Later, as the blastoderm expands vegetally to surround the yolk cell, some of the yolk syncytial nuclei will move under the blastoderm to form the **internal YSL (iYSL)**, and others will move vegetally, staying ahead of the blastoderm margin, to form the **external YSL (eYSL; FIGURE 11.36A,B)**. The YSL will be important for directing some of the cell movements of gastrulation.

The second cell population distinguished at the mid-blastula transition is the **enveloping layer (EVL)**. It is made up of the most superficial cells from the blastoderm, which form an epithelial sheet a single cell layer thick. The EVL is a protective covering that is sloughed off after 2 weeks. It allows the embryo to develop in a hypotonic solution (such as fresh water) that would otherwise burst the cells (Fukazawa et al. 2010). Between the EVL and the YSL is the third set of blastomeres, the **deep cells**, that give rise to the embryo proper.

The fates of the early blastoderm cells are not determined, and cell lineage studies (in which a nondiffusible fluorescent dye is injected into a cell so that its descendants can be followed) show that there is much cell mixing during cleavage. Moreover, any one of these early blastomeres can give rise to an unpredictable variety of tissue descendants (Kimmel and Warga 1987; Helde et al. 1994). A fate map of the blastoderm cells can be made shortly before gastrulation begins. At this time, cells in specific regions of the embryo give rise to certain tissues in a highly predictable manner (**FIGURE 11.36C**; see also Figure 1.11), although they remain plastic, and cell fates can change if tissue is grafted to a new site.

VADE MECUM

The segment on zebrafish development includes time-lapse movies of the beautiful and rapid development of this organism.

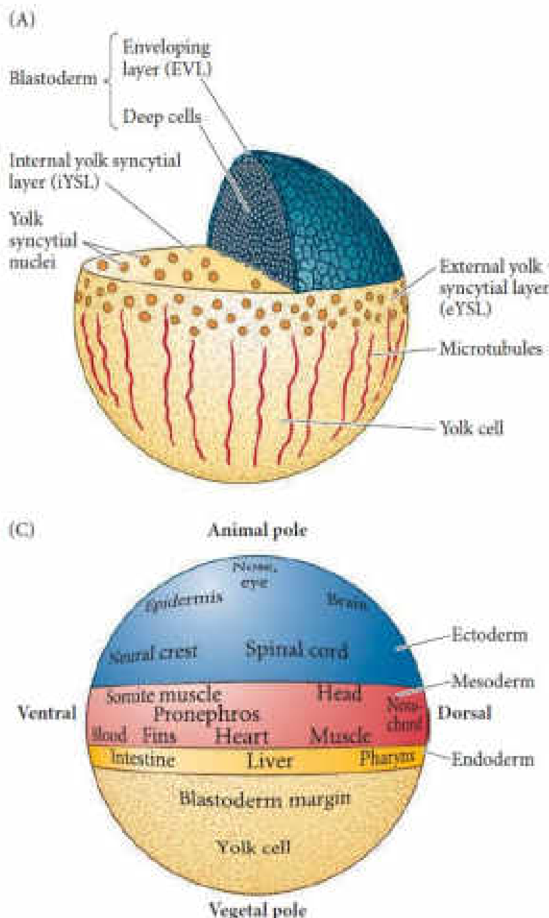

FIGURE 11.36 Fish blastula. (A) Prior to gastrulation, the deep cells are surrounded by the enveloping layer (EVL). The animal surface of the yolk cell is flat and contains the nuclei of the yolk syncytial layer (YSL). Microtubules extend through the yolky cytoplasm and the external YSL. (B) Late-blastula-stage embryo of the minnow *Fundulus*, showing the external YSL. The nuclei of these cells were derived from cells at the margin of the blastoderm, which released their nuclei into the yolky cytoplasm. (C) Fate map of the deep cells after cell mixing has stopped. This is a lateral view; for the sake of clarity, not all organ fates are labeled. (A,C after Langeland and Kimmel 1997; B from Trinkaus 1993, courtesy of J. P. Trinkaus.)

Gastrulation and Formation of the Germ Layers

All three layers of the zebrafish blastoderm undergo epiboly. The first cell movement of fish gastrulation is the epiboly of the blastoderm cells over the yolk, and this is thought to be controlled both by maternal proteins (such as Eomesodermin) and by new proteins transcribed from the YSL nuclei (Du et al. 2012). In the initial phase of this movement, the deep cells of the blastoderm move outward to intercalate with the cells closer to the surface of the embryo, and the yolk cell (with its syncytial nuclei) pushes upward (Warga and Kimmel 1990). This intercalation of cells causes a flattening of the "dome" of the blastoderm cells (**FIGURE 11.37A**).

PROGRESSION OF EPIBOLY When about half the yolk is covered, a new set of movements is initiated. The YSL nuclei divide such that some nuclei (constituting the external YSL, or eYSL) remain in the upper cortex of the yolk cell, while the iYSL (internal YSL) lies beneath the blastoderm. The enveloping layer is tightly joined to the iYSL by E-cadherin and tight junctions (Shimizu et al. 2005a; Siddiqui et al. 2010) and is dragged ventrally as the iYSL nuclei migrate "downward." That the vegetal migration of the blastoderm margin is dependent on the epiboly of the YSL can be demonstrated by severing the attachments between the YSL and the EVL. When this is done, the

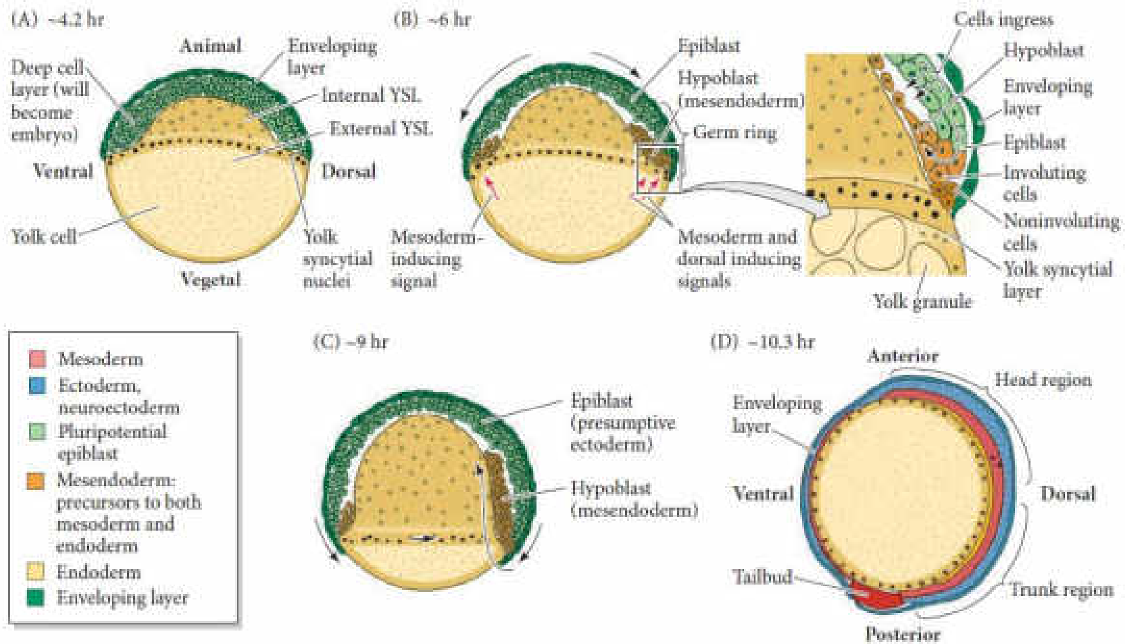

FIGURE 11.37 Cell movements during zebrafish gastrulation. (A) The blastoderm at 30% completion of epiboly (about 4.7 hours). (B) Formation of the hypoblast, either by involution of cells at the margin of the epibolizing blastoderm or by delamination and ingression of cells from the epiblast (6 hr). A close-up of the marginal region is at the right. (C) As ectodermal epiboly nears completion, the hypoblast, carrying the mesoderm and endoderm precursors, begins to cover the yolk. (D) Completion of gastrulation (10.3 hr). The germ layers (yellow endoderm, blue ectoderm, red mesoderm) are present. (After Driever 1995; Langeland and Kimmel 1997; Carvalho and Heisenberg 2010; Lepage and Bruce 2010.)

EVL and the deep cells spring back to the top of the yolk, while the YSL continues its expansion around the yolk cell (Trinkaus 1984, 1992).

The migration of the YSL ventrally depends partially on the expansion of this layer by cell division and intercalation, and partly on the cytoskeletal network within the yolk cell (see Lepage and Bruce 2010). An actomyosin band forms in the eYSL, at the boundary between the YSL and the EVL. This pulls down the YSL/EVL at its vegetal connection by means of contraction and friction (Behrndt et al. 2012). Meanwhile, the eYSL nuclei appear to migrate along the microtubles aligned along the animal-vegetal axis of the yolk cell, presumably pulling the iYSL and its accompanying EVL over the yolk cell. (Radiation or drugs that block the polymerization of tubulin slow epiboly; Strahle and Jesuthasan 1993; Solnica-Krezel and Driever 1994.) At the end of gastrulation, the entire yolk cell is covered by the blastoderm.

INTERNALIZATION OF THE HYPOBLAST After the blastoderm cells have covered about half the zebrafish yolk cell, a thickening occurs throughout the margin of the deep cells. This thickening, called the **germ ring**, is composed of a superficial layer, the epiblast (which will become the ectoderm); and an inner layer, the **hypoblast** (which will become endoderm and mesoderm). The hypoblast forms in a synchronous "wave" of internalization (Keller et al. 2008) that has some characteristics of ingression (especially in the dorsal region; see Carmany-Rampey and Schier 2001) and some elements of involution (especially in the future ventral regions). Thus, as the cells of the blastoderm undergo epiboly around the yolk, they are also internalizing cells at the blastoderm margin to form the hypoblast. The epiblast cells (presumptive ectoderm) do not involute, whereas the deep cells—the future mesoderm and endoderm—do (Figure 11.37 B,C). As the hypoblast cells internalize, the future mesoderm cells (the majority of the hypoblast cells) initially migrate vegetally while proliferating to make new mesoderm cells. Later, they alter direction and proceed toward the animal pole. The endodermal precursors, however, appear to move randomly over the yolk (Pézeron et al. 2008). The coordination of migration and cell specification

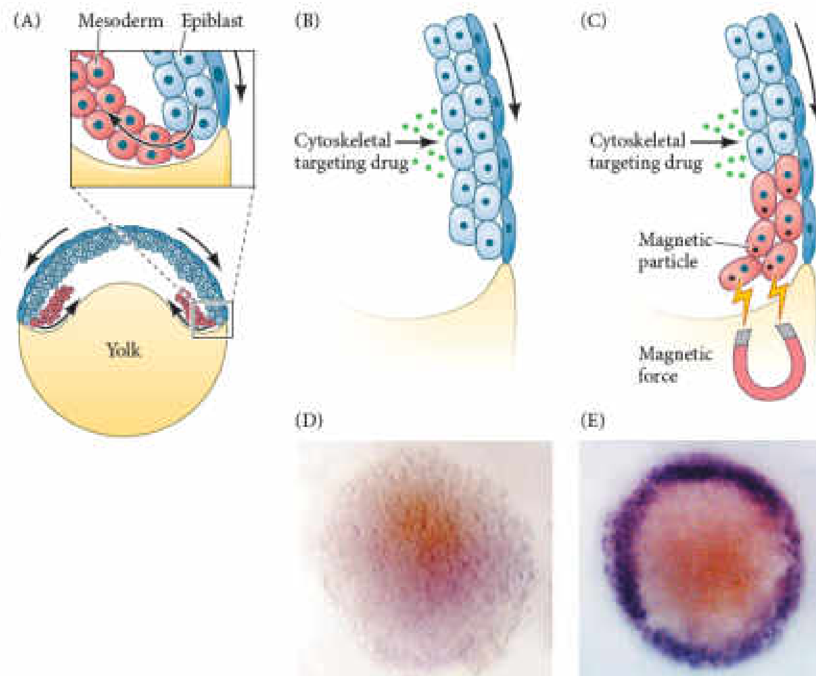

FIGURE 11.38 Stretching the zebrafish epiblast cells generates mesoderm. (A) During epiboly, cells at the border undergo structural changes and involute. As they do, mesodermal genes (red) are activated. (B) When the cortical cytoskeleton is prevented from contracting, the animal cap cells remain ectodermal and do not involute. (C) However, if these cells are pulled by a magnetic field, the mesodermal genes become expressed. (D,E) Circumpolar views of B and C, respectively, visualize expression of the mesodermal gene *Notail* (the zebrafish homologue of the *Brachyury* gene). (D) *Notail* expression is blocked by the lack of involution. (E) *Notail* expression induced by stretching and subsequent involution. (A–C after Piccolo 2013; D,E from Brunet et al. 2013.)

is accomplished by physical forces rather than by chemicals. When the cortical cytoskeleton is disrupted by drugs, the cells fail to turn and the mesodermal genes are not activated. However, if the cells are injected with magnetic particles before being hit with the drugs, they can be mechanically towed around the embryo. The cells don't involute, but the mesodermal genes do turn on. Thus, during normal development, epiboly and cell specification may be coordinated by the mechanical stress of involution (**FIGURE 11.38**; Brunet et al. 2013).

THE EMBRYONIC SHIELD AND THE NEURAL KEEL Once the hypoblast has formed, cells of the epiblast and hypoblast intercalate on the future dorsal side of the embryo to form a localized thickening, the **embryonic shield** (Schmitz and Campos-Ortega 1994). Here, the cells converge and extend anteriorly, eventually narrowing along the dorsal midline (**FIGURE 11.39A**). This convergent extension in the hypoblast forms the chordamesoderm, the precursor of the notochord (Trinkaus 1992; **FIGURE 11.39B,C**). This convergent extension is similar to that discussed in *Xenopus*, and is similarly accomplished by the Wnt-mediated planar cell polarity pathway (see Vervenne et al. 2008).

WATCH DEVELOPMENT 11.6 Watch convergent extension happen as the "ball" of cells is converted into a structure with a definite elongated anterior-posterior axis.

As we will see, the embryonic shield is functionally equivalent to the dorsal blastopore lip of amphibians, since it can organize a secondary embryonic axis when transplanted to a host embryo (Oppenheimer 1936; Ho 1992). The cells adjacent to the chordamesoderm—the paraxial mesoderm cells—are the precursors of the mesodermal somites (see Chapter 17). Concomitant convergence and extension in the epiblast bring presumptive neural cells from the epiblast into the dorsal midline, where they form the **neural keel**. The neural keel, a band of neural precursors that extends over the axial

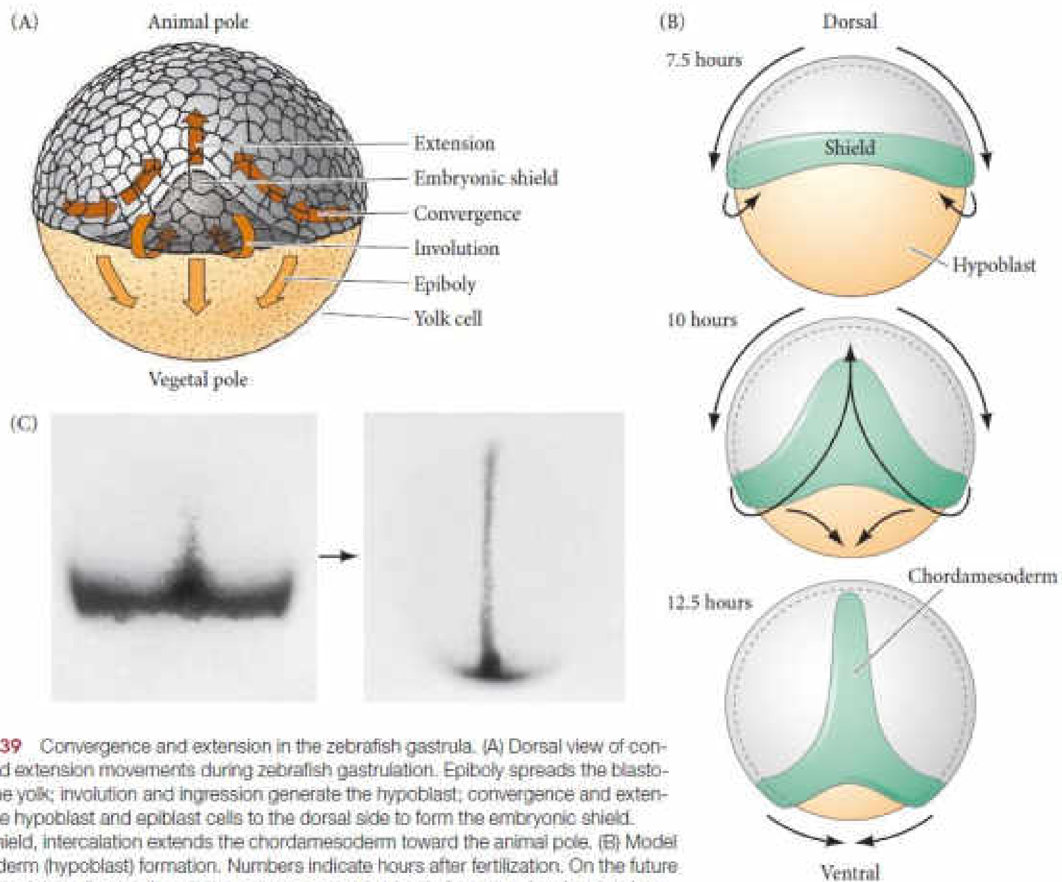

FIGURE 11.39 Convergence and extension in the zebrafish gastrula. (A) Dorsal view of convergence and extension movements during zebrafish gastrulation. Epiboly spreads the blastoderm over the yolk; involution and ingression generate the hypoblast; convergence and extension bring the hypoblast and epiblast cells to the dorsal side to form the embryonic shield. Within the shield, intercalation extends the chordamesoderm toward the animal pole. (B) Model of mesendoderm (hypoblast) formation. Numbers indicate hours after fertilization. On the future dorsal side, the internalized cells undergo convergent extension to form the chordamesoderm (notochord) and the paraxial (somitic) mesoderm adjacent to it. On the ventral side, the hypoblast cells migrate with the epibolizing epiblast toward the vegetal pole, eventually converging there. (C) Convergent extension of the chordamesoderm of the hypoblast cells. These cells are marked by their expression of the *no-tail* gene (dark areas) encoding a T-box transcription factor. (A,C from Langeland and Kimmel 1997, courtesy of the authors; B after Keller et al. 2001.)

and paraxial mesoderm, eventually develops a slitlike lumen to become the neural tube and to enter into the embryo.[7] Those cells remaining in the epiblast become the epidermis. On the ventral side (see Figure 11.39B), the hypoblast ring moves toward the vegetal pole, migrating directly beneath the epiblast that is epibolizing itself over the yolk cell. Eventually, the ring closes at the vegetal pole, completing the internalization of those cells that will become mesoderm and endoderm (Keller et al. 2008).

WATCH DEVELOPMENT 11.7 Watch two separate views of zebrafish neurulation, as well as Dr. Rolf Karlson's stunning video showing zebrafish development.

By different mechanisms, the *Xenopus* egg and zebrafish egg have reached the same state: they have become multicellular; they have undergone gastrulation; and they have

[7] This is different from the formation of the neural tube in frog embryos and is probably equivalent to "secondary" neural tube formation in the posterior of mammalian embryos (see Chapter 13).

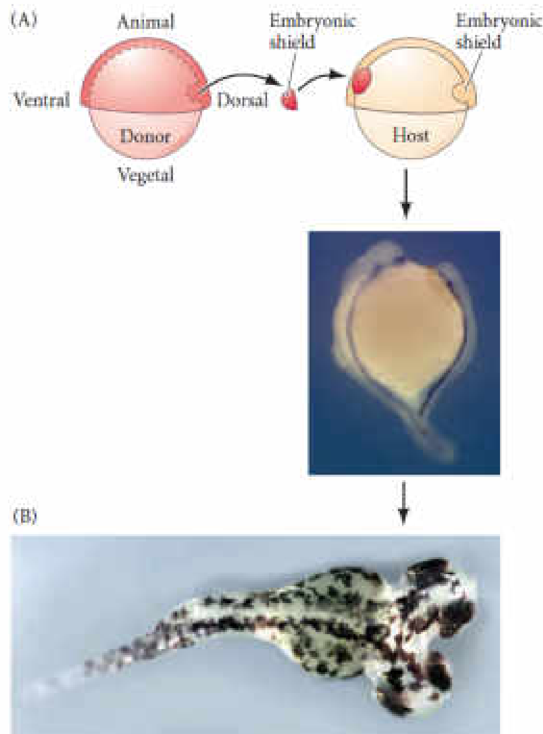

FIGURE 11.40 The embryonic shield as organizer in the fish embryo. (A) A donor embryonic shield (about 100 cells from a stained embryo) is transplanted into a host embryo at the same early-gastrula stage. The result is two embryonic axes joined to the host's yolk cell. In the photograph, both axes have been stained for *sonic hedgehog* mRNA, which is expressed in the ventral midline. (The embryo to the right is the secondary axis.) (B) The same effect can be achieved by activating nuclear β-catenin in embryos at sites opposite where the embryonic shield will form. (A after Shinya et al. 1999, photograph courtesy of the authors; B courtesy of J. C. Izpisúa-Belmonte.)

positioned their germ layers such that the ectoderm is on the outside, the endoderm is on the inside, and the mesoderm lies between them. We will now see that zebrafish form their body axes in ways very similar to those of *Xenopus*, and using very similar molecules.

Dorsal-ventral axis formation

As mentioned above, the embryonic shield of fish is homologous to the dorsal blastopore lip of amphibians, and it is critical in establishing the dorsal-ventral axis. Shield tissue can convert lateral and ventral mesoderm (blood and connective tissue precursors) into dorsal mesoderm (notochord and somites), and it can cause the ectoderm to become neural rather than epidermal. This transformative capacity was shown by transplantation experiments in which the embryonic shield of an early-gastrula embryo was transplanted to the ventral side of another (**FIGURE 11.40**; Oppenheimer 1936; Koshida et al. 1998). Two axes formed, sharing a common yolk cell. Although the prechordal plate and notochord were derived from the donor embryonic shield, the other organs of the secondary axis came from host tissues that would normally form ventral structures. The new axis had been induced by the donor cells.

Like the amphibian blastopore lip, the embryonic shield forms the prechordal plate and the notochord of the developing embryo. The prechordal plate cells are the first to involute, and they migrate toward the animal pole (Dumortier et al. 2012). The presumptive prechordal plate and notochord are responsible for inducing ectoderm to become neural ectoderm, and they appear to do this in a manner very much like the homologous structures in amphibians.[5] Like amphibians, fish induce the epidermis by BMPs (especially BMP2B) and Wnt proteins (especially Wnt8) made in the ventral and lateral regions of the embryo (see Schier 2001; Tucker et al. 2008). The notochords of both zebrafish and *Xenopus* secrete factors (the homologues of *chordin*, *noggin*, and *follistatin*) that block this induction, thereby allowing the ectoderm to become neural (Dal-Pra 2006). Like in amphibians, FGFs made in the dorsal side of the embryo also inhibit BMP gene expresson (Fürthauer et al. 2004; Tsang et al. 2004; Little and Mullins 2006). In the caudal region of the embryo, FGF signaling is probably the predominant neural specifier (Kudoh et al. 2004). And as in *Xenopus*, insulin-like growth factors (IGFs) also play a role in the production of the anterior neural plate. Zebrafish IGFs appear to upregulate *chordin* and *goosecoid* while restricting the expression of *bmp2b*. Although IGFs appear to be made throughout the embryo, during gastrulation the IGF *receptors* are found predominantly in the anterior portion of the embryo (Eivers et al. 2004). Also, Wnt inhibitors appear to play roles in head formation. When antisense morpholinos are used to downregulate Wnt3a and Wnt8 throughout gastrulating zebrafish embryos, the trunk structures become anteriorized (Shimizu et al. 2005b).

But in fish, there may be another important source of organization: the entire blastopore lip. Recall that the blastopore of a fish extends around the entire yolk cell. The dorsal lip (the shield) will induce head structures when placed into the ventral region of the blastopore margin. However, it will not induce any structures from the neighboring

[5]Another similarity between the amphibian and fish organizers is that they can be duplicated by rotating the egg and changing the orientation of the microtubules (Fluck et al. 1998). One difference in the axial development of these groups is that in amphibians, the prechordal plate is necessary for inducing the anterior brain to form. In zebrafish, although the prechordal plate appears to be necessary for forming ventral neural structures, the anterior regions of the brain can form in its absence (Schier et al. 1997; Schier and Talbot 1998).

tissue when placed onto the animal cap of a blastula, which contains thoroughly undifferentiated cells. When a graft from the *ventral* blastopore lip is placed on animal cap cells, a well-organized tail structure is formed, having epidermis, somites, neural tube, but no dorsal mesoderm (Agathon et al. 2003). Much of this structure is induced from the host tissue. So the ventral blastopore lip in zebrafish is a "tail organizer." Cells from the *lateral* blastopore lips will induce trunk and posterior head structures, having notochord tissue. Moreover, these transplanted tissues do not express BMPs, Wnts, or their antagonists.

So it appears that, in addition to the classical shield organizer, the entire blastopore lip appears involved in forming posterior head, trunk and tail, through another means. This second set of axis-determining factors appears to be a dual gradient of Nodal and BMP proteins (Fauny et al. 2009; Thisse and Thisse 2015). Along the blastopore lip, from the ventral to the dorsal margin, there forms a continuous gradation in the ratio of BMP to Nodal activity. BMP is highest at the ventral margin, low dorsolaterally, and approaches 0 in the dorsalmost domain, where only Nodal is active. Thus, each region of the blastopore lip is characterized by a specific BMP/Nodal ratio of activity. Remarkably, an entire ectopic axis can be made by injecting one of the animal cap blastomeres with Nodal mRNA and another animal cap cell with BMP mRNA (Xu et al., 2014). A gradient is formed between them, and the neighboring cells respond by constructing a new axis (**FIGURE 11.41**).

Moreover, by injecting different amounts of BMP and Nodal mRNAs into a single blastula animal cap cells, one can mimic the effect of the blastopore lip. Injections of mRNAs with a high BMP to Nodal ratio induce the formation of new tails growing from the animal pole of the embryo. Wnt8, a posterior morphogen, is produced in these cells. Injection of mRNAs with decreasing BMP to Nodal ratios induces the formation of secondary trunks from these animal pole cells. When Nodal and BMP are injected in the same amounts, a posterior head is induced (Thisse et al. 2000). As mentioned in *Xenopus*, Nodal proteins are critical for the formation of the Organizer; and in zebrafish, ectopic expression of Nodal in the ventral blastopore margin will convert the ventral blastopore lip into a shield, inducing an entire secondary axis. The shield in zebrafish may be the "head organizer," while the cells 180° away become the "tail organizer."

The engine for integrating the BMP-Chordin and BMP-Nodal axes appears to be β-catenin. As in *Xenopus*, β-catenin activates the *Nodal* genes. In addition, β-catenin activates the genes encoding FGFs and other factors that repress BMP and Wnt expression on the dorsal side of the embryo while activating the genes for *goosecoid*, *noggin*, and *dickkopf* there (Solnica-Krezel and Driever 2001; Sampath et al. 1998; Gritsman et al. 2000; Schier and Talbot 2001; Fürthauer et al. 2004; Tsang et al. 2004). As in *Xenopus*, β-catenin accumulates specifically in the nuclei destined to become the dorsal cells (Langdon and Mullins 2011). And, as

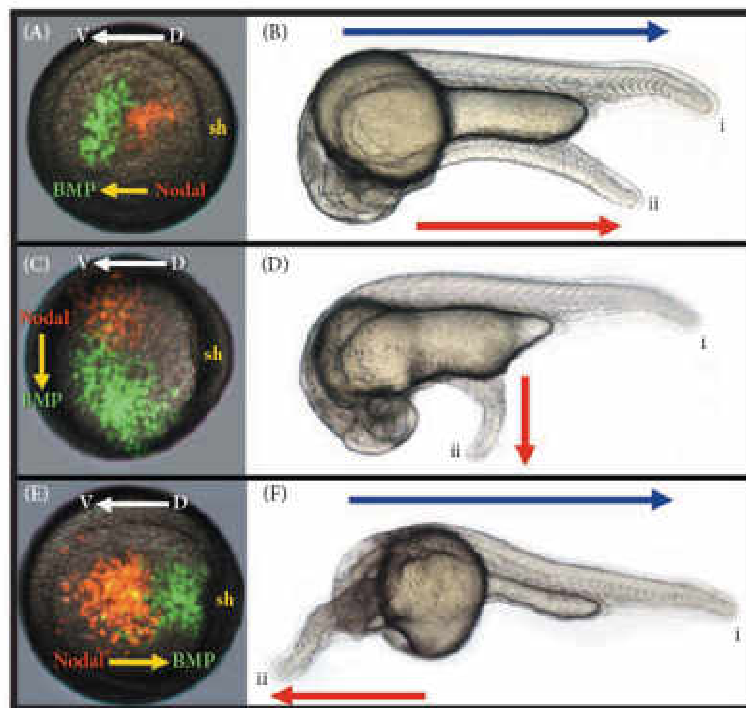

FIGURE 11.41 Correlation between the relative position of BMP- and Nodal-secreting clones and the orientation of the secondary embryonic axis induced at the animal cap. (A,B) When the Nodal-BMP vector (yellow arrow in A) is parallel to the D-V axis (white arrow) of the embryonic margin (where Nodal is strong dorsally and BMP strong ventrally), the original axis (blue arrow in B) and the secondary axis (red arrow) axes are parallel. (C,D) When the Nodal-BMP vector is perpendicular to the original dorsal-ventral axis, the secondary embryonic axis grows perpendicular to the primary axis. (E,F) When the Nodal-BMP vector is against that of the original dorsal-ventral axis, the primary and secondary axes grow in opposite directions. sh, embryonic shield. A, C, and E are animal pole views at the shield stage; B, D, and F are lateral views at 30 hours after fertilization. (From Xu et al. 2014, courtesy of C. Thisse.)

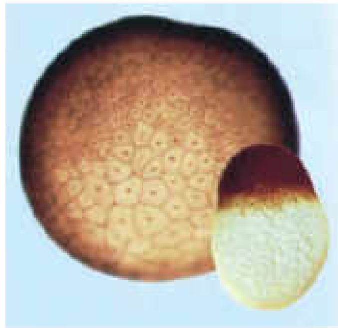

FIGURE 11.42 β-Catenin activates organizer genes in the zebrafish. (A) Nuclear localization of β-catenin marks the dorsal side of the *Xenopus* blastula (larger image) and helps form its Nieuwkoop center beneath the organizer. In the zebrafish late blastula (smaller image), nuclear localization of β-catenin is seen in the yolk syncytial layer nuclei beneath the future embryonic shield. (A courtesy of S. Schneider.)

VADE MECUM

Within the segment on zebrafish development you can see a visualization of the teratogenic effects of retinoic acid.

in *Xenopus*, this appears to be regulated by a maternal Wnt protein, in this case Wnt8a (Lu et al. 2011). The presence of β-catenin distinguishes dorsal YSL from the lateral and ventral YSL regions[9] (**FIGURE 11.42**; Schneider et al. 1996), and inducing β-catenin accumulation on the *ventral* side of the egg results in its dorsalization and a second embryonic axis (Kelly et al. 1995).

SCIENTISTS SPEAK 11.4 Dr. Bernard Thisse discusses experiments leading to the notion that the dorsal-ventral axis of the zebrafish is specified by a gradient of Nodal and BMP. Dr. Christine Thisse discusses the evidence that an entire embryo can be generated from pluripotent cells having two opposite gradient activities.

Anterior-posterior axis formation

The patterning of the neural ectoderm along the anterior-posterior axis in the zebrafish appears to be the result of the interplay of FGFs, Wnts, and retinoic acid, similar to that seen in *Xenopus*. In fish embryos, there seem to be two separate processes. First a Wnt signal represses the expression of anterior genes; then Wnts, retinoic acid, and FGFs are required to activate the posterior genes.

This regulation of anterior-posterior identity appears to be coordinated by **retinoic acid-4-hydroxylase**, an enzyme that degrades RA (Kudoh et al. 2002; Dobbs-McAuliffe et al. 2004). The gene encoding this enzyme, *cyp26*, is expressed specifically in the region of the embryo destined to become the anterior end. Indeed, this gene's expression is first seen during the late blastula stage, and by the time of gastrulation it defines the presumptive anterior neural plate. Retinoic acid-4-hydroxylase prevents the accumulation of RA at the embryo's anterior end, blocking the expression of the posterior genes there. This inhibition is reciprocated, since the posteriorly expressed FGFs and Wnts inhibit the expression of the *cyp26* gene, as well as inhibiting the expression of the head-specifying gene *Otx2*. This mutual inhibition creates a border between the zone of posterior gene expression and the zone of anterior gene expression. As epiboly continues, more and more of the body axis is specified to become posterior.

Retinoic acid acts as a morphogen, regulating cell properties depending on its concentration. Cells receiving very little RA express anterior genes; cells receiving high levels of RA express posterior genes; and those cells receiving intermediate levels of RA express genes characteristic of cells between the anterior and posterior regions. This morphogen is extremely important in the hindbrain, where different levels of RA specify different types of cells along the anterior-posterior axis (White et al. 2007).

Left-right axis formation

In all vertebrates studied, the right and left sides differ both anatomically and developmentally. In fish, the heart is on the left side and there are different structures in the left and right regions of the brain. Moreover, as in other vertebrates, the cells on the left side of the body are given that information by Nodal signaling and by the Pitx2 transcription factor. The ways the different vertebrate classes accomplish this asymmetry differ, but recent evidence suggests that the currents produced by motile cilia in the node may be responsible for left-right axis formation in all the vertebrate classes (Okada et al. 2005).

In zebrafish, the Nodal structure housing the cilia that control left-right asymmetry is a transient fluid-filled organ called **Kupffer's vesicle**. As mentioned earlier, Kupffer's vesicle arises from a group of dorsal cells near the embryonic shield shortly after gastrulation. Essner and colleagues (2002, 2005) were able to inject small beads into Kupffer's vesicle and see their translocation from one side of the vesicle to the other. Blocking

[9] Some of the endodermal cells that accumulate β-catenin will become the precursors of the ciliated cells of Kupffer's vesicle (Cooper and D'Amico 1996). As we will discuss in the final section of this chapter, these cells are critical in determining the left-right axis of the embryo.

ciliary function by preventing the synthesis of dynein or by ablating the precursors of the ciliated cells resulted in abnormal left-right axis formation. Cilia are responsible for the left-side specific activation of the Nodal signaling cascade. Nodal target genes are critically important in instructing asymmetric organ migration and morphogenesis in the body (Rebagliati et al. 1998; Long et al. 2003).

WATCH DEVELOPMENT 11.8 See the rotary motion of cilia in the Kupffer's vesicle of zebrafish.

Next Step Investigation

The BMP-Nodal gradient so vital to amphibian and fish development may be critically important in other vertebrates (including humans) as well. Moreover, can *any* field of pluripotent cells (such as human embryonic stem cells) respond to gradients of BMP and Nodal signals? If this is the case, it may be possible to induce morphogenesis in vitro and to organize the pluripotent cells into fully functional structures. Knowing the events that generate patterned organs from the cues in gradients could be an important breakthrough for regenerative medicine.

Closing Thoughts on the Opening Photo

The zebrafish embryo in the photo has two body axes, a normal one and a second axis (arrow) that was induced by adding a region of an embryo containing high amounts of Nodal (see p. 375). New theories concerning conjoined twinning hypothesize that during gastrulation, ectopic expression of signaling molecules such as Nodal might lead to new axis formation. Human conjoined twins will be discussed in more detail in Chapter 12. (Photograph courtesy of Christine Thisse.)

11 Snapshot Summary
Early Development in Amphibians and Fish

1. Amphibian cleavage is holoblastic, but it is unequal because of the presence of yolk in the vegetal hemisphere.
2. Amphibian gastrulation begins with the invagination of the bottle cells, followed by the coordinated involution of the mesoderm and the epiboly of the ectoderm. Vegetal rotation plays a significant role in directing the involution.
3. The driving forces for ectodermal epiboly and the convergent extension of the mesoderm are the intercalation events in which several tissue layers merge. Fibronectin plays a critical role in enabling the mesodermal cells to migrate into the embryo.
4. The dorsal lip of the blastopore forms the organizer tissue of the amphibian gastrula. This tissue dorsalizes the ectoderm, transforming it into neural tissue, and it transforms ventral mesoderm into lateral and dorsal mesoderm.
5. The organizer consists of pharyngeal endoderm, head mesoderm, notochord, and dorsal blastopore lip tissues. The organizer functions by secreting proteins (Noggin, Chordin, and Follistatin) that block the BMP signal that would otherwise ventralize the mesoderm and activate the epidermal genes in the ectoderm.
6. Dorsal-ventral specification begins with maternal messages and proteins stored in the vegetal cytoplasm. These include Nodal-like paracrine factors, transcription factors (such as VegT), and agents that protect β-catenin from degradation.
7. The organizer is itself induced by the Nieuwkoop center, located in the dorsalmost vegetal cells. This center is formed by the translocation of the Disheveled protein and Wnt11 to the dorsal side of the egg to stabilize β-catenin in the dorsal cells of the embryo.
8. The Nieuwkoop center is formed by the accumulation of β-catenin, which can complex with Tcf3 to form a transcription factor complex that can activate the transcription of the *siamois* and *twin* genes on the dorsal side of the embryo.

9. The Siamois and Twin proteins collaborate with activated Smad2 transcription factors generated by the TGF-β pathway (Nodal, Vg1) to activate genes encoding BMP inhibitors. These inhibitors include the secreted factors Noggin, Chordin, and Follistatin, as well as the transcription factor Goosecoid.
10. In the presence of BMP inhibitors, ectodermal cells form neural tissue. The action of BMP on ectodermal cells causes them to become epidermis.
11. In the head region, an additional set of proteins (Cerberus, Frzb, Dickkopf, Tiki) blocks the Wnt signal from the ventral and lateral mesoderm.
12. Wnt signaling causes a gradient of β-catenin along the anterior-posterior axis of the neural plate that appears to specify the regionalization of the neural tube.
13. Insulin-like growth factors (IGFs) help transform the neural tube into anterior (forebrain) tissue.
14. The left-right axis appears to be initiated by the activation of a Nodal protein solely on the left side of the embryo. In *Xenopus*, as in other vertebrates, Nodal protein activates expression of *pitx2*, which is critical in distinguishing left-sidedness from right-sidedness.
15. Cleavage in fish is meroblastic. The deep cells of the blastoderm form between the yolk syncytial layer and the enveloping layer. These deep cells migrate over the top of the yolk, forming the hypoblast and epiblast.
16. On the future dorsal side, the hypoblast and epiblast intercalate to form the embryonic shield, a structure homologous to the amphibian organizer. Transplantation of the embryonic shield into the ventral side of another embryo will cause a second embryonic axis to form.
17. In both amphibians and fish, neural ectoderm is permitted to form where the BMP-mediated induction of epidermal tissue is prevented. The fish embryonic shield, like the amphibian dorsal blastopore lip, secretes the BMP antagonists. Like the amphibian organizer, the shield receives its abilities by being induced by β-catenin and by underlying endodermal cells expressing Nodal-related paracrine factors.