Chapter 32
Axis Formation and Its Evolution in Ray-Finned Fish

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Abstract In teleost embryos, the formation of the body axes is controlled by both maternal and zygotic factors. During oogenesis, the formation of the oocyte's animal–vegetal polarity is maternally controlled. A mature oocyte contains a set of factors (dorsal determinants) involved in dorsal determination at the vegetal pole. After fertilization, a parallel array of microtubules forms briefly at the yolk's vegetal pole to transport the dorsal determinants to the prospective dorsal side. The dorsal determinants activate Wnt/β-catenin signaling and induce expression of dorsal-specific genes required for forming the dorsal organizer. The molecules expressed in the dorsal organizer antagonize the signaling of ventralizing or posteriorizing factors such as Bmps and Wnts, thereby establishing the signaling gradients that are subsequently required to properly form the dorsoventral (DV) and anteroposterior (AP) axes. Genetic analyses of zebrafish mutants have identified the maternal and zygotic genes that control formation of the body axes. Comparative studies of zebrafish, the primitive ray-finned fish bichir, the basal vertebrate lamprey, and the amphibian *Xenopus* indicate that bichir embryogenesis is a good model for understanding the evolution of DV axis formation. This chapter focuses on the genetic control of DV and AP axis formation, and its evolution in ray-finned fish.

Keywords Axis formation · Wnt · Bmp · Dorsal organizer · Zebrafish · Bichir

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32.1 Introduction

In vertebrate embryogenesis, the dorsoventral (DV), anteroposterior (AP), and other body axes are formed through an intricate developmental process involving signaling, transcriptional regulation, organelle transport, and cell differentiation and movement. The molecular mechanisms that form these axes have been relatively well studied in zebrafish and other model animals. A zebrafish oocyte has clear animal–vegetal (AV) polarity in which the blastodisc and the yolk are located at the animal (A) and vegetal (V) poles, respectively, but there is no apparent DV axis until the early gastrula stage, at around 6 hours postfertilization (hpf), when the embryo's dorsal side is marked by the embryonic shield (the dorsal organizer structure). However, the program that forms the DV axis is thought to be initiated soon after fertilization. Recent studies have revealed that oocyte AV polarity is linked to the formation of the embryonic DV axis. Dorsal determinants, which are deposited at the vegetal pole of the egg during oogenesis, are thought to be transferred after fertilization to the dorsal blastomeres, where they induce the dorsal-specific genes that establish the dorsal organizer. The organizer factors interact with ventralizing and posteriorizing signals to establish the embryonic DV and AP axes. In this chapter, we first describe the molecular mechanisms in zebrafish by which (1) oocyte AV polarity is established and linked to the embryo's DV axis; (2) the dorsal organizer is formed; and (3) interactions between the organizer factors and ventralizing factors determine the DV and AP body axes. Next, we compare the developmental processes of zebrafish with those of other vertebrate species—including bichir, lamprey, and *Xenopus*—and discuss the evolution of the germ layer and DV axis formation in ray-finned fish.

32.2 Embryonic Development of Zebrafish

Zebrafish sperm enters the oocyte at the animal pole. After fertilization, the chorion is detached from the egg surface, and cytoplasmic materials in the yolk are transported toward the animal pole to form the blastodisc. After the first blastodisc cleavage at 45 minutes postfertilization (mpf), synchronous cleavages occur every 15 min until the midblastula transition (MBT) at the 512-cell stage, at around 3 hpf (Kimmel et al. 1995). Zygotic gene expression is initiated at the MBT. Cell differentiation and morphogenetic processes start concomitantly, including (1) formation of the enveloping layer (EVL), which is an epithelial cell sheet covering the blastoderm; (2) formation of the yolk syncytial layer (YSL), which occurs when marginal blastomeres (connected to the yolk) collapse and release their nuclei into the yolk; and (3) epiboly, in which the blastomeres and EVL move to the vegetal pole to cover the yolk; this event starts slightly later than the MBT (Kimmel et al. 1995). Although the YSL is an extraembryonic structure, it is important in forming the germ layer. Transplanting the YSL to the animal pole of the blastoderm ectopically induces the

endoderm and mesoderm (Mizuno et al. 1996; Ober and Schulte-Merker 1999; Rodaway et al. 1999), and depletion of RNAs from the yolk inhibits mesoderm and endoderm formation (Chen and Kimelman 2000), indicating that the YSL functions to induce the endoderm and mesoderm (Sakaguchi et al. 2002). The YSL attaches to the EVL, possibly through E-cadherin, and takes the EVL and the blastoderm to the vegetal pole during epiboly (Shimizu et al. 2005b; Solnica-Krezel and Driever 1994).

During gastrulation, the embryonic structure is further established with a series of cell movements: involution/ingression of the mesendoderm, convergent extension, and migration of the axial mesoderm, in addition to epiboly (Solnica-Krezel and Sepich 2012). Mesoderm convergence and extension bring more cells to the dorsal side to form the dorsal thick (axial) mesendoderm. The axial mesendoderm elongates in the AP axis to become the prechordal plate and notochord. Various signaling pathways, including the Wnt/PCP (planar cell polarity) pathway, are involved in convergent extension [see review by Solnica-Krezel and Sepich (2012)]. Signaling molecules generated from the embryo's dorsal and ventral regions act in coordination with gastrulation movements to establish the DV and AP axes.

32.3 Establishment of Oocyte Animal–Vegetal Polarity During Oogenesis in Zebrafish

Mature zebrafish oocytes contain dorsal determinants at the vegetal pole (see Sect. 32.4.2). Thus, the formation of AV polarity in oocytes is important for forming the embryonic DV and AP axes. A zebrafish oocyte develops through four stages until it is mature and competent for fertilization (Lessman 2009; Marlow 2010; Nagahama and Yamashita 2008). Oocyte AV polarity begins to form during stage I with the appearance of the Balbiani body (Fig. 32.1a), which is composed of a variety of messenger RNAs (mRNAs), proteins, and organelles, including mitochondria. The Balbiani body initially localizes adjacent to the nucleus on the future vegetal pole side and then moves to the vegetal pole and releases its contents (e.g., mRNAs) to the vegetal cortex (Bontems et al. 2009; Marlow and Mullins 2008). In *Xenopus* and zebrafish oocytes, some germline-specific transcripts are transported via the Balbiani body to the vegetal pole (Wilk et al. 2005; Kloc et al. 1996, 2001; Kloc and Etkin 1995; Kosaka et al. 2007).

Genetic studies with a maternal mutant have revealed that formation of the Balbiani body is controlled by Bucky ball, a protein that lacks obvious sequence similarities to other proteins (Bontems et al. 2009; Marlow and Mullins 2008). Oocytes with a *bucky ball* mutation fail to form the Balbiani body, to localize germline mRNAs to the vegetal pole, and to establish AV polarity (Bontems et al. 2009; Marlow and Mullins 2008). In these *bucky ball*–mutant oocytes, mRNAs that normally localize to the vegetal pole localize radially instead (Bontems et al. 2009; Dosch et al. 2004; Nojima et al. 2010). The vegetal pole localization of the transcripts

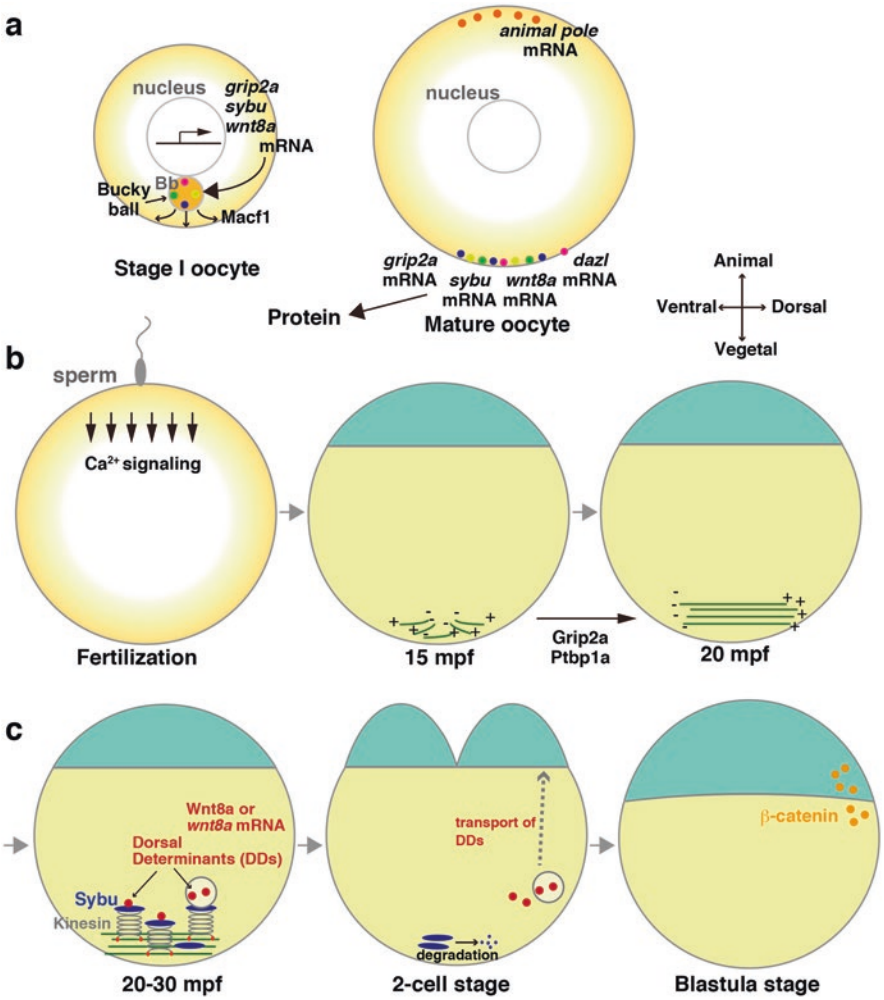

Fig. 32.1 Maternally controlled formation of the oocyte animal–vegetal (AV) axis and embryonic dorsoventral (DV) axis in zebrafish. (**a**) Formation of the oocyte AV axis. In the stage I oocyte, messenger RNAs (mRNAs) involved in dorsal determination and germ cell development are transcribed in the nucleus and deposited in the Balbiani body (Bb); these include glutamate receptor interacting protein 2a (*grip2a*), *syntabulin* (*sybu*), *wnt8a*, and *daz-like* (*dazl)* mRNAs. During oocyte maturation, mRNAs are transferred from the Balbiani body to the vegetal cortex. (**b**) Microtubule-dependent dorsal determination. Sperm entry activates Ca^{2+} signaling, which travels from the animal pole to the vegetal pole, where it induces microtubule formation. The microtubules are bundled to form an array at around 20 minutes postfertilization. (**c**) Dorsal determinants (DDs) or vesicles containing DDs are transported through the vegetal microtubule array. At the early cleavage stage, an unknown mechanism transports DDs (or vesicular DDs) to prospective dorsal blastomeres, where they activate canonical Wnt signaling, which causes β-catenin to accumulate at the blastula stage (Figure modified from Hibi et al. 2002; Langdon and Mullins 2011; Nojima et al. 2010)

for glutamate receptor interacting protein 2a (Grip2a) and Syntabulin, which are reported to be involved in dorsal determination, is dependent on Bucky ball (Nojima et al. 2010; Ge et al. 2014); *wnt8a* transcripts, which are probably involved in dorsal determination (Sect. 3.2), are also transferred from the Balbiani body to the vegetal pole (Lu et al. 2011). These data suggest that the localization of mRNAs to the vegetal pole, which depends on the Balbiani body, may be indispensable in dorsal determination. Study of another maternal mutant, *magellan*, has revealed that microtubule–actin crosslinking factor 1a (Macf1a) regulates oocyte AV polarity, possibly through microtubule-dependent transport of Balbiani body components (Gupta et al. 2010).

32.4 Microtubule-Dependent Dorsal Determination in Zebrafish

32.4.1 Microtubule Array Formation

An array of parallel microtubules forms at the vegetal pole of the zebrafish embryo at around 20 mpf (Jesuthasan and Stahle 1997) (Fig. 32.1b). Disruption of the microtubules by nocodazole treatment, cold temperatures, or ultraviolet irradiation at this point causes a loss of the dorsal organizer and ventralization of the embryo (Jesuthasan and Stahle 1997). Removal of the vegetal yolk mass at the early one-cell stage also severely ventralizes the embryo (Mizuno et al. 1999; Ober and Schulte-Merker 1999). These data have established the hypothesis that dorsal determinants initially localize to the vegetal pole and are then transported along the vegetal microtubules to the prospective dorsal side, where they activate the genetic program(s) that induce dorsal tissue.

The relevance of the vegetal microtubule array in dorsal determination was initially proposed for *Xenopus* embryogenesis. After fertilization, the *Xenopus* egg cortex rotates relative to the sperm entry point during the first cell cycle (cortical rotation). Microtubules initially appear to be randomly oriented at the vegetal cortex. However, during cortical rotation, the cortical microtubules become aligned with the plus ends toward the prospective dorsal side (Olson et al. 2015). During this process, organelles are transported along the microtubule array. Inhibition of microtubule formation disrupts dorsal tissue formation (Elinson and Rowning 1988; Houliston and Elinson 1991; Rowning et al. 1997), supporting the microtubule array's role in transporting dorsal determinants (or organelles containing the dorsal determinants) in *Xenopus* embryos. In *Xenopus*, sperm entry is proposed to control the orientation of vegetal microtubules by providing nascent microtubules originating from the sperm-derived centrosome (aster) (Houliston and Elinson 1991; Schroeder and Gard 1992).

In zebrafish, the micropyle is located at the animal pole region of the chorion, and the sperm can enter only into the animal pole of the egg. No microtubules are

seen in the area between the pronucleus (its associated centrosome) and the vegetal cortex. Therefore, the sperm entry provides little, if any, information regarding the bias of vegetal microtubule orientation. Nevertheless, the plus ends of the vegetal microtubules that form at around 20–30 mpf are oriented to the prospective dorsal side (within 30° of the embryonic shield) (Tran et al. 2012) (Fig. 32.1b). Vegetal microtubule formation depends on Ca^{2+} signaling, which is potentially activated by sperm entry (Tran et al. 2012) (Fig. 32.1b). Vesicular structures (called cortical granules) are transported along the dorsal-oriented microtubules to the prospective dorsal side (Tran et al. 2012). Some mRNAs that localize to the vegetal pole, such as *wnt8a* mRNA, are translocated slightly to the prospective dorsal side. Although it is not clear how the vegetal microtubules are oriented to the prospective dorsal side, these data suggest that microtubule-dependent transport and a cortical rotation–like movement take place in zebrafish. These events are likely to be involved in dorsal determination in zebrafish, as in *Xenopus* (Fig. 32.1c). However, the vegetal microtubules do not reach the blastodisc (Tran et al. 2012), implying that the vegetal microtubule-dependent mechanism only provides a bias in positioning the dorsal determinants at the vegetal pole, and that other mechanism(s) translocate the dorsal determinants to the dorsal blastomeres.

32.4.2 Mechanisms That Control Microtubule Array Formation and Transport of Dorsal Determinants

Studies of maternal effect mutations in zebrafish that affect the initial dorsal determination have identified molecules that function in microtubule-dependent dorsal determination (Table 32.1, Fig. 32.1b). The *hecate* mutants are deficient in genes that encode Grip2a, an adaptor protein that contains multiple PDZ domains (Ge et al. 2014), and whose mRNA localizes to the vegetal pole. The *hecate* mutant embryos fail to bundle microtubules at the vegetal pole and do not form a parallel microtubule array, implying that Grip2a is involved in bundling the vegetal microtubules (Ge et al. 2014). Embryos with the maternal effect mutant *brom bones*, which are deficient in the gene encoding polypyrimidine tract binding protein 1a (Ptbp1a, also known as hnRNPI), are also severely ventralized. Embryos with a *brom bones* mutation do not activate inositol 1,4,5-triphosphate (IP_3)-mediated Ca^{2+} release, do not undergo exocytosis of the cortical granules, and do not form a vegetal microtubule array (Mei et al. 2009), suggesting that Ptbp1a is involved in processing the pre-RNA for IP_3–Ca^{2+} signaling components. It has been suggested that the persistent cortical granules in the vegetal cortex of *brom bones* mutants secondarily affect the formation of the vegetal microtubule array (Mei et al. 2009). However, it is also possible that the IP_3-dependent activation of Ca^{2+} signaling plays a role in formation of the vegetal microtubule array. In any case, these data provide genetic evidence that the parallel microtubule array plays a pivotal role in dorsal determination. Embryos of the maternal effect mutant *tokkaebi* also show

Table 32.1 Zebrafish maternal effect mutants and the role of the mutant loci in axis formation

Gene	Mutant	Gene product	Role in axis formation	Other function
(A) Regulation of oocyte animal–vegetal axis				
bucky ball	*bucky ball*	Cytoplasmic, no known homologues	Balbiani body formation	Germ cell development
macf1a	*magellan*	Microtubule–actin crosslinking factor 1a	Microtubule-dependent transport of Balbiani body	
(B) Regulation of embryonic dorsoventral axis				
ptbp1a	*brom bones*	Polypyrimidine tract binding protein 1a	Formation of vegetal microtubule array	IP_3-dependent Ca^{2+} release
grip2a	*hecate*	Glutamate receptor interacting protein 2a, PDZ-containing adaptor	Bundling of vegetal microtubules	Germ cell development in *Xenopus*[a]
syntabulin[b]	*tokkaebi*	Syntaxin-interacting, linker for kinesin-1 motor protein	Microtubule-dependent transport	Axonal transport, germ cell development
β-catenin 2	*ichabod*	One of the two β-catenin genes in fish, downstream from the canonical Wnt pathway	Regulation of dorsal-specific genes	
kif5Ba	*kif5Ba*	Heavy chain of kinesin-1	Bundling of vegetal microtubules	Microtubule-dependent transport

IP_3 inositol 1,4,5-triphosphate

[a]Knocking down *Xenopus* Grip2.1 (XGRIP2.1) reduces the number of primordial germ cells (Tarbashevich et al. 2007)

[b]Syntabulin is involved in microtubule-dependent axonal transport of synaptic vesicles and mitochondria in cultured rat neurons (Cai et al. 2005; Su et al. 2004). Syntabulin is also involved in dorsal axis formation; *syntabulin* messenger RNA localizes to the germ plasm and is expressed later in primordial germ cells in *Xenopus* (Colozza and De Robertis 2014)

ventralization but do not display abnormalities in formation of the vegetal microtubule array (Nojima et al. 2004). The *tokkaebi* locus encodes Syntabulin, which is a linker for the kinesin motor protein and is involved in microtubule-dependent transport of organelles in neurons (Cai et al. 2005; Su et al. 2004). Since *syntabulin* mRNA is localized to the vegetal pole, Syntabulin protein is also localized to the vegetal pole until the vegetal microtubules form, after which Syntabulin is translocated from the vegetal pole in a microtubule-dependent manner and is degraded at the two-cell stage (Nojima et al. 2010). These data suggest that Syntabulin assists in transporting the dorsal determinants—or the organelles (e.g., vesicles) containing the dorsal determinants—along the vegetal microtubules and in releasing them from the vegetal microtubules after the two-cell stage. It was recently reported that maternal effect mutants of *kif5Ba*, which encodes a heavy chain of Kinesin-1, showed abnormal formation of the vegetal microtubules (random orientation or nonbundled), aberrant localization of Syntabulin and *wnt8a* mRNA, and ventralized

phenotypes (Campbell et al. 2015), suggesting that Kinesin-1 plays an important role in the vegetal microtubule formation and the subsequent microtubule-dependent dorsal determination.

32.4.3 Dorsal Determinants

The canonical Wnt pathway, which results in β-catenin accumulation, is believed to play an essential role in dorsal determination in *Xenopus* and zebrafish embryos. In these animals, activating the canonical Wnt pathway elicits ectopic formation or expansion of the dorsal organizer, whereas its inhibition impairs dorsal axis formation (Hibi et al. 2002). Furthermore, the maternal effect mutant *ichabod*, which lacks the expression of maternal β-catenin 2, fails to establish dorsal tissues (Kelly et al. 2000). In zebrafish, β-catenin accumulation is detected in the nuclei of dorsal blastomeres by the 128-cell stage, and in the dorsal blastoderm and dorsal YSL of midblastula-stage embryos (Dougan et al. 2003; Schneider et al. 1996). These data suggest that dorsal determinants activate the canonical Wnt pathway to induce dorsal-specific genes. It was initially thought that Wnt molecules may not be directly involved in dorsal determination, since inhibition of Wnt molecules by either dominant negative Wnt1/8 or a secreted Frizzled-related protein, Frzb1, did not suppress dorsal axis formation in *Xenopus* embryos (Tao et al. 2005; Hoppler et al. 1996; Leyns et al. 1997; Wang et al. 1997). However, maternal Wnt11 was shown to activate the canonical Wnt pathway, and the Wnt11/5a complex has been suggested to be a dorsal determinant in *Xenopus* (Cha et al. 2008; Cha et al. 2009). Neither *wnt11* nor *wnt5a* can activate the canonical Wnt pathway and induce dorsal-specific genes in zebrafish (Lu et al. 2011; Nojima et al. 2010). In zebrafish, *wnt8a* mRNA localizes to the egg's vegetal pole (Lu et al. 2011) and then is translocated from the vegetal pole in a microtubule-dependent manner. Expression of a dominant negative Wnt8a abolishes the expression of the dorsal-specific gene *chordin* (Lu et al. 2011; Ge et al. 2014; Tran et al. 2012), suggesting that *wnt8a* mRNA is a dorsal determinant. Many vegetal mRNAs and proteins are translocated by the movement accompanying cortical rotation. It is not clear whether it is the *wnt8a* mRNA or Wnt8a protein that acts as a dorsal determinant. It also remains to be elucidated how Wnt8a is transported to the dorsal blastoderm. Before the 128-cell stage, *wnt8a* mRNA is not detected in dorsal blastomeres, yet Wnt8a protein may be translated and transported to the dorsal blastomeres before that stage. Genetic analyses of maternal *wnt8a* mutants and detailed localization analyses of *wnt8a* mRNA or Wnt8a protein should provide compelling evidence for the role of Wnt8a in dorsal determination. In any case, Wnt8a may play a major role in activating the canonical Wnt pathway (Fig. 32.1c).

32.5 Program for Forming the Dorsal Organizer in Zebrafish

32.5.1 Wnt Signaling and Dharma

The canonical Wnt pathway induces dorsal-specific genes by binding a complex of Tcf/Lef family protein and β-catenin to their promoter/enhancer regions at the MBT; this process is controlled by both positive and negative regulators. Caveolin-1 is reported to inhibit the nuclear translocation of β-catenin, and Tob1a inhibits the formation of the Tcf/Lef and β-catenin complex (Mo et al. 2010; Xiong et al. 2006).

The dorsal-specific gene *dharma* (also known as *nieuwkoid*) encodes a homeodomain-containing transcriptional repressor, which harbors an Engrailed homology 1 (Eh1) repressor motif (Koos and Ho 1998; Yamanaka et al. 1998), The *dharma*-defective mutant *bozozok* exhibits various degrees of defects in the dorso-anterior tissues, including defects in organizer formation (Fekany et al. 1999; Koos and Ho 1999). At the MBT, *dharma* is expressed in the dorsal blastoderm; thereafter, its expression is confined to the dorsal YSL at the early gastrula stage (Koos and Ho 1998; Yamanaka et al. 1998). The canonical Wnt pathway induces *dharma* expression. The *dharma* promoter/enhancer contains many Tcf/Lef-binding sites involved in dorsal-specific *dharma* expression (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000), indicating that *dharma* is a direct target of the maternal canonical Wnt pathway (Fig. 32.2a). Dharma represses the ventral expression of the homeobox genes *vox*, *vent*, and *ved* (Kawahara et al. 2000a, b; Imai et al. 2001; Shimizu et al. 2002). The expression of *vox*, *vent*, and *ved* is positively regulated by the maternal factor Runx2b type2 (Runx2bt2) (Flores et al. 2008). Vox, Vent, and Ved are also transcriptional repressors, which repress dorsal organizer genes such as *goosecoid* and *chordin* (Shimizu et al. 2002; Imai et al. 2001; Melby et al. 2000). Hence, the Dharma-mediated repression of *vox*, *vent*, and *ved* releases the expression of dorsal organizer genes in the dorsal blastomeres. Vox, Vend, and Ved also repress *dharma* expression. Thus, the mutual repression of *vox/vent/ved* and *dharma* refines the dorsal organizer domain (Kawahara et al. 2000a, b; Imai et al. 2001; Shimizu et al. 2002). The SoxB1 transcription factors Sox3 and Sox19b also restrict *dharma* expression (Shih et al. 2010). Dharma not only represses *vox/vent/ved* expression but also directly represses the gene expression of the ventralizing factors Bmp2b and Wnt8a (Erter et al. 2001; Leung et al. 2003a). The Dharma-mediated inhibition of Bmp and Wnt signals may contribute to Dharma's non-cell-autonomous role in forming dorsal and anterior tissues (Koos and Ho 1998; Yamanaka et al. 1998). The stability of the Dharma protein is regulated by protein degradation mediated by the E3 ubiquitin ligase Lnx2b (Ro and Dawid 2009). The regulation of *dharma* expression and its role in DV axis formation are summarized in Fig. 32.2a.

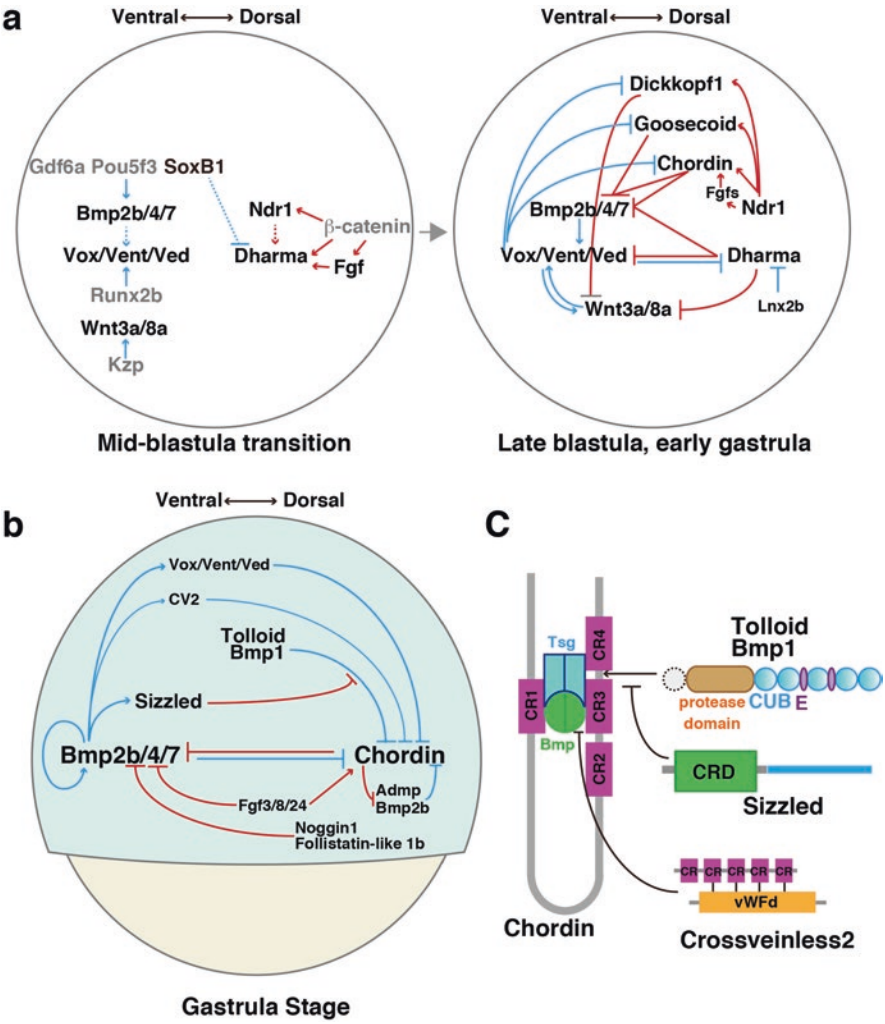

Fig. 32.2 Molecular mechanisms controlling dorsoventral (DV) axis formation in zebrafish. (**a**) Control of DV axis formation at the midblastula transition (*left panel*) and at the late blastula to early gastrula stages (*right panel*). Expression of the ventralizing factors Bmp2b/4/7, Vox/Vent/Ved, and Wnt3a/8a is regulated by the maternal factors Gdf6a/Pou5f3, Runx2b, and Kzp, respectively. Expression of the dorsalizing factors Dharma and Ndr1 is regulated by maternal Wnt signaling. The mutual repressive interaction between Dharma and Vox/Vent/Ved defines the DV axis. Dharma releases the expression of dorsal organizer genes by suppressing Vox/Vent/Ved-mediated repression of these genes. (**b**) Regulation of DV axis formation at the gastrula stage. Dorsalizing and ventralizing signals are marked by *blue lines* and *red lines*, respectively; maternal factors are indicated by *gray letters*. (**c**) Regulation of Chordin and Bmps. Chordin has four cysteine-rich (CR) domains. Tolloid and Bmp1—which have proteinase, CUB, and epidermal growth factor–like domains (E)—cleave Chordin protein. Sizzled, which has a CR domain similar to that of Frizzled, binds Bmp1 and inhibits Bmp1-dependent (and possibly Tolloid-dependent) Chordin degradation. Crossveinless 2 (CV2) contains CR domains and a von Willebrand factor domain (vWFd). The cleaved form of CV2 suppresses Chordin-mediated Bmp inhibition and thus displays pro-Bmp activity. Twisted gastrulation (Tsg) binds Chordin and Bmps, and promotes Bmp signaling. Tsg enhances Tolloid-dependent Chordin degradation (Langdon and Mullins 2011; Muraoka et al. 2006; Shimizu et al. 2000)

32.5.2 Nodal-Related Genes

The Nodal-related gene *ndr1* (also known as *squint*) is also thought to be a target of the maternal canonical Wnt pathway (Shimizu et al. 2000; Kelly et al. 2000). The dorsal expression of *dharma* and *ndr1* is lost in *ichabod* mutants, which are defective in β-catenin 2; β-catenin rescues the expression of these two genes (Kelly et al. 2000), supporting the idea that *dharma* and *ndr1* function downstream from the maternal Wnt pathway. Ndr1 functions with another Nodal-related gene, *ndr2* (known as *cyclops*), to form the endoderm and the dorsal mesoderm (Dougan et al. 2003; Erter et al. 1998; Rebagliati et al. 1998a, b; Sampath et al. 1998; Feldman et al. 1998; Schier and Talbot 2005). A combined deficiency of *dharma* and *ndr1* (zygotic combined mutations) severely reduces the dorsoanterior tissues (Shimizu et al. 2000; Sirotkin et al. 2000), revealing that *dharma* and *ndr1* play major roles in forming the dorsal organizer and the dorsoanterior tissues (Fig. 32.2a). In addition to this zygotic Ndr1 function, a role has also been proposed for maternal *ndr1* transcripts in dorsal determination. Maternally deposited *ndr1* mRNA is distributed to the two prospective dorsal blastomeres at the four-cell stage; morpholino-mediated *ndr1* knockdown causes severe ventralization (Gore et al. 2005), suggesting that maternal Ndr1 functions in dorsal determination. However, arguing against this role of Ndr1, dorsal axis formation is not severely defective in maternal effect *ndr1* mutants that cannot generate the Ndr1 protein (Erter et al. 2001; Feldman et al. 1998; Heisenberg and Nusslein-Volhard 1997; Amsterdam et al. 2004). It was recently suggested that a noncoding function of *ndr1* RNA might activate the maternal canonical Wnt pathway, although the mechanism remains elusive (Lim et al. 2012).

32.5.3 Fgf Signaling

The fibroblast growth factors (Fgf) *fgf3*, *fgf8*, and *fgf24*—which are expressed in the dorsal marginal blastomeres in the blastula period—control dorsal axis formation by repressing *bmp* gene expression (Furthauer et al. 1997, 2004). In *ichabod* mutants, β-catenin-dependent expression of the organizer genes depends on Fgf signaling. Fgf signaling is also involved in Ndr1-dependent *chordin* expression and in maintaining *dharma* expression (Maegawa et al. 2006) (Fig. 32.2a). Therefore, the *fgf* genes function in dorsal axis formation downstream from the maternal canonical Wnt pathway. The Fgf signaling in dorsal axis formation is negatively regulated by Sef, Sprouty2, and Dusp6 (Mkp3, a mitogen-activated protein kinase [MAPK] phosphatase), which function as feedback regulators (Furthauer et al. 2002, 2004; Tsang et al. 2002, 2004). Precise control of the Fgf signaling gradient should contribute to the regulation of Bmp signaling along the DV axis. Fgf signaling, together with Wnt and retinoic acid signaling, also controls the posteriorization of embryos (Koshida et al. 1998, 2002; Shimizu et al. 2005a, 2006; Kudoh et al. 2002, 2004) (Fig. 32.3c, d).

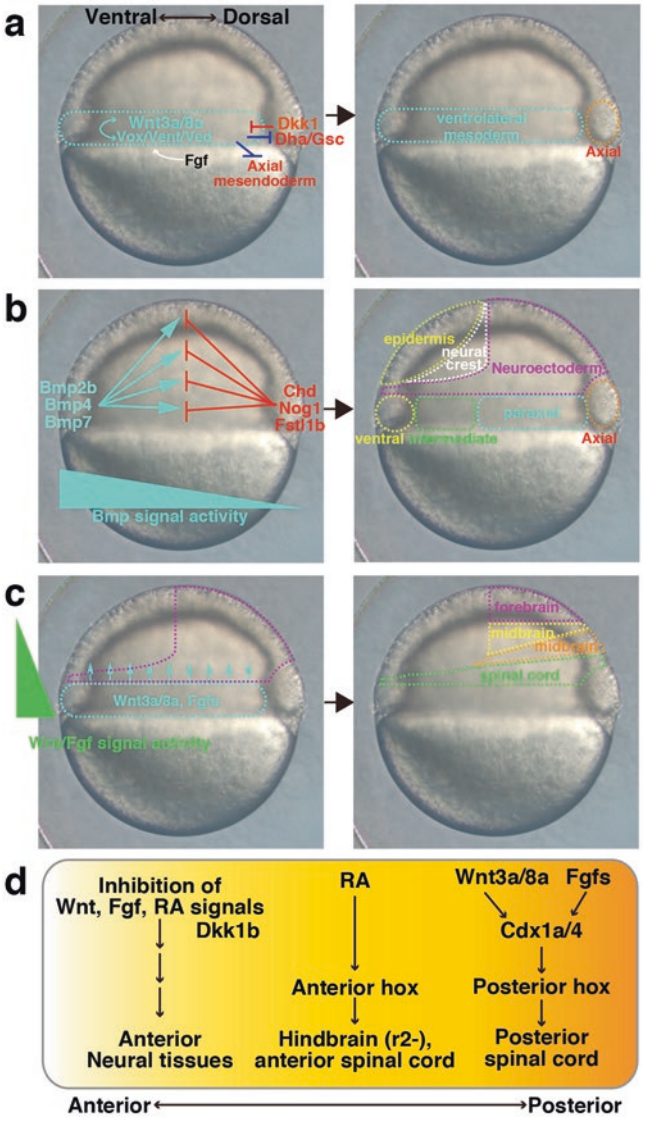

Fig. 32.3 Fate determination according to dorsoventral (DV) and anteroposterior (AP) positional information in zebrafish. (**a**) Wnt signaling controls the fates of the ventrolateral versus axial mesendoderm. Wnts and Vox/Vent/Ved are involved in forming the ventrolateral mesoderm. Dkk1, Dharma, and Goosecoid control the formation of axial mesendoderm tissue by inhibiting Wnts and Vox/Vent/Ved. (**b**) A Bmp signal gradient controls cell fates on the DV axis. Strong Bmp activity is required for formation of the ventral mesoderm and epidermis. Bmp inhibition is required for neuroectoderm formation. (**c**) Control of AP axis formation in the neuroectoderm. The posterior region of the neuroectoderm, which receives Wnt and fibroblast growth factor (Fgf) signaling from the nonaxial mesoderm, is fated to become posterior neural tissue such as the spinal cord or hindbrain. The anterior (animal pole) region of the neuroectoderm, which does not receive posterior-

32.6 Interactions Between the Dorsal Organizer and Ventralizing/Caudalizing Factors in Zebrafish

32.6.1 Goosecoid

The Wnt target genes (e.g., *dharma*, *ndr1*, and *fgf*s) cooperatively regulate the expression of dorsal organizer genes such as *goosecoid*, *chordin*, and *dickkopf1* (*dkk1*) (Hashimoto et al. 2000; Maegawa et al. 2006; Shimizu et al. 2000; Sirotkin et al. 2000) (Fig. 32.2a). The expression of *goosecoid* and *dkk1* depends on both Dharma and Nodal signaling (Hashimoto et al. 2000; Shimizu et al. 2000). However, *chordin* expression depends more on Dharma than on Nodal signaling, as *chordin* expression is relatively well maintained in Nodal signal–deficient embryos, such as the maternal zygotic *one-eyed pinhead* (*oep*) mutant (Gritsman et al. 1999; Shimizu et al. 2000). Goosecoid is a homeodomain-containing transcription factor, which harbors an Eh1 repressor motif and exhibits sequence similarity to Dharma (Cho et al. 1991; Stachel et al. 1993). Goosecoid can inhibit Bmp signaling even in the absence of the secreted Bmp inhibitors Chordin, Noggin1, and Follistatin-like 1b (Dixon Fox and Bruce 2009) (Fig. 32.2a). Thus, it is possible that Goosecoid directly binds *bmp* gene promoters/enhancers to negatively regulate their expression in dorsal blastomeres, like Dharma. Although the *goosecoid* gene exists in most, if not all, vertebrate genomes, *dharma* is found only in teleost genomes. It is conceivable that *goosecoid* was duplicated during teleost-specific whole-genome duplication (WGD), and that the expression and function of the two genes were diversified during teleost evolution: *dharma* came to be expressed earlier, *goosecoid* came to function at a later stage, and the expression of *goosecoid* came to depend on *dharma*. This hypothesis could explain the variable expressivity and penetrance of the *bozozok* mutants (Fekany et al. 1999) because *dharma* may partly function redundantly with *goosecoid*.

32.6.2 Chordin and Other Bmp Antagonists Regulate Bmp Signaling

Chordin, which is a secreted Bmp inhibitor containing four cysteine-rich domains, binds Bmp dimers, thereby inhibiting Bmp's binding to its receptors (Piccolo et al. 1996; Sasai et al. 1994; De Robertis 2009). Among the known Bmp inhibitors,

Fig. 32.3 (continued) izing signals, becomes anterior neural tissues such as the forebrain and midbrain. (**d**) Molecular mechanisms controlling AP axis formation during early neural development. Wnts and Fgfs from the nonaxial mesoderm regulate the expression of the caudal-related homeodomain proteins Cdx1a and Cdx4, which induce the posterior *hox* genes that determine the fate of the posterior spinal cord. Retinoic acid (RA), generated by the anterior paraxial mesoderm, controls expression of the anterior *hox* genes required for fate determination of the hindbrain (posterior from rhombomere 2) and the anterior spinal cord. Posteriorizing signals (Wnt, Fgf, and RA) must be inhibited to induce anterior neural tissues

Chordin functions nonredundantly in DV axis formation (Dal-Pra et al. 2006; Hammerschmidt et al. 1996; Schulte-Merker et al. 1997). The genes encoding the Bmp inhibitors Noggin1 and Follistatin-like 1b are also expressed in the dorsal organizer region at the blastula and early gastrula stages (Dal-Pra et al. 2006; Furthauer et al. 1999). These genes function redundantly with Chordin: knocking the genes down separately does not cause ventralization, but knockdown of the two genes along with *chordin* results in strong ventralization (Dal-Pra et al. 2006; Furthauer et al. 1999). Compared with Noggin1 and Follistatin-like 1b, Chordin has unique features: (1) *chordin* is expressed more broadly than other Bmp inhibitor genes and is negatively controlled by Bmp signaling (Miller-Bertoglio et al. 1997; Schulte-Merker et al. 1997); and (2) the stability of the Chordin protein is precisely regulated by a mechanism involving Tolloid/Bmp1 family proteinase, Sizzled, Twisted gastrulation, and other proteins (Blader et al. 1997; Connors et al. 1999, 2006; Jasuja et al. 2006; Little and Mullins 2004; Muraoka et al. 2006; Xie and Fisher 2005; Yabe et al. 2003) (Fig. 32.2b, c). These features make it likely that Chordin is a nonredundant factor that regulates Bmp signaling and DV axis formation.

In addition to Chordin, Crossveinless 2 (CV2)—a Chordin family protein that has von Willebrand factor C domains and is expressed ventrally—also modulates Bmp activity. Noncleaved CV2 functions as a Bmp inhibitor, but the cleaved form binds both Bmp and Chordin and suppresses Chordin-mediated Bmp inhibition (pro-Bmp activity) (Rentzsch et al. 2006; Zhang et al. 2010). The expression of the *bmp* genes *bmp2b*, *bmp4*, and *bmp7a* is regulated by the maternal factors Gdf6a (also known as Radar, a Bmp-related cytokine), Pou5f3 (also known as Pou2 or Spiel ohne grenzen, an Oct3/4 orthologue or paralogue) (Reim and Brand 2006; Sidi et al. 2003), and the zygotic SoxB1 transcription factors Sox2/3/19a/19b (Okuda et al. 2010) (Fig. 32.2a). *bmp* gene expression is also self-regulated by Bmp signaling (De Robertis 2009). Interactions between Chordin, the Chordin regulators, ventrally expressed Bmps (Bmp2b, Bmp4, and Bmp7a), and dorsally expressed Bmps (Bmp2b and Admp) define a clear Bmp signaling gradient that is required for cell differentiation along the DV axis (Fig. 32.3b). The control of Bmp signaling is beyond the scope of this chapter; for excellent reviews on the regulation of Bmp signaling in zebrafish axis formation, see Langdon and Mullins (2011) and Bier and De Robertis (2015).

32.6.3 Role of Wnt Inhibition in Forming the DV and AP Axes

Although maternal canonical Wnt signaling is involved in initiating dorsal axis formation, zygotic canonical Wnt signaling negatively regulates dorsal axis formation and positively regulates formation of posterior tissues. The mechanism by which maternal and zygotic Wnt signals regulate a different set of genes and exhibit opposite functions in axis formation has not been defined. During gastrulation, *wnt8a*

and *wnt3a* are expressed throughout the blastoderm margin except in the most dorsal region, which corresponds to the embryonic shield; this expression persists in the tailbud at the end of gastrulation (Kelly et al. 1995; Lekven et al. 2001; Shimizu et al. 2005a). The expression of *wnt8a* is at least partly regulated by the maternal transcription factor Kzp (Kaiso zinc finger–containing protein) (Yao et al. 2010) (Fig. 32.2a). The zebrafish *wnt8a* gene has two open reading frames (ORFs), which are located tandemly in the zebrafish genome and can function as a bicistronic transcript (Lekven et al. 2001). Inhibition of both *wnt8a* ORFs reduces the ventrolateral mesoderm and tail structure, and enlarges the head structure (Lekven et al. 2001). Wnt3a functions redundantly with Wnt8a. Inhibition of both Wnt3a and the Wnt8a ORFs causes more severe phenotypes than inhibition of Wnt8a alone: the ventrolateral mesoderm is reduced, while the axial mesoderm expands, and the tail structure is lost, while the head structure expands (Shimcizu et al. 2005a; Thorpe et al. 2005). Inhibition of β-catenins 1 and 2 expands *chordin* expression and causes severe dorsalization (Varga et al. 2007). These data suggest that zygotic (gastrula) canonical Wnt signaling plays two roles in axis formation: (1) it controls the fate of the axial versus ventrolateral mesoderm for the DV axis, since Wnt induces the ventrolateral mesoderm; and (2) it functions as a posteriorizing signal for the AP axis (Fig. 32.3a, c). The ventral expression of *vox*, *vent*, and *ved* is regulated by Wnt8a/Wnt3a signaling, and *wnt8a* and *wnt3a* expression is regulated by Vox/Vent/Ved (Ramel and Lekven 2004; Shimizu et al. 2005a). The cross-regulation between Wnt signaling and Vox/Vent/Ved may refine the fate determination of axial versus ventrolateral mesoderm tissue; Dharma restricts the axial mesoderm by repressing these ventralizing signals (Figs. 32.2a and 32.3a). For posteriorization, the caudal-related genes *cdx1a* and *cdx4* function downstream from Wnt signaling; inhibition of Cdx1a and Cdx4 severely truncates the posterior, as with inhibition of Wnt8a/Wnt3a (Shimizu et al. 2005a) (Fig. 32.3d). The loss of Cdx1a/Cdx4 also leads to ectopic formation of hindbrain tissue (Shimizu et al. 2006; Skromne et al. 2007), indicating that Cdx1a and Cdx4 function downstream from Wnt8a/3a to control posterior tissue formation and repress anterior tissues (Fig. 32.3d). The Sp1 family transcription factors Sp5a and Sp5l also function downstream from Wnt signaling, for both the DV and AP axes (Thorpe et al. 2005; Weidinger et al. 2005). Further studies are necessary to reveal the relationships between Sp5/5l and Vox/Vent/Ved, or Sp5/Sp5l and Cdx1a/Cdx4, for DV and AP axis formation (Table 32.2).

The dorsal organizer expresses the Wnt inhibitor Dkk1 (Glinka et al. 1998), which binds the Wnt lipoprotein receptor proteins 5 and 6 (LRP5/6) and Kremen, and downregulates Wnt signaling (Davidson et al. 2002; Mao et al. 2002). Zebrafish have two *dkk1* genes (Untergasser et al. 2011). Initially, *dkk1b* is expressed in the dorsal marginal blastoderm and the dorsal YSL at the blastula stage, and then in the prechordal plate at the gastrula stage (Hashimoto et al. 2000; Shinya et al. 2000). Canonical Wnt signaling also controls *dkk1b*, thus functioning as a negative feedback regulator (Shinya et al. 2000). In the blastoderm margin, *dkk1b* expression may be regulated by Wnt8a and Wnt3a (Hashimoto et al. 2000; Shinya et al. 2000). Thus, the Wnt8a/Wnt3–Dkk1 system is part of a reaction–diffusion mechanism that forms a Wnt signal gradient; a similar role has been proposed for Dkk in hair follicle

Table 32.2 Molecular players in dorsoventral and anteroposterior axis formation in zebrafish

Molecule	Nature of molecule	Role in axis formation
Admp	Secreted protein	Ventralization, a Bmp family member
Bmp2b/4/7	Secreted protein	Ventralizing factor
Caveolin-1	Transmembrane protein	Inhibition of nuclear translocation of β-catenin
Chordin	Secreted protein	Inhibition of Bmp signaling
Cdx1a/4	Transcription factor	Posteriorization
CV2	Secreted protein	Inhibition and promotion of Bmp signaling
Dharma	Transcriptional repressor	Repression of ventral genes
Dickkopf1	Secreted protein	Inhibition of Wnt signaling
Dusp6	Cytoplasmic protein	Inhibition of Fgf signaling, a MAPK phosphatase
Eomesa	Transcription factor	Endoderm formation
Fgf3/Fgf8/Fgf24	Secreted protein	Dorsalization, posteriorization, and mesoderm formation
Follistatin-like 1b	Secreted protein	Inhibition of Bmp signaling
Gdf6a	Secreted protein	Ventralization (maternal factor for *bmp* expression)
Goosecoid	Transcriptional repressor	Repression of ventral genes
Kremen1	Transmembrane protein	Dkk-mediated inhibition of Wnt signaling
Kzp	Transcription factor	Regulation of *wnt3a/8a* expression
Lnx2b	Ubiquitin ligase	Degradation of Dharma
LRP5/6	Transmembrane protein	Coreceptor for Wnt signaling
Ndr1/2	Secreted protein	Nodal-related molecules, organizer and mesendoderm induction
Noggin1	Secreted protein	Inhibition of Bmp signaling
One-eyed pinhead	Membrane-attached protein (GPI anchored)	Coreceptor for Nodal-related molecules
Pou5f3	Transcription factor	Ventralization (maternal factor for *bmp* expression)
Retinoic acid	Signaling molecule	Posteriorization
Runx2bt2	Transcription factor	Maternal regulator for *vox/vent/ved* expression
Sef	Transmembrane protein	Inhibition of Fgf signaling
Sizzled	Secreted protein	Inhibition of Tolloid/Bmp1 family protein (stabilization of Chordin)
Smad1/5	Signaling and transcription factor	Mediator of Bmp signaling
Sox2/3/19a/19b	Transcription factor	Ventralization (promotes *bmp* expression and represses *dharma*)
Sp5a/5l	Transcription factor	Mediator of Wnt signaling
Sprouty2	Cytoplasmic protein	Inhibition of Fgf signaling
Tbx16	Transcription factor	Paraxial mesoderm formation

(continued)

Table 32.2 (continued)

Molecule	Nature of molecule	Role in axis formation
Tcf/Lef family	Transcription factor	Involved in the canonical Wnt pathway, interacting with β-catenin
Tob1a	Transcription factor	Inhibition of formation of Tcf/Lef and β-catenin complex
Tolloid/Bmp1 family	Secreted protein, metalloprotease	Degradation of Chordin (promotion of Bmp signaling)
Vox/Vent/Ved	Transcriptional repressor	Repression of dorsal genes and mediator of Bmp signaling
Wnt3a	Secreted protein	Posteriorization and axial mesoderm formation
Wnt8a	Secreted protein	Maternal: dorsal determination Zygotic: posteriorization and axial mesoderm formation

Molecules involved in dorsoventral and anteroposterior axis formation are listed alphabetically. Molecules listed in Table 32.1 are not described in this table
Fgf fibroblast growth factor, *GPI* glycophosphatidylinositol, *MAPK* mitogen-activated protein kinase

formation (Sick et al. 2006). This mechanism may be similar to that in the Nodal–Lefty (Nodal inhibitor) system for mesoderm and endoderm differentiation (Muller et al. 2012; Schier 2009). As nonaxial mesendoderm expressing Wnt8a and Wnt3a is suggested to provide posteriorizing signals (Koshida et al. 1998; Woo and Fraser 1997), Dkk1b may generate a high Wnt signal area near the margin and a low Wnt signal area in the animal pole (Fig. 32.3c, d). This Wnt signaling gradient is required to establish AP embryonic polarity. At the late gastrula stage, Dkk1b from the prechordal plate may ensure the anterior neural fate. Since Dkk1b can rescue the formation of not only the anterior neuroectoderm but also the axial mesoderm in *dharma*-deficient *bozozok* embryos, Dkk1 may also be involved in axial mesendoderm formation (Hashimoto et al. 2000) (Fig. 32.3a). Although genetic analysis has revealed that Dkk1 is essential for head development in the mouse (Mukhopadhyay et al. 2001), the role of Dkk1 in formation of the AP axis in zebrafish has not been genetically proven. Combination knockouts of *dkk* family members expressed in zebrafish gastrula embryos (*dkk1a*, *dkk1b*, and *dkk3*) (Lu et al. 2011) should reveal the role of Dkk family proteins in body axis formation.

32.6.4 Nodal/Bmp/Fgf/Wnt Signaling Interactions for DV and AP Axis Formation

Zygotic Nodal, Bmp, Fgf, and Wnt signaling cooperatively regulate axis formation. DV patterning along the AP axis is reported to be temporally coordinated: inhibition of Bmp signaling at the onset of gastrulation controls the formation of the anterior neuroectoderm, whereas at a later stage it regulates the formation of the posterior

neuroectoderm (Tucker et al. 2008). In this process, Bmp-mediated DV patterning is temporally coordinated with the posteriorizing signals Fgf, Wnt, and retinoic acid (Hashiguchi and Mullins 2013). Fgf negatively regulates Bmp signaling by phosphorylating Smad1/5, which are Bmp signal transducers; this mechanism is involved in coordination of Bmp and Fgf signaling, at least in part (Hashiguchi and Mullins 2013).

The ventral blastoderm margin is proposed to function as the tail organizer independently of the dorsal organizer. The activation of Nodal/Bmp/Wnt signaling mimics tail organizer activity (Agathon et al. 2003). The dorsal organizer, the tail organizer, and the entire blastoderm margin are proposed to function as an organizing center, which depends on the ratio of Nodal/Bmp activity: Nodal is high on the dorsal side, and Bmp is high on the ventral side (Fauny et al. 2009). Moreover, Nodal and Bmp alone are sufficient to organize uncommitted naïve cells of the blastula animal pole into a well-organized embryo, both in vivo and in vitro (Xu et al. 2014). These data suggest that Nodal and Bmp signaling are minimal requirements for providing embryonic cells with DV and AP axis information, and that Nodal/Bmp activity gradients play a pivotal role in axis formation. Although genetic evidence may be required to prove that endogenous Nodal and Bmp levels are sufficient to generate the embryonic axis, the data imply that Nodal and Bmp signaling function as hubs in the program for DV and AP axis formation. Other signaling pathways and transcription factors that control the expression of Nodal/Bmp molecules and inhibitors function downstream from (and possibly in parallel with) Nodal/Bmp signaling in axis formation, as discussed earlier. Mathematical modeling will help to explain the intricate programs that shape the DV and AP axes.

32.7 Evolution of Axis Formation in Ray-Finned Fish (Actinopterygii)

32.7.1 Bichir Provides a Good Model for Evolutionary Developmental Biology (Evo–Devo) Studies

Although the mechanisms controlling axis formation in zebrafish are relatively well understood, the degree to which these mechanisms are conserved among fish—and whether these mechanisms are also shared by other vertebrate species—is not clear. Ancient jawless fish (Agnatha) that existed about 600 million years ago were the ancestors of all vertebrates (Blair and Hedges 2005). The extant jawless fish are in the class Cyclostomata, which includes lampreys and hagfish; other vertebrates (Gnathostomata) are derived from an ancient lineage of jawed fish that diverged from the Agnatha. The extant jawed fish are categorized into two groups: cartilaginous fish (Chondrichthyes) and bony fish (Osteichthyes); the bony fish are further classified into two subgroups: lobe-finned fish (Sarcopterygii—e.g., coelacanths, lungfish, and tetrapods) and ray-finned fish (Actinopterygii). The ray-finned fish

include several taxa: Polypteriformes (bichirs and reedfish), Chondrostei (sturgeons and paddlefishes), Lepisosteiformes (gars), Amiiformes (*Amia calva*), and Teleostei (teleosts, Fig. 32.4). Most extant fish, including zebrafish and medaka model fish, are teleosts. Early embryogenesis is similar in various teleosts, but teleost embryogenesis is quite different from that of nonteleost fish (Bolker 1993a, b; Cooper and Virta 2007). This difference may be rooted in the WGD that is proposed to have taken place in the teleosts (Amores et al. 1998; Jaillon et al. 2004). The teleost-specific WGD may have generated gene variations that contribute to teleost-specific developmental processes (Kuraku et al. 2009).

Thus, to understand the evolution of fish embryogenesis, it is important to study the development of nonteleost fish. Bichirs (order: Polypteriformes, family; Polypteridae), which live in African rivers and lakes, have been used in recent studies of developmental biology (Takeuchi et al. 2009a). The bichir lineage diverged from ray-finned fish about 400 million years ago, soon after bony fish diverged into lobe-finned and ray-finned fish (Inoue et al. 2003). Thus, bichirs are considered to be one of the most primitive ray-finned fish. Furthermore, bichirs did not undergo teleost-specific WGD. Therefore, comparative analysis of zebrafish and bichir development should provide insights into the evolution of mechanisms for embryonic axis formation.

32.7.2 Morphogenetic Processes of Bichir Embryos

A teleost embryo undergoes meroblastic cleavages, whereas a bichir embryo undergoes holoblastic cleavages, like the *Xenopus* embryo, cleaving from the animal to the vegetal pole. In *Xenopus*, the first cleavage furrow demarcates the DV axis. When one of the two blastomeres is labeled at the two-cell stage, the interface between the labeled and unlabeled halves coincides with the midsagittal plane (Klein 1987). In contrast, the first cleavage plane in zebrafish is not related to either the DV axis or the left–right (LR) axis, and the blastomeres are intermingled during the blastula and gastrula stages (Kimmel and Law 1985; Kimmel and Warga 1987), indicating that *Xenopus* and zebrafish use distinct developmental strategies for cleaving and mixing the blastomeres. In bichir embryos, injection of rhodamine dextran into one of the two blastomeres at the two-cell stage marks cells in either the left or right half of the embryo (Fig. 32.5a) (Takeuchi et al. 2009a). Therefore, the first cleavage plane in bichir embryos demarcates the DV axis, and the blastomeres are not intermingled as they are in *Xenopus* embryos. Holoblastic cleavage is associated with formation of embryonic cavities (the blastocoel and archenteron) and absence of the EVL and YSL. Bichir embryos have a blastocoel and an archenteron, and no EVL or YSL (Fig. 32.5), suggesting they have inherited an amphibian-type morphogenetic process. The embryonic structure of bichirs is also similar to that of the agnathan lamprey (Takeuchi et al. 2009b). It is tempting to speculate that the morphogenetic process of the bichir embryo may be similar to that of ancestral fish; the EVL and YSL might have evolved in the ray-finned fish lineage after the bichir had diverged from the ray-finned fish.

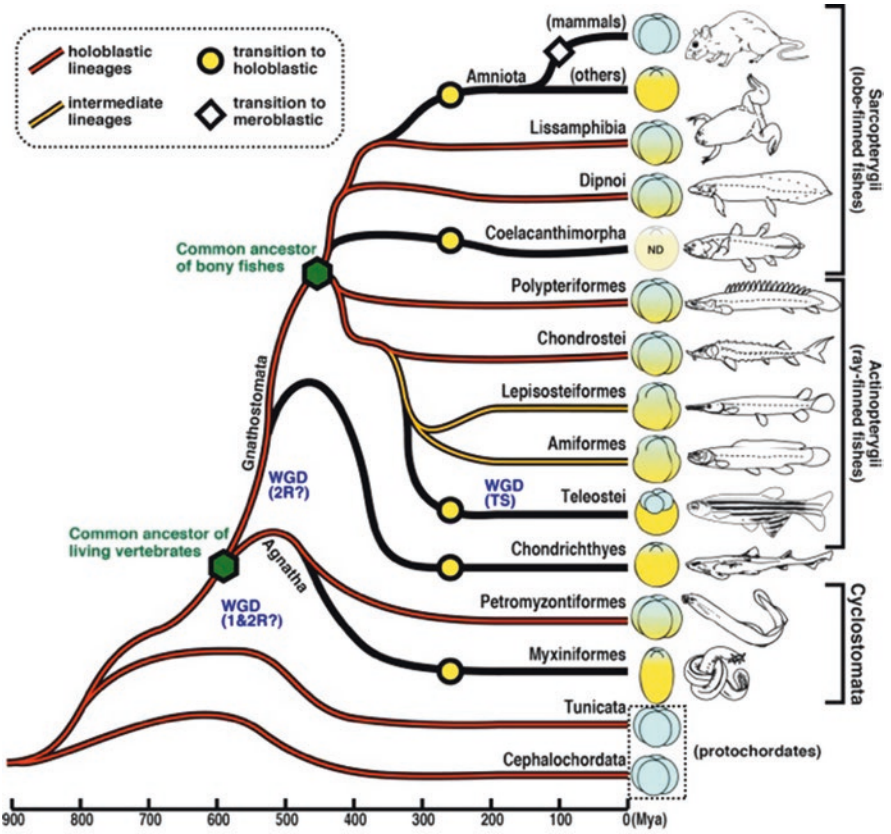

Fig. 32.4 Vertebrate phylogenic tree and cleavage patterns. Basal chordate embryos undergo holoblastic cleavages. As the common ancestor of vertebrates evolutionarily acquired a massive yolk (indicated by the yellow color in the embryos), their cleavage patterns were highly diversified. The holoblastic cleavage pattern is retained in various vertebrate lineages (indicated by red lines). The transition from a holoblastic to a meroblastic cleavage pattern occurred independently several times in the vertebrate lineages (yellow circles). Bichirs (Polypteriformes)—the most primitive ray-finned fish (Actinopterygii)—undergo holoblastic cleavages, as do amphibians. Bichirs diverged from all other ray-finned fish about 400 million years ago (Mya) during the Devonian period, before the teleost-specific (TS) whole-genome duplication (WGD) and soon after an ancestral bony fish diverged into ray-finned and lobe-finned fish (Sarcopterygii)

Fig. 32.5 (continued) midline region, fluorescent image). Dotted lines indicate the midline. (**b**) Germ layer patterning in the bichir and lamprey during gastrulation. Bichir and lamprey embryos have a blastocoel and an archenteron but no enveloping layer (EVL) or yolk syncytial layer (YSL). Both the mesoderm (red) and endoderm (green) are formed in the marginal zone of the bichir embryo and in the conical eminence of the lamprey embryo during early gastrulation. In the bichir embryo, the vegetal cell mass (VCM) is an extraembryonic structure, which does not contribute to the endoderm. The archenterons are marked by *asterisks*. (**c**) Evolution of endoderm and mesoderm induction in vertebrates. In the vertebrate ancestor, vegetal cells might have induced the endoderm (green arrow) and mesoderm (pink arrow) in the embryo; the bichir VCM retains this activity. With the holoblastic-to-meroblastic transition, this activity was retained in the YSL of teleost embryos. The activity was incorporated into the endoderm cells of amphibian embryos as the vegetal cells became the endoderm. In amniote embryos, the activity was retained in the visceral endoderm or the hypoblast, which are extraembryonic structures

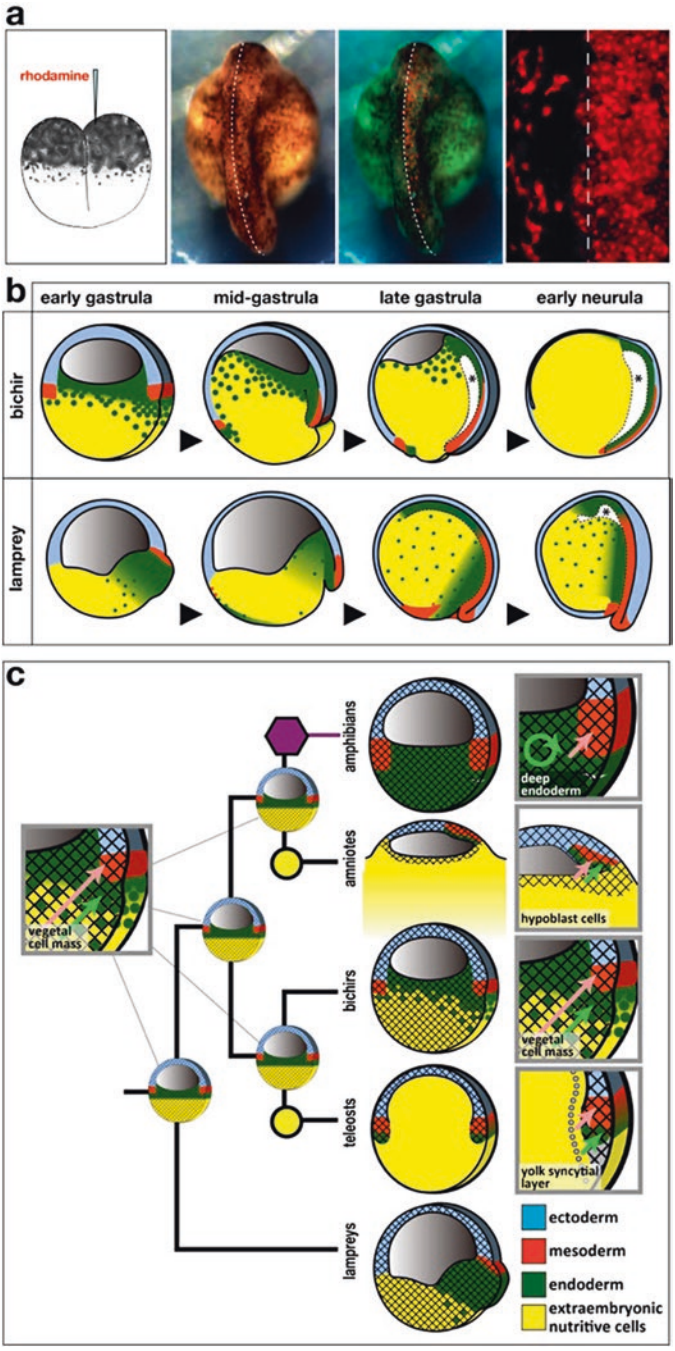

Fig. 32.5 Early bichir and lamprey embryogenesis. (**a**) In bichirs, as in amphibians, the first cleavage plane of the embryo demarcates the dorsoventral (DV) axis. However, there is no correlation between the first cleavage and the body axes in teleost embryos. In bichirs, rhodamine injection into one of the blastomeres of a two-cell-stage embryo (left panel) results in fluorescence mostly in either the left or right half of the embryo at the tailbud stage (middle left panel: bright-field image; *middle right panel*: merged images from bright-field and fluorescent images; right panel: high-magnification view of the

32.7.3 Molecular Mechanisms Controlling Bichir Embryonic Development

It is thought that a cytoplasmic fusion of vegetal blastomeres during the evolution of ray-finned fish brought about the teleost YSL (Figs. 32.4 and 32.5). The zebrafish YSL and the *Xenopus* vegetal endoderm are strikingly similar in their ability to induce the mesoderm and endoderm. However, the cellular identity of the teleost YSL is distinct from that of the *Xenopus* vegetal endoderm. The *Xenopus* vegetal endoderm expresses endodermal markers and gives rise to endoderm tissues such as the pancreas, liver, and gut, whereas the teleost YSL is an extraembryonic tissue that does not express endoderm markers. Bichir embryos, like *Xenopus* embryos, have vegetal blastomeres, which raises the question of whether the vegetal blastomeres in bichir embryos are endoderm cells.

However, recent histological analyses of bichir and lamprey embryos, using molecular markers, has revealed that the vegetal blastomeres in bichirs and lampreys are extraembryonic nutritive cells that do not express endoderm markers (Takeuchi et al. 2009b). The endoderm and mesoderm form in the equatorial (marginal) zone (the conical eminence in lampreys). The Nodal-related gene *ndr1*, which is expressed in the zebrafish YSL, is also expressed in the equatorial zone of bichir embryos (Takeuchi et al. 2009b). Although bichir and lamprey embryos are morphologically similar to *Xenopus* embryos, the localization of the mesoderm and endoderm in bichir and lamprey embryos is similar to that in zebrafish (teleost) embryos (Fig. 32.5).

The T-box transcription factor Tbx16/VegT is required for endoderm formation and for vegetal cell induction of the mesoderm in *Xenopus*. Tbx16/VegT mRNA is maternally deposited at the vegetal pole of the *Xenopus* egg (Zhang et al. 1998). In contrast to *Xenopus*, bichirs do not express *tbx16* maternally or in vegetal blastomeres (Takeuchi et al. 2009b). There is no *tbx16* homologue in the lamprey genome. The mRNA of Eomesodermin homologue a (Eomesa, Eomes, Tbr2)—another T-box transcription factor involved in endoderm formation—is maternally deposited and zygotically expressed in the prospective endoderm in zebrafish (Bjornson et al. 2005). As with zebrafish, *eomes* transcripts are maternally deposited and zygotically expressed in the endoderm region in bichir and lamprey embryos (Takeuchi et al. 2009b). These data suggest that although bichirs and lampreys follow the amphibian-type morphogenetic process, they use the teleost-type mechanism for germ layer formation. The lamprey and bichir lineages are phylogenetically distant but use similar mechanisms for embryonic morphogenesis and germ layer formation. This developmental strategy—amphibian-type (holoblastic) morphogenesis and teleost-type germ layer formation—might have been used by the common ancestors of the vertebrates (the stem lineage). As maternal *tbx16/VegT* expression is common to at least some amphibians, Tbx16/VegT-dependent differentiation of the endoderm of the vegetal cells might have evolved in the amphibian lineage (Takeuchi et al. 2009b).

32.7.4 Bichir DV Axis Formation

Injection of *Xenopus β-catenin* mRNA into both blastomeres of a two-cell-stage bichir embryo can induce a secondary axis, as in *Xenopus* (Takeuchi et al. 2009a) (Fig. 32.6). This observation indicates that the maternal canonical Wnt pathway also plays a key role in dorsal determination in the bichir embryo. What molecule(s) function downstream from the Wnt pathway to control the expression of dorsal-specific genes in bichirs? Nodal-related genes are regulated by the Wnt pathway in both *Xenopus* and zebrafish (Sect. 32.5.2). In bichir embryos, *ndr1* is expressed in the dorsal equatorial zone (Takeuchi et al. 2009b), suggesting that *ndr1* may also be involved in inducing the dorsal organizer, as proposed for zebrafish. The transcriptional repressor Dharma functions downstream from the Wnt pathway to induce dorsal tissues in zebrafish (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000), whereas the transcriptional activator Siamois and its paralog Twin mediate this process in *Xenopus* (Ishibashi et al. 2008; Kessler 1997; Laurent et al. 1997; Lemaire et al. 1995). Injection of the mRNA of the bichir Siamois-related transcription activator into *Xenopus* embryos can induce a secondary axis (Masaki Takeuchi, unpublished data). These data suggest that bichirs use the amphibian-type mechanism—at least in part—for Wnt signal–mediated induction of dorsal tissues. Although *siamois*-related genes have not been identified in lampreys or other nonbichir fish, the presence of the *siamois*-related gene in bichirs suggests that common ancestors of the bony fish might have used Siamois-related transcription factor(s) for dorsal axis formation (Fig. 32.6). During teleost evolution, the *siamois*-related gene was lost, and *dharma* might have evolved (or diverged from *goosecoid*) in the teleost lineage (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000).

32.8 Perspectives

Although we understand many aspects of the molecular mechanisms that control axis formation in zebrafish, there are many unanswered questions, including how the oocyte AV polarity is initially established during oogenesis, how the vegetal pole mRNAs are transferred through the Balbiani body to the vegetal pole, what initiates the formation of the vegetal microtubules, how the vegetal microtubules are oriented to the prospective dorsal side, and how patterning signals (Wnt, Bmp, Nodal, Fgf, etc.) are coordinated to control axis formation.

Many loss-of-function studies of zygotic genes have used antisense morpholinos. Recent genome-editing techniques, such as the CRISPR/Cas9 and TALEN systems, can generate mutants of genes of interest, which will allow us to determine the function of genes whose mutants were not isolated by forward genetic screening. Germline replacement with the genetic mutants will enable us to understand the role of maternal factors (Ciruna et al. 2002). It has been reported that antisense morpholino-mediated knockdown and genetic knockout (mutation) often lead to

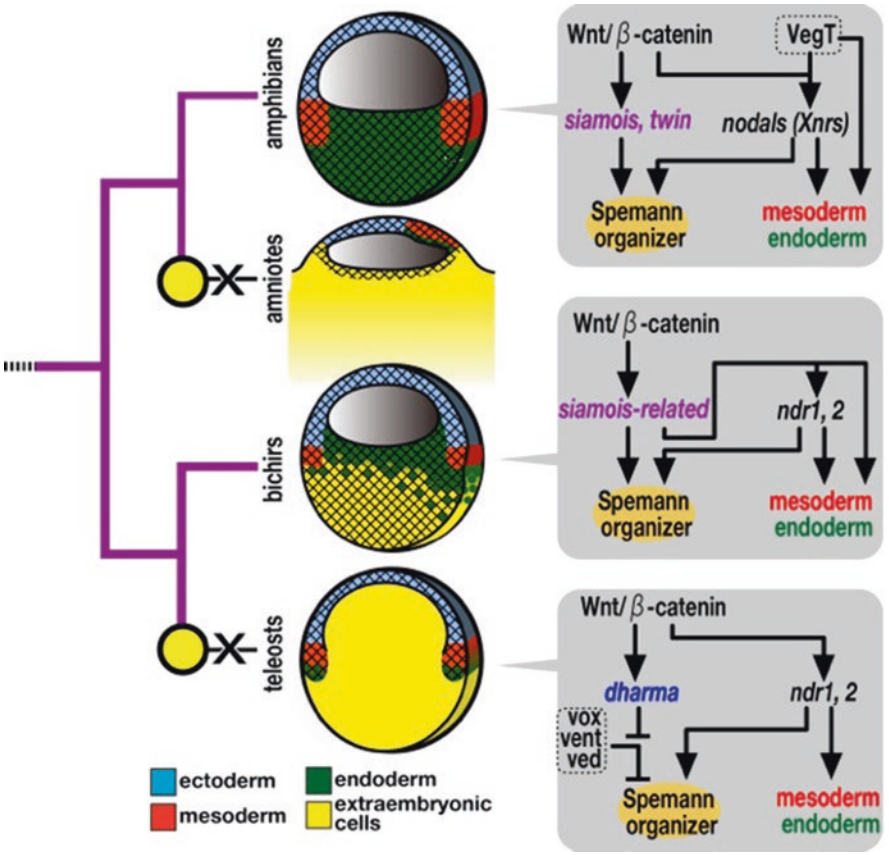

Fig. 32.6 Axis formation in bichirs and its evolution in vertebrates. A *siamois*-related gene might have mediated the canonical Wnt pathway to induce the dorsal axis in the common ancestor of the bony fish. During evolution, the *siamois*-related gene was retained in the amphibian and bichir genomes, but it was lost in the amniote and teleost genomes. In contrast, *dharma* evolved to function as a Wnt target for dorsal axis formation in teleosts

different phenotypes (Kok et al. 2015; Stainier et al. 2015). Although genetic compensation (upregulation of genes that compensate for loss of target genes) may explain such discrepancies (Rossi et al. 2015), we may need to reevaluate data obtained from antisense morpholino experiments.

In dorsal determination, regulation of microtubule formation and microtubule-dependent transport play essential roles. Visualization of the components involved in dorsal determination, along with time-lapse analysis, will reveal the molecular dynamics associated with DV axis formation. Mathematical modeling with precise transcriptome (single-cell transcriptome) data should help us understand the intricate processes that coordinate multiple signals.

There are also questions with respect to evolution, including to what degree the axis formation mechanisms are conserved among fish; whether the bichir-type developmental mechanism (amphibian-type morphogenetic process, teleost-type germ layer formation, and amphibian-type dorsal axis formation) is used by Cyclostomata (lamprey and hagfish), cartilaginous fish, lobe-finned fish, and nonteleost ray-finned fish; and how stem lineage axis formation is adapted for the amniote lineage. Sequencing with next- and third-generation sequencers enables us to reveal the genome sequences and transcriptomes of nonmodel animals, and whole-genome sequencing of bichir is in progress. Genome-editing techniques also allow us to study gene function in these animals. In the future, we will be able to discuss the details of the molecular mechanisms that form the body axes in many different vertebrate species, and find the blueprint for the evolution of axis formation in vertebrates.

Note We have recently reported that maternal *wnt8a* is dispensable for the initial dorsal determination but cooperates with zygotic *wnt8a* for ventrolateral and posterior tissue formation. Maternal *wnt6a* is an alternative dorsal determinant candidate (Hino et al. Dev Biol 434(1), 96–107, 2018). The data suggest that Wnt8a, Wnt6a, and possibly other Wnts that are expressed maternally may cooperate to activate the canonical Wnt pathway for the dorsal axis formation.

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