TGF-β Family Signaling in Early Vertebrate Development

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TGF-β family ligands function in inducing and patterning many tissues of the early vertebrate embryonic body plan. Nodal signaling is essential for the specification of mesendodermal tissues and the concurrent cellular movements of gastrulation. Bone morphogenetic protein (BMP) signaling patterns tissues along the dorsal–ventral axis and simultaneously directs the cell movements of convergence and extension. After gastrulation, a second wave of Nodal signaling breaks the symmetry between the left and right sides of the embryo. During these processes, elaborate regulatory feedback between TGF-β ligands and their antagonists direct the proper specification and patterning of embryonic tissues. In this review, we summarize the current knowledge of the function and regulation of TGF-β family signaling in these processes. Although we cover principles that are involved in the development of all vertebrate embryos, we focus specifically on three popular model organisms: the mouse *Mus musculus,* the African clawed frog of the genus *Xenopus,* and the zebrafish *Danio rerio,* highlighting the similarities and differences between these species.

EVOLUTIONARY CONTEXT OF TGF-β FAMILY SIGNALING IN EARLY VERTEBRATE DEVELOPMENT

Transforming growth factor (TGF)-β family signaling acts in establishing or patterning multiple tissues of the three axes of the vertebrate body plan early in development. These axial patterning events form the basis for the correct positioning and patterning of all subsequent tissues. Nodal signaling specifies and patterns mesendodermal tissues along an axis, sometimes referred to as the oral–aboral axis or, often in *Xenopus* and zebrafish, as the animal–vegetal axis (Conlon et al. 1994; Jones et al. 1995; Feldman et al. 1998; Schier 2003; Shen 2007). At the same stages, bone morphogenetic protein (BMP) signaling patterns tissues along a perpendicular axis, the dorsal–ventral (DV) axis of the blastula and gastrula embryo. This axis is distinct from the later DV axis of the fully developed embryo, because of the massive cell movements and cell rearrangements that occur during gastrulation, dorsal convergence, and neurulation (Hammerschmidt et al. 1996b; Holley et al. 1996; De Robertis and Kuroda 2004; Little and Mullins 2006; Ramel and Hill 2012). Shortly after gastrulation, Nodal functions in breaking the symmetry of the embryo along the third, left–right (LR) axis of the em-

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bryo (Lohr et al. 1997; Rebagliati et al. 1998a; Lowe et al. 2001; Saijoh et al. 2003; Blum et al. 2014a; Shiratori and Hamada 2014). These roles in development are deeply conserved within the animal kingdom. It was first thought that Nodal was a vertebrate innovation, because of its absence in *Drosophila* and *Caenorhabditis elegans* (Schier 2009). However, Nodal and other TGF-β ligands have been found to predate Bilateria and have conserved roles in development. Five major families of TGF-β ligands, Nodal, BMP-2 and BMP-4, BMP-5–8, TGF-β, and Activin, are conserved with cnidarians (Watanabe et al. 2014a,b). Additionally, the core elements of the TGF-β family signaling pathway, including the type I and type II receptors, Smad intracellular signal transducers, and the Noggin antagonist, are also conserved and have also been found in the more evolutionary distant sponges (Riesgo et al. 2014).

The Nodal signaling pathway plays conserved ancestral functions in specifying the mesendoderm that forms the germ layers during gastrulation (Conlon et al. 1994; Jones et al. 1995; Feldman et al. 2000; Tremblay et al. 2000). The Nodal signaling pathway in conjunction with Wnt signaling defines the dorsal organizer, a key feature of vertebrate embryonic axis formation and DV axial patterning. Within the dorsal organizer, Nodal acts downstream from Wnt signaling (Norris and Robertson 1999; Hashimoto-Partyka et al. 2003; Fan and Dougan 2007; Fan et al. 2007), inducing expression of the pan-mesodermal gene *brachyury* (Wilkinson et al. 1990; Smith et al. 1991; Cunliffe and Smith 1992; Schulte-Merker et al. 1994; Rodaway et al. 1999; Loose and Patient 2004). Surprisingly, these genes play analogous roles during Hydra (phylum Cnidaria) budding, a method of asexual reproduction in which a new body axis sprouts from the existing body axis. The expression of *nodal* defines the oral region of the bud before it sprouts (Watanabe et al. 2014b). The prospective bud region is known as the head organizer and has striking similarities to the vertebrate dorsal organizer, expressing many of the same genes as the vertebrate developing mesendoderm (reviewed in Technau and Steele 2011). Consistent with this, the Hydra *brachyury* gene can induce mesoderm in *Xenopus* (Marcellini et al. 2003). The cnidarian head organizer also expresses an ortholog of the vertebrate BMP antagonist *chordin* (Rentzsch et al. 2007), a gene expressed in the vertebrate dorsal organizer. Remarkably, Hydra Chordin can antagonize vertebrate BMPs and dorsalize zebrafish embryos (Rentzsch et al. 2007), indicating a conserved function.

Nodal signaling is also required for gastrulation in other invertebrates. In the sea urchin, Nodal acts downstream from Wnt signaling (Range et al. 2007) to specify oral fates (Duboc et al. 2004). In the snail, disruption of Nodal signaling early in development blocks gastrulation (Grande and Patel 2009). A TGF-β family ligand also seems to play a role in specifying the single oral–aboral axis of sponge embryos (Adamska et al. 2007), although here it acts in apparent opposition to Wnt signaling, and the ligand itself is more similar to the TGF-β family ligand antidorsalizing morphogenetic protein (ADMP) than to Nodal.

Studies in invertebrates also suggest a conserved role for Nodal signaling in LR asymmetry. Nodals are important for LR asymmetry in all deuterostomes (Lohr et al. 1997; Rebagliati et al. 1998a; Lowe et al. 2001; Morokuma et al. 2002; Yu et al. 2002; Saijoh et al. 2003; Duboc et al. 2005). Nodal signaling controls shell chirality in snails (Grande and Patel 2009), acting upstream of the homeodomain transcription factor gene *pitx2*, homologous to its role in vertebrate LR patterning (Piedra et al. 1998). This suggests that the role of Nodal in LR asymmetry is an ancestral trait of Bilateria, and that ecdysozoans, including *Drosophila* and *C. elegans*, have lost *nodal*. Nodal function in Hydra also resembles vertebrate LR patterning, in which it acts upstream of *pitx2* (Watanabe et al. 2014b). The preservation of the *nodal-pitx2* genetic circuit and its shared role in introducing asymmetry between vertebrates and cnidarians suggests that the LR program may be the original Nodal signaling circuit (Watanabe et al. 2014b).

BMPs are expressed in all three branches of Bilateria in which they show a conserved role in DV axial patterning. Although BMP expression defines ventral regions in chordates such as ver-

tebrates (Holley et al. 1995; Hammerschmidt et al. 1996b) and cephalochordates (Yu et al. 2007), it instead defines the dorsal regions in protostomes, such as flies (Irish and Gelbart 1987; St Johnston and Gelbart 1987), annelids (Denes et al. 2007), and flatworms (Molina et al. 2007, 2011), consistent with a general inversion of the body plan between protostomes and deuterostomes (Lacilli 1995; De Robertis and Sasai 1996; Gerhart 2000, 2002; Sander and Schmidt-Ott 2004). In most of these systems, BMP represses neural ectoderm (Sasai et al. 1995; Biehs et al. 1996; Holley et al. 1996; Miya et al. 1997; Denes et al. 2007; Molina et al. 2011; Kozmikova et al. 2013), and the domains of BMP ligand and BMP antagonist expression oppose each other along the DV axis (Ferguson and Anderson 1992; Francois et al. 1994; Sasai et al. 1994, 1995; Miller-Bertoglio et al. 1997; Onai et al. 2010; Molina et al. 2011). Notable exceptions to this include *C. elegans*, which does not use BMPs in DV patterning, instead using intracellular determinants (reviewed in Gonczy and Rose 2005), and echinoderms, which express BMPs and their antagonists on the same side of the embryo (Angerer et al. 2000; Duboc et al. 2004). Unexpectedly, in echinoderms, this coexpression of BMPs and their antagonists limits BMP signaling activity to the dorsal side, even though the transcripts themselves localize ventrally in the embryo (Lapraz et al. 2009). In cnidarians, BMPs are expressed along the same oral–aboral axis as Nodal (Rentzsch et al. 2006; Watanabe et al. 2014b), and, like echinoderms, they are expressed in the same domain as their inhibitors.

Together, these findings suggest that many of the TGF-β family proteins in vertebrate development retain the same roles as in the last common ancestor of Bilateria. Furthermore, many of the gene expression networks used to specify the axes in bilateral organisms seem to predate the bilateral body plan.

THE ROLE OF TGF-β FAMILY SIGNALING IN MESENDODERM SPECIFICATION AND PATTERNING

One of the first roles of TGF-β signaling in vertebrate development is the specification of mesendodermal cell fates by Nodal signaling (Zhou et al. 1993; Conlon et al. 1994; Jones et al. 1995; Rodaway et al. 1999). In amniotes, this process occurs within the primitive streak (Fig. 1A) (Bellairs 1953; Conlon et al. 1994; Skromne and Stern 2002; Kimura et al. 2006), whereas in amphibians mesendoderm is specified around the circumference of the blastopore lip (Fig. 1A′) (Cooke 1985; Lustig et al. 1996; Kurth and Hausen 2000), and in teleosts around the germ ring (Fig. 1A″) (Kimmel et al. 1990; Rodaway et al. 1999; Warga and Nusslein-Volhard 1999). In all vertebrates tested, *Nodal* expression defines these structures (Conlon et al. 1994; Ecochard et al. 1995; Feldman et al. 1998; Skromne and Stern 2002) (Fig. 1C–C″) and is required for the specification and subsequent involution or ingression movements of mesodermal and endodermal cells during gastrulation (Conlon et al. 1994; Osada and Wright 1999; Feldman et al. 2000).

The Initiation of Nodal Signaling during Gastrulation and Early Morphogenesis

In both *Xenopus* and zebrafish, *nodal* expression initiates within the vegetal tissues of the embryo (Feldman et al. 1998; Fan et al. 2007; Hong et al. 2011). In the zebrafish, this is an extraembryonic tissue consisting of a single polynucleated yolk cell (Fig. 1A″) (Kimmel and Law 1985). In *Xenopus*, yolk is distributed throughout all embryonic cells, but vegetal cells are particularly yolky and form the vegetal cell mass. The vegetal cell mass is somewhat analogous to the zebrafish yolk cell, although it is not extraembryonic and ultimately contributes to the endoderm (Fig. 1A′). In both zebrafish and *Xenopus*, the initial expression of *nodal* is triggered by dorsally localized nuclear β-catenin (Feldman et al. 1998; Kofron et al. 1999; Kelley et al. 2000; Maegawa et al. 2006) (discussed further in the section on regulation of TGF-β family gene expression during axial patterning). In zebrafish and frogs, β-catenin binds a *cis*-regulatory element at the 5′ end of the *nodal* first exon, which is conserved in nonvertebrate deuterostomes such as sea urchins (Norris and Robertson 1999; Fan and Dougan 2007; Range et al. 2007; Granier et al.

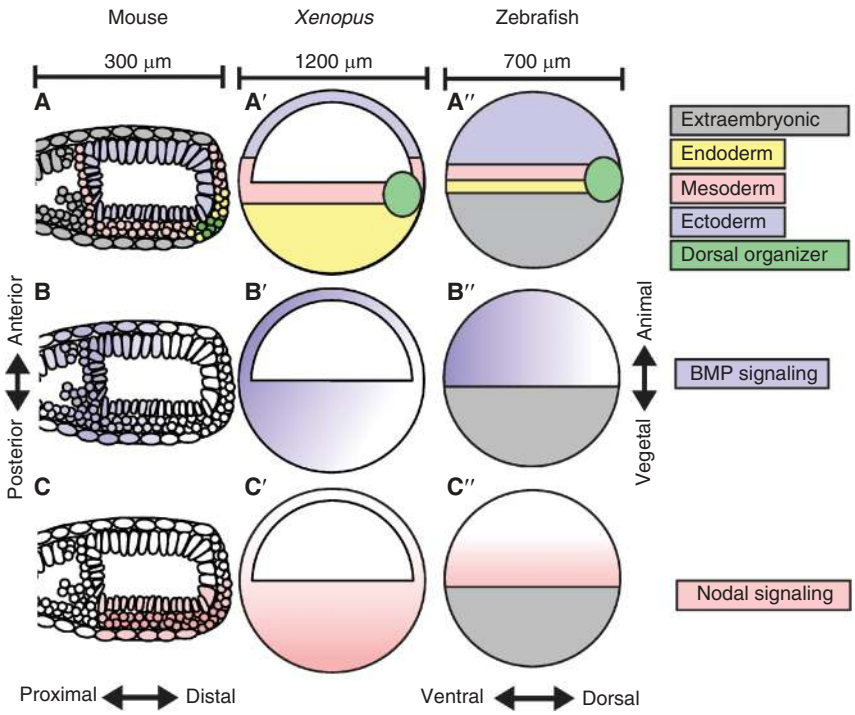

Figure 1. TGF-β family signaling gradients during gastrulation. (*A*) Embryonic tissues patterned by Nodal signaling during gastrulation in mouse, *Xenopus*, and zebrafish. (*B*) The bone morphogenetic protein (BMP) signaling gradient during gastrulation. (*C*) The Nodal signaling gradient during gastrulation.

2011). Nodal then activates its own expression in the adjacent marginal cells (Feldman et al. 1998; Fan et al. 2007; Hong et al. 2011) using a deeply conserved *nodal* autoregulatory element within the first intron. This regulatory element is known as the asymmetric enhancer element or ASE, which contains a binding site for the Smad2 cofactor FoxH1. FoxH1 binding sites are found in the first intron of all mammalian, *Xenopus*, zebrafish, ascidian, and sea urchin *nodal* genes (Osada et al. 2000; Fan and Dougan 2007; Range et al. 2007; Papanayotou et al. 2014). Moreover, the function of these binding sites in *nodal* autoregulation has been confirmed in both mice (Yamamoto et al. 2001; Norris et al. 2002) and *Xenopus* (Osada et al. 2000).

In mammals, there are no known maternally localized determinants, but the extraembryonic tissues and the activation of the WNT pathway both retain their importance. Unlike in *Xenopus* and zebrafish, mouse *Nodal* is initially expressed throughout the epiblast, possibly through activation of a specific enhancer regulating *Nodal* expression, the HBE (Papanayotou et al. 2014). The HBE is a mammal-specific *Nodal cis*-regulatory element that responds to OCT4, SOX2, NANOG and KLF4 (Papanayotou et al. 2014). NODAL signals from the epiblast to the extraembryonic ectoderm activating BMP-4 signaling within the extraembryonic ectoderm, which in turn activates Wnt signaling. WNT signaling then directly activates *Nodal* expression in the adjacent epiblast, through a motif 12 kb upstream of the transcriptional start site called the proximal epiblast enhancer, or PEE (Norris and Robertson 1999), forming a positive feedback loop (Ben-Haim et al. 2006). This positive feedback loop is essential to maintain *Nodal* expression in the proximal (closer to the site of implantation) posterior region of the epiblast, as negative feed-

back suppresses *Nodal* expression elsewhere in the epiblast. When BMP signaling is deficient in extraembryonic tissue, NODAL signaling is not maintained, and mice do not form a primitive streak (Waldrip et al. 1998; Tallquist and Soriano 2000; Fujiwara et al. 2002; Mishina et al. 2002; Davis et al. 2004; Miura et al. 2006).

In addition to maintaining *Nodal* expression, extraembryonic BMP signaling proximal to the epiblast and NODAL signaling from the epiblast are important to maintain the extraembryonic ectoderm, which becomes trophoblast in the absence of BMP or NODAL signaling (Guzman-Ayala et al. 2004). Unique to the mouse, BMP signaling within the extraembryonic tissue induces the expression of secreted NODAL convertases (Beck et al. 2002; Ben-Haim et al. 2006). It is presumed that these convertases act extracellularly, as they are expressed in extraembryonic tissues, whereas *Nodal* is expressed within the epiblast (Ben-Haim et al. 2006). Human embryonic stem cells will recapitulate these basic processes in cell culture (Warmflash et al. 2014). Remarkably, when these stem cells are grown on micropatterned plates that restrict them to forming circular colonies, they form an outer trophectoderm-like region (corresponding to proximal in the mouse embryo), which surrounds a *NODAL*-expressing, primitive-streak-like region, itself surrounding a central ectoderm region (similar to the inner part of the mouse epiblast).

In mouse, NODAL signaling specifies an important extraembryonic tissue known as the anterior visceral endoderm (AVE) at the distal end of the embryo (Fig. 2A) (Rosenquist and Martin 1995; Varlet et al. 1997; Takaoka et al. 2006; Takaoka and Hamada 2012). Once specified, the AVE cells migrate anteriorly and secrete the NODAL antagonists LEFTY, CERBERUS, and DICKKOPF-1 (DKK1) (Fig. 2B) (Takaoka and Hamada 2012; Li et al.

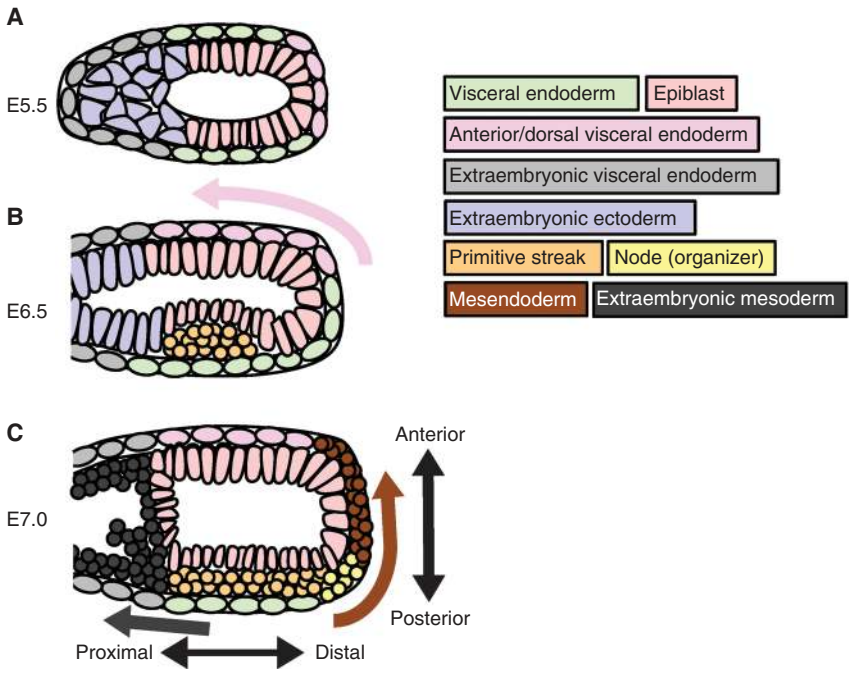

Figure 2. Morphogenetic movements of mouse tissues at the onset of gastrulation. (*A*) NODAL signaling specifies the anterior visceral endoderm (AVE) before gastrulation. (*B*) The AVE migrates anteriorly as the primitive streak forms. (*C*) Mesendodermal cells ingress from the primitive streak and intercalate with extraembryonic tissues during gastrulation.

2013). In contrast, *Xenopus* and zebrafish embryos express the Nodal antagonist Lefty in the same domain as *nodal* (Thisse and Thisse 1999; Cheng et al. 2000). Mouse gastrulation is reviewed elsewhere in detail (Robertson 2014).

During gastrulation, Nodal specifies the cells that will become mesendoderm and triggers the involution or ingression of these cells to form the germ layers (Conlon et al. 1994; Osada and Wright 1999; Feldman et al. 2000). In *Xenopus* and zebrafish, presumptive mesendodermal cells move from the exterior of the embryo to the interior whereas the more animally located cells migrate vegetally over the vegetal yolk cells, internalizing them (Holtfreter 1944; Warga and Kimmel 1990; Winklbauer 1990; Shih and Fraser 1995; Wilson et al. 1995; Winklbauer and Damm 2012). In mouse, the presumptive mesendodermal cells delaminate or ingress from the epiblast to form mesoderm and definitive endoderm (Lawson and Pedersen 1987). In mouse, a failure of ingression completely halts gastrulation (Conlon et al. 1994). Similarly, tissue explant experiments show that Nodal signaling is required for normal gastrulation movements in *Xenopus* (Osada and Wright 1999). In zebrafish *nodal* pathway loss-of-function mutants, the mesendoderm is also not specified and fails to ingress, but concurrent *nodal*-independent epiboly movements still progress (Gritsman et al. 1999; Feldman et al. 2000; Carmany-Rampey and Schier 2001; Woo et al. 2012).

TGF-β Family Pathway Components Acting in Mesendoderm Specification

A complete loss of Nodal signaling results in the failure to form most or all mesendodermal tissues (Zhou et al. 1993; Conlon et al. 1994; Feldman et al. 1998; Rodaway et al. 1999). In the mouse, this phenotype is observed in zygotic homozygous mutants for the single mammalian *Nodal* gene (Zhou et al. 1993; Conlon et al. 1994). In the zebrafish, to completely eliminate Nodal signaling during mesendoderm specification, two of the three zebrafish *nodal* genes, *ndr1* (*squint*) and *ndr2* (*cyclops*), must be eliminated (Feldman et al. 1998; Rodaway et al. 1999). A third zebrafish *nodal* gene, *southpaw*, is not expressed during gastrulation (Long et al. 2003) but is essential later for LR patterning (discussed in the section on the role of TGF-β family in left–right patterning). *Xenopus* embryos express four Nodals during gastrulation that are encoded by *xnr1, xnr2, xnr3*, and *xnr4* (Agius et al. 2000; Onuma et al. 2002; Kuroda et al. 2004; Sudou et al. 2012). Similar to zebrafish Southpaw, only one *Xenopus* Nodal ligand, Xnr1, is required for LR axis patterning (Toyoizumi et al. 2005).

During mesendodermal specification, Nodal signals through the type I receptor Acvr1b (ALK-4) (Gu et al. 1998), the type II receptors Acvr2a (ActRII or ActRIIA) and Acvr2b (ActRIIB) (Song et al. 1999), and the EGF-CFC coreceptor(s) known as CRIPTO and CRYPTIC in the mouse, FRL-1, Xcr2, and Xcr3 in *Xenopus*, and Oep in zebrafish (Ding et al. 1998; Gritsman et al. 1999; Dorey and Hill 2006; Chu and Shen 2010). The elimination of the EGF-CFC coreceptor(s) causes a failure of mesendoderm to form, recapitulating the complete Nodal loss-of-function phenotype. Although the type I receptor Acvr1c (ALK-7) has been shown to bind Nodal (Reissmann et al. 2001), it is not required for embryonic development in the mouse (Jörnvall et al. 2004).

Several intracellular Nodal signal transducers and cofactors function in mesendodermal patterning. After Nodal binds its receptor complex, the type I receptor phosphorylates the signal transducers Smad2 and Smad3. Loss of *smad2* function in zebrafish, and loss of function of both *Smad2* and *Smad3* in mice abolishes mesendodermal specification (Hoodless et al. 1999; Dunn et al. 2004; Dubrulle et al. 2015). Because Smad2 does not bind DNA directly, it requires a cofactor to associate with DNA and regulate transcription (Chen et al. 1996; Weisberg et al. 1998; Liu et al. 1999; Yeo et al. 1999). In mesendodermal patterning, the most important of these is FoxH1, and mouse and zebrafish *foxh1* mutants and *Xenopus foxh1* morphants (embryos injected with antisense *foxh1* morpholino oligonucleotides) partially recapitulate the *Nodal* loss-of-function phenotype, resulting in a truncation of the body axis, the loss of

anterior mesoderm, and impaired formation of craniofacial structures (intermediate *Nodal* phenotypes are discussed further in the section on tissues patterned by different levels of Nodal signaling) (Hoodless et al. 2001; Kofron et al. 2004; Slagle et al. 2011). The ability of Nodal to pattern some mesendodermal tissues in the absence of FoxH1 suggests it also acts through other cofactors. In the zebrafish, this is evident by the observation that FoxH1 is essential for specifying the axial mesoderm, but dispensable for specifying other mesodermal tissues where the transcriptional cofactors eomesodermin and Mixl1 play larger roles (Slagle et al. 2011). Also required for the induction of mesendoderm in the mouse is the E3 ubiquitin ligase ARKADIA, which enhances NODAL signaling by ubiquitylating the inhibitory SMAD7 and SNON (Episkopou et al. 2001; Niederlander et al. 2001; Koinuma et al. 2003; Levy et al. 2007; Mavrakis et al. 2007). Similarly, in zebrafish, the E3 ubiquitin ligase Siah2 enhances Nodal signaling activity (Szeto and Kimelman 2006; Kang et al. 2014).

In zebrafish *ndr1*;*ndr2* double loss-of-function mutants, mesodermally derived tail somites are still specified within the tail bud through a different process (Gritsman et al. 1999; Szeto and Kimelman 2006). Posterior somitic mesoderm is derived from ventral regions of the gastrula embryo that are specified by BMP signaling (Mullins et al. 1996; Holley 2006; Szeto and Kimelman 2006). After specification of the tailbud, a region of high Wnt signaling and *brachyury* expression maintains a population of neuromesodermal progenitors, which can give rise to mesodermal and neurectodermal tissues (reviewed in Kimelman 2016). The exact mechanism specifying posterior mesodermal cell fates in zebrafish remains unclear, but it appears to require the action of Wnt and Brachyury (reviewed in Szeto and Kimelman 2006; Kimelman 2016).

Tissues Patterned by Different Levels of Nodal Signaling

Intermediate *nodal* loss-of-function phenotypes reveal that Nodal patterns distinct tissues in a dose-dependent manner. Partial *nodal* loss of function is achieved through hypomorphic ligand alleles (Lowe et al. 2001), partial silencing with morpholino oligonucleotides (Feldman and Stemple 2001; Karlen and Rebagliati 2001; Yabe 2003a), small molecule kinase inhibitors of Acvr1b (Sun et al. 2006b; Hagos and Dougan 2007), dominant-negative versions of Nodal pathway components (Hemmati-Brivanlou and Melton 1992; Hoodless et al. 1999; Osada and Wright 1999; Reissmann et al. 2001; Aoki et al. 2002; Onuma et al. 2002; Jia et al. 2008), mosaic *nodal* loss of function (Lu and Robertson 2004), overexpression of the Nodal inhibitors Lefty or Cerberus (Meno et al. 1999; Agius et al. 2000; Cheng et al. 2000; Gritsman et al. 2000; Takahashi et al. 2000; Thisse et al. 2000), zygotic loss of function of genes with maternal and zygotic contributions (Schier et al. 1997; Feldman et al. 1998; Dubrulle et al. 2015), or through the elimination of individual, partially redundant signaling components (Hatta et al. 1991; Matzuk et al. 1995a; Heisenberg and Nusslein-Volhard 1997; Oh and Li 1997; Feldman et al. 1998; Rebagliati et al. 1998a; Song et al. 1999; Pogoda et al. 2000; Hoodless et al. 2001; Dougan 2003; Tian et al. 2003; Chu and Shen 2010). Mild disruption of Nodal signaling only disrupts LR patterning, suggesting that LR patterning is the most sensitive process to Nodal depletion, with defects ranging from benign isomerisms, through lethal circulatory and cardiac deformities, to gross organ positioning defects (Heisenberg and Nusslein-Volhard 1997; Oh and Li 1997; Song et al. 1999; Lowe et al. 2001; Lu and Robertson 2004). LR patterning occurs after mesendodermal patterning, and is discussed in the section om the role of the TGF-β family in left–right patterning.

More severe reductions in Nodal signaling reveal that the endoderm and the most anterior mesodermal tissue require more Nodal signaling than more posterior mesoderm (Heisenberg and Nusslein-Volhard 1997; Feldman et al. 1998; Song et al. 1999; Gritsman et al. 2000; Thisse et al. 2000; Lowe et al. 2001; Onuma et al. 2002; Dougan 2003; Vincent et al. 2003; Sun et al. 2006b; Hagos and Dougan 2007; Jia et al. 2008). The progressive depletion of

Nodal signaling results first in the loss of endoderm and anterior mesodermal tissues such as the prechordal plate, followed by more posterior mesodermal tissues, such as the somites, notochord, and muscle (Osada and Wright 1999; Gritsman et al. 2000; Thisse et al. 2000; Aoki et al. 2002; Onuma et al. 2002; Dougan 2003; Tian et al. 2003; Tanegashima et al. 2004; Sun et al. 2006b; Hagos and Dougan 2007; Jia et al. 2008). The prechordal plate and notochord secrete Sonic hedgehog (Shh), which acts in axial midline, neural, and craniofacial patterning (Sampath et al. 1998; Song et al. 1999; Muller et al. 2000; Rubinstein et al. 2000; Lowe et al. 2001; Tian et al. 2003). Nodal pathway component deficiencies that reduce the prechordal plate mesoderm show a range of defects in Shh-dependent processes up to and including cyclopia and reduction of the forebrain and facial structures (Osada and Wright 1999; Song et al. 1999; Thisse et al. 2000; Lowe et al. 2001; Reissmann et al. 2001; Rohr et al. 2001; Dougan 2003; Tian et al. 2003). Increasingly severe disruptions of Nodal signaling lead to dramatic gastrulation phenotypes, such as turning defects and primitive streak truncation in the mouse, and the dramatic truncation of the anterior–posterior (AP) axis in *Xenopus* (Song et al. 1999; Takahashi et al. 2000; Lowe et al. 2001; Onuma et al. 2002; Yabe 2003a).

Genes Activated by Nodal Signaling during Mesendoderm Specification

At least two levels of Nodal signaling induce the expression of distinct gene sets, consistent with the different Nodal levels acting in tissue patterning discussed above. High levels of Nodal signaling induce the endodermal markers *Sox17*, *Foxa2*, *Casanova* (*Sox32*), and *Hex* (Dickmeis et al. 2001; Aoki et al. 2002; Dougan 2003; Hagos and Dougan 2007; Jia et al. 2008). Reflecting the dependence of the most anterior mesoderm also on high Nodal signaling, tissue induction studies in cell culture and in vivo experiments show that the organizer/prechordal plate marker *goosecoid* is induced by high levels of Nodal signaling in *Xenopus*, zebrafish, and mouse (Meno et al. 1999; Osada and Wright 1999; Agius et al. 2000; Gritsman et al. 2000; Takahashi et al. 2000; Thisse et al. 2000; Dougan 2003; Sun et al. 2006b; Hagos and Dougan 2007; Jia et al. 2008; Harvey and Smith 2009). Lower levels of Nodal signaling induce the more posterior mesodermal marker *brachyury/notail* in several model organisms (Gurdon et al. 1994, 1995; Meno et al. 1999; Osada and Wright 1999; Agius et al. 2000; Gritsman et al. 2000; Tanegashima et al. 2000; Thisse et al. 2000; Dougan 2003; Sun et al. 2006b; Hagos and Dougan 2007; Jia et al. 2008; Harvey and Smith 2009).

Activating Nodal signaling by overexpressing Nodal (Wittbrodt and Rosa 1994; Jones et al. 1995; Erter et al. 1998; Osada and Wright 1999; Agius et al. 2000; Gritsman et al. 2000; Tanegashima et al. 2000; Thisse et al. 2000; Pfendler et al. 2005; Harvey and Smith 2009; Slagle et al. 2011), deficiency of the antagonist Lefty (Meno et al. 1999; Chen and Schier 2002; Tanegashima et al. 2004), or expressing an activated Acvr1b type I receptor (Aoki et al. 2002; Poulain and Lepage 2002) expands the domains with Nodal-dependent expression of genes, including *goosecoid*, *brachyury*, and *floating head*, a marker of notochord. Genes most sensitive to Nodal depletion, like *goosecoid*, require more Nodal signaling to be induced than genes that respond to less Nodal signaling, such as *brachyury*. Nodal signaling also plays a role in DV patterning. For example, Goosecoid induces the expression of the BMP antagonist genes *noggin* and *chordin* (Jones et al. 1995; Kurth and Hausen 2000), which dorsalizes the embryo (Erter et al. 1998; Harvey and Smith 2009). The effect of Nodal signaling on DV patterning is covered in the section on the role of the TGF-β family in DV axis patterning. In addition to dorsalizing the embryo, Nodal overexpression enlarges the notochord (Erter et al. 1998; Rebagliati et al. 1998b), and, in the zebrafish, it also enlarges the hatching gland, a prechordal plate derivative (Erter et al. 1998). Nodal overexpression can also induce a secondary body axis (Toyama et al. 1995; Armes and Smith 1997; Erter et al. 1998; Tanegashima et al. 2000). The ectopic overexpression of Nodal leads to secondary

axis formation in zebrafish (Toyama et al. 1995; Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015), frog (Lustig et al. 1996), and chick (Bertocchini and Stern 2002). Whether Nodal overexpression leads to axis duplication or the enlargement of specific Nodal-induced tissues appears to depend on the distribution of *nodal* expression.

Several efforts have been made to identify direct and indirect transcriptional target genes regulated by Nodal signaling. Numerous mesendodermal genes have been shown to respond to Nodal signaling such as *goosecoid, mixl1, mezzo, sox32, brachyury, eomes, foxa2, sox17, floating head*, and *fgf8* (Dickmeis et al. 2001; Poulain and Lepage 2002; Kurth et al. 2005; Bennett et al. 2007; Guzman-Ayala et al. 2009; Lee et al. 2011b). In addition to these, experiments with microarrays as well as Smad2 and FoxH1, and chromatin immunoprecipitation-sequencing (ChIP-seq) have identified a diverse array of Nodal signaling pathway target genes. These include several Nodal pathway genes, *nodal* itself, *cripto*, *foxh1*, and *pitx2*, which encode a transcription factor associated with both mesendodermal and LR patterning and are a direct target of Nodal signaling (Bennett et al. 2007; Lee et al. 2011b). Nodal also activates the expression of many of its own inhibitors, including the extracellular antagonists Lefty and Cerberus (Dickmeis et al. 2001; Bennett et al. 2007; Lee et al. 2011b), as well as the intracellular inhibitor Smad7 (Lee et al. 2011b). Thus, both positive and negative feedback are invoked during Nodal patterning of the mesendoderm. Suppression of translation by cycloheximide shows that *chordin* and *noggin*, which encode BMP inhibitors, are also direct targets of the Nodal signaling pathway (Kurth et al. 2005; Dubrulle et al. 2015), making them both direct and indirect targets through *goosecoid*. Cycloheximide treatment paired with RNA-seq has been used to identify and quantify the expression of direct targets of Nodal signaling (Dubrulle et al. 2015). The same study confirms 47 direct transcriptional targets of Nodal signaling activity including *brachyury* and *goosecoid*, which are activated sequentially with increasing amounts of Nodal exposure.

Mechanisms of Tissue Patterning during Mesendoderm Specification

The mechanism by which Nodal functions as a morphogen has been an area of significant debate and study. Early models posited that Nodal patterns multiple tissue types through a simple spatial gradient generated by diffusion of Nodal ligands away from their source, their reception through signaling, and their interactions with diffusible inhibitors like Lefty (Chen and Schier 2001, 2002; Muller et al. 2012, 2013). Supporting this model, ectopic point sources of Nodal produce a spatially nested pattern of Nodal-dependent gene expression (Chen and Schier 2001). Visualization of Smad2 in zebrafish and *Xenopus* embryos also shows a gradient of nuclear Smad2, reflecting a presumptive ligand concentration gradient (Harvey and Smith 2009). Moreover, models that take into account the diffusion rates of Nodal ligands and Lefty in the zebrafish (Muller et al. 2012) can explain observed nuclear Smad2 levels as a classical reaction–diffusion system.

Although there is a wealth of evidence supporting the spatial concentration gradient model, several studies suggest that this is an incomplete picture of Nodal signaling. Cell culture and *Xenopus* explant studies show that duration of exposure to Nodal or Activin could also play a role, as higher threshold genes can be activated by either increased ligand concentration or longer duration of exposure (Green and Smith 1990; Gurdon et al. 1995; Guzman-Ayala et al. 2009). Experiments in zebrafish, which express two Nodal ligands, Ndr1 and Ndr2, during gastrulation (Hatta et al. 1991; Heisenberg and Nusslein-Volhard 1997; Feldman et al. 1998) further challenge a strictly spatial action of Nodal signaling. Ndr1 acts at a greater distance and can behave as a morphogen when expressed ectopically, but Ndr2 cannot (Chen and Schier 2001). Nevertheless, Ndr2 still patterns the mesendoderm in an *ndr1* null mutant, albeit more slowly, and the *ndr2* loss-of-function phenotype is more severe than *ndr1* loss of function (Hatta et al. 1991; Heisenberg and Nusslein-Volhard 1997; Feldman et al. 1998; Dougan 2003), indicating that it plays a greater role than Ndr1 in

mesendoderm induction. Because Ndr2 cannot act at a long range and pattern tissues spatially in the same manner as Ndr1 (Chen and Schier 2001; Cheng et al. 2004), this suggests that the duration of Nodal signaling may be more important than a Nodal spatial concentration gradient.

Further supporting a role for Nodal duration acting in mesodermal patterning, studies using timed inhibition of Nodal signaling by SB-431542, a small molecule kinase inhibitor of Acvr1b, TβRI, and Acvr1c (ALK-4, -5, -7), revealed that distinct cell types are patterned during different time frames of blastula and early gastrula stages (Hagos and Dougan 2007). Somites are specified first, requiring the shortest duration of signaling, followed by the notochord, Kupffer's vesicle, blood, heart, and hatching gland. These investigators further show that a decrease in *nodal* expression levels in *ndr1* single mutants delayed the specification of these tissues, whereas Nodal overexpression accelerated their specification. This suggests that the nested patterning of these tissues relies on cumulative exposure to Nodal over time rather than a fixed window of competence. It has been proposed that although Nodal can act over long range in some contexts, the observed gradient of phosphorylated Smad2 and Smad3, and the nested gene expression domains induced during mesendodermal patterning can be explained exclusively by the duration of Nodal exposure and relays with fibroblast growth factor (FGF) signaling (van Boxtel et al. 2015).

In addition to the spatial gradient and duration of exposure models, another intriguing mechanism has been proposed that Nodal patterns the mesendoderm via a ratchet model (Gurdon et al. 1995; Dyson and Gurdon 1998; Bourillot et al. 2002), in which cells retain a memory of their highest level of ligand exposure. This is supported by the observation in *Xenopus* tissue explant systems that the transcription of Nodal target genes can persist long after a short pulse of ligand exposure. The longevity of receptor complexes at the cell surface provides a potential mechanism for this (Jullien and Gurdon 2005).

Several investigators have proposed that both Nodal concentration and duration are important (Hagos and Dougan 2007; Harvey and Smith 2009; Dubrulle et al. 2015; Sako et al. 2016). One potential mechanism for this is that differential transcription rates can account for both the concentration- and time-dependent sensitivity of different Nodal pathway target genes, as slowly transcribed genes will be boosted by both increased concentration and duration, whereas rapidly transcribed genes will respond swiftly to even low concentrations. Indeed, the Nodal targets, *brachyury* and *goosecoid* are transcribed at different rates (Dubrulle et al. 2015). Long-range targets of Nodal signaling, such as *brachyury*, are expressed rapidly in response to low levels of Nodal signaling, whereas short-range targets, such as *goosecoid*, are transcribed slowly and require high levels of Nodal signaling (Dubrulle et al. 2015).

Experiments in zebrafish with light-activated Nodal receptors that dimerize on blue light exposure, provide a direct means to test the effect of Nodal signal duration on gene expression (Sako et al. 2016). In this study, the investigators found that *goosecoid* requires a longer duration of Nodal signaling exposure than the endodermally expressed gene *sox32*. Because both genes are known to require high concentrations of Nodal ligand, the investigators posit that in some cases Nodal concentration and duration may have independent effects. They propose a gene network in which *sox32* activates expression of the endodermal marker *sox17*, whereas *goosecoid* specifies prechordal plate and represses *sox17*. The resulting system allows both concentration and duration to be exploited for the induction of different tissues, with cells exposed briefly to high Nodal concentrations producing endoderm, and those exposed for longer producing prechordal plate.

GDF-1 (Vg1), Activin, and Other Signaling in Mesendoderm Patterning

Although the role of Nodal as a morphogen in specifying mesendodermal cell types is firmly established, Nodal also synergizes with several other signaling molecules in this process, both

within and outside the TGF-β family. The TGF-β ligands Activin and growth and differentiation factor 1 (GDF-1, or Vg1) can both induce mesoderm. In fact, the first mesoderm inducing experiments reporting thresholds of gene induction, now attributed to Nodal, used Activin, which shares the signal transducers Smad2 and Smad3 with the Nodal pathway (Green and Smith 1990; Smith et al. 1990; Green et al. 1992; Gurdon et al. 1995). Although ACTIVIN was initially thought to act in this patterning in vivo, the absence of a mesendodermal defect in mouse mutants (Matzuk et al. 1995a) or in response to the Activin inhibitor Follistatin in *Xenopus* (Schulte-Merker et al. 1994), coupled with the lack of Activin expression during gastrulation (Albano et al. 1994; Feijen et al. 1994) suggested that Activin plays little or no role in this process. Morpholino oligonucleotide-mediated depletion experiments of the Activin B (Inhibin β_B chain dimer) in *Xenopus*, however, support a role in mesendodermal patterning (Piepenburg et al. 2004; Bates et al. 2013). In particular, Activin B may be important for regulating the proliferation of mesendodermal cells (Ramis et al. 2007).

GDF-1 (also called Gdf-3 in zebrafish, and Vg1 in zebrafish and *Xenopus*) is required for the specification of mesendoderm, and likely forms a heterodimer with NODAL during mesendodermal specification (Fuerer et al. 2014). *gdf1* expression overlaps with *nodal* during mesendodermal patterning (Weeks and Melton 1987; Tannahill and Melton 1989; Helde and Grunwald 1993; Wall et al. 2000; Cheng et al. 2003; Andersson et al. 2007; Fleming et al. 2013), and disruptions of GDF-1 signaling show mesendodermal patterning defects in mouse and frog (Joseph and Melton 1998; Wall et al. 2000; Andersson et al. 2006; Fleming et al. 2013). GDF-1 can also induce mesendodermal tissue, and like NODAL depends on EGF-CFC cofactors to do so (Dale et al. 1993; Thomsen and Melton 1993; Kessler and Melton 1995; Dohrmann et al. 1996; Shah et al. 1997; Cheng et al. 2003; Fleming et al. 2013). *Gdf1* loss of function also compounds *Nodal* loss of function (Andersson et al. 2006). Moreover, GDF-1-NODAL heterodimers are dramatically more effective at inducing endoderm in vitro than NODAL homodimers (Fuerer et al. 2014).

Both FGF and Wnt can induce mesoderm in cell culture (Godsave and Slack 1989; Slack et al. 1990; Green et al. 1992; Isaacs et al. 1992; LaBonne and Whitman 1994; Cui et al. 1996; Rodaway et al. 1999; Zorn et al. 1999; Finley et al. 2003; Cao et al. 2004; Mathieu et al. 2004; Lindsley et al. 2006; Hansson et al. 2009; Luxardi et al. 2010; Payne et al. 2011; Rankin et al. 2011; Engert et al. 2013; Toivonen et al. 2013) and are required for the differentiation of specific mesendodermal tissues in vivo (Amaya et al. 1991; LaBonne and Whitman 1994; Zorn et al. 1999; Wills et al. 2008; Engert et al. 2013). Activation of the WNT and FGF pathways has also been shown to enhance NODAL or ACTIVIN induction of mesoderm and endoderm in embryonic stem cells (Lindsley et al. 2006; Sumi et al. 2008; Payne et al. 2011; Toivonen et al. 2013). FGF-8, in particular, has been shown to function in a relay with Nodal signaling, and induces many of the same target genes, including *goosecoid* and *chordin*, and loss of *fgf8* exacerbates hypomorphic *nodal* phenotypes (Mathieu et al. 2004). FGF-8 also drives cells away from an endodermal fate and toward a mesodermal one, suggesting a role for FGF-8 in the distinction between these two Nodal-induced tissues (Rodaway et al. 1999; Mizoguchi et al. 2006). BMP also patterns mesendodermal fates along the DV axis, with more ventral and posterior fates requiring higher BMP signaling activity (Tiso et al. 2002; Sumi et al. 2008; Wills et al. 2008). BMP signaling also restricts the size of a retinoic acid signaling center, which patterns mesendodermal tissues along the AP axis later in development (Naylor et al. 2016). Although Nodal is key to the induction of mesendoderm, and specifies different fates along its axis of activity, the integration of multiple embryonic signaling pathways is necessary to specify the full range of mesendodermal tissues.

THE ROLES OF TGF-β FAMILY SIGNALING IN DV AXIAL PATTERNING

The DV axis of all vertebrates is patterned by a gradient of BMP signaling (Fig. 1B) (Gourronc et al. 2007; De Robertis 2008). Axis patterning in mice takes place from about E5.5–E8.5,

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5 days after the transition from maternal to zygotic transcription (Beddington and Robertson 1999). In contrast, the AP and DV axes of zebrafish and *Xenopus* are patterned within hours following the transition to widespread zygotic transcription, called the midblastula transition (MBT) (De Robertis and Kuroda 2004; Schier and Talbot 2005). In vertebrates, high levels of BMP signaling induce ventral tissue fates, such as epidermis and blood, intermediate levels induce lateral tissue, such as neural crest, whereas BMP signaling must be blocked for dorsal tissue development into notochord, brain, and prechordal plate tissues (De Robertis and Sasai 1996; Schier and Talbot 2005; Little and Mullins 2006). In all vertebrates investigated, multiple BMP ligands are secreted ventrally (proximally in mice), and then move through the extracellular space, to ultimately activate signaling by binding to two type I and two type II receptors (Waldrip et al. 1998; Arnold and Robertson 2009; Robertson 2014). The formation of this receptor complex allows the constitutively active type II receptors to phosphorylate the type I receptors (Wrana et al. 1994). The type I receptors then phosphorylate Smad1, Smad5, and Smad8 (Liu et al. 1996; Abdollah et al. 1997), which form complexes with Smad4 and accumulate in the nucleus (Schmierer and Hill 2005), inducing BMP target gene expression.

The BMP Ligands and Receptors Required in DV Patterning

The constellation of BMP ligands and ligand dimers that are required during DV axial patterning differ somewhat in zebrafish, *Xenopus*, and mouse. In zebrafish, BMP signaling is induced solely by Bmp2–7 heterodimers, and whereas Bmp2 and Bmp7 homodimers are produced, they do not signal (Little and Mullins 2009). Consistent with a requirement for Bmp2–7 heterodimers, the loss of either *bmp2* (Kishimoto et al. 1997; Nguyen et al. 1998; Schmid et al. 2000) or *bmp7* (Dick et al. 2000; Schmid et al. 2000) causes a loss of all ventral tissue leading to embryonic lethality during somitogenesis. Both *bmp2* and *bmp7* are expressed ventrally in the late blastula and gastrula (Hammerschmidt et al. 1996a; Nguyen et al. 1998; Schmid et al. 2000; Sidi et al. 2003; Furthauer et al. 2004; Ramel and Hill 2013; Xue et al. 2014). *bmp4* is also expressed ventrally in the zebrafish gastrula (Nikaido et al. 1997; Stickney et al. 2007), possibly forming homo- and heterodimers with Bmp2 and Bmp7, but the loss of *bmp4* has a much milder effect on DV patterning, only affecting tail patterning (Stickney et al. 2007). In *Xenopus*, Bmp2, Bmp4, Bmp7, and the BMP-related ligand ADMP all contribute to BMP signaling and ventral tissue formation, and only depleting the expression of all four ligands using morpholino oliginucleotides causes a complete loss of ventral cell fates (Reversade and De Robertis 2005; Reversade et al. 2005). However, more work is needed to determine which homo- or heterodimer combinations of Bmp2, Bmp4, Bmp7, and ADMP form and signal. *bmp4* and *bmp7* are expressed ventrally in the blastula and gastrula, whereas *bmp2* is expressed ubiquitously, and *admp* is expressed in the dorsal organizer (Hemmati-Brivanlou and Thomsen 1995; Moos et al. 1995; Knochel et al. 2001; Marom et al. 2005).

In mouse, both *Bmp2* and *Bmp4* are needed to establish extraembryonic structures such as the allantois, but only *Bmp4* is required to drive AVE migration (Coucouvanis and Martin 1999; Soares et al. 2008; Miura et al. 2010) and pattern the axis of the epiblast (Winnier et al. 1995; Lawson et al. 1999; Ying and Zhao 2001). *Bmp2* mutants have impaired allantois and cardiac development (Zhang and Bradley 1996). *Bmp2* and *Bmp4* are expressed predominantly in the extraembryonic ectoderm (Winnier et al. 1995; Zhang and Bradley 1996; Coucouvanis and Martin 1999; Lawson et al. 1999; Ying et al. 2000; Ying and Zhao 2001; Danesh et al. 2009; Madabhushi and Lacy 2011). Whether homo- or heterodimers are required during mouse DV patterning has not yet been established, but the loss of either *Bmpr1a* (Mishina et al. 1995) or *Acvr1* (Gu et al. 1999; Mishina et al. 1999) alone causes significant disruption of primitive steak formation, suggesting that BMPRIA (ALK-3) and ACVRI (ALK-2) form a heteromeric receptor complex with a BMP heterodimer in signaling in the AVE. Mutating

individual members of the 60A subgroup of BMP ligands, encoded by *Bmp5* (Kingsley et al. 1992; King et al. 1994), *Bmp6* (Solloway et al. 1998), or *Bmp7* (Dudley et al. 1995; Luo et al. 1995; Karsenty et al. 1996; Wawersik et al. 1999), does not disrupt early embryonic patterning. However, $Bmp5^{-/-}$;$Bmp7^{-/-}$ mutants show severe cell proliferation defects leading to lethality by E10.5, suggesting that the 60A members act redundantly in early development (Solloway and Robertson 1999).

Similar BMP receptors are required during axis patterning in zebrafish, *Xenopus*, and mice. During zebrafish DV patterning, Bmp2–7 heterodimers signal through the type I receptors Bmpr1a and/or Bmpr1b (Alk3 and Alk6) and Acvr1 (Bauer et al. 2001), and through Smad5 (Hild et al. 1999; Kramer et al. 2002). These three type I receptors are expressed ubiquitously during DV patterning in zebrafish (Hild et al. 1999), but it is unclear which of the six known type II receptors contribute to DV patterning (Albertson et al. 2005; Monteiro et al. 2008; Yadin et al. 2016). Similarly, during *Xenopus* DV patterning, Bmp2, Bmp4, Bmp7, and ADMP signal through the type I receptors Bmpr1a/b (Fritz and Sheets 2001; Schille et al. 2016) and Acvr1 (Armes and Smith 1997; Fritz and Sheets 2001), Acvr2a and/or Acvr2b (New et al. 1997), Bmpr2 (Frisch and Wright 1998), and Smad1 (Thomsen 1996; Fritz and Sheets 2001). *bmpr1a* and *bmpr1b* are expressed animally (Fritz and Sheets 2001; Schille et al. 2016), whereas *acvr1* is expressed ubiquitously (Armes and Smith 1997; Fritz and Sheets 2001). However, little is known about the spatial expression of the type II receptors in *Xenopus*. During mouse axial patterning, BMP-2 and BMP-4 signal through the type I receptors ACVRI (Gu et al. 1999; Yoshikawa et al. 2000) and BMPRIA (Roelen et al. 1994; Dewulf et al. 1995; Mishina et al. 1995; Davis et al. 2004; Di-Gregorio et al. 2007; Danesh et al. 2009), and SMAD1, 5, and 8 (Tremblay et al. 2001; Arnold et al. 2006). The loss of type II receptor *Bmpr2* (Beppu et al. 2000), or the combined loss of *Acvr2a* and *Acvr2b* (Manova et al. 1995; Song et al. 1999) disrupts primitive streak formation, suggesting that they mediate Nodal and/or BMP signaling during axial patterning. In the mouse, *Bmpr1a*, *Bmpr2*, *Acvr2a*, and *Acvr2b* are expressed ubiquitously along the proximal–distal axis in wild-type embryos (Manova et al. 1995; Beppu et al. 2000; Danesh et al. 2009). *Bmpr1b* is expressed at very low levels during early embryonic patterning (Dewulf et al. 1995; Danesh et al. 2009). *Acvr1* is expressed proximally in the extraembryonic ectoderm but not distally (Gu et al. 1999; Yoshikawa et al. 2000). In contrast, the BMP ligands and their extracellular regulators are asymmetrically expressed along the proximal–distal and AP axes throughout early embryonic patterning (Zhao 2003; Little and Mullins 2006).

The BMP Signaling Gradient Patterns DV Axial Tissues in Vertebrates

The BMP signaling gradient is established by the asymmetric expression of BMP ligands, agonists, and antagonists, whereas the expression of the BMP receptors and Smads is ubiquitous. In mouse, zebrafish, and *Xenopus*, the majority of BMP ligands are expressed ventrally, whereas the majority of extracellular antagonists are expressed dorsally, near and within the dorsal organizer (Figs. 3 and 4) (Niehrs 2004; Kishigami and Mishina 2005; Little and Mullins 2006; Carron and Shi 2016). Also referred to as the Spemann–Mangold organizer in *Xenopus* and zebrafish or the Node in mouse, the dorsal organizer is the region where gastrulation movements begin. The dorsal organizer expresses a common suite of extracellular BMP antagonists and transcriptional repressors essential to repressing BMP signaling in the dorsal region of the embryo (Nieto 1999; Niehrs 2004; Thisse and Thisse 2015). BMP antagonists such as Chordin, Noggin, and Follistatin bind to BMP ligands in the extracellular space, preventing BMP signaling dorsally. These antagonists are opposed by the ventrally expressed metalloproteases Tolloid and Bmp1, which cleave Chordin and release the BMP ligand. A complex network of other extracellular proteins regulates antagonist binding and decay, including BMP endothelial regulator (BMPER, also called Crossveinless-2, CV2), Twisted gastrula-

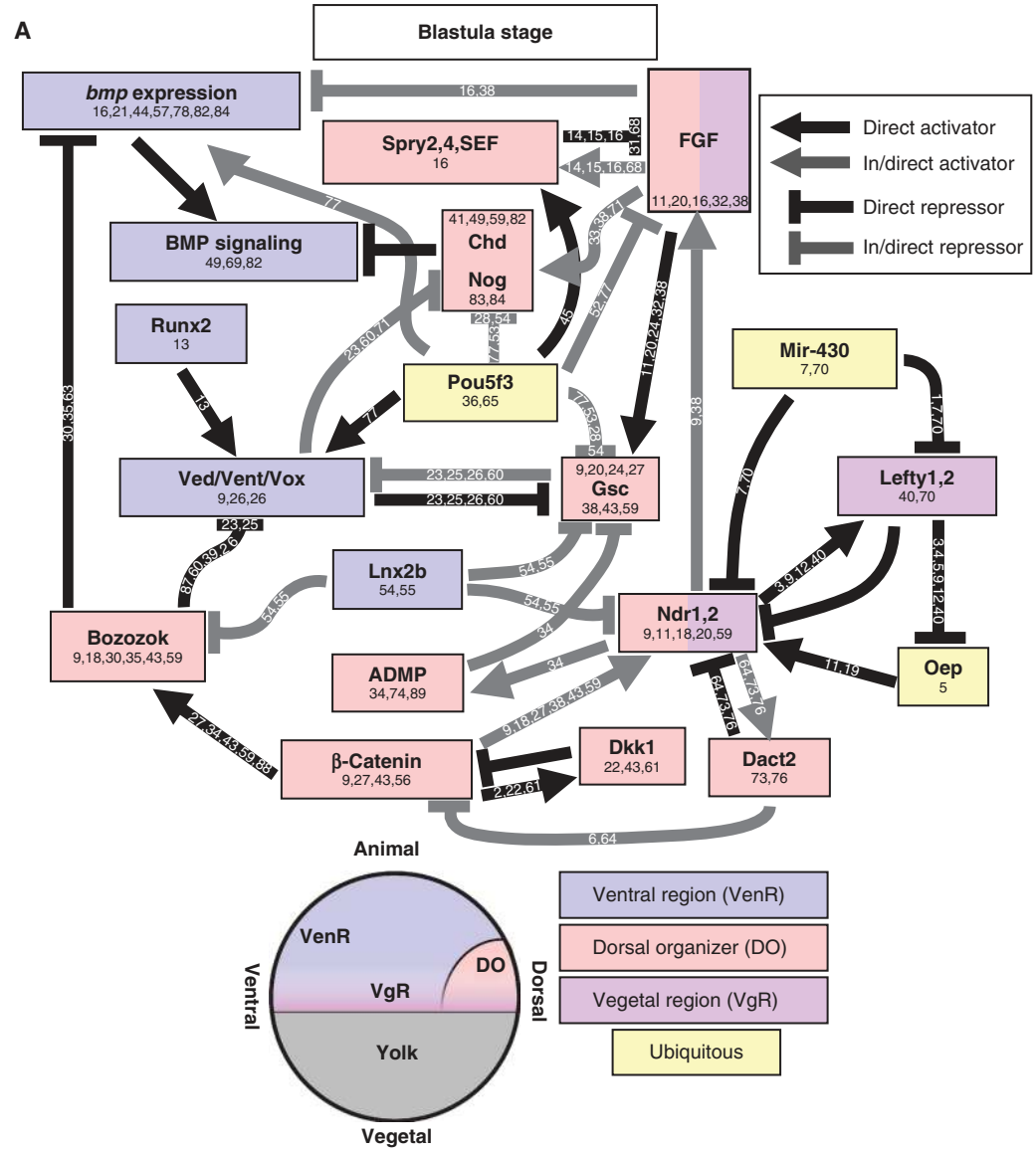
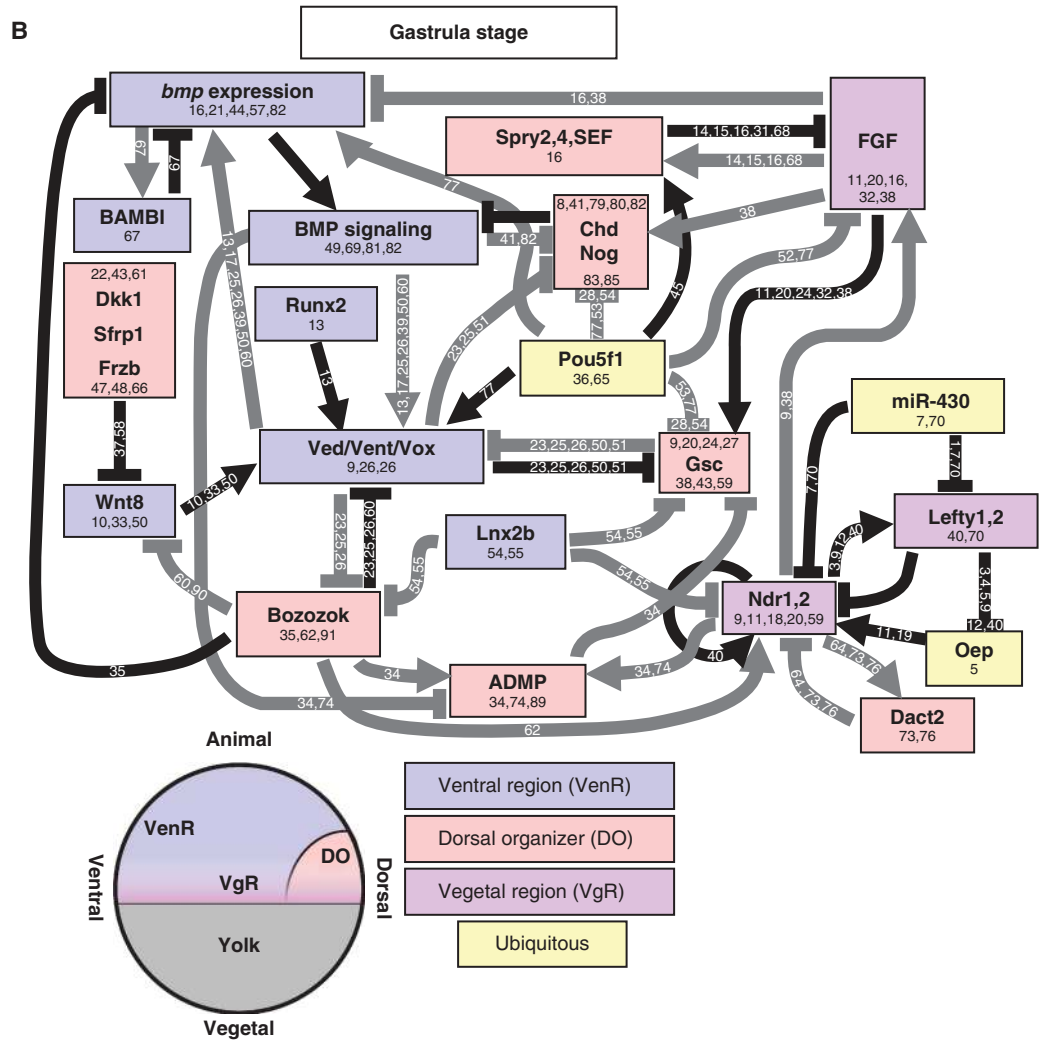


Figure 3. Regulation of TGF-β family expression during axis patterning. Gene activation and repression in (*A*) early blastula (sphere to shield stage) and (*B*) the gastrula (after shield stage). Direct activators and repressors are marked by black lines. Indirect activators and repressors, and activators and repressors without sufficient evidence to prove a direct relationship, are marked by gray lines. Genes are color coded by their expression domains. References providing evidence for each relationship or expression domain are listed on the line connecting two genes or in the box with the gene name. Chd, Chordin; Dact2, Dapper homolog 2; Gsc, Goosecoid; Nog, Noggin; Spry, Sprouty. Numbers 1–91 in panels *A* and *B* refer to the following references: 1, Bassett et al. 2014; 2, Chamorro et al. 2005; 3, Chen and Schier 2002; 4, Chen and Shen 2004; 5, Cheng et al. 2004; 6, Cheyette et al. 2002; 7, Choi et al. 2007; 8, Dal-Pra et al. 2006; 9, Dougan 2003); 10, Erter et al. 2001; 11, Feldman et al. 1998; 12, Feldman et al. 2002; 13, Flores et al. 2008; 14, Furthauer et al. 2001; 15, Furthauer et al. 2002; 16, Furthauer et al. 2004; 17, Gilardelli et al. 2004; 18, Gore et al. 2005; 19, Gritsman et al. 1999; 20, Gritsman et al. 2000; 21, Hammerschmidt et al. 1996a; 22, Hashimoto et al. 2000; 23, Imai et al. 2001; 24, Joore et al. 1996; 25, Kawahara et al. 2000b; 26, Kawahara et al. 2000a; 27, Kelley et al. 2000; (*Continued on following page.*)



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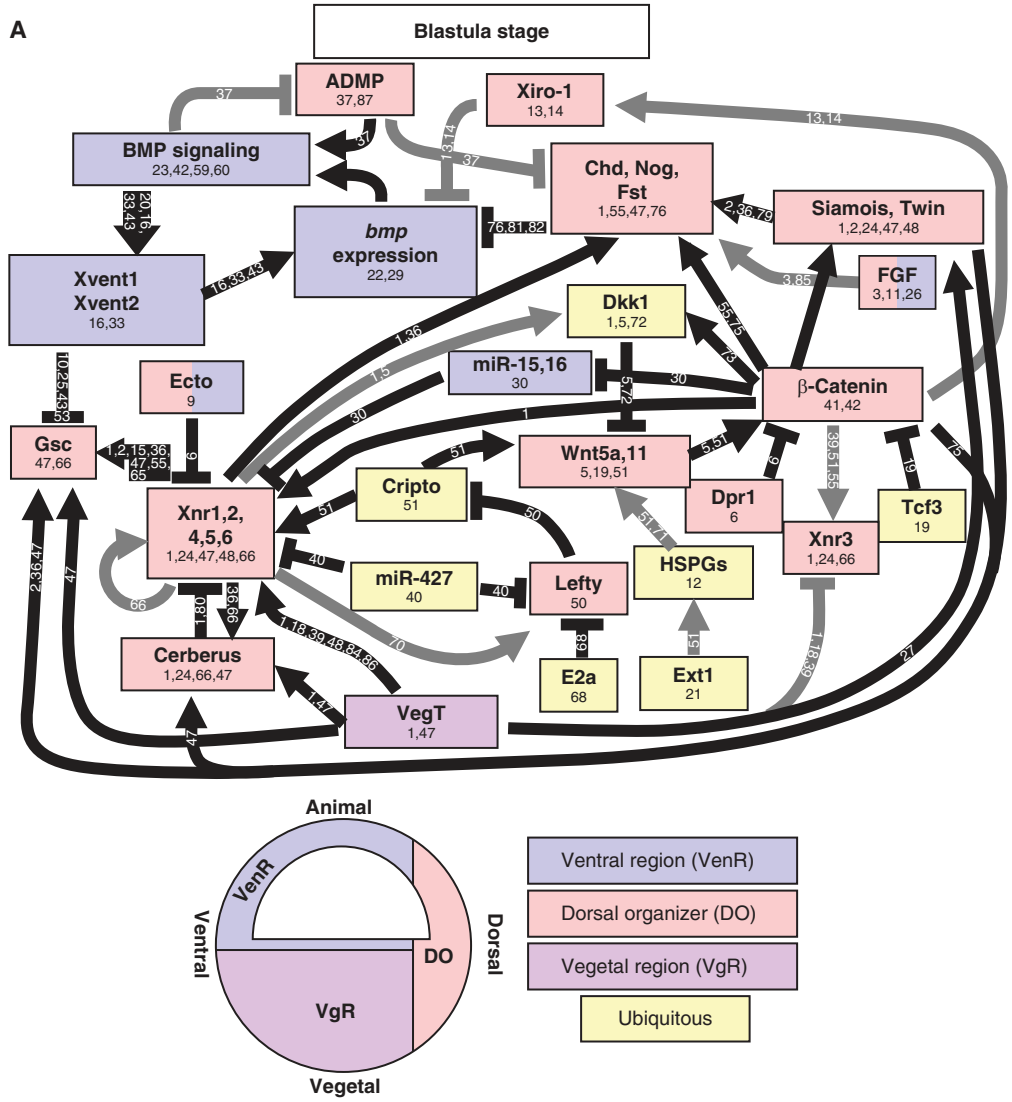

Figure 4. Regulation of TGF-β family expression during axis patterning in *Xenopus*. Gene activation and repression in the early blastula (*A*) and gastrula (*B*). Direct activators and repressors are marked by black lines. Indirect activators and repressors, and activators and repressors without sufficient evidence to prove a direct relationship are marked by gray lines. Genes are color coded by their expression domains. References providing evidence for each relationship or expression domain are listed on the line connecting two genes or in the box with the gene name. Chd, Chordin; Dpr1, Dapper1; Ecto, Ectodermin; Fst, Follistatin; Gsc, Goosecoid; Nog, Noggin. Numbers 1 through 92 in panels *A* and *B* refer to the following references: 1, Agius et al. 2000; 2, Bae et al. 2011; 3, Branney et al. 2009; 4, Carnac et al. 1996; 5, Cha et al. 2008; 6, Cheyette et al. 2002; 7, Chiu et al. 2014; 8, de Almeida et al. 2008; 9, Dupont et al. 2005; 10, Eimon and Harland 1999; 11, Fletcher and Harland 2008; 12, Galli et al. 2003; 13, Glavic et al. 2001; 14, Gómez-Skarmeta et al. 2001; 15, Hashimoto-Partyka et al. 2003; 16, Hemmati-Brivanlou and Thomsen 1995; Hikasa et al. 2010; 17, Hoppler and Moon 1998; 18, Houston et al. 2002; 19, Houston 2012; 20, Karaulanov et al. 2004; 21, Katada et al. 2002; 22, Knochel et al. 2001; 23, Kurata et al. 2000; 24, Kuroda et al. 2004; 25, Laurent and Cho 1999; 26, Lea et al. 2009; 27, Li et al. 2015; 28, Marom et al. 1999; 29, Marom et al. 2005; 30, Martello et al. 2007; 31, Mochizuki et al. 2000; 32, Murakami et al. 2003; 33, Onichtchouk et al. 1996; 34, Onichtchouk et al. 1996; (*Continued on following page.*)

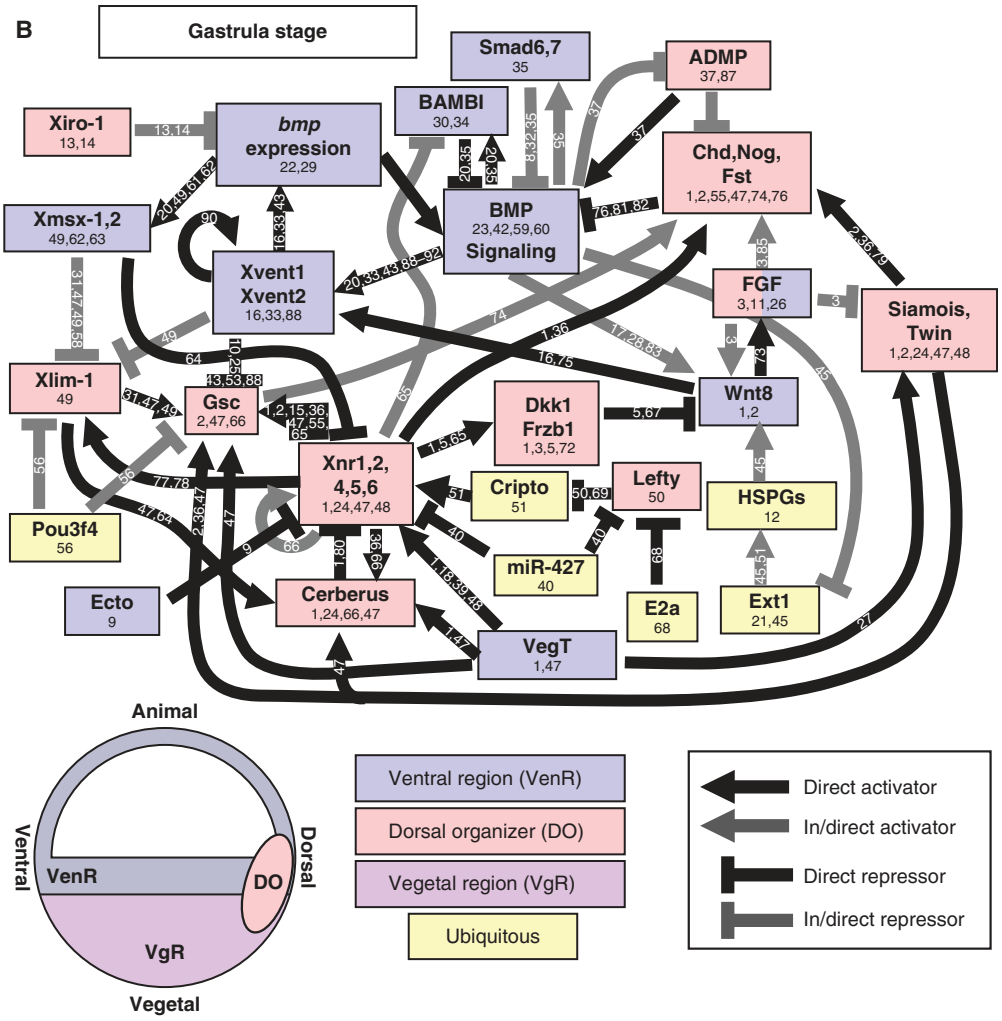

Figure 4. 35, Paulsen et al. 2011; 36, Reid et al. 2012; 37, Reversade and De Robertis 2005; 38, Reversade et al. 2005; 39, Rex et al. 2002; 40, Rosa et al. 2009; 41, Schneider et al. 1996; 42, Schohl and Fagotto 2003; 43, Schuler-Metz et al. 2000; 44, Sekiya et al. 2004; 45, Shieh et al. 2014; 46, Schmidt et al. 1995; 47, Sudou et al. 2012; 48, Takahashi et al. 2000; 49, Takeda et al. 2000; 50, Tanegashima et al. 2004; 51, Tao et al. 2005; 53, Trindade et al. 1999; 54, Vonica and Gumbiner 2007; 55, Wessely et al. 2001; 56, Witta and Sato 1997; 57, Xanthos et al. 2002; 58, Yamamoto et al. 2000; 59, Cho et al. 2013; 60, Plouhinec et al. 2013; 61, Maeda et al. 1997; 62, Onitsuka et al. 2000; 63, Suzuki et al. 1997; 64, Yamamoto et al. 2001; 65, Chiu et al. 2014; 66, Onuma et al. 2002; 67, Leyns et al. 1997; 68, Wills and Baker 2015; 69, Branford and Yost 2002; 70, Cheng et al. 2000; 71, Ohkawara 2003; 72, Glinka et al. 1998; 73, Chamorro et al. 2005; 74, Sander et al. 2007; 75, Nakamura et al. 2016; 76, Khokha et al. 2005; 77, Watanabe et al. 2003; 78, Rebbert and Dawid 1997; 79, Collart et al. 2005; 80, Piccolo et al. 1999; 81, Piccolo et al. 1996; 82, Zimmerman et al. 1996; 83, Schmidt et al. 1995; 84, Kofron et al. 1999; 85, Delaune et al. 2005; 86, Hyde and Old 2000; 87, Dickmeis et al. 2001; 88, Gawantka et al. 1995; 89, Hata et al. 2000; 90, Henningfeld et al. 2002; 91, Lee et al. 2002; 92, Lee et al. 2011a.

tion (Tsg), Ont1, Sizzled, and Crescent. These proteins and their interactions are covered in detail in the section on extracellular regulation of the TGF-β family (see Fig. 6).

It remains unclear how the BMP signaling gradient informs the expression of BMP target genes along the DV axis. It is postulated that cells along the BMP gradient sense the amount of signal, which determines their DV tissue fate as a morphogen. The known direct targets of BMP signaling during DV patterning are *msx1b* (Maeda et al. 1997; Tribulo et al. 2003; Esteves et al. 2014), *p63* (Bakkers et al. 2002), *foxi1* (Hans et al. 2007), *Xvent2* (Hata et al. 2000; Schuler-Metz et al. 2000; Henningfeld et al. 2002; Lee et al. 2002; Karaulanov et al. 2004), *Xvent1* (Lee et al. 2011a), *bambi* (Karaulanov et al. 2004), *tsg* (Karaulanov et al. 2004), *bmpr2* (Karaulanov et al. 2004), *smad6* (Karaulanov et al. 2004), and *smad7* (Karaulanov et al. 2004), and there are likely more yet to be identified. However, it is not known whether different BMP direct targets along the DV axis are induced by different thresholds of BMP signaling, different durations of BMP signaling, or some combination of the two. It is also not known how many distinct domains and signaling thresholds are patterned by the gradient of BMP signaling. Deciphering these mechanisms has been hindered by the lack of quantitative measurements of BMP signaling activity and BMP target gene expression. The BMP signaling gradient has been visualized using antibodies against phosphorylated Smad5 in mouse (Di-Gregorio et al. 2007), zebrafish (Tucker et al. 2008; Hashiguchi and Mullins 2013; Ramel and Hill 2013; Xue et al. 2014), and *Xenopus* embryos (Faure et al. 2000; Kurata et al. 2000; Schohl and Fagotto 2003; Cho et al. 2013; Plouhinec et al. 2013), but these visualizations have only been qualitative. The development of quantitative readouts for target gene expression and BMP signaling could reveal how the BMP target genes read and respond to the BMP signaling gradient.

In zebrafish and *Xenopus*, the AP and DV axes are patterned simultaneously in a coordinated fashion (Tuazon and Mullins 2015). Wnt, FGF, and Nodal signaling pattern the AP axis at the same time that BMP signaling patterns the DV axis (reviewed by Tuazon and Mullins 2015). The AP and DV axes are both patterned progressively starting with anterior tissues and progressing to posterior tissues during blastula and gastrula stages (Gamse and Sive 2001; Kudoh et al. 2002; Maves and Kimmel 2005; Tucker et al. 2008; Hashiguchi and Mullins 2013; Tuazon and Mullins 2015). Posterior tissues are not patterned by BMP signaling during blastula and early gastrula periods, although these cells are responding to the BMP signal (Tucker et al. 2008; Hashiguchi and Mullins 2013). Conversely, the loss of BMP signaling in midgastrula stages does not affect anterior tissues because they were patterned before the loss of BMP signaling (Tucker et al. 2008). Wnt and FGF signals cooperate with BMP signaling by phosphorylating the Smad5 linker region to modulate its stability and activity (Eivers et al. 2009; Hashiguchi and Mullins 2013; Tuazon and Mullins 2015). Nodal also induces mesendoderm (covered in the previous section) (Thisse et al. 2000; Brennan et al. 2001), and the relative ratio of BMP to Nodal in ectopic expression studies can inform the DV and AP fate of cells in the gastrula (Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015) (discussed further in the next section). In these experiments, clonal injections of *bmp* and *nodal* RNA were sufficient to induce an intact secondary axis or even pattern the AP and DV cell fates of an animal cell explant from the zebrafish blastula (Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015). However, whether AP and DV patterning are similarly coordinated in mice remains to be seen (Beddington and Robertson 1999; Kishigami and Mishina 2005; Takaoka and Hamada 2012).

The role of BMP signaling in axis patterning in mice differs somewhat from its role in *Xenopus* and zebrafish axis patterning. Although primarily responsible for patterning the DV axis in *Xenopus* and zebrafish, BMP signaling in the mouse also drives AVE migration (Mishina et al. 1995; Winnier et al. 1995; Coucouvanis and Martin 1999; Soares et al. 2008; Yamamoto et al. 2009; Miura et al. 2010), specifies the primordial germ cells (Lawson et al. 1999; Chang and Matzuk 2001; Ying and Zhao 2001;

Ying et al. 2001), and acts in allantois development (Chang et al. 1999; Fujiwara et al. 2001). BMP ligands are expressed predominantly in ventrally (proximally) located extraembryonic tissue in the mouse (Winnier et al. 1995; Zhang and Bradley 1996; Lawson et al. 1999; Ying et al. 2000; Ying and Zhao 2001; Danesh et al. 2009; Madabhushi and Lacy 2011), in contrast to zebrafish (Hammerschmidt et al. 1996a; Nguyen et al. 1998; Schmid et al. 2000; Furthauer et al. 2004; Ramel and Hill 2013) and *Xenopus* (Hemmati-Brivanlou and Thomsen 1995; Knochel et al. 2001; Marom et al. 2005) where BMPs are expressed embryonically. Interestingly, chimeras expressing *Bmp4* only in extraembryonic tissues form a primitive streak, suggesting that extraembryonic BMP-4 is sufficient for primitive streak formation (Fujiwara et al. 2001). Similarly, although the loss of *Bmpr1a* in the entire embryo disrupts AVE migration and gastrulation, causing early lethality (Mishina et al. 1995), the loss of *Bmpr1a* in the embryonic tissues alone does not (Tallquist and Soriano 2000; Mishina et al. 2002). The embryos survive long enough to show an enlargement of the forebrain, prechordal plate, early definitive endoderm, and AVE (Davis et al. 2004). Disruption of BMP signaling disrupts dorsal and AVE formation and migration (Fig. 2) (Mishina et al. 1995; Coucouvanis and Martin 1999; Soares et al. 2008; Yamamoto et al. 2009; Miura et al. 2010), without which the primitive streak and the dorsal organizer (known as the Node in mouse) fail to form (Mishina et al. 1995; Beddington and Robertson 1999; Takaoka and Hamada 2012). In contrast, in *Xenopus* and zebrafish the dorsal organizer still forms and embryos gastrulate even in the absence of BMP signaling (Kishimoto et al. 1997; Dick et al. 2000; Schmid et al. 2000; Reversade and De Robertis 2005; Reversade et al. 2005).

Tail and Trunk Patterning by Relative Levels of Nodal and BMP Signaling

Experiments in the zebrafish show that adjacent sources of Nodal and BMP signaling are sufficient to recapitulate the function of the intact organizer and duplicate the entire embryonic axis. The dorsal organizer has long been known to be capable of generating a secondary axis when transplanted into an ectopic location of another embryo, and this structure is defined by *nodal* expression, discussed above in the section on the role of TGF-β family signaling in mesendoderm specification and patterning and the section on regulation of TGF-β family gene expression during axis patterning (Spemann and Mangold 1924; Toyama et al. 1995; Lustig et al. 1996; Agathon et al. 2003; Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015). Moreover, ectopic expression of *nodal* recapitulates many of the functions of the dorsal organizer itself, and is capable of generating a secondary body axis (Spemann and Mangold 1924; Toyama et al. 1995; Lustig et al. 1996; Agathon et al. 2003; Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015). In the zebrafish, the introduction of Nodal to the animal pole, which is competent to respond but normally is beyond the range of Nodal signaling, induces gastrulation but ultimately only specifies dorsal and axial tissues. One study found that animal pole expression of Nodal can induce a complete secondary axis, only in the presence of an adjacent patch of BMP-expressing cells (Xu et al. 2014; Thisse and Thisse 2015).

The combined action of adjacent Nodal and BMP signaling centers can pattern all tissues of the zebrafish embryo (Xu et al. 2014). In particular, the ratio between Nodal and BMP appears to be important for the specification of different tissues along the zebrafish AP axis (Fauny et al. 2009). Tissues along most of the germ ring (marginal zone) of the developing zebrafish embryo express and are exposed to both Nodal and BMP signaling, and can induce axial structures when transplanted to the animal pole. Regions of the germ ring expressing high Nodal and low BMP induce anterior tissues, such as the head, whereas regions expressing high BMP and low Nodal contribute to the tail, and regions with intermediate levels of both signals contribute to the trunk (Fauny et al. 2009). This suggests that the entire germ ring has some degree of organizer function, with different portions of the germ ring orga-

nizing different segments of the zebrafish body axis (Fauny et al. 2009). The ability of these signals to recapitulate the whole body axis likely arises from their ability to both pattern tissue, and direct important morphogenetic movements, with Nodal specifying mesendoderm and inducing gastrulation, and BMP patterning the DV axis and inducing both convergence and extension, and the migration of cells toward the tail bud (Agathon et al. 2003; Szeto and Kimelman 2006; Fauny et al. 2009; Xu et al. 2014; Thisse and Thisse 2015).

BMP, Nodal, and a second signal, possibly FGF, direct mesodermal cells to their relative AP somitic position along the zebrafish embryonic axis (Szeto and Kimelman 2006). Specifically, maternal-zygotic (MZ, embryos lacking both maternally and zygotically supplied gene function) *oep* (the zebrafish EGF-CFC coreceptor gene) null mutant cells, which are blind to Nodal signaling, when transplanted at 5 hours postfertilization (hpf, late blastula) into a wild-type embryo can only contribute to somitic tissue of the tail—that is, somites posterior to somite number 15 (Szeto and Kimelman 2006), similar to that observed in MZ*oep*- or Nodal-deficient zebrafish embryos. Intriguingly, these cells could contribute to caudal trunk somites, absent in MZ*oep* loss-of-function embryos, if the wild-type recipient was one hour younger at 4 hpf. This implies the existence of a second, Nodal-dependent trunk signal, which does not require the EGF-CFC coreceptor to signal, possibly FGF-8. When these MZ*oep* cells overexpress BMP and are transplanted into 4 hpf wild-type recipients, there is a shift in the mesodermal progenitors now toward the tail somites. The specification of tail somitic mesoderm in the absence of Nodal signaling is discussed in the section on TGF-β family pathway components involved in mesendoderm specification. These studies are broadly consistent with the above studies of adjacent ectopic Nodal and BMP centers. In both studies, distinct AP axial regional tissues are induced by high Nodal and low BMP signaling, which generates anterior tissues (rostral trunk somites), and low or no Nodal and high BMP signaling generating tail tissues (somites).

THE REGULATION OF TGF-β FAMILY GENE EXPRESSION DURING AXIAL PATTERNING

The regulation of BMP and Nodal expression during vertebrate DV patterning is intertwined, so they are discussed together in the following sections. Nodal is a key dorsal determining factor induced by β-catenin in the dorsal organizer. In turn, Nodal signaling acts to induce the expression of numerous BMP antagonists. The regulation of BMP and Nodal expression in zebrafish and *Xenopus* are discussed separately to highlight the different approaches used in studies of early development, as each system uses distinct strengths. Early patterning studies in zebrafish have relied heavily on genetic analysis, whereas studies in *Xenopus* use explants to analyze gene expression and map target gene promoter regions. Together, these analyses have generated very similar epistatic maps of gene interactions during axis patterning (Figs. 3 and 4), although there are some minor differences.

Regulation of *bmp* Gene Expression during Zebrafish Axial Patterning

BMP signaling acts in patterning ventrolateral cell fates and must be inhibited dorsally for neural tissue formation; however, BMP genes are initially expressed ubiquitously in the zebrafish embryo before being cleared from the dorsal region. The maternal expression of the BMP ligand gene *gdf6a* (also known as *radar* in zebrafish) is implicated in inducing zygotic *bmp2* and *bmp7* expression (Sidi et al. 2003; Wilm and Solnica-Krezel 2003), along with several other maternal factors (reviewed in Langdon and Mullins 2011). The *bmp2* and *bmp7* genes are expressed ubiquitously after MBT at 3 hpf until ~4 hpf, when their transcripts are cleared from the dorsal region by a complex network of regulatory factors (Schier and Talbot 2005).

The genes encoding the two major dorsalizing factors, *bozozok* and *nodal*, are induced dorsally by β-catenin through a maternal Wnt signaling pathway in zebrafish (Fig. 3A) (Kelley et al. 2000; Shimizu et al. 2000; Ryu et al. 2001; Dougan 2003; Nojima et al. 2004; Gore et al. 2005; Maegawa et al. 2006). Maternally depos-

ited β-catenin accumulates in the nuclei of dorsal marginal cells as early as the 512-cell stage (2.75 hpf) (Schneider et al. 1996; Dougan 2003). Sox3 opposes the action of β-catenin, inhibiting *nodal*, *bozozok*, *chordin*, and *noggin* expression (Shih et al. 2010; Kuo et al. 2013). Mutants that disrupt nuclear accumulation of β-catenin, such as *ichabod* (β-*catenin2*) and *syntabulin*, fail to induce *bozozok* and *nodal* gene expression, along with other dorsal factors, which in turn leads to the ubiquitous expression of *bmp2, bmp7*, and genes for other ventral factors (Kelley et al. 2000; Nojima et al. 2004), ventralizing the embryonic axis.

The expression of *bmp2* and *bmp7* dorsally is directly repressed by two partially redundant factors: the transcription factor Bozozok (Koos and Ho 1999; Shimizu et al. 2000; Solnica-Krezel and Driever 2001; Leung 2003) and the Nodal ligands Ndr1 (Squint) and Ndr2 (Cyclops) (Fig. 3A) (Shimizu et al. 2000; Furthauer et al. 2004; Maegawa et al. 2006). Both *bozozok* and *nodal* are induced by dorsal nuclear-localized, maternal β-catenin (Kelley et al. 2000; Shimizu et al. 2000; Ryu et al. 2001; Dougan 2003; Nojima et al. 2004; Gore et al. 2005; Maegawa et al. 2006) and are inhibited by the ubiquitin ligase Lnx2b (Ro and Dawid 2009, 2010). Bozozok inhibits BMP signaling dorsally by directly repressing *bmp2b* transcription (Koos and Ho 1999; Solnica-Krezel and Driever 2001; Leung 2003) and repressing the expression of the ventralizing factors, *vox*, *vent*, and *ved* (Kawahara et al. 2000a,b; Melby et al. 2000; Imai et al. 2001; Shimizu et al. 2002). Nodal signaling represses *bmp* expression dorsally by inducing *fgf8* expression (Dougan 2003; Furthauer et al. 2004; Maegawa et al. 2006) (further discussed below). Interestingly, Bozozok does not induce *nodal* expression (Shimizu et al. 2000), consistent with it acting as a transcriptional repressor (Leung 2003). The Nodal ligands promote the expression of *admp*, a gene encoding a BMP ligand that acts as a ventralizing factor in dorsal regions (Dickmeis et al. 2001; Lele et al. 2001; Willot et al. 2002). ADMP limits the size of the dorsal organizer by repressing *goosecoid* (Lele et al. 2001). The initial expression of *admp* is induced dorsally by Nodal and Wnt signaling in the early blastula. *admp* expression is then maintained by Nodal signaling during gastrulation while being repressed by BMP signaling (Lele et al. 2001; Willot et al. 2002).

FGF signaling represses *bmp2b*, *bmp4*, and *bmp7* expression (Furthauer et al. 2004) and directly activates *goosecoid* (Fig. 3A,B) (Joore et al. 1996; Feldman et al. 1998; Gritsman et al. 2000; Maegawa et al. 2006; Kuo et al. 2013) and *chordin* (Maegawa et al. 2006; Varga et al. 2007; Kuo et al. 2013), encoding dorsalizing factors. FGFs indirectly repress *vox*, *vent*, and *ved*, encoding related ventralizing factors, by activating Goosecoid expression, which then inhibits *vox*, *vent*, and *ved* expression (Yamanaka et al. 1998; Kawahara et al. 2000a,b; Imai et al. 2001). FGF signaling induces the expression of *sprouty2*, *sprouty4*, and *sef*, which encode extracellular FGF inhibitors, forming a negative feedback loop that limits FGF expression and signaling (Furthauer et al. 2001, 2002, 2004; Tsang et al. 2002; Kovalenko et al. 2006).

The ubiquitously expressed maternal transcription factor Pou5f3 (also called Pou5f1, Oct4) promotes BMP expression and inhibits dorsalizing factors (Fig. 3A,B) (Takeda et al. 1994; Lippok et al. 2014). Maternal-zygotic *pou5f3* mutants (*spiel ohne grenzen* or MZ*spg*) lack endoderm, show gastrulation defects, and are dorsalized (Schier et al. 1996; Reim et al. 2004; Reim and Brand 2006; Belting et al. 2011). Pou5f3 induces *bmp2b* expression by inhibiting *fgf8a* expression (Reim and Brand 2006; Belting et al. 2011), potentially by directly inducing the expression of *sprouty4*, which encodes an FGF inhibitor (Onichtchouk et al. 2010). Pou5f3 directly induces *vox* and *vent* expression (Belting et al. 2011), which either directly or indirectly inhibit *goosecoid*, *chordin*, and *noggin* expression ventrolaterally (Reim et al. 2004; Reim and Brand 2006; Belting et al. 2011; Khan et al. 2012). However, reports conflict as to whether Pou5f3 enhances *nodal* and *bozozok* expression (Reim et al. 2004; Reim and Brand 2006; Belting et al. 2011; Khan et al. 2012). Ints6 similarly promotes ventral and inhibits dorsal genes, but its mechanism of action has yet to be determined (Kapp et al. 2013).

During organizer patterning, Vox, Vent, and Ved act redundantly to repress *goosecoid* and *chordin* gene expression ventrolaterally (Fig. 3B) (Kawahara et al. 2000a,b; Imai et al. 2001; Shimizu et al. 2002; Ramel and Lekven 2004; Ramel et al. 2005; Varga et al. 2007). Consistent with this function, loss of *vox*, *vent*, and *ved*, which act partially redundantly to each other, severely dorsalizes the embryo (Imai et al. 2001; Shimizu et al. 2002; Gilardelli et al. 2004). All evidence points to Vent and Vox inhibiting *goosecoid* expression directly as they bind the *goosecoid* promoter (Kawahara et al. 2000a,b). Interestingly, Vent and Vox can also physically interact with Goosecoid protein when coexpressed in cell culture, suggesting potential direct antagonism between these proteins (Kawahara et al. 2000b). *ved* is directly activated by the maternally expressed transcription factor Runx2 (Flores et al. 2008). Vent and Vox repress *ved* expression, possibly forming a negative feedback loop to limit its expression (Gilardelli et al. 2004). Bozozok and Goosecoid inhibit *vox*, *vent*, and *ved* expression dorsally (Yamanaka et al. 1998; Kawahara et al. 2000a,b; Melby et al. 2000; Imai et al. 2001; Shimizu et al. 2002). Interestingly, Bozozok promotes *goosecoid* expression by directly repressing *vox*, *vent*, and *ved*, rather than by activating *goosecoid* directly (Imai et al. 2001; Shimizu et al. 2002). The mutual transcriptional antagonism between *goosecoid* and *vox, vent*, and *ved* produces largely complementary expression domains between these genes (Fig. 3).

The transcriptional network regulating *bmp* expression changes at or shortly after the onset of gastrulation, initiating a feedback loop that regulates *bmp* transcription (Fig. 3B). At this stage, *bmp2, bmp7*, and *bmp4* expression becomes dependent on BMP signaling, evident by the marked loss of *bmp* expression in mutants for *bmp2, bmp7*, or *smad5* (Hammerschmidt et al. 1996a; Nguyen et al. 1998; Schmid et al. 2000). BMP signaling feeds back on its own expression by positively regulating *vox*, *vent*, and *ved* expression ventrally (Kawahara et al. 2000a,b; Melby et al. 2000; Imai et al. 2001; Shimizu et al. 2002; Gilardelli et al. 2004; Ramel and Lekven 2004). Vox, Vent, and Ved in turn repress the expression of dorsalizing factors *goosecoid*, *chordin*, and *noggin* (Kawahara et al. 2000a,b; Imai et al. 2001; Ramel and Lekven 2004; Ramel et al. 2005). In *Xenopus*, Vox, Vent, and Ved can directly induce *bmp4* expression (Schuler-Metz et al. 2000), but it is not yet known if they directly induce *bmp2, bmp4*, or *bmp7* expression in zebrafish as well.

Wnt signaling undergoes a dramatic shift from being a dorsalizing factor in the midblastula to being a ventralizing factor during gastrulation (Fig. 3A,B). Although maternal Wnt signaling activates dorsal genes to repress BMP expression (Nojima et al. 2004; Lu et al. 2011), zygotic Wnt8 directly activates the ventrally expressed genes *vox*, *vent*, and *ved* to maintain *bmp2, bmp4*, and *bmp7* gene expression (Erter et al. 2001; Lekven et al. 2001; Ramel and Lekven 2004). Consistent with this, *wnt8* is expressed ventrally in the late blastula and during gastrulation (Erter et al. 2001; Lekven et al. 2001; Ramel and Lekven 2004). Expression of the Wnt inhibitor Dkk1 is induced by Wnt signaling in the dorsal organizer (Hashimoto et al. 2000), repressing Wnt signaling and possibly contributing to the shift in Wnt function and expression. *dkk1* is initially induced dorsally by maternal dorsal Wnt signaling (Hashimoto et al. 2000; Shinya et al. 2000; Nojima et al. 2004; Chamorro et al. 2005). Its expression then expands around the germ ring before becoming restricted to the dorsal organizer (Hashimoto et al. 2000; Shinya et al. 2000; Nojima et al. 2004). In addition to *dkk1*, the genes encoding Wnt inhibitors, *sfrp1* and *frzb*, are also expressed dorsally, possibly inhibiting maternal Wnt signaling dorsally along with Dkk1 (Peng and Westerfield 2006; Pezeron et al. 2006; Seiliez et al. 2006; Tendeng and Houart 2006; Lu et al. 2011). The transcription factor Kaiso zinc finger-containing protein (Kzp) is necessary to initiate zygotic *wnt8* expression (Yao et al. 2010), but the signaling pathways regulating *kzp* expression are not known.

ADMP, a member of the BMP subfamily, is expressed dorsally and helps to limit the size of the dorsal organizer (Lele et al. 2001; Willot et al. 2002). It can also promote BMP signaling and ventral cell fates when overexpressed (Lele

et al. 2001; Willot et al. 2002). However, unlike other BMPs that are expressed ventrally, *admp* is expressed dorsally and regulated by Nodal signaling and Bozozok (Fig. 3A,B) (Lele et al. 2001). ADMP represses the dorsal organizer gene *goosecoid*, forming a negative feedback loop potentially limiting dorsal organizer gene expression (Lele et al. 2001). In addition to being expressed ventrally, *bmp2b* is also expressed in the dorsal organizer after gastrulation begins (Nguyen et al. 1998; Schmid et al. 2000; Furthauer et al. 2004; Xue et al. 2014). Additionally, dorsally expressed Bmp2b is reported to inhibit *goosecoid* and *chordin* expression, thereby limiting organizer size in a similar way as ADMP (Xue et al. 2014). However, although *chordin* expression is known to be inhibited by BMP signaling (Miller-Bertoglio et al. 1997) and could therefore be repressed by dorsal Bmp2b expression, *goosecoid* expression has not been reported to respond to BMP signaling, as *goosecoid* expression does not change in many BMP pathway component mutants (Hammerschmidt et al. 1996a; Miller-Bertoglio et al. 1997; Nguyen et al. 1998; Little and Mullins 2009), or in fully dorsalized embryos overexpressing *chordin* (Tucker et al. 2008). More work is needed to resolve how dorsally expressed Bmp2b and ADMP limit the expression not only of genes responsive to BMP signaling such as *chordin*, but also dorsal organizer genes like *goosecoid* that are not usually affected by changes in BMP signaling.

In addition to the dorsal organizer, a few negative feedback loops exist to limit BMP signaling. BMP signaling induces the expression of the gene encoding the pseudoreceptor BAMBI, which can inhibit BMP signaling by acting as an inhibitory receptor to form a negative feedback loop (Tsang et al. 2000). BMP signaling induces Sizzled expression, which indirectly inhibits BMP signaling by blocking the metalloproteases that cleave Chordin, which is covered in detail in a later section (see Fig. 6) (Yabe 2003b; Lee et al. 2006). These feedback loops help the system self-regulate after gastrulation begins, balancing the positive feedback loop formed by BMP signaling maintaining *bmp2*, *bmp4*, and *bmp7* expression with negative feedback loops.

Regulation of *nodal* Expression during Zebrafish Axial Patterning

The expression of *nodal* is induced dorsally by maternal Wnt–β-catenin signaling (Kelley et al. 2000; Shimizu et al. 2000; Ryu et al. 2001; Dougan 2003; Nojima et al. 2004; Gore et al. 2005; Maegawa et al. 2006), but *nodal* (*ndr1*, *sqt*) transcript itself is also maternally deposited in the egg and dorsally enriched (Fig. 3A) (Gore et al. 2005). After initial induction by β-catenin, Nodal signaling is regulated by both intracellular and extracellular factors. The two Nodal ligands, Ndr1 and Ndr2, are important to induce mesendoderm and the dorsal organizer (Feldman et al. 1998; Rebagliati et al. 1998a; Sampath et al. 1998). As discussed in the mesendoderm patterning section, Nodal signaling regulates itself by inducing *ndr1* and *ndr2* transcription (Meno et al. 1999; Chen and Schier 2002; Feldman et al. 2002; Dougan 2003). Ndr1 can signal at a distance whereas Ndr2 does not (Chen and Schier 2001) because of the higher diffusivity of Ndr1 ($D = 3.2\ \mu m^2/sec$) as compared with Ndr2 ($D = 0.7\ \mu m^2/sec$) (Muller et al. 2012). Nodal also promotes the expression of *lefty1* and *lefty2*, which encode secreted extracellular Nodal antagonists that bind and inhibit both Nodal ligand and the EGF-CFC coreceptor Oep (Meno et al. 1999; Chen and Schier 2002; Feldman et al. 2002; Dougan 2003; Chen and Shen 2004; Cheng et al. 2004). The loss of *oep* phenocopies the loss of both *ndr1* and *ndr2* (Gritsman et al. 1999, 2000; Schier 2009). Lefty2 has a higher diffusion rate than Nodal ligands ($D = 18.9\ \mu m^2/sec$) (Muller et al. 2012), allowing it to inhibit Nodal signaling in cells more distant from the site of Nodal production. The induction of *lefty* by Nodal was previously thought to form a Turing reaction–diffusion mechanism, whereby Nodal would negatively regulate its own expression during axial patterning (Schier 2009; Hamada 2012). However, later work has shown that the translation of *lefty* messenger RNA (mRNA) is delayed by miR-430 (van Boxtel et al. 2015) and that the amount of Nodal present is insufficient to predict target gene response (Dubrulle et al. 2015), suggesting

that a Turing reaction–diffusion mechanism is not needed for *nodal* to regulate its own expression during axial patterning.

Nodal signaling is inhibited by the miR-430/427/302 family of micro-RNAs (miRNAs) (Fig. 3A,B) (Choi et al. 2007; Bassett et al. 2014; van Boxtel et al. 2015). *lefty1* and *2* as well as *ndr1* mRNA translation is inhibited by miR-430 (Choi et al. 2007; Bassett et al. 2014; van Boxtel et al. 2015). Nodal up-regulates *dapper1* and *dapper*2, which inhibit Nodal signaling by targeting type I Nodal receptors for degradation (Zhang et al. 2004; Waxman 2005; Su et al. 2007). Dapper1 and Dapper2 have also been reported to interact with Wnt, but reports conflict as to whether it acts as an antagonist by promoting Disheveled degradation (Cheyette et al. 2002; Zhang et al. 2006; Su et al. 2007) or acts as an agonist (Waxman et al. 2004).

Regulation of *bmp* Expression during *Xenopus* Axial Patterning

In *Xenopus bmp2*, *bmp4*, *bmp7*, and the *admp* expression all contribute to axial patterning (Reversade and De Robertis 2005; Reversade et al. 2005). In *Xenopus*, *bmp2* and *bmp7* are ubiquitously expressed maternally, but transcription rapidly diminishes during the blastula stage. Conversely, *bmp4* is not expressed maternally and peaks in the early gastrula (Knochel et al. 2001; Marom et al. 2005). *gdf6a*, which encodes a homolog of zebrafish Radar, is expressed starting at the midblastula transition in *Xenopus*, but does not appear to induce initial *bmp2*, *bmp4*, and *bmp7* expression, as reported for *radar* in zebrafish (Chang and Hemmati-Brivanlou 1999; Hanel and Hensey 2006).

Like in zebrafish, maternal dorsally activated Wnt–β-catenin signaling acts to push back the BMP expression domain out of dorsal regions (Fig. 4A) (Hemmati-Brivanlou and Thomsen 1995; Schneider et al. 1996; Kurata et al. 2000; Schohl and Fagotto 2003; Tao et al. 2005). The vegetally localized *wnt11* and *wnt5a* transcripts in the egg translocate asymmetrically to the prospective dorsal region by a microtubule-dependent process known as cortical rotation (Tao et al. 2005; Cha et al. 2008; Houston 2012). Also required are Wnt receptors, inhibitors, and signal transducers (Houston 2012). The maternal loss of Wnt receptors and intracellular pathway components disrupt organizer formation (Houston 2012). Maternal exostosin glycosyltransferase 1 (Ext1) facilitates dorsal Wnt signaling by glycosylating heparin sulfate proteoglycans (HSPGs) (Katada et al. 2002; Tao et al. 2005). Consequently, the loss of maternal *ext1* down-regulates dorsal genes and ventralizes the embryo (Tao et al. 2005). However, only one HSPG, Glypican4, has been studied thus far, and *glyp4* loss of function has no DV phenotype, suggesting that Ext1 must act either through multiple redundant HSPGs or on a different HSPG altogether (Galli et al. 2003). Wnt signaling promotes the expression of the dorsal transcription factor encoding gene *Xiro1*, which inhibits BMP signaling dorsally (Glavic et al. 2001; Gómez-Skarmeta et al. 2001). The Wnt inhibitor Dkk1 inhibits Wnt signaling outside the dorsal organizer region (Houston et al. 2002; Cha et al. 2008). Another factor that inhibits the expression of Wnt target genes outside of the organizer is the transcription factor Tcf3 (Houston et al. 2002). Wnt signaling is also down-regulated by Dapper1, which is expressed in the dorsal organizer region (Cheyette et al. 2002). After the midblastula transition, β-catenin induces the expression of a network of factors to form the dorsal organizer, a signaling center that opposes BMP signaling and *bmp4* expression (Fig. 4) (Kuroda et al. 2004; Sudou et al. 2012), as in the zebrafish. β-catenin directly induces *nodal* (*xnr*) expression (as in zebrafish), as well as the gene encoding transcription factor *siamois* (not found in zebrafish) (Carnac et al. 1996; Agius et al. 2000; Wessely et al. 2001; Houston et al. 2002; Tao et al. 2005; Vonica and Gumbiner 2007). Together, β-catenin, Nodal, and Siamois induce expression of the BMP antagonist genes *chordin* (Wessely et al. 2001; Nakamura et al. 2016), *noggin* (Wessely et al. 2001; Nakamura et al. 2016) and *follistatin* (Khokha et al. 2005). These BMP antagonists repress BMP signaling and subsequently limit the expression domains of *bmp2, bmp4*, and *bmp7*, as BMP

signaling feeds back to promote their expression (Khokha et al. 2005), similar to zebrafish.

Like in zebrafish, the ventralizing factor ADMP acts in a negative feedback loop that limits the size of the dorsal organizer. *admp* expression is activated by low BMP signaling in the dorsal organizer (Moos et al. 1995; Dosch and Niehrs 2000; Reversade and De Robertis 2005). ADMP represses the expression of dorsal organizer genes such as *chordin*, *noggin*, *follistatin*, and *goosecoid* (Moos et al. 1995; Dosch and Niehrs 2000; Reversade and De Robertis 2005). It has been proposed that ADMP represses dorsal genes by binding to Chordin and furthermore that Chordin–ADMP shuttles to the ventral region of the embryo where Chordin is cleaved and ADMP is released, thereby increasing BMP signaling ventrally (Ben-Zvi et al. 2008, 2014). This shuttling mechanism may explain how the *Xenopus* DV axis can scale effectively—that is, maintain proportional patterning in adapting to changes in embryo size (Ben-Zvi et al. 2008, 2014).

Dkk1 and Dkk3 play distinct roles in *Xenopus* axial patterning. Dkk1 inhibits Wnt signaling whereas Dkk3 is required for Nodal signaling (Fig. 4). Like in zebrafish, Nodal signaling promotes the expression of *dkk1* to form a negative feedback loop, which may limit the expression of dorsal organizer genes (Agius et al. 2000; Cha et al. 2008). In the gastrula, Dkk1 begins to inhibit Wnt signaling dorsally, coinciding with the transition of Wnt signaling from a dorsalizing factor in the midblastula to a ventralizing factor in the gastrula (Hoppler and Moon 1998; Marom et al. 1999; Chamorro et al. 2005; Cha et al. 2008). Dkk3 is required for Nodal signaling and for the dorsal mesoderm to form (Pinho and Niehrs 2007), but where it is expressed in the blastula and gastrula has not been reported.

BMP signaling enhances its own expression during both the late blastula and gastrula stages in *Xenopus* through multiple positive feedback loops. Like in zebrafish, BMP signaling forms a positive feedback loop with Xvent1 and Xvent2 (Fig. 4B). BMP signaling promotes the expression of the homeobox genes *Xvent1* and *Xvent2* (Onichtchouk et al. 1996; Schuler-Metz et al. 2000; Karaulanov et al. 2004; Hikasa et al. 2010). Xvent1 and Xvent2 in turn positively regulate *bmp4* and *bmp7* expression during gastrulation, while repressing *goosecoid* expression (Eimon and Harland 1999; Laurent and Cho 1999; Trindade et al. 1999; Schuler-Metz et al. 2000). In the gastrula, Goosecoid induces the expression of *chordin*, which encodes a BMP antagonist. Thus, by repressing *goosecoid*, Xvent1 and Xvent2 promote BMP ligand expression and signaling (Sander et al. 2007). In the gastrula, BMP ligand expression indirectly represses dorsally expressed genes such as *goosecoid* and Nodal ligands by inducing the expression of the muscle segment homeobox genes *xmsx1* and *xmsx2* (Maeda et al. 1997; Onitsuka et al. 2000; Takeda et al. 2000). Xmsx-1 and Xmsx-2 are ventrally expressed transcriptional repressors (Suzuki et al. 1997; Yamamoto et al. 2000, 2001). They directly repress the expression of Nodal ligands, which activate *goosecoid* expression (Suzuki et al. 1997; Yamamoto et al. 2000, 2001). Xmsx-1 and Xmsx-2 also repress *goosecoid* expression by inhibiting the expression of the homeobox gene *xlim1*. xLim1 is a direct transcriptional activator of *goosecoid* (Mochizuki et al. 2000; Takeda et al. 2000; Sudou et al. 2012). *xlim1* and *goosecoid* are both repressed by Pou3f4, a POU-domain transcription factor expressed across the entire marginal zone (Fig. 4B) (Witta and Sato 1997). Pou3f4 promotes *bmp2* expression, likely by indirectly repressing *chordin* and *noggin* expression through repression of *goosecoid* expression (Witta and Sato 1997). In the gastrula, BMP signaling induces the expression of BMP ligands by regulating Wnt signaling (Fig. 4B). BMP signaling promotes *wnt8* expression (Schmidt et al. 1995; Hoppler and Moon 1998; Marom et al. 1999), which in turn directly activates *Xvent2* expression (Hikasa et al. 2010; Nakamura et al. 2016), and Xvent2 then induces the expression of BMP ligands.

Like in zebrafish, BMP signaling also feeds back negatively onto itself. Potentially to balance the positive feedback loops described in the paragraph above, BMP signaling forms a negative feedback loop by inhibiting the expression of *exotosin1*. Exotosin1 decreases the gly-

cosylation of HSPGs and ultimately leads to diminished Wnt signaling (Fig. 4B) (Shieh et al. 2014). Decreases in *exotosin* expression indirectly reduce BMP ligand expression and BMP signaling by reducing Wnt8 signaling (Hikasa et al. 2010; Nakamura et al. 2016). BMP signaling also forms a negative feedback loop with itself by up-regulating *bambi*, which encodes the BMP pseudo-receptor that inhibits BMP signaling (Fig. 4B) (Karaulanov et al. 2004; Sekiya et al. 2004; Paulsen et al. 2011). BMP signaling forms a similar negative feedback loop with the inhibitory Smads, Smad6, and Smad7 (Murakami et al. 2003; de Almeida et al. 2008; Paulsen et al. 2011). BMP signaling induces the production of the extracellular BMP antagonist Sizzled (see Fig. 6), as in zebrafish (discussed further in a later section) (Collavin 2003; Lee et al. 2006). These negative feedback loops may act to balance the levels of BMP ligand expression during gastrulation to ensure proper patterning.

Regulation of *nodal* Expression during *Xenopus* Axial Patterning

After the midblastula transition, β-catenin activates the expression of a network of factors that promote *nodal* expression and Nodal signaling (Kuroda et al. 2004; Sudou et al. 2012). β-catenin directly induces Nodal ligand expression and expression of the gene encoding the transcription factor Siamois (Fig. 4A) (Carnac et al. 1996; Agius et al. 2000; Wessely et al. 2001; Houston et al. 2002; Tao et al. 2005; Vonica and Gumbiner 2007). β-catenin also induces *fgf20* (Chamorro et al. 2005), *xnr3* (Wessely et al. 2001; Kuroda et al. 2004; Tao et al. 2005), and *dkk1* (Chamorro et al. 2005). β-catenin induces *xnr3* expression in the dorsal organizer, but, in the DV Nieuwkoop center, β-catenin functions synergistically with the vegetally expressed transcription factor VegT to induce *xnr1, 2, 4, 5*, and *6* expression and inhibit *xnr3* expression (Kofron et al. 1999; Agius et al. 2000; Takahashi et al. 2000; Houston et al. 2002; Rex et al. 2002; Hashimoto-Partyka et al. 2003). Also, essential to the early activation of Nodal signaling is the TGF-β family ligand GDF-1 (Vg1), which is maternally supplied and vegetally localized (Birsoy et al. 2006; Levine et al. 2009).

The transcription factor VegT directly activates numerous dorsal genes that regulate *nodal* expression (Fig. 4). Maternal *vegT* transcript is localized to the vegetal pole of the egg and is expressed before the midblastula transition (Agius et al. 2000; Sudou et al. 2012). VegT directly binds and activates the *siamois* promoter, synergizing with Wnt signaling to activate *siamois* expression dorso-vegetally (Li et al. 2015). VegT both promotes and inhibits Nodal signaling by directly activating *nodal* expression (Agius et al. 2000; Takahashi et al. 2000; Houston et al. 2002; Rex et al. 2002), while simultaneously promoting the dorsal expression of the extracellular Nodal antagonist gene *cerberus* (Agius et al. 2000; Reid et al. 2012; Sudou et al. 2012). The dorsal expression of *cerberus* may limit Nodal signaling activity. Cerberus also inhibits BMP signaling dorsally, playing an important role in head formation and DV patterning (Bouwmeester et al. 1996; Silva et al. 2003). VegT, Nodal, Twin, and Siamois all synergistically and directly activate *goosecoid* (Bae et al. 2011; Reid et al. 2012; Sudou et al. 2012). VegT, Twin, and Siamois do so by binding the promoter region of *goosecoid* (Reid et al. 2012; Sudou et al. 2012).

Like in zebrafish, Nodal signaling is essential to both establish mesendodermal tissue and activate dorsal organizer genes necessary for repressing BMP ligand expression (see section on the role of TGF-β family signaling in mesendoderm specification and patterning). However, in contrast to zebrafish DV patterning, in which Nodal signaling activates dorsal genes indirectly by inducing FGF expression (Maegawa et al. 2006; Kuo et al. 2013), there is no evidence that Nodal ligands activate the expression of FGF genes in *Xenopus*. Although FGF signaling does contribute to *Xenopus* DV patterning, reports vary as to how FGF ligand gene expression is regulated and what genes are regulated by FGF signaling (discussed further below) (Schohl and Fagotto 2003; Fletcher and Harland 2008; Branney et al. 2009; Lee et al. 2011c). Instead, the dorso-vegetally expressed Nodal ligands directly

induce dorsal genes. Nodal signaling induces *goosecoid* expression by binding of Smad2, the Nodal signal transducer, to its promoter region (Agius et al. 2000; Wessely et al. 2001; Hashimoto-Partyka et al. 2003; Chiu et al. 2014). Nodal ligands promote their own expression (Onuma et al. 2002), whereas they induce negative feedback by inducing the expression of the extracellular Nodal inhibitor *cerberus* (Fig. 4) (Reid et al. 2012), possibly functioning to balance one another. Nodal ligands inhibit BMP ligand expression dorsally by promoting the expression of the BMP antagonist genes *chordin* and *noggin* (Agius et al. 2000; Reid et al. 2012). Nodal signaling is inhibited by Ectodermin (also known as TRIM33 or TIF1γ), a RING-type ubiquitin ligase for Smad4 that limits nuclear Smad accumulation (Dupont et al. 2005).

The epistatic relationship between FGF and TGF-β family ligands and regulators along the DV axis is unclear. FGF signals around the equator of the blastula and is reported to be high dorsally and lower ventrally (Schohl and Fagotto 2003; Branney et al. 2009). Paradoxically, the FGF ligands are expressed ubiquitously along the DV axis, so it is unclear how a gradient of FGF signaling emerges (Lea et al. 2009). The expression of *fgf20* is positively regulated around the margin by zygotic Wnt signaling (Chamorro et al. 2005). When FGF signaling is disrupted, trunk and tail tissues fail to form (Amaya et al. 1991, 1993). Microarray and whole-mount in situ analyses suggest that FGF signaling activates, either directly or indirectly, *sprouty1* and *sprouty2* (Nutt et al. 2001; Branney et al. 2009), *fgf4* (Fletcher and Harland 2008), *wnt8* (Branney et al. 2009), *dkk1* (Branney et al. 2009), *goosecoid* (Fletcher and Harland 2008; Branney et al. 2009), and *noggin* (Fletcher and Harland 2008; Branney et al. 2009) expression, while repressing the expression of *siamois* (Branney et al. 2009) and *xnr4* (Branney et al. 2009). Reports conflict on whether FGF signaling affects *chordin* expression (Fletcher and Harland 2008; Branney et al. 2009). More experiments are needed to determine the epistatic relationship of FGF to the TGF-β ligands and effectors.

Like in zebrafish, the EGF-CFC Nodal coreceptor Cripto is necessary for Nodal signaling in the *Xenopus* blastula. In *Xenopus*, three genes encode the EGF-CFC proteins FRL-1/Xcr1, Xcr2, and Xcr3 (Kinoshita et al. 1995; Dorey and Hill 2006; Onuma et al. 2006). Maternal FRL-1 protein binds to Wnt5 and Wnt11 extracellularly to promote Wnt signaling (Tao et al. 2005). The dorsally expressed Nodal inhibitor Lefty inhibits Nodal signaling by binding directly to Nodal and to FRL-1 (Lee et al. 2001; Tanegashima et al. 2004). The Nodal signaling pathway directly activates *lefty* expression during LR patterning, which negatively feeds back on Nodal signaling (Cheng et al. 2000), and may also do so in the blastula to self-limit the organizer. *lefty* expression is directly inhibited by the transcription factor E2a (Wills and Baker 2015).

Regulation of TGF-β Family Gene Expression during Mouse Axial Patterning

The regulation of *Bmp* and *Nodal* expression during axial patterning in mice has proven more difficult to study because of the early embryonic lethality of embryos lacking BMP signaling and the functional redundancy of some of the pathway components in the system (Zhao 2003; Kishigami and Mishina 2005). During mouse gastrulation, both BMP and NODAL facilitate communication between the epiblast and extraembryonic tissues, guiding the formation of the primitive steak (Robertson 2014). BMP and NODAL signaling are involved in a positive feedback regulatory loop during early gastrulation, which is required for the specification of the primitive streak (Ben-Haim et al. 2006). This interdependence, however, complicates the discernment of a precise gene regulatory network during gastrulation, and the requirement of BMP signaling during mouse gastrulation largely obscures any later role in development (Winnier et al. 1995; Lawson et al. 1999). Equivalent or similar phenotypes for *Nodal*, *Bmp4, Acvr1, Bmpr1a, Acvr1b, Bmpr2*, and *Acvr2a/Acvr2b* double mutants make it difficult to distinguish between the receptors that are used by BMP versus by NODAL signaling (Mishina et al. 1995; Gu et al. 1998;

Zhao 2003; Kishigami and Mishina 2005; Carron and Shi 2016). This is particularly the case for the type II receptors, which have not yet been inactivated at the gene level in a nonmammalian vertebrate. These limitations have hindered the assembly of an epistatic map of genes regulating *Bmp* and *Nodal* expression. Nonetheless, some aspects of NODAL and BMP regulation during primitive streak specification have been discerned, and are discussed within the context of their associated tissues in the section on the initiation of NODAL signaling during gastrulation and mesendoderm specification.

THE ROLE OF TGF-β FAMILY SIGNALING IN LEFT–RIGHT PATTERNING

Following mesendoderm specification and DV patterning during gastrulation, TGF-β family signaling plays a crucial role in defining LR asymmetry. In vertebrates, this asymmetry is established by a specialized structure called the LR organizer (reviewed in Blum et al. 2014a). In amniotes, this is known as the node (Levin et al. 1995; Collignon et al. 1996), in amphibians it is the gastrocoele roofplate (Schweickert et al. 2007), and in zebrafish it is called Kupffer's vesicle (Essner et al. 2002; Hashimoto et al. 2004; Blum et al. 2014a). TGF-β family ligands function in both the establishment of this structure, the interpretation of symmetry breaking, and the transmission of this information to adjacent tissues. Disruption of Nodal (Levin et al. 1995; Collignon et al. 1996; Lohr et al. 1997; Rebagliati et al. 1998b; Long et al. 2003), GDF-1 (known as Gdf3 in zebrafish and Vg1 in zebrafish and *Xenopus*) (Rankin et al. 2000; Tanaka et al. 2007; Peterson et al. 2013), or BMP (Branford et al. 2000; Piedra and Ros 2002; Schlange et al. 2002; Kishigami and Mishina 2005; Chocron et al. 2007; Mine et al. 2008; Komatsu et al. 2011; Lenhart et al. 2011; Smith et al. 2011; Katsu et al. 2012, 2013) signaling disrupts LR patterning. The gene circuitry that controls the vertebrate LR asymmetry establishment involves Nodal auto-induction (Osada et al. 2000; Saijoh et al. 2000; Long et al. 2003; Ohi and Wright 2007; Oki et al. 2007; Wang and Yost 2008), inhibition by the antagonists Cerberus and Lefty (Meno et al. 1996, 1998, 1999; Yokouchi et al. 1999; Branford et al. 2000; Cheng et al. 2000; Hashimoto et al. 2004; Marques et al. 2004; Vonica and Brivanlou 2007; Wang and Yost 2008; Schweickert et al. 2010; Katsu et al. 2012), and the activation of the transcription factor Pitx2 (Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998; St Armand et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Essner et al. 2000). The binding of these antagonists to Nodal and BMP is covered in the section on extracellular antagonists of TGF-β family signaling (Table 1). Much of this circuitry is conserved in invertebrates, with similar pathways regulating asymmetric budding in Hydra (Watanabe et al. 2014b), and shell chirality in snails (Grande and Patel 2009; Blum et al. 2014a).

Mechanisms of Symmetry Breakage

Left-sided, asymmetric expression of *nodal* has been observed in all vertebrates tested (Levin et al. 1995; Collignon et al. 1996; Lohr et al. 1997; Rebagliati et al. 1998b; Long et al. 2003), and analogous asymmetric *nodal* expression has been observed in related processes in invertebrates as divergent as echinoderms (Duboc et al. 2005), gastropods (Grande and Patel 2009), and cnidarians (Watanabe et al. 2014b). The upstream mechanisms that create this asymmetric expression, however, are not as conserved and remain an area of considerable debate within the field. The predominant model is that asymmetry in vertebrates is established within the ciliated LR organizer (reviewed in Matsui and Bessho 2012; Blum et al. 2014b; Shiratori and Hamada 2014; Yoshiba and Hamada 2014). The alignment of the cells of the LR organizer along the AP axis, coupled with the inherent chirality of polarized cilia induces a leftward flow that is responsible for breaking symmetry (Fig. 5A, inset) (Hashimoto and Hamada 2010; Blum et al. 2014b). This model is supported by a vast array of evidence showing that the presence of the LR organizer (Dufort et al. 1998; Davidson et al. 1999; Essner et al. 2005; Stubbs et al. 2008; Blum et al. 2009; Matsui et al. 2011), along with the proper formation of cilia (Chen et al. 1998; Marszalek et al. 1999;

Table 1. BMP extracellular modulators acting as an agonist or antagonist, general region of expression, mechanism of action, organisms that possess a homolog, and known binding interactions and affinities

Name	BMP agonist or antagonist	Dorsal or ventral expression	Mechanism	Organism	Binding affinities	References
Tolloid	Agonist	Ventral	Cleaves Chd	Human, zebrafish, frog, mouse	[2]BMP-7 = Binds [2]BMP-4 = 20 nM [1]Chd ≈ 10 nM [1]Szl = 19 nM [3]Collagen IV = Binds	[1]Lee et al. 2006; [2]Lee et al. 2009; [3]Winstanley et al. 2015
Bmp1	Agonist	Ventral	Cleaves Chd	Human, zebrafish, frog, mouse	[2]BMP-4 = 16 nM [1]Szl = 14 nM [3]Ont1 = Binds	[1]Bijakowsky et al. 2012; [2]Lee et al. 2009; [3]Inomata et al. 2008
BMP Ligands	Agonist	Ventral	Signal through type I and type II receptors to activate Smad1, 5, 8	Human, zebrafish, frog, mouse	See inhibitors [1]HSPG = Binds	[1]Jasuja et al. 2004
Twisted Gastrulation	Agonist and antagonist	Ventral	Enhances Chd cleavage by Tld/BMP-1; enhances the binding of Chd to BMP; binds and inhibits BMP; has another yet-unknown Chd-independent BMP agonist function	Human, zebrafish, frog, mouse	[1]BMP-7 = 28 nM [2,3]BMP-4 = 2.5 nM [1]BMP-2 = 50 nM [1]GDF-5 = 53 nM [5]HSPGs = NB [2,3,6]Chd = 3 nM [4]Bmper = Binds [6]Tld = NB	[1]Zhang et al. 2007; [2]Oelgeschlager et al. 2000, 2003; [3]Chang et al. 2001; [4]Ambrosio et al. 2008; [5]Jasuja et al. 2004; [6]Troilo et al. 2016
Bmper	Agonist and antagonist	Ventral	Binds and Inhibits Chd; binds and inhibits BMP	Human, zebrafish, frog, mouse	[7]BMP-9 = Binds [2,5]BMP-7 = 3.5–7 nM [1,2]BMP-4 = 2.0 nM [2,4,5]BMP-2 = 1.2–22 nM [1,2,4]Chd = 1.4–175 nM [2,3]HSPGs = Binds [5]GDF-5 = 34 nM [6]LRP1 = Binds	[1]Ambrosio et al. 2008; [2]Rentzsch et al. 2006; [3]Serpe et al. 2008; [4]Zhang et al. 2010; [5]Zhang et al. 2007; [6]Pi et al. 2012; [7]Yao et al. 2012
Sizzled	Antagonist	Ventral	Inhibits Chd cleavage by Tld/BMP-1	Zebrafish, frog	[1]BMP-1 = 14 nM [2]Tld = 19 nM	[1]Bijakowsky et al. 2012; [2]Lee et al. 2006

Continued

Table 1. *Continued*

Name	BMP agonist or antagonist	Dorsal or ventral expression	Mechanism	Organism	Binding affinities	References
ADMP	Agonist	Dorsal	Signals through type I and type II receptors to activate Smadl, 5, 8	Zebrafish, frog	See inhibitors	
Ontl	Agonist and antagonist	Dorsal	Enhances Chd cleavage by Tld/BMP-1; binds to BMP ligand and Chd	*Xenopus*	[1]Chd = Binds [1]BMP-4 = Binds	[1]Inomata et al. 2008
Gremlin-1	Antagonist	Dorsal	Binds and Inhibits BMP	Human, zebrafish, frog, mouse	[3]BMP-7 = 88 nM [3]BMP-6 = 76 nM [3,6]BMP-4 = 28 nM [3]BMP-2 = 32 nM [1]BMP-2 = 5.6 nM [2]HSPGs = 20 nM [5]GDF-5 = Binds [4]VEGFR1 = 47 nM	[1]Kisonaite et al. 2016; [2]Chiodelli et al. 2011; [3]Church et al. 2015; [4]Mitola et al. 2010; [5]Dionne et al. 2001; [6]Sun et al. 2006a
Chordin	Antagonist	Dorsal	Binds and Inhibits BMP	Human, zebrafish, frog, mouse	[27]BMP-7 = 8–46 nM [1212]BMP-4 = 0.3–5.8 nM [37]BMP-2 = 12–37 nM [4]Tld ≈ 10 nM [3,5,6]Bmper = 1.4–175 nM [8]HSPG = Binds [9]Tsg1 = 3 nM [10]Ont1 = Binds [11]Integrins = Binds	[1]Piccolo et al. 1996; [2]Troilo et al. 2014; [3]Rentzsch et al. 2006; [4]Lee et al. 2006; [5]Zhang et al. 2010; [6]Ambrosio et al. 2008; [7]Zhang et al. 2007; [8]Jasuja et al. 2004; [9]Troilo et al. 2016; [10]Inomata et al. 2008; [11]Larrain et al. 2000;

Continued

Table 1. *Continued*

Name	BMP agonist or antagonist	Dorsal or ventral expression	Mechanism	Organism	Binding affinities	References
Noggin	Antagonist	Dorsal	Binds and inhibits BMP, Nodal, and Activin	Human, zebrafish, frog, mouse	[5]ADMP = Binds [1]BMP-10 = NB [1]BMP-9 = NB [2,7]BMP-7 = Binds [2,5,7]BMP-4 = 0.02 nM [2]BMP-2 = Binds [3,4,6]HSPGs = Binds [1,8] GDF-5 = 2 nM [5]Activin = Binds [5]Xnr2 = Binds [5]Xnr4 = Binds [5]Wnt8 = Binds	[1]Seemann et al. 2009; [2]Zimmerman et al. 1996; [3]Nesterenko et al. 2015; [4]Viviano et al. 2004; [5]Bayramov et al. 2011; [6]Paine-Saunders et al. 2002; [7]Groppe et al. 1998, 2003; [8]Degenkolbe et al. 2013
Follistatin	Antagonist	Dorsal	Binds 2:1 (follistatin:BMP) and inhibits BMP[1]	Human, zebrafish, frog, mouse	[5]BMP-15 ≈ 30 nM [6,7,8]BMP-7 = 35 nM [7,8]BMP-6 = 5.4 nM [6,7,8,11]BMP-4 = 2.9–23 nM [10]BMP2 = 136 nM [2,9]HSPG = 5–56 nM [10]GDF-11 = 4.95 nM [3,4,7,8,12]Activin = 0.02–0.28 nM [6]TGF-p = NB	[1]Thompson et al. 2005; [2]Nakamura et al. 1991; [3]Chang et al. 2003; [4]Schneyer et al. 1994; [5]Otsuka et al. 2001; [6]Iemura et al. 1998; [7]Glister et al. 2004, 2015; [8]Sidis et al. 2006; [9]Zhang et al. 2012; [10]Takehara-Kasamatsu et al. 2007; [11]Geng et al 2011;
Crescent	Antagonist	Dorsal	Inhibits Chd cleavage by Tld/BMP-1[1]; can also inhibit Wnt5a, 8, 11[2]	frog	[1]BMP-1 = 11 nM [2]Wnt11 = Binds [2]Wnt8 = Binds [2]Wnt5a = Binds	[1]Ploper et al. 2011; [2]Shibata et al. 2005

Continued

Table 1. *Continued*

Name	Nodal agonist or antagonist	Animal or vegetal expression	Mechanism	Organism	Binding affinities	References
Nodal	Agonist	Dorsal and vegetal	Signals through type I and type II receptors to activate Smad2 and Smad3	Human, zebrafish, frog, mouse	See inhibitors	
Lefty	Antagonist	Dorsal and vegetal	Inhibits nodal signaling by binding nodal ligand and the nodal receptor Cripto (EGF-CFC)	Human, zebrafish, frog, mouse	[1]Sqnt = 29 nM [1]Cyc = 50 nM [4]Nodal = Binds [3,4]EGF-CFC = Binds	[1]Wang et al. 2016; [2]Cheng et al. 2000; [3]Tanegashima et al. 2004; [4]Chen and Shen 2004
Cerberus	Antagonist	Dorsal and animal	Binds and inhibits nodal, Wnt, and BMP. Human Cerberus does not bind BMP or Wnt.	Frog, mouse, human	[5]Activin A = NB [1]Activin B = 0.096 nM [1]BMP-7 = 19.1 nM [1]BMP-6 = 17.3 nM [2,4,6]BMP-4 = 0.6–23.4 nM [1,3]BMP-2 = 3000 nM (hCER) [2,4]BMP-2 = 3.4 nM [2,5]Xnr1 = Binds [5]Xnr3 = NB [1,3]Nodal = 0.3 nM [2]Xwnt8 = Binds [3]GDF-8,9 = NB	[1]Aykul and Martinez-Hackert 2016; [2]Piccolo et al. 1999; [3]Aykul et al. 2015; [4]Chi et al. 2011; [5]Agius et al. 2000; [6]Belo et al. 2000
Cerberus-like proteins (Coco DAND5, Charon)	Antagonist	Animal	Coco and Dand5 enhance TGF-p signaling, but inhibit Nodal, BMP, and Activin	Zebrafish, frog, mouse, human	[1,2,4,5]BMP-4 = 1.1 nM [2,3]Xnr1 = Binds [4,5]Nodal = 1.1 nM [2]Wnt8 = Binds [2]Activin = Binds [3]Derriere = Binds	[1]Deglincerti et al. 2015; [2]Bell 2003; [3]Vonica and Brivanlou 2007; [4]Katsu et al. 2012; [5]Marques et al. 2004
Tomoregulin-1	Antagonist	Animal	Inhibits nodal and BMP signaling	Frog, mouse, human	[2]Cripto = Binds [1]Activin = NB [2]Xnr1 = NB	[1]Chang et al. 2003; [2]Harms and Chang 2003

In second column: green, agonists; yellow, agonist and antagonist; peach, antagonist. In third column: green, ventrally expressed; peach, dorsally expressed; pink, dorsally and vegetally expressed; blue, animally expressed.

NB, no binding; Binding, has been shown to bind, but the affinity has not been measured; hCER, human Cerberus; Chd, Chordin; Szl, Sizzled; Tld, Tolloid.

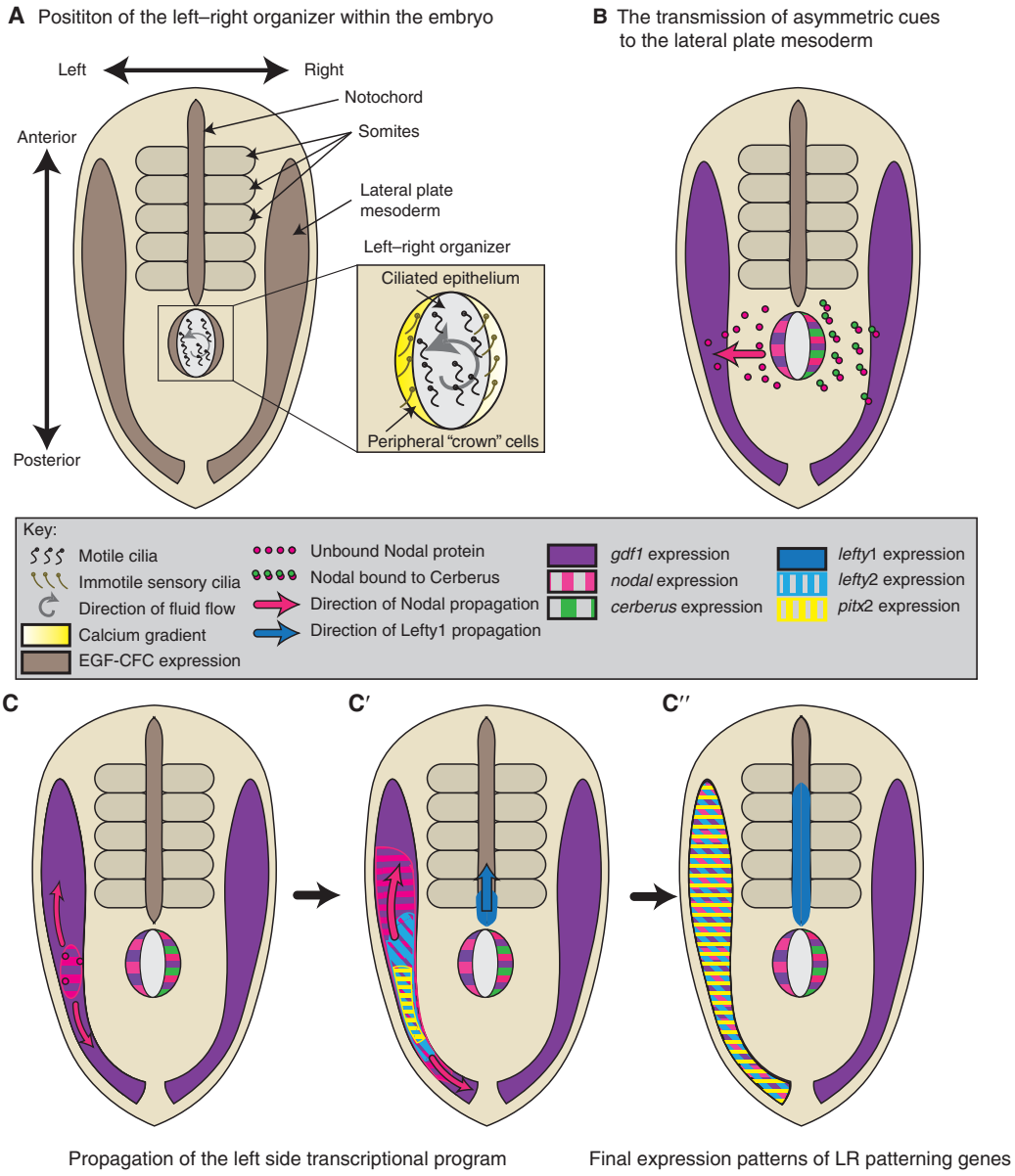

Figure 5. Expression of Nodal pathway components during symmetry breaking and patterning of the left–right (LR) axis in a generic vertebrate embryo. (*A*) Physical structures involved in LR patterning or proximal to the LR organizer. Structures receptive to Nodal signaling expressing EGF-CFC receptors are shown in brown. (*inset*) Close-up of the LR organizer showing the relative positions of motile and nonmotile mechanosensory cilia, the direction of fluid flow, and the gradient of calcium. (*B*) Expression of *nodal* and *cerberus* around the LR organizer. This panel also shows *gdf1/vg1* expression, and the diffusion of Nodal ligand and Cerberus antagonists to the lateral plate mesoderm. (*C*) Expansion of *nodal*, *lefty*, and *pitx2* expression domains within the lateral plate mesoderm and notochord. The *left* panel shows the initial patch of nodal expression within the left lateral plate mesoderm, proximal to the LR organizer. (*C′*) Advance of *nodal* expression toward the anterior and posterior, followed by *lefty2* and *pitx2* expression within the lateral plate mesoderm, and *lefty1* expression within the notochord. (*C″*) Ultimate expression domain of *nodal*, *lefty2*, and *pitx2* encompassing the entire left lateral plate mesoderm.

Supp et al. 1999; Brody et al. 2000; Taulman et al. 2001; Watanabe et al. 2003; Rana et al. 2004; Bisgrove et al. 2005; Essner et al. 2005; Kramer-Zucker et al. 2005; Oishi et al. 2006; Stubbs et al. 2008; Neugebauer et al. 2009; Tian et al. 2009; Lopes et al. 2010; Hatayama et al. 2011; Matsui and Bessho 2012; Manning et al. 2013; Walentek et al. 2013; Wang et al. 2013) and the fluid flow generated within it (Okada et al. 1999; Nonaka et al. 2002; Essner et al. 2005; Kramer-Zucker et al. 2005; Schweickert et al. 2007; Blum et al. 2009; Nonaka 2009; Vick et al. 2009) are all necessary for establishing proper laterality in the vast majority of vertebrates tested. Additionally, several human ciliopathies, such as primary ciliary dyskinesia, result in LR patterning defects supporting a conserved role in humans as well (reviewed in Sharma et al. 2008).

Several argue that this model does not explain the initial establishment of LR asymmetry (Aw et al. 2010; Vandenberg and Levin 2013). Evidence against the fluid flow model includes the fact that several genes appear to be localized along the LR axis well before development of the LR organizer (Fukumoto et al. 2005; Adams et al. 2006; Aw et al. 2010; Vandenberg et al. 2013). There is also a notable absence of motile, mesodermal cilia in the LR organizer of some vertebrate species, such as the chick and the pig (Gros et al. 2009; Blum et al. 2014a). Additionally, there are simpler, possibly more widely conserved mechanisms of symmetry breaking in several groups of invertebrates (Vandenberg and Levin 2009). An ion-flux model has also been proposed in which serotonin and an ATP-sensitive K^+ pump are asymmetrically distributed within the first few cell divisions establishing a voltage gradient across the embryo, which is required for asymmetry (Aw et al. 2010). Advocates of the fluid flow model argue that all the components required for the ion flux model, are actually required for the correct formation and activity of cilia, or downstream aspects of fluid flow (Blum et al. 2014b). Advocates of the ion flux model argue conversely that elements required for cilia formation are also required for the ion flux (Vandenberg and Levin 2013). Fluid flow advocates agree that fluid flow is unique to vertebrates, or perhaps deuterostomes (Blum et al. 2014a; Takemoto et al. 2016; Tisler et al. 2016) and not an ancestral bilaterian mechanism. Nevertheless, they argue that the vertebrate exceptions to this model have lost their cilia, and present novel, rather than ancestral mechanisms of symmetry breaking (Blum et al. 2014a). Moreover, they posit that these exceptions can be explained without invoking the ion flux model. As the advocates of the ion flux model still recognize a role for the LR organizer in the "amplification" of asymmetry (Vandenberg and Levin 2013), and the mechanism of the ion flux model falls outside the realm of TGF-β signaling, we will focus on the fluid flow model for the remainder of this review. A number of reviews give a more detailed account of the arguments on both sides (see Vandenberg and Levin 2009, 2013; Burdine and Caspary 2013; Blum et al. 2014a,b).

TGF-β Family Proteins in the Specification of the LR Organizer

The LR organizer manifests in a variety of forms across the vertebrate phylum, but the basic structure involves an epithelium of monociliated cells (Fig. 5A, inset) (Blum et al. 2014a). In *Xenopus*, it is a flat triangular epithelium called the gastrocoel roof plate (Stubbs et al. 2008), in mouse it is an indented pit at the anterior tip of the primitive streak, called the node (Davidson et al. 1999), and in zebrafish it is a fully enclosed vesicle called Kupffer's vesicle (Essner et al. 2005). TGF-β family ligands have important roles in the development of the LR organizer, the breakage of symmetry within it, and in the transmission of symmetry breakage from it to the lateral plate mesoderm.

During gastrulation, Nodal signaling specifies the cells that will become the LR organizer. The zebrafish LR organizer Kupffer's vesicle forms posterior to the notochord from cells known as the dorsal forerunner cells (Cooper and D'Amico 1996; Essner et al. 2005). Dorsal forerunner cells are evident in the early gastrula, as a handful of cells that lie ahead of the shield and migrate vegetally during epiboly (Cooper and D'Amico 1996). Dorsal forerunner cells

are restricted to a dorsal marginal region, but unlike other dorsal marginal cells, they do not involute (Cooper and D'Amico 1996; Melby et al. 1996; D'Amico and Cooper 1997). These cells are specified by the Nodal signaling pathway (Essner et al. 2005; Hagos and Dougan 2007); mutants of the Nodal coreceptor Oep (Essner et al. 2005) or of the Smad2 transcription cofactor FoxH1 (Pogoda et al. 2000) fail to form dorsal forerunner cells or Kupffer's vesicle, and fail to form LR asymmetry. The zebrafish dorsal forerunner cells also require *sox32* and *brachyury*, which are known Nodal signaling transcriptional targets, for their specification (Essner et al. 2005; Gourronc et al. 2007).

Similar to the zebrafish, the mouse LR organizer, the node, also depends on NODAL signaling during gastrulation, with *Foxh1* and *Brachyury* expression also important for proper node formation (Rashbass et al. 1991; Yamamoto et al. 2001). The *Xenopus* LR organizer, the gastrocoel roof plate, forms from superficial mesodermal cells. Like the dorsal forerunner cells, these cells reside posterior to the developing notochord and rely on *brachyury* to form the gastrocoel roof plate, suggesting a dependence on Nodal signaling during gastrulation (Blum et al. 2014b). Moreover, the critical Nodal acting in LR patterning in *Xenopus*, Xnr1 (Toyoizumi et al. 2005) is expressed in the gastrocoel roof plate, but its expression requires an earlier Xnr5 Nodal signal (Tadjuidje et al. 2016). In mice, zebrafish, and *Xenopus*, the LR organizer is in the same relative position of the embryo, lying posterior to the notochord and developing somites (Fig. 5A) (Sulik et al. 1994; Cooper and D'Amico 1996; Schweickert et al. 2007; Shook et al. 2004; Basu and Brueckner 2008). After symmetry breaking, the LR organizer eventually contributes to the notochord and somites (Davidson and Tam 2000; Norris et al. 2002; Shook et al. 2002).

BMP signaling is also necessary for the formation of the LR organizer in the mouse, with reduced levels of BMP signaling disrupting LR organizer formation (Shiratori and Hamada 2014). The type I BMP receptor ACVR1 (ALK-2) was found to be essential in the epiblast for proper node cilia formation in the mouse (Komatsu et al. 2011) and epiblast-specific loss of *Bmp4* in the mouse embryo causes a lack of *Nodal* expression within the node, which is necessary for LR patterning (Fujiwara et al. 2002). In the mouse, expression of the NODAL coreceptor CRYPTIC also depends on BMP-4 expression (Fujiwara et al. 2002). Although BMP-4 is required for node formation and *Nodal* expression within the node, BMP overexpression also disrupts LR patterning. However, this is likely because of its roles in other aspects of LR patterning, such as the formation of the midline barrier, and the repression of *Nodal* expression within the lateral plate mesoderm, which will be discussed later. In the zebrafish, BMP antagonists are also required within Kupffer's vesicle for LR patterning, as shown by the results of depleting Chordin in the dorsal forerunner cells, which randomizes laterality (Aamar and Dawid 2010).

TGF-β Family during Symmetry Breaking

During symmetry breaking, *nodal* (Long et al. 2003; Zhou et al. 1993; Blum et al. 2007) and *gdf1* (*vg1*) (Hyatt and Yost 1998; Rankin et al. 2000; Peterson et al. 2013) are expressed bilaterally around the periphery of the LR organizer (Fig. 5B). Both ligands are essential for proper LR patterning in all vertebrates tested (Levin et al. 1995; Collignon et al. 1996; Hyatt et al. 1996; Lohr et al. 1997; Rebagliati et al. 1998b; Rankin et al. 2000; Peterson et al. 2013). It has been postulated that Nodal acts as a heterodimer with GDF-1 during LR patterning. Interestingly, Nodal–GDF-1 heterodimers are more potent in cell culture (Fuerer et al. 2014), and coexpression with *gdf1* has been found to be essential for Nodal to function during LR patterning in both mice and *Xenopus* (Tanaka et al. 2007). These investigators also observed Nodal–GDF-1 heterodimers in co-immunoprecipitation experiments. Others, however, did not observe heterodimers and suggest that GDF-1 and Nodal must mutually enhance each other's activity through other mechanisms (Peterson et al. 2013). Expression of *Nodal* specifically within the LR organizer is essential for proper LR patterning in mouse (Brennan et al. 2001; Saijoh et al. 2003),

yet this remains untested in other vertebrates. In *Xenopus*, which has 5 *nodal* genes, and zebrafish, which has 3, the task of breaking and transducing LR asymmetry has been subfunctionalized to one gene, *xnr1* in *Xenopus* (Sampath et al. 1997; Toyoizumi et al. 2005), and *southpaw* in zebrafish (Long et al. 2003). These *nodal* genes are only required for LR patterning; however, other *nodal* genes important for mesendodermal patterning also affect LR patterning when disrupted, likely because they function in LR organizer formation (discussed earlier and in the mesendoderm-patterning section).

In the mouse, *Nodal* expression in the LR organizer is activated by NOTCH signaling through an intronic, node-specific enhancer, the NDE (Norris and Robertson 1999; Krebs et al. 2003). Although this enhancer sequence does not appear to be conserved in nonmammalian vertebrates (Alten et al. 2012), the importance of Notch signaling is conserved, as disruption of Notch signaling disrupts zebrafish LR asymmetry (Raya et al. 2003; Takeuchi et al. 2007). The expression of *Gdf1* also depends on NOTCH signaling, with NOTCH inhibitors suppressing *Gdf1* expression within the node (Kitajima et al. 2013). Additionally, SHH signaling is required in the mouse for *Gdf1* expression in the LR organizer (Zhang et al. 2001).

The interaction of Nodal with its antagonist Cerberus is central to the mechanism of symmetry breaking within the LR organizer. The cells within the LR organizer display planar cell polarity (PCP) that is aligned with the AP axis (Nonaka et al. 2005; Okada et al. 2005; Schweickert et al. 2007; Antic et al. 2010; Borovina et al. 2010; Hashimoto and Hamada 2010; May-Simera et al. 2010). These cells are also monociliated with the cilium tilted toward the posterior. Cilia in the center of the LR organizer are motile and produce a leftward fluid flow (Fig. 5A, inset) (Sulik et al. 1994; Nonaka et al. 1998; Essner et al. 2005; Kramer-Zucker et al. 2005; Schweickert et al. 2007). At the periphery of the LR organizer, cells with nonmotile, mechanosensory cilia sense this fluid flow and experience intracellular calcium oscillations (Fig. 5A, inset) (McGrath et al. 2003; Sarmah et al. 2005; Kreiling et al. 2008; Francescatto et al. 2010; Yoshiba et al. 2012; Yuan et al. 2015). Cells on the left side of the organizer respond to this flow by degrading the RNA of the Nodal antagonist, *cerberus* (Vick et al. 2009; Nakamura et al. 2012; Tingler et al. 2014). These are the same cells that express Nodal and its likely heterodimeric partner GDF-1, and like Nodal and GDF-1, the peripheral expression of Cerberus depends on Notch signaling (Gourronc et al. 2007; Kitajima et al. 2013). Initially all three genes are expressed symmetrically around the LR organizer, but the left-sided degradation of *cerberus* transcript confines Cerberus to the right side (Fig. 5B) (Hashimoto et al. 2004; Lopes et al. 2010; Schweickert et al. 2010; Kawasumi et al. 2011; Nakamura et al. 2012; Inacio et al. 2013). The right-sided expression of Cerberus suppresses Nodal signaling on the right side of the LR organizer (Hashimoto et al. 2004; Marques et al. 2004; Schweickert et al. 2010), resulting in a left-sided bias in downstream Nodal signaling, evidenced in the mouse by higher Smad2 activation on the left side (Kawasumi et al. 2011; Nakamura et al. 2012). There is also evidence that the initial degradation of *cerberus* mRNA is amplified on the left side of the node by the up-regulation of *wnt3* (Nakamura et al. 2012), and that *wnt3* and *cerberus* exist in a bistable double negative feedback loop.

TGF-β Family Signaling Transfers LR Asymmetry to the Lateral Plate Mesoderm

Asymmetric Nodal activity in the LR organizer is shortly followed by the expression of *nodal* in the left lateral plate mesoderm (Fig. 5C) (Collignon et al. 1996; Lustig et al. 1996; Rebagliati et al. 1998a,b; Long et al. 2003; Blum et al. 2007). The node and lateral plate mesoderm are separated by the presomitic mesoderm, which does not appear to respond to LR asymmetries (Blum et al. 2014b). Several lines of research suggest that Nodal itself or Nodal–GDF-1 heterodimers diffuse between the LR organizer and the lateral plate mesoderm (Fig. 5B) (reviewed in Shiratori and Hamada 2014). Nodal can initiate its own expression within the lateral plate mesoderm (Saijoh et al. 2000; Norris et al. 2002; Yamamoto et al. 2004;

Toyoizumi et al. 2005; Ohi and Wright 2007; Wang and Yost 2008), through its autoregulatory "asymmetric" enhancer, the ASE (Norris and Robertson 1999; Osada et al. 2000; Brennan et al. 2002; Saijoh et al. 2005; Fan et al. 2007). In the mouse, there is an additional asymmetric enhancer, the "left sided enhancer" or LSE. These enhancers are necessary and sufficient for the expression of *Nodal* within the lateral plate mesoderm (Adachi et al. 1999; Saijoh et al. 2005).

Much evidence suggests that Nodal diffuses directly from the node to the lateral plate mesoderm. In mice, the NODAL coreceptor CRYPTIC is required for the initiation of *Nodal* expression within the lateral plate mesoderm (Gaio et al. 1999), but it is not required to initiate *Nodal* expression in the LR organizer itself. The other mouse EGF-CFC receptor, CRIPTO, is required for node formation, but is not expressed during symmetry breakage (Ding et al. 1998; Oki et al. 2007). Moreover, the transgenic rescue of *Cryptic* specifically in the lateral plate mesoderm is enough to rescue axial patterning, eliminating the possibility that node cells are translating asymmetric Nodal activity within the LR organizer into some other signaling relay mechanism (Oki et al. 2007). Similarly, the three *Xenopus* EGF-CFC receptors (Xcr) are subfunctionalized, with Xcr3 important for early gastrulation and Xcr2 essential for axial patterning (Onuma et al. 2006). Similar to mouse *Cryptic* mutants, *xcr2* silencing disrupts *nodal* expression within the lateral plate mesoderm, but not the LR organizer, and left side–specific rescue of *xcr2* can restore left-sided *nodal* expression (Onuma et al. 2006). Cryptic and FoxH1 are not found in tissues adjacent to the LR organizer such as the endoderm or the presomitic mesoderm (Agathon et al. 2001; Onuma et al. 2006; Oki et al. 2007), making these unlikely candidates to relay the asymmetric Nodal signal. There is evidence that the mechanosensory calcium flux experienced on the left side of the node is transferred to adjacent endodermal tissues (Saund et al. 2012; Saijoh et al. 2014), and the integrity of this endodermal tissue is necessary for initiation of *nodal* expression within the lateral plate mesoderm. However, this flux only affects cells immediately adjacent to the LR organizer. This suggests that the endoderm supports the transfer of Nodal to the lateral plate mesoderm, rather than transferring asymmetric cues via a calcium flux (reviewed in Shiratori and Hamada 2014).

In the direct diffusion model, Nodal itself or Nodal–GDF-1 heterodimers are secreted by peripheral cells of the LR organizer. On the right side of the LR organizer, these ligands are quickly bound and inactivated by Cerberus (Fig. 5B) (Matsui and Bessho 2012; Shiratori and Hamada 2014), and active Nodal ligand diffuses toward the left lateral plate mesoderm, whereas inactive Cerberus-bound Nodal diffuses toward the right lateral plate mesoderm. In mice and *Xenopus*, Nodal diffuses in this process (Oki et al. 2007) by interacting with sulfated glycosaminoglycans in the extracellular matrix. These are expressed by both endodermal and mesodermal tissues between the LR organizer and the lateral plate mesoderm, and are required for the expression of *nodal* within the lateral plate mesoderm (Marjoram and Wright 2011). On reaching the lateral plate mesoderm, Nodal initiates its own expression and propagates throughout the left lateral plate mesoderm via a positive feedback mechanism (Fig. 5C).

TGF-β Family Signaling in the Lateral Plate Mesoderm

Within the lateral plate mesoderm, TGF-β family ligands and antagonists play key roles in both the amplification of LR asymmetry, and the confinement of left-specific cues to the left side of the organism. When Nodal reaches the left lateral plate mesoderm, it initiates its own expression (Saijoh et al. 2000; Norris et al. 2002; Yamamoto et al. 2004; Ohi and Wright 2007; Wang and Yost 2008), as well as the expression of *pitx2*, encoding a transcription factor (Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Yan et al. 1999; Long et al. 2003), and *lefty2* within the lateral plate mesoderm (Heymer et al. 1997; Meno et al. 1997, 1998, 1999; Adachi et al. 1999;

Bisgrove et al. 1999; Gaio et al. 1999; Cheng et al. 2000; Liang et al. 2000; Long et al. 2003). It also activates *lefty1* expression along the midline of the embryo (Fig. 5C′) (Meno et al. 1998, 1999; Long et al. 2003; Ohi and Wright 2007; Wang and Yost 2008). The expression of *nodal* and *pitx2* begins toward the posterior end of the lateral plate mesoderm, somewhat proximal to the LR organizer (Lohr et al. 1997; Long et al. 2003; Ohi and Wright 2007; Wang and Yost 2008), and propagates anteriorly, eventually covering the whole left side of the lateral plate mesoderm (Fig. 5C–C′′). The expression of *lefty* along the midline also progresses anteriorly following the expression of *nodal* (Meno et al. 1999; Ohi and Wright 2007; Wang and Yost 2008) and plays a critical role in confining *nodal* expression to the left side of the lateral plate mesoderm (Meno et al. 1998; Wang and Yost 2008; Smith et al. 2011). The left-sided expression of *pitx2* persists beyond that of *nodal* expression (Campione et al. 1999; Schweickert et al. 2000, 2001; Shiratori et al. 2001, 2006; Long et al. 2003; Ohi and Wright 2007), and is then translated into the proper orientation of asymmetric organs, such as the brain, heart, and gut (Piedra et al. 1998; Ryan et al. 1998; Campione et al. 1999; Branford et al. 2000).

Before the arrival of asymmetric cues from the LR organizer, both the right and left lateral plate mesoderm are primed to receive and propagate Nodal signals. The ectopic introduction of Nodal to either side of the lateral plate mesoderm can activate the entire left-sided transcriptional (Saijoh et al. 2000) cascade (i.e., *nodal*, *lefty*, and *pitx2* [Heymer et al. 1997; Levin et al. 1997; Campione et al. 1999; Ohi and Wright 2007; Smith et al. 2011; Peterson et al. 2013]), with right-sided *nodal* expression reliably producing *situs inversus*. Just as Activin can replicate Nodal's ability to induce mesoderm earlier in development, the ectopic expression of Activin in the lateral plate mesoderm of *Xenopus* can also activate *nodal*, *lefty*, and *pitx2* expression (Campione et al. 1999). Both sides and the midline express the Nodal EGF-CFC cofactor genes (*cryptic* or *cripto* [*oep*]) (Shen et al. 1997; Zhang et al. 1998; Thisse et al. 2004; Onuma et al. 2006) and GDF-1 (Rankin et al. 2000; Thisse et al. 2004; Peterson et al. 2013), which likely acts as a heterodimer with Nodal (Tanaka et al. 2007) and is required for the propagation of *nodal* expression within the lateral plate mesoderm (Rankin et al. 2000; Tanaka et al. 2007; Peterson et al. 2013).

Nodal activates its own expression within the lateral plate mesoderm through the autoregulatory, FoxH1-dependent ASE enhancer in all vertebrates (Norris and Robertson 1999; Osada et al. 2000; Saijoh et al. 2000), and additionally through another FoxH1-dependent enhancer, the LSE in mammals (Saijoh et al. 2005). Moreover, elements resembling the ASE have been found in *nodal* genes of organisms as divergent as ascidians and sea urchins (Osada et al. 2000; Range et al. 2007). This enhancer is not only conserved across species, but similar, left side–specific FoxH1 binding enhancers are shared by *lefty* and *pitx2* (Norris and Robertson 1999; Saijoh et al. 2000; Shiratori et al. 2001, 2006).

On reaching the lateral plate mesoderm, Nodal activates expression of the Lefty antagonists in both the lateral plate mesoderm and along the midline of the embryo, in the prospective floor plate and notochord (Meno et al. 1997, 1999; Adachi et al. 1999; Bisgrove et al. 1999; Gaio et al. 1999; Cheng et al. 2000; Liang et al. 2000; Long et al. 2003; Toyoizumi et al. 2005). *Xenopus* has a single *lefty* gene, which is expressed in both these regions (Cheng et al. 2000). In mouse and zebrafish, two *lefty* genes have subfunctionalized expression domains, with *lefty1* expressed primarily along the midline and *lefty2* expressed primarily in the lateral plate mesoderm (Meno et al. 1997; Long et al. 2003; Chocron et al. 2007; Wang and Yost 2008; Smith et al. 2011). Although *lefty* expression propagates throughout the entire lateral plate mesoderm in mouse and *Xenopus*, *lefty2* expression in zebrafish is limited to the left heart field (Meno et al. 1996, 1997; Cheng et al. 2000; Long et al. 2003). Complete loss of Lefty expression causes earlier defects in development (see mesendodermal patterning section); however, loss of *lefty1* alone allows *nodal* expression to spread from the left lateral plate mesoderm to the right lateral mesoderm, resulting in bilateral *nodal*

expression and laterality defects (Meno et al. 1998; Wang and Yost 2008). These results suggest that Lefty creates a midline barrier, restricting the spread of Nodal activity to the left side of the embryo. In support of this midline barrier hypothesis, physical removal of midline tissues, or mutants that disrupt their formation, allow the spread of left asymmetric cues to the right lateral plate mesoderm (Danos and Yost 1996; Lohr et al. 1997; Melloy et al. 1998; Burdine and Grimes 2016). Down-regulation of Lefty expression also accelerates the spread of *nodal* expression within the left lateral plate, inferring a role for Lefty in regulating the timing of *nodal* expansion. In *Xenopus*, overexpression of Lefty on the left side of the embryo (Cheng et al. 2000) also disrupts left-sided *nodal* expression. Several investigators have proposed that Lefty and Nodal function as a classical Turing reaction–diffusion, or "self-enhancement lateral inhibition" system (Sakuma et al. 2002; Nakamura et al. 2006; Marjoram and Wright 2011; Muller et al. 2012), in which Nodal enhances its own activity locally, while inhibiting its activity laterally, through the activity of a faster diffusing antagonist Lefty. Supporting this model, zebrafish and *Xenopus* Lefty diffuses faster than Nodal (Marjoram and Wright 2011; Muller et al. 2012).

BMPs also play crucial roles in both facilitating Nodal signaling and restricting Nodal activity within the lateral plate mesoderm. In the mouse, signaling by BMP-4 is required for the expression of the EGF-CFC NODAL coreceptors within the lateral plate mesoderm (Fujiwara et al. 2002). *Bmp4* is expressed in the lateral plate mesoderm before and during *Nodal* expression, and its removal prevents the propagation of NODAL signaling within the lateral plate mesoderm, and BMP overexpression activates NODAL signaling in the chick lateral plate mesoderm (Piedra and Ros 2002). On the other hand, overexpression of BMP during LR symmetry breaking represses Nodal activity in mouse, *Xenopus*, and zebrafish (Ramsdell and Yost 1999; Chocron et al. 2007; Furtado et al. 2008; Mine et al. 2008). It seems likely that while BMP is required for the formation of the LR organizer and the lateral plate mesoderm earlier in development, BMP antagonizes *nodal* as it propagates through the lateral plate mesoderm. One study further suggests that a positive role for BMP-4 during Nodal propagation within the lateral plate mesoderm may be artifactual, caused by the methodology, which exposes all tissues of the embryo, not just the lateral plate mesoderm to Noggin (Mine et al. 2008). Supporting an anti-Nodal role for BMP signaling, the reduction of BMP antagonists such as Chordin and Noggin represses Nodal activity in the lateral plate mesoderm (Chocron et al. 2007; Mine et al. 2008), whereas the local overexpression of these antagonists (Chocron et al. 2007; Mine et al. 2008), the loss of Acvr1 (Ramsdell and Yost 1999; Constam and Robertson 2000; Kishigami et al. 2004), or the local elimination of BMP-activated Smads (Chang et al. 2000; Constam and Robertson 2000; Furtado et al. 2008) within the lateral plate mesoderm activates Nodal signaling. In *Xenopus*, the disruption of BMP signaling on the right side of the embryo with the truncated BMP type I receptor Acvr1 results in ectopic *nodal* expression and reversed morphology, whereas the overactivation of BMP signaling on the left side with constitutively active Acvr1 disrupts *nodal* expression and also reverses heart orientation (Ramsdell and Yost 1999).

One explanation for the antagonism of BMP and Nodal within the lateral plate mesoderm, is that the two signaling pathways are competing for the shared co-Smad, Smad4 (reviewed in Shiratori and Hamada 2014). Supporting this model, the overexpression of SMAD4 in the right lateral plate mesoderm leads to bilateral expression of *Pitx2*, an effect that can be rescued with the simultaneous right-sided overexpression of BMP-4 (Furtado et al. 2008). Alternatively, BMP-4 may antagonize Nodal activity by activating the expression of Lefty. In zebrafish, BMP signaling is necessary to activate *lefty* expression in the midline, enhancing the expression of *lefty* independently of Nodal in both the midline and the lateral plate mesoderm (Chocron et al. 2007; Smith et al. 2011). BMP signaling is also required for midline *Lefty1* expression in the mouse (Fujiwara et al. 2002; Kishigami et al. 2004). The

expression of *lefty1* in zebrafish *bmp4* mutants is normal, but they still have expanded Nodal activity (Lenhart et al. 2011), suggesting that Lefty is not the only means by which BMPs regulate Nodal signaling within the lateral plate mesoderm and that BMP creates an additional midline barrier confining Nodal to the left lateral plate mesoderm. *bmp4* expression starts symmetrically in the lateral plate mesoderm, but develops a left-sided bias in the zebrafish when Nodal signaling initiates (Chocron et al. 2007); this asymmetry may play a role in heart morphology (Chocron et al. 2007; Smith et al. 2008).

Expression of *nodal* in the left lateral plate mesoderm leads to the asymmetric expression of *pitx2* (Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Yan et al. 1999; Long et al. 2003). In *Xenopus* and mice, *pitx2* expression persists long after *nodal* expression terminates, being maintained by *nkx2* expression (Shiratori et al. 2001, 2006). Cells expressing *pitx2* generally adopt a left-sided morphology (Piedra et al. 1998; Ryan et al. 1998; Campione et al. 1999; Lin et al. 1999; Essner et al. 2000). In *Xenopus* and mice, ectopic or atypical *pitx2* expression is capable of altering the laterality of the heart (Ryan et al. 1998; Campione et al. 1999; Lin et al. 1999; Okada et al. 1999), lungs (Lin et al. 1999), gut (Ryan et al. 1998; Campione et al. 1999), and brain (Garric et al. 2014). The mouse *Pitx2* mutant shows laterality defects of the lungs, in particular a duplication of right-sidedness or right isomerism (Gage et al. 1999; Lin et al. 1999; Lu et al. 1999; Liu et al. 2001; Shiratori et al. 2006), as well as defects in heart morphology and embryo turning.

Although zebrafish *pitx2* is expressed in the left lateral plate mesoderm in a Nodal-dependent manner, and has long been thought to contribute to organ laterality in the same way as in other vertebrates, it has been found in zebrafish that *pitx2* is not required for normal organ laterality (Ji et al. 2016). The investigators suggest that the gene adjacent to *pitx2*, *elovl6*, a fatty acid elongase is instead important for zebrafish laterality. The investigators show that this gene is expressed in the left lateral plate mesoderm and is dependent on Nodal activity but not on Pitx2. It will be interesting to see more studies on the role of non-Pitx2, but Nodal-dependent left side expressed genes in the future.

Nodal signaling guides laterality of several organ systems, including the heart, which loops to the right early in development (Stainier et al. 1993; Nieuwkoop and Faber 1994), the gut, which folds asymmetrically within the abdominal cavity (Cook 1965; Nieuwkoop and Faber 1994), the brain, which includes several asymmetric structures (Kolb et al. 1982; Bisgrove et al. 1999; Concha et al. 2000; Essner et al. 2000; Liang et al. 2000), and the lungs, the left lung being smaller to accommodate the heart (Cook 1965; Kolb et al. 1982). Manipulations of the aforementioned processes (i.e., establishment of the LR organizer, symmetry breaking, and the propagation of Nodal signaling within the lateral plate mesoderm) alter the positioning of the organs, *situs solitus*, in several ways. Some manipulations, usually those upstream of LR symmetry breaking, such as the reversal of flow within the LR organizer (Piedra et al. 1998; Okada et al. 1999; Nonaka et al. 2002; Barth et al. 2005; Toyoizumi et al. 2005; Kim et al. 2013) can completely reverse the normal LR positioning of organs, known as *situs inversus*. Overactive NODAL or loss of LEFTY can lead to a duplication of left-sided morphologies, known as left-isomerism (Meno et al. 1998). Conversely, reducing or eliminating NODAL signaling can result in a duplication of right-sided morphologies, known as right-isomerism (Brennan et al. 2002). Sometimes, organ lateralities are altered independently of each other; for example, the initial gut orientation of the mouse *Pitx2* mutant is oriented normally, even though it shows a right isomerism of the lungs (Gage et al. 1999; Lin et al. 1999; Lu et al. 1999; Liu et al. 2001; Shiratori et al. 2006). Other organs, such as the dorsal diencephalon within the brain, do not depend on Nodal signaling for their intrinsic asymmetry, but require Nodal for the correct orientation (Concha et al. 2000). The zebrafish dorsal diencephalon contains two prominent asymmetric structures, the habenular nuclei, the left of which is larger, and the

parapineal, which is displaced to the left side. The disruption of Nodal signaling randomizes the positioning of these organs with respect to the body axis of the zebrafish, but the large habenular nucleus and the parapineal always end up on the same side, and these structures are not isomerized. This suggests that the dorsal diencephalon has a separate symmetry breaking mechanism that is informed by, but not dependent on Nodal signaling. The heart is another organ where its position and orientation are informed by Nodal signaling, but also contains intrinsic Nodal-independent asymmetries (Ramsdell 2005; Baker et al. 2008; Bakkers 2011; Noel et al. 2013). As proper formation of the heart and major arteries is essential for circulation, laterality defects in the heart are often lethal.

The mechanisms by which the left-sided identity of the lateral plate mesoderm is translated into organ asymmetry remains poorly understood, and may be distinct for different organ systems. In mammals, chicks, and zebrafish, it appears that left lateral plate cells adopt a more compact morphology, express different extracellular matrix proteins, and migrate (Horne-Badovinac et al. 2003; Muller et al. 2003; Davis et al. 2008; Welsh et al. 2013). These morphological cues are then transferred via the forming mesentery to the forming gut tube (Kurpios et al. 2008).

ROLES OF TGF-β FAMILY PROTEINS IN DORSAL CONVERGENCE

Both Nodal and BMP signaling contribute to convergence and extension movements during gastrulation. In zebrafish, initially cells are uniformly distributed in the blastoderm along the DV axis. During gastrulation stages, lateral cells begin migrating dorsally to form the developing body axis (Myers et al. 2002; von der Hardt et al. 2007; Naylor et al. 2016). Cells receiving the highest levels of BMP signaling in the ventralmost 30% of the embryo do not migrate dorsally, forming a zone of "no convergence, no extension" (Myers et al. 2002; von der Hardt et al. 2007; Naylor et al. 2016). In embryos deficient in BMP signaling, the rate of cell migration dorsally is decreased (Myers et al. 2002; von der Hardt et al. 2007; Naylor et al. 2016). Consistent with this, dorsally migrating cells are elongated and extended, whereas ventral cells are not (Myers et al. 2002; von der Hardt et al. 2007). Interestingly, BMP-coated beads can induce convergence and extension movements even in the absence of noncanonical Wnt or FGF signaling, suggesting that BMP regulation of cell adhesion is, at least in part, independent of the canonical PCP pathway (von der Hardt et al. 2007). Instead, the absence of BMP signaling stabilizes lamellipodia-mediated cell–cell adhesions, which cause cells to converge dorsally into regions that lack BMP signaling (von der Hardt et al. 2007). These cell–cell adhesions are mediated by N-cadherin (von der Hardt et al. 2007), and, accordingly, dorsal convergence is disrupted in loss-of-function mutants that lack E-catenin (Han et al. 2016). Whether BMP signaling plays a similar role in convergence and extension movements during *Xenopus* and mouse gastrulation is not yet known.

Nodal signaling contributes to involution, cell migration, and convergence and extension movements during gastrulation. In inducing mesoderm formation, Nodal signaling in turn drives involution and gastrulation via activation of the canonical PCP pathway genes (Feldman et al. 2000; Luu et al. 2008; Shindo et al. 2008; Roszko et al. 2009), as discussed in the section on the role of TGF-β family signaling in mesendoderm specification and patterning. However, Nodal signaling also contributes to convergence and extension movements independently of mesoderm induction. In *Xenopus*, Xnr1 and Xnr2 contribute to dorsal convergence and extension movements, whereas Xnr5 and Xnr6 induce mesoderm (Luxardi et al. 2010). In zebrafish, Nodal signaling induces the expression of miR-206, a short noncoding RNA that drives convergence and extension by modulating c-Jun amino-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) signaling and *prickle* expression (Liu et al. 2012, 2013). In mice, NODAL signaling induces the formation and migration of the AVE, a process that depends heavily on the WNT/PCP path-

way (Stower and Srinivas 2014). However, it is not clear whether NODAL signaling induces PCP-independent cell movement in the mouse.

EXTRACELLULAR REGULATION OF TGF-β

BMP and Nodal signaling components interact with a network of extracellular regulators that can antagonize or promote their signaling. Many of these extracellular regulators play pivotal roles in DV, LR, and mesendodermal patterning, which were discussed in previous sections. Here, we will discuss the binding interactions of these extracellular modulators (Table 1), along with their place in the network of extracellular regulation of TGF-β family proteins (Fig. 6).

Antagonism of BMP by Chordin: An Overview

The extracellular BMP antagonist Chordin and its homologs are essential to properly regulate BMP signaling in mouse, zebrafish, and frog development. Chordin is the central node of a network of regulators that modulate BMP function in the extracellular space. Chordin inhibits BMP signaling by binding BMP ligand, rendering it unable to bind its receptors (Fig. 6A; Table 1) (Piccolo et al. 1996; Zhang et al. 2007; Troilo et al. 2014). Chordin is expressed in dorsal tissues, including the dorsal organizer, throughout early development (Miller-Bertoglio et al. 1997; Schulte-Merker et al. 1997; Bachiller et al. 2000; Shimizu et al. 2000; Bachiller 2003; Kuroda et al. 2004; Branam et al. 2010; Ramel and Hill 2013; Abe et al. 2014, 2016; Xue et al. 2014). In zebrafish, the loss of *chordin* causes a modest expansion of ventral mesodermal and ectodermal structures such as blood and tail and a concomitant reduction of dorsal structures such as the somites, eyes, and brain (Hammerschmidt et al. 1996a; Fisher et al. 1997; Schulte-Merker et al. 1997). A similar expansion of ventral mesodermal markers and ventral

Figure 6. (*Figure on following page.*) Extracellular agonism and antagonism of bone morphogenetic protein (BMP) and Nodal during axis patterning. (*A*) References supporting and defining agonism and antagonism listed next to each connector. Expression domain of each species during axis patterning denoted by box color. (*B–M*) Conserved domains in each agonist and antagonist along with known binding domains. Note that additional binding partners that do not have a known binding domain determined by a structure–function analysis may exist. References to structure–function analysis shown for each binding domain. AA, amino acid; CR, cysteine-rich domain; Pro, Pro-domain; CC, coil–coil domain; DAN, differentially screening-selected gene arbitrative in neuroblastoma domain; Olfactomedin, olfactomedin domain; TM, transmembrane domain; Partial vWFD, Von Willebrand factor type D domain; Kaz, Kazal domain family Follistatin module; E, EGF domain; CUB, complement C1r/C1s-sea urchin epidermal growth factor-BMP-1; Protease, protease, Nog domain, Noggin domain; SFRP-1L, secreted frizzled-related protein domain; Chd, chordin; Chrd, chordin domain; TgfB-L, TGF-β-like domain; TIL, trypsin inhibitor-like cysteine-rich domain. Numbers 1 through 75 in panels *A* and *B* refer to the following references: 1, Agius et al. 2000; 2, Aykul and Martinez-Hackert 2016; 3, Aykul et al. 2015; 4, Ambrosio et al. 2008; 5, Bates et al. 2013; 6, Bayramov et al. 2011; 7, Bell 2003; 8, Belo et al. 2000; 9, Bijakowski et al. 2012; 10, Blader 1997; 11, Blitz et al. 2000; 12, Blitz et al. 2003; 13, Chang et al. 2001; 14, Chang et al. 2003; 15, Chen and Shen 2004; 16, Church et al. 2015; 17, Collavin 2003; 18, Connors et al. 1999; 19, Connors et al. 2006; 20, Dal-Pra et al. 2006; 21, Degenkolbe et al. 2013; 22, Feldman et al. 2002; 23, Geng et al. 2011; 24, Geach and Dale 2008; 25, Glister et al. 2004; 26, Glister et al. 2015; 27, Goodman et al. 1998; 28, Groppe et al. 1998; 29, Groppe et al. 2002; 30, Groppe et al. 2002; 31, Harms and Chang 2003; 32, Iemura et al. 1998; 33, Inomata et al. 2008; 34, Inomata et al. 2013; 35, Jasuja et al. 2006; 36, Katsu et al. 2012; 37, Khokha et al. 2005; 38, Kisonaite et al. 2016; 39,40, Larrain et al. 2000; 41, Larrain et al. 2001; 42, Lee et al. 2006; 43, Lee et al. 2009; 44, Marques et al. 2004; 45, Miller-Bertoglio et al. 1999; 46, Muraoka et al. 2006; 47, Oelgeschlager et al. 2000; 48, Oelgeschlager 2003; 49, Paine-Saunders et al. 2002; 50, Piccolo et al. 1997; 51, Piccolo et al. 1999; 52, Ploper et al. 2011; 53, Rentzsch et al. 2006; 54, Cha et al. 2006; 55, Salic et al. 1997; 56, Scott et al. 1999; 57, Scott et al. 2001; 58, Seemann et al. 2009; 59, Serpe et al. 2008; 60, Shibata et al. 2005; 61, Sidis et al. 2006; 62, Sun et al. 2006a; 63, Tanegashima et al. 2004; 64, Troilo et al. 2014; 65, Troilo et al. 2016; 66, Viviano et al. 2004; 67, Vonica and Brivanlou 2007; 68, Wardle et al. 1999; 69, Winstanley et al. 2015; 70, Xie and Fisher 2005; 71, Yabe 2003a; 72, Zhang et al. 2007; 73, Zhang et al. 2010; 74, Zimmerman et al. 1996; 75, Cheng et al. 2004.

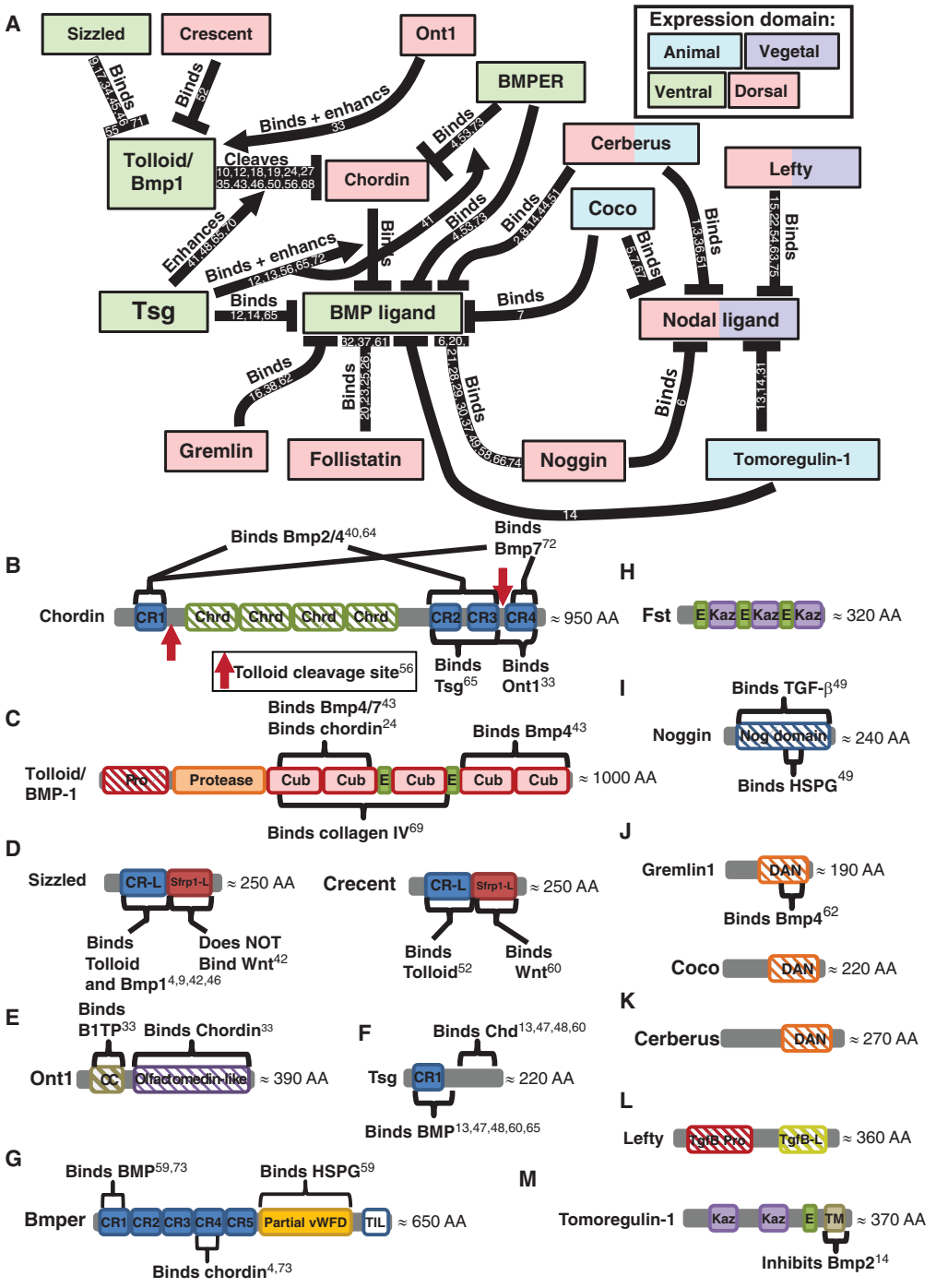

Figure 6. (*See legend on previous page.*)

structures is observed in *Xenopus* embryos deficient for Chordin (Oelgeschlager et al. 2003). In the mouse, the loss of *Chordin* alone causes a less severe phenotype, an expansion of the allantois at the expense of the embryonic mesoderm, along with mild pharyngeal and bone defects (Bachiller 2003).

Genes encoding the motifs, CXXCXC and CCXXC, which are found in Chordin, and antagonize BMP signaling are referred to as "*chordin-like*" genes (Garcia Abreu et al. 2002). Although one *chordin-like* gene has been suggested to act redundantly with *chordin* during gastrulation in zebrafish, the limited early expression of *chordin-like* genes in mouse and *Xenopus* suggests they only play a role later in development (Nakayama et al. 2001, 2004; Branam et al. 2010; Pfirrmann et al. 2015). Other nonhomologous BMP antagonists play partially redundant roles to Chordin, as discussed further below.

Chordin binds to BMP and other modulators via multiple conserved cysteine-rich repeats known as CR domains or Von Willebrand type C domains (Fig. 6B) (Larrain et al. 2000; Zhang et al. 2007). One molecule of Chordin binds one dimer of BMP ligand (Piccolo et al. 1996; Zhang et al. 2007; Troilo et al. 2014). Chordin curves around the BMP dimer, binding one half with its CR1 domain and the other with its CR2-CR3-CR4 domains (Troilo et al. 2014). Chordin can also bind numerous other BMP extracellular modulators. The CR2-CR3 domains of Chordin bind the BMP extracellular modulator Tsg (Table 1; Fig. 6B) (Troilo et al. 2016). Chordin binds the BMP extracellular regulator BMPER (Crossveinless-2) and HSPGs through undetermined domains (Fig. 6B; Table 1) (Jasuja et al. 2004; Lee et al. 2006; Ambrosio et al. 2008; Zhang et al. 2010). Tsg and BMPER can both inhibit and enhance BMP activity, and do so by binding independently to Chordin and to BMP ligand, or by binding both Chordin and BMP in a tripartite complex (Fig. 6A,F,G; Table 1, discussed further below) (Chang et al. 2001; Scott et al. 2001; Blitz et al. 2003; Rentzsch et al. 2006; Zhang et al. 2007, 2010; Ambrosio et al. 2008; Troilo et al. 2016). The primary regulators of Chordin protein stability are the highly homologous metalloproteases Tolloid (also called Xolloid in *Xenopus*) and BMP-1, as well as the metalloprotease inhibitors Sizzled and Crescent (Fig. 6A–D) (Salic et al. 1997; Miller-Bertoglio et al. 1999; Collavin 2003; Yabe 2003b; Muraoka et al. 2006; Ploper et al. 2011; Bijakowski et al. 2012; Inomata et al. 2013; De Robertis and Moriyama 2016) (discussed in next subsection). Together, this network of extracellular factors regulates BMP signaling by modulating activity and stability of the antagonist Chordin.

Tolloid and BMP-1 Antagonize Chordin

Tolloid and BMP-1 are metalloproteases that regulate Chordin stability by cleaving Chordin at two locations near the amino- and carboxy-terminal region of the protein (Fig. 6B) (Blader 1997; Piccolo et al. 1997; Scott et al. 1999; Wardle et al. 1999; Muraoka et al. 2006). The cleavage of Chordin blocks the ability of Chordin to bind and inhibit BMP ligand (Larrain et al. 2000; Lee et al. 2006; Piccolo et al. 1997). The cleavage of Chordin by Tolloid leaves the individual BMP binding domains (CR domains) intact, which can still bind BMP (Troilo et al. 2014). However, these fragments bind BMP with a lower affinity than full-length Chordin (Larrain et al. 2000), are cleared from the extracellular space faster (Larrain et al. 2001; Xie and Fisher 2005; Kelley et al. 2009), and can be competed away by the extracellular BMP agonist Tsg (Larrain et al. 2001). Tolloid is composed of "complement 1r/s, Uegf and BMP-1" (CUB) domains and epidermal growth factor (EGF) domains that are needed for effective cleavage of Chordin (Canty et al. 2006; Geach and Dale 2008). The first two CUB domains bind to BMP ligand, and may also be responsible for its high-affinity to Chordin (Fig. 6C; Table 1) (Lee et al. 2006, 2009; Geach and Dale 2008). The first three CUB domains are also needed for Tolloid to bind collagen IV (Winstanley et al. 2015), which enhances Chordin cleavage by Tolloid (Fig. 6A,C; Table 1) (Winstanley et al. 2015). Ont1 also acts as a scaffold to enhance the cleavage of Chordin by

Tolloid and Bmp1 (Fig. 6A,B,C,E) (Inomata et al. 2008).

Bmp1 and Tolloid enhance BMP signaling and thus promote the formation of ventral cell fates in the developing embryo (Table 1). In zebrafish and *Xenopus*, *tolloid* and *bmp1* are first ubiquitously expressed in the early gastrula before becoming ventrally restricted in the late gastrula (Table 1) (Goodman et al. 1998; Connors et al. 1999; Dale et al. 2002; Jasuja et al. 2006). In zebrafish, the loss of either *bmp1* or *tolloid* alone only mildly dorsalizes the most posterior portions of the embryo, whereas the loss of both leads to a severe loss of all ventral tissues (Blader 1997; Connors et al. 1999, 2006; Jasuja et al. 2006; Muraoka et al. 2006). A similar level of dorsalization is seen in *Xenopus* injected with RNA encoding a dominant-negative form of Bmp1 or Tolloid (Piccolo et al. 1997; Wardle et al. 1999; Blitz et al. 2000; Geach and Dale 2008). In the early mouse gastrula, *Bmp1* and *Tolloid* are expressed ubiquitously, whereas *Tolloid-like1* is expressed laterally and *Tolloid-like2* is expressed anteriorly (Scott et al. 1999). However, mice mutant for *Bmp1* and *Tolloid* show no early DV patterning phenotype, possibly because of functional redundancy between TOLLOID, BMP-1, and the TOLLOID-LIKE proteins (Suzuki et al. 1996; Pappano et al. 2003).

Sizzled and Crescent Antagonize Tolloid and BMP-1

Sizzled and Crescent, two members of the secreted Frizzled receptor (SFRP) family, competitively inhibit the metalloprotease activity of Bmp1 and Tolloid (Fig. 6A) (Lee et al. 2006; Muraoka et al. 2006; Ambrosio et al. 2008; Ploper et al. 2011; Bijakowski et al. 2012). Like other SFRPs, Crescent is able to bind Wnt ligand (Fig. 6D; Table 1) (Pera and De Robertis 2000; Shibata et al. 2005; Ploper et al. 2011). In contrast, Sizzled cannot bind Wnt ligand or inhibit Wnt signaling (Fig. 6D) (Lee et al. 2006) and is only known to inhibit BMP signaling. The amino-terminal cysteine-rich Frizzled domain of both Sizzled and Crescent tightly binds to the active site of Tolloid and Bmp1, abrogating the ability of Tolloid and Bmp1 to bind and cleave Chordin (Fig. 6A,D) (Lee et al. 2006; Muraoka et al. 2006; Ambrosio et al. 2008; Ploper et al. 2011; Bijakowski et al. 2012). Homologs of *sizzled* and *crescent* are not present among human and mouse *SFRP* genes, and other human or mouse SFRPs cannot inhibit TOLLOID- or BMP-1-mediated proteolysis of CHORDIN (Kobayashi et al. 2009; Bijakowski et al. 2012). Crescent and Frizzled-related protein (Frzb) also greatly enhance the diffusion of Wnt in *Xenopus* embryos, transporting Wnts and allowing them to signal at considerable distances from where they are secreted (Mii and Taira 2009).

By inhibiting Tolloid and Bmp1, Sizzled and Crescent increase the amount of Chordin that can block BMP signaling, thus promoting dorsal cell fate specification in the early embryo. *sizzled* is expressed ventrally and its expression depends on BMP signaling, acting as a negative feedback inhibitor during DV patterning (Fig. 6A; Table 1). In contrast, *crescent* is expressed dorsally in *Xenopus* (Table 1) (Pera and De Robertis, 2000; Yabe, 2003a; Lee et al. 2006; Ploper et al. 2011). Loss of *sizzled* causes an expansion of ventral mesodermal and ectodermal cell fates, which depends on the presence of Tolloid and/or Bmp1 (Hammerschmidt et al. 1996a; Miller-Bertoglio et al. 1999; Collavin 2003; Yabe 2003b; Lee et al. 2006). The loss of *sizzled* does not further ventralize *chordin* mutant embryos (Miller-Bertoglio et al. 1999; Lee et al. 2006). Together, these results show that Sizzled acts entirely by inhibiting Tolloid/BMP-1 degradation of Chordin during axis patterning (Miller-Bertoglio et al. 1999; Lee et al. 2006). The loss of *crescent* ventralizes *Xenopus* embryos, whereas the injection of *crescent* RNA dorsalizes them (Pera and De Robertis 2000; Ploper et al. 2011). Despite the important roles of Sizzled and Crescent during zebrafish and *Xenopus* DV patterning, mammals do not express Sizzled or Crescent homologs (Kuraku and Kuratani 2011), and the related members of the SFRP family do not appear to inhibit Chordin metalloprotease activity (Kobayashi et al. 2009; Bijakowski et al. 2012).

Sizzled stands out as an antagonist of BMP signaling that is expressed ventrally in a similar

domain as the BMP ligands (Yabe 2003b; Lee et al. 2006). *sizzled* expression is promoted by BMP signaling (Figure 3) (Lee et al. 2006; Inomata et al. 2013), thereby forming a negative feedback loop on BMP signaling activity. It has been postulated that this feedback loop provides stability to the system. If BMP signaling were to only induce the expression of BMP agonists and repress the expression of antagonists, the system could be easily thrown out of balance. The negative feedback of Sizzled helps BMP limit its own expression domain through a transcriptional autoregulatory loop, stabilizing the system (Collavin 2003; Inomata et al. 2013). There is also evidence that this negative feedback loop helps properly shape the BMP gradient in different sized embryos, a phenomenon referred to as scaling (Inomata et al. 2013).

Antagonism and Agonism of BMP by Twisted Gastrulation

Twisted gastrulation (Tsg) is a small but multifunctional extracellular modulator capable of promoting or antagonizing BMP signaling depending on embryonic context. Tsg can antagonize BMP signaling in either the absence or presence of Chordin (Fig. 6A). In the absence of Chordin, Tsg inhibits BMP signaling by binding the BMP ligand with an affinity ranging between 2.5 nM and 50 nM depending on the ligand (Table 1) (Oelgeschlager et al. 2000; Chang et al. 2001; Oelgeschlager 2003; Zhang et al. 2007; Troilo et al. 2016). Tsg binds BMP ligand with its amino-terminal CR domain (Fig. 6F) (Oelgeschlager 2003; Zhang et al. 2007). Tsg can also antagonize BMP signaling by forming a ternary complex with BMP and Chordin, thereby enhancing the binding of Chordin to BMP ligand (Oelgeschlager et al. 2000; Chang et al. 2001; Scott et al. 2001; Oelgeschlager 2003; Zhang et al. 2007; Troilo et al. 2016). Consistent with this, the overexpression of *tsg* mRNA antagonizes BMP signaling in the absence or presence of Chordin (Chang et al. 2001; Blitz et al. 2003; Little and Mullins 2004; Troilo et al. 2016). Conversely, in the presence of both Chordin and the metalloprotease Tolloid, Tsg acts as a BMP agonist by enhancing the degradation of Chordin by Tolloid (Fig. 6A) (Scott et al. 2001; Xie and Fisher 2005; Troilo et al. 2016). Tsg exerts this effect by binding Chordin and pulling its CR domains 2–4 away from the BMP ligand, thus making this domain more accessible to Tolloid and/or BMP-1 cleavage (Fig. 6A,B,F; Table 1) (Larrain et al. 2001; Little and Mullins 2004; Xie and Fisher 2005; Troilo et al. 2016). Tsg also enhances the binding of the extracellular BMP modulator BMPER to Chordin (Fig. 6A) (Ambrosio et al. 2008). Therefore, Tsg can enhance or inhibit BMP signaling depending on the presence and concentration of BMP ligand, Chordin, BMPER, and the metalloproteases Tolloid and Bmp1.

Loss of Tsg suggests both promoting and antagonizing effects on BMP signaling. In *Xenopus*, *tsg* is ventrally expressed in a similar domain as BMP ligand during DV patterning (Table 1) (Oelgeschlager et al. 2000). In mouse, *Tsg* is expressed in the AVE and the primitive streak in the late blastula and throughout the mesoderm in the early gastrula (Zakin and De Robertis 2004). In zebrafish, the depletion of Tsg causes a retraction of ventral gene markers, an expansion of dorsal somites, and loss of tail structures (Little and Mullins 2004; Xie and Fisher 2005). Conversely, Tsg depletion in *Xenopus* has an opposite effect during DV patterning (Blitz et al. 2003). Despite the strong conservation between zebrafish, *Xenopus*, and mouse *tsg* genes, the loss of *Tsg* in mouse does not alter early patterning, manifesting only as subtle defects in the vertebrae and thymus (Nosaka et al. 2003; Zakin and De Robertis 2004). However, the loss of *Tsg* in conjunction with one allele of *Bmp4* causes forebrain, eye, and further skeletal defects suggesting that *Tsg* acts as a BMP agonist in mouse as well (Zakin and De Robertis 2004). Although Tsg has been shown in some contexts to act as a BMP agonist in vivo, it is likely that Tsg exerts different effects on BMP signaling in different embryonic contexts.

Antagonism and Agonism of BMP by BMPER

Like Tsg, BMPER (Crossveinless-2) is a multifunctional extracellular modulator capable of promoting or antagonizing BMP signaling

depending on embryonic context. BMPER can antagonize BMP signaling in the absence or presence of Chordin, but can only act as an agonist when Chordin is present (Fig. 6A; Table 1). BMPER acts as a BMP agonist by binding to Chordin, reducing its ability to bind and inhibit BMP (Fig. 6G; Table 1) (Rentzsch et al. 2006; Ambrosio et al. 2008; Zhang et al. 2010). BMPER interacts with the extracellular matrix by binding HSPGs (Fig. 6G; Table 1) (Serpe et al. 2008), and this interaction is thought to enhance BMP signaling during vertebral field patterning by concentrating BMP ligand in the vertebral body where *Bmper* is expressed (Zakin et al. 2008, 2010). Paradoxically, BMPER also increases CHORDIN protein levels in the vertebral body, suggesting that CHORDIN, BMPER, and BMP ligand may form a ternary complex. Alternatively, BMPER may sequester CHORDIN extracellularly facilitating the release of BMP from CHORDIN. Additional studies are needed to fully resolve the mechanism by which BMPER enhances BMP signaling. The antagonism of BMP signaling by BMPER is more clear. BMPER binds directly to the BMP ligand (Fig. 6G; Table 1), and thus interferes with the interaction of the BMP ligand and its type I receptor (Rentzsch et al. 2006; Ambrosio et al. 2008; Zhang et al. 2010). In cell culture, the BMP–BMPER complex binds to low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) and is endocytosed more rapidly than BMP alone, suggesting that BMPER may also antagonize BMP ligand by clearing it from the extracellular space (Table 1) (Pi et al. 2012). Tsg enhances the ability of BMPER to bind BMP ligand and inhibit signaling (Fig. 6A) (Ambrosio et al. 2008), and it is possible that BMPER and TSG act synergistically, as suggested by their genetic interaction in mouse kidney and vertebral field formation (Zakin et al. 2008; Ikeya et al. 2010).

BMPER acts as either a BMP agonist or antagonist depending on the developmental context and organism. During zebrafish DV patterning, BMPER enhances BMP signaling by acting as a competitive inhibitor of Chordin, and the knockdown of *bmper* dorsalizes the embryo (Rentzsch et al. 2006; Zhang et al. 2010). Conversely, during *Xenopus* DV patterning BMPER inhibits BMP signaling by binding BMP ligand directly, and the inactivation of *bmper* ventralizes the embryo (Ambrosio et al. 2008). In both systems, overexpression of *bmper* dorsalizes the embryo by binding directly to the BMP ligand (Moser et al. 2003; Rentzsch et al. 2006; Zhang et al. 2010). In mouse, the loss of BMPER function has no effect on axis patterning, instead causing skeletal and kidney defects later in development (Ikeya et al. 2006). The loss of *Bmper* and *Tsg* together does not affect axis patterning either (Ikeya et al. 2008; Zakin et al. 2008).

Noggin and the Follistatin Family Antagonize BMP

Noggin, Follistatin, and Follistatin-like are extracellular BMP inhibitors that bind to BMP ligand and inhibit BMP ligand–receptor interaction. Noggin homodimerizes to form a butterfly-shaped complex capable of binding some, but not all, BMP ligands with a high affinity (Fig. 6A,I; Table 1) (Groppe et al. 2002). Noggin can also bind the BMP-related GDFs, and to a lesser extent ADMP, Wnt8, and Activin (Table 1) (Seemann et al. 2009; Bayramov et al. 2011; Degenkolbe et al. 2013). Gene inactivation studies suggest that the binding of Noggin to Wnt8, and Activin or Nodal plays a role during embryonic patterning in *Xenopus* (Bayramov et al. 2011). Noggin also strongly binds HSPGs (Fig. 6I), and this interaction is thought to limit Noggin dimer mobility in the extracellular space (Paine-Saunders et al. 2002; Viviano et al. 2004; Inomata et al. 2013; Nesterenko et al. 2015). Follistatin similarly binds numerous BMPs, GDFs and Activins (Fig. 6A) (Nakamura et al. 1991; Shimonaka et al. 1991; Schneyer et al. 1994; Iemura et al. 1998; Otsuka et al. 2001; Glister et al. 2004, 2015; Sidis et al. 2006; Takehara-Kasamatsu et al. 2007; Geng et al. 2011). Unlike Noggin, Follistatin does not dimerize, although two Follistatin proteins can bind to a single BMP dimer (Thompson et al. 2005). Like Noggin, Follistatin strongly binds HSPGs, which may limit its diffusivity in the extracellular space (Table 1) (Nakamura et al. 1991;

Zhang et al. 2012). Interestingly, Follistatin–Activin complexes bind HSPGs more tightly than Follistatin or Activin alone (Zhang et al. 2012).

Noggin, Follistatin, and Follistatin-like proteins act as BMP antagonists during axis patterning, promoting dorsal fates by binding BMP ligand. *noggin*, and *follistatin* or *follistatin-like1b* (called *follistatin* herein) are expressed in the dorsal organizer during axis patterning (Table 1) (Bachiller et al. 2000; Bachiller 2003; Khokha et al. 2005; Dal-Pra et al. 2006). Interestingly, the loss of either *noggin* or *follistatin* or both *noggin* and *follistatin* together has little effect on embryonic DV patterning (Matzuk et al. 1995b; McMahon et al. 1998; Khokha et al. 2005; Dal-Pra et al. 2006; Geng et al. 2011; Lana-Elola et al. 2011; Sylva et al. 2013; Stafford et al. 2014). Only in the absence of *chordin* does knockdown of *noggin* and *follistatin* further ventralize zebrafish and *Xenopus* embryos, indicating that these three proteins act partially redundantly to promote dorsal cell fates (Khokha et al. 2005; Dal-Pra et al. 2006). The triple *Chordin;Noggin;Follistatin* loss-of-function phenotype is not yet known for mice, but double mutants for *Chordin* and *Noggin* fail to form forebrain (Bachiller et al. 2000). It is possible that additional BMP antagonists such as Gremlin, Cerberus, and Chordin-like also function redundantly to compensate for the loss of Chordin, Noggin, and Follistatin during axis patterning.

Dan Family Proteins Cerberus, Gremlin, and Cerberus-Like Proteins Antagonize BMP and Nodal

Cerberus, Gremlin, and Cerberus-like proteins (DAND5, zCharon, Coco) are "differentially screening-selected gene arbitrative in neuroblastoma" (DAN) family extracellular proteins capable of inhibiting BMPs as well as other ligands such as Activin, Wnt, and Nodal (Fig. 6A,J,K; Table 1). Cerberus and Cerberus-like proteins can bind numerous BMP, Wnt, and Nodal ligands (Table 1) (Piccolo et al. 1999; Agius et al. 2000; Belo et al. 2000; Chang et al. 2003; Marques et al. 2004; Chi et al. 2011; Katsu et al. 2012; Aykul et al. 2015; Aykul and Martinez-Hackert 2016). However, only *Xenopus* Cerberus can bind and inhibit Wnt ligand (Belo et al. 2000; Piccolo et al. 1999). Notably, although *Xenopus* and mouse Cerberus have been shown to bind BMP ligands with high affinity (Piccolo et al. 1999; Belo et al. 2000; Chi et al. 2011), human Cerberus binds BMP ligands with a far lower affinity than it does Nodal (Aykul et al. 2015; Aykul and Martinez-Hackert 2016). Mouse and chick DAN proteins are able to bind BMP-2, BMP-4, and GDF-5 (BMP-14) (Table 1) (Katsu et al. 2012). The Cerberus-like protein Coco binds and inhibits Activin, BMP, Nodal, and Wnt ligands, but also enhances canonical TGF-β signaling (Bell 2003; Bates et al. 2013; Deglincerti et al. 2015) by interacting with its receptor TβRI/Alk5 (Fig. 6A,J; Table 1) (Deglincerti et al. 2015). This array of ligand interactions allows Cerberus and Cerberus-like proteins to contribute to both AP and LR patterning.

Gremlin binds and inhibits numerous BMP ligands as well as GDF-5 (Fig. 6A,J; Table 1) (Dionne et al. 2001; Sun et al. 2006a; Church et al. 2015; Kisonaite et al. 2016). Interestingly, Gremlin also belongs to the cystine-knot superfamily, which includes vascular endothelial growth factor (VEGF) (Vitt et al. 2001). Because of its similarity to VEGF, Gremlin can activate VEGF receptors and promote angiogenesis (Mitola et al. 2010). Gremlin binds strongly to HSPGs, likely limiting its effective diffusivity (Table 1) (Chiodelli et al. 2011). Mice lacking *Gremlin* suffer from malformed limbs, lungs, and kidneys (Khokha et al. 2003; Michos et al. 2004). The phenotype for the loss of *gremlin1* has not been determined in zebrafish or *Xenopus*, but *gremlin1* is expressed dorsally during axis patterning in zebrafish (Nicoli et al. 2005).

Cerberus and Cerberus-like proteins play a role in both AP and LR axis patterning (Belo et al. 2009). Overexpression of *cerberus* induces ectopic head formation (Bouwmeester et al. 1996). In *Xenopus*, the depletion of *cerberus* has no axis patterning phenotype but sensitizes the embryo to a lower amount of BMP, Nodal, or Wnt overexpression needed to disrupt head formation (Silva et al. 2003). The zebrafish gene *charon*, which encodes a Cerberus-like protein,

restricts Nodal to the left side of the embryo during LR patterning (Hashimoto et al. 2004). In *Xenopus*, Coco inhibits endoderm and mesoderm formation by inhibiting Activin and Nodal signaling (Bell 2003; Bates et al. 2013). Coco also acts in establishing the fate of the right side of the embryo by inhibiting Nodal signaling (Vonica and Brivanlou 2007; Schweickert et al. 2010).

The mouse has multiple genes encoding Cerberus-like proteins. Similar to *Xenopus cerberus*, the mouse genes encoding Cerberus-like proteins are expressed in the AVE and the dorsal organizer (Bouwmeester et al. 1996; Perea-Gomez et al. 2001, 2002; Kuroda et al. 2004). However, unlike *Xenopus* Cerberus, mouse CERBERUS-LIKE1 cannot bind WNT ligand, and the loss of CERBERUS1 function has only a mild kidney malformation phenotype with no axis patterning phenotype, whereas the loss of DAND5 function, a homolog of *Xenopus coco*, shows a LR patterning defect (Belo et al. 2000; Shawlot et al. 2000; Marques et al. 2004; Chi et al. 2011). The loss of *Cerberus1* does not enhance the *Noggin* or *Goosecoid* loss-of-function phenotypes (Borges et al. 2001, 2002; Perea-Gomez et al. 2002). However, the loss of *Cerberus1* in conjunction with *Lefty* induces the formation of multiple AVEs, indicating that CERBERUS inhibits NODAL during axis patterning in mouse (Perea-Gomez et al. 2001, 2002; Yamamoto et al. 2004). The loss of Cerberus-like2 function results in numerous LR axis defects in the mouse, with DAND5 needed to inhibit NODAL in the node during LR axis patterning (Marques et al. 2004; Oki et al. 2009; Inacio et al. 2013).

Lefty Antagonizes Nodal

Lefty (also called Antivin) is an extracellular antagonist of Nodal signaling that binds to both Nodal ligands and receptors (Fig. 6A; Table 1). Lefty is a highly divergent relative of Nodal (Fig. 4L) (Meno et al. 1996; Thisse and Thisse 1999). Lefty binds directly to Nodal ligands, but not to Activin or BMP (Fig. 6A; Table 1) (Cheng et al. 2000; Tanegashima et al. 2004; Chen and Shen 2004; Wang et al. 2016). Lefty also binds the Nodal coreceptor Cripto (Oep, Tdgf1, FRL), but not the Nodal receptors Acvr2b or Acvr1b (Alk4, Table 1) (Chen and Shen 2004; Cheng et al. 2004; Tanegashima et al. 2004). This allows Lefty to antagonize Vg1 and Nodal, but not Activin signaling, which does not require the Cripto coreceptor to signal (Cheng et al. 2004; Cha et al. 2006). Lefty binds HSPGs, which are thought to facilitate its transport, although how it does so is unclear (Marjoram and Wright 2011).

Lefty proteins play a role in AP axis patterning, mesendoderm specification, DV patterning, and LR patterning. Two Lefty genes have been identified in vertebrates, *lefty1/leftyB/antivin* and *lefty2/leftyA/EBAF*. *Xenopus* has only one identified *lefty*, *lefty1*. During gastrulation, *lefty1* in *Xenopus* and *lefty1* and *lefty2* in zebrafish are expressed around the entire margin and are strongest in the dorsal organizer (Bisgrove et al. 1999; Meno et al. 1999; Thisse and Thisse 1999; Branford et al. 2000; Branford and Yost 2002; Cha et al. 2006). Similarly, during gastrulation in the mouse, *Lefty2* is expressed throughout the primitive streak and is strongest in the node (the mouse dorsal organizer), whereas *Lefty1* is expressed in the AVE (Meno et al. 1999; Kimura et al. 2000; Perea-Gomez et al. 2002; Yamamoto et al. 2004). During LR patterning, *lefty* is coexpressed with *nodal* to the left of the midline in the left lateral plate mesoderm (Bisgrove et al. 1999; Thisse and Thisse 1999; Branford et al. 2000; Meno et al. 2001; Kramer et al. 2002). The individual loss of *lefty1* or *lefty2* causes LR patterning defects such as a bilateral LR patterning (Meno et al. 1998; Nakamura et al. 2006; Wang and Yost 2008; Lenhart et al. 2011). The loss of *lefty* also increases mesoderm at the expense of ectoderm and disrupts AP patterning (Agathon et al. 2001; Chen and Schier 2002).

Tomoregulin Antagonizes Nodal, Vg1, and BMP

Tomoregulin (TMEFF) is a membrane-bound, Follistatin-related protein that inhibits Nodal and BMP signaling (Fig. 6A,M; Table 1) (Chang et al. 2003; Harms and Chang 2003). Tomoregulin inhibits Nodal signaling not by binding

to Nodal ligands, but instead by directly binding to the Nodal coreceptor Cripto (Table 1) (Harms and Chang 2003). Like Follistatin, Tomoregulin can also inhibit BMP signaling (Fig. 6A). The mechanism by which Tomoregulin inhibits BMP signaling is unknown, but interestingly the Follistatin domains of Tomoregulin are dispensable for BMP inhibition, whereas the carboxy-terminal transmembrane and intracellular region are required (Fig. 6M) (Chang et al. 2003). It is possible that Tomoregulin interacts with BMP receptors as well. Tomoregulin can also inhibit GDF-1 (Vg1), which requires the Cripto coreceptor, but it is unable to inhibit Activin or bind the type I receptor of Activin and Nodal, Acvr1b (Harms and Chang 2003).

Little is known about the role of Tomoregulin in mesodermal patterning or LR axis patterning. Although it is present in mouse, zebrafish, and *Xenopus*, mutant phenotypes have not been reported in zebrafish or *Xenopus*. *Tmeff2*$^{-/-}$ mice, which do not express one of the two tomoregulin genes in mouse, show no major axis patterning defects, but are diminished in size and die shortly after birth (Chen et al. 2012). *Tomoregulin* is ubiquitously expressed during axis patterning in mice (De Groot et al. 2000). In *Xenopus*, overexpression of *tomoregulin-1* interferes with mesoderm and endoderm formation (Chang et al. 2003). However, *tmeff1* is not strongly expressed until midgastrulation, suggesting that it plays little role in the initial induction of mesendoderm (Chang et al. 2003). The *tmeff1 and tmeff2* genes have not been studied in zebrafish.

CONCLUSION

TGF-β family ligands and their antagonists establish many of the first asymmetric cues in the developing vertebrate embryo. These cues are necessary for gastrulation, and the correct positioning and patterning of every organ within the adult organism. These programs represent not only the first roles of TGF-β family ligands in animal development, but are also arguably the most conserved throughout the animal kingdom. Moreover, it is often these fundamental processes, which are disrupted or hijacked in the diseased state. Therefore, studies of TGF-β family signaling in vertebrate development not only inform the systems biology of organism form and function, but also illuminate the roles of TGF-β family signaling more broadly in normal physiology and disease, as well as in metazoan evolution.

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Signaling Cross Talk between TGF-β/Smad and Other Signaling Pathways
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