Cold Spring Harbor Perspectives in Biology
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Wnt Signaling in Vertebrate Axis Specification

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The Wnt pathway is a major embryonic signaling pathway that controls cell proliferation, cell fate, and body-axis determination in vertebrate embryos. Soon after egg fertilization, Wnt pathway components play a role in microtubule-dependent dorsoventral axis specification. Later in embryogenesis, another conserved function of the pathway is to specify the anteroposterior axis. The dual role of Wnt signaling in *Xenopus* and zebrafish embryos is regulated at different developmental stages by distinct sets of Wnt target genes. This review highlights recent progress in the discrimination of different signaling branches and the identification of specific pathway targets during vertebrate axial development.

Wnt pathways play major roles in cell-fate specification, proliferation and differentiation, cell polarity, and morphogenesis (Clevers 2006; van Amerongen and Nusse 2009). Signaling is initiated in the responding cell by the interaction of Wnt ligands with different receptors and coreceptors, including Frizzled, LRP5/6, ROR1/2, RYK, PTK7, and proteoglycans (Angers and Moon 2009; Kikuchi et al. 2009; MacDonald et al. 2009). Receptor activation is accompanied by the phosphorylation of Dishevelled (Yanagawa et al. 1995), which appears to transduce the signal to both the cell membrane and the nucleus (Cliffe et al. 2003; Itoh et al. 2005; Bilic et al. 2007). Another common pathway component is β-catenin, an abundant component of adherens junctions (Nelson and Nusse 2004; Grigoryan et al. 2008). In response to signaling, β-catenin associates with T-cell factors (TCFs) and translocates to the nucleus to stimulate Wnt target gene expression (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996).

This β-catenin-dependent activation of specific genes is often referred to as the "canonical" pathway. In the absence of Wnt signaling, β-catenin is destroyed by the protein complex that includes Axin, GSK3, and the tumor suppressor APC (Clevers 2006; MacDonald et al. 2009). Wnt proteins, such as Wnt1, Wnt3, and Wnt8, stimulate Frizzled and LRP5/6 receptors to inactivate this β-catenin destruction complex, and, at the same time, trigger the phosphorylation of TCF proteins by homeodomain-interacting protein kinase 2 (HIPK2) (Hikasa et al. 2010; Hikasa and Sokol 2011). Both β-catenin stabilization and the regulation of TCF protein function by phosphorylation appear to represent general strategies that are conserved in multiple systems (Sokol 2011). Thus, the signaling

Editors: Roel Nusse, Xi He, and Renee van Amerongen
Additional Perspectives on Wnt Signaling available at www.cshperspectives.org

 doi: 10.1101/cshperspect.a007955
Cite this article as *Cold Spring Harb Perspect Biol* 2013;5:a007955

pathway consists of two branches that together regulate target gene expression (Fig. 1).

Other Wnt proteins, such as Wnt5a or Wnt11, strongly affect the cytoskeletal organization and morphogenesis without stabilizing β-catenin (Torres et al. 1996; Angers and Moon 2009; Wu and Mlodzik 2009). These "noncanonical" ligands do not influence TCF3 phosphorylation (Hikasa and Sokol 2011), but may use distinct receptors such as ROR1/2 and RYK instead of or in addition to Frizzled (Hikasa et al. 2002; Lu et al. 2004; Mikels and Nusse 2006; Nishita et al. 2006, 2010; Schambony and Wedlich 2007; Grumolato et al. 2010; Lin et al. 2010; Gao et al. 2011). In such cases, signaling mechanisms are likely to include planar cell polarity (PCP) components, such as Vangl2, Flamingo, Prickle, Diversin, Rho GTPases, and c-Jun amino-terminal kinases (JNKs), which do not directly affect β-catenin stability (Fig. 1) (Sokol 2000; Schwarz-Romond et al. 2002; Schambony and Wedlich 2007; Komiya and Habas 2008; Axelrod 2009; Itoh et al. 2009; Tada and Kai 2009; Sato et al. 2010; Gao et al. 2011). This simplistic dichotomy of the Wnt pathway does not preclude some Wnt ligands from using both β-catenin-dependent and -independent routes in a context-specific manner.

Despite the existence of many pathway branches, only the β-catenin-dependent branch

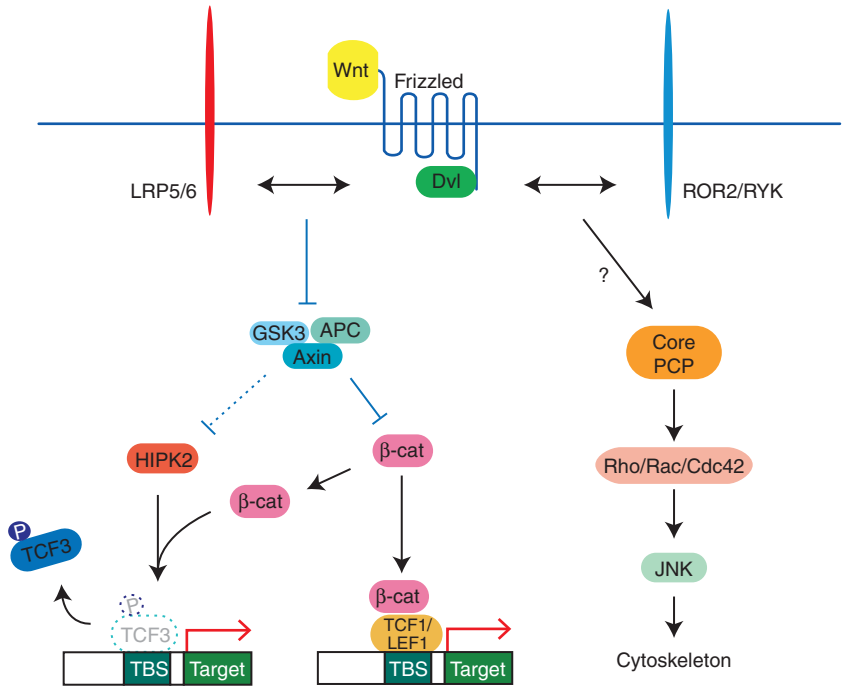

Figure 1. Conserved Wnt pathway branches and components. In the absence of Wnt signals, glycogen synthase kinase 3 (GSK3) binds Axin and APC to form the β-catenin destruction complex. Some Wnt proteins, such as Wnt8 and Wnt3a, stimulate Frizzled and LRP5/6 receptors to inhibit GSK3 activity and stabilize β-catenin (β-cat). Stabilized β-cat forms a complex with T-cell factors (e.g., TCF1/LEF1) to activate target genes. Moreover, GSK3 inhibition leads to target gene derepression by promoting TCF3 phosphorylation by homeodomain-interacting protein kinase 2 (HIPK2) through an unknown mechanism, for which β-catenin is required as a scaffold. This phosphorylation results in TCF3 removal from target promoters and gene activation. Other Wnt proteins, such as Wnt5a and Wnt11, use distinct receptors such as ROR2 and RYK, in addition to Frizzled, to control the the cytoskeletal organization through core planar cell polarity (PCP) proteins, small GTPases (Rho/Rac/Cdc42), and c-Jun amino-terminal kinase (JNK).

has been implicated in body-axis specification. Recent experiments in lower vertebrates have identified additional pathway components and targets and provided new insights into the underlying mechanisms.

ROLE OF CYTOPLASMIC DETERMINANTS AND WNT SIGNALING IN DORSOVENTRAL AXIS SPECIFICATION

The specification of the dorsoventral axis coincides with the formation of an essential embryonic signaling center known as the Spemann organizer in amphibians, the shield in zebrafish, and the node in the mouse (Harland and Gerhart 1997; Schier and Talbot 2005; Marlow 2010). During egg fertilization, sperm entry triggers microtubule-dependent rearrangement of the cortical cytoplasm from the vegetal pole toward the future dorsal side, a process known as the cortical rotation (Houliston and Elinson 1992). Experiments with cytoplasm microinjections or removal revealed vegetally localized dorsalizing activity that moves to the dorsal region before the first cleavage in *Xenopus* or zebrafish eggs (Fujisue et al. 1993; Holowacz and Elinson 1993; Mizuno et al. 1999; Ober and Schulte-Merker 1999). Treatment of fertilized *Xenopus* or zebrafish eggs with microtubule-depolymerizing agents, such as UV irradiation or nocodazole, causes embryo ventralization (Chung and Malacinski 1980; Scharf and Gerhart 1980; Jesuthasan and Stahle 1997). These observations indicated that the movement of the dorsalizing activity is essential for the establishment of the primary dorsoventral axis in both *Xenopus* and zebrafish.

One of the important outcomes of the cortical rotation is the accumulation of β-catenin in dorsal nuclei of early blastulae, whereas β-catenin remains largely cytoplasmic and cortical in ventral cells (Schneider et al. 1996; Schohl and Fagotto 2002). This difference in β-catenin localization can be detected as early as the 2- to 4-cell stage (Larabell et al. 1997). Supporting the critical role of this localization for dorsal development, several maternal effect mutants in zebrafish such as *hecate, ichabod*, and *tokkaebi* result in the reduction of β-catenin dorsal accumulation and defects in dorsal development (Kelly et al. 2000; Nojima et al. 2004; Lyman Gingerich et al. 2005; Bellipanni et al. 2006). The *ichabod* mutation has been mapped to the proximity of the gene encoding β-catenin-2, a second zebrafish β-catenin that is essential for maternal Wnt signaling (Bellipanni et al. 2006). Consistent with the ventralized phenotype of the *ichabod* embryos, maternal β-catenin-2 RNA is down-regulated. *Hecate* is another zebrafish mutation that is manifested by deficient dorsal development and enhanced intracellular calcium release; however, the mutated gene has not yet been identified (Lyman Gingerich et al. 2005). Positional cloning revealed that the *tokkaebi* gene encodes syntabulin, a microtubule-binding protein connecting kinesin I to syntaxin-containing cargo vesicles (Nojima et al. 2010). This finding further implicates microtubule-dependent trafficking in dorsoventral axis specification. Reminiscent of the key function of β-catenin in *Xenopus* and zebrafish, mouse embryos lacking β-catenin reveal abnormal visceral endoderm patterning before gastrulation and do not form the primitive streak (Haegel et al. 1995; Huelsken et al. 2000).

An essential role for Wnt/β-catenin signaling in dorsoventral axis determination has been initially suggested in gain-of-function experiments, in which Wnt and Dishevelled messenger RNAs (mRNAs) triggered ectopic organizer formation (McMahon and Moon 1989; Smith and Harland 1991; Sokol et al. 1991, 1995; Rothbacher et al. 1995). The requirement for β-catenin has been established by antisense oligonucleotide-mediated depletion of β-catenin in *Xenopus* and zebrafish embryos (Heasman et al. 1994; Bellipanni et al. 2006). β-catenin activates several major targets in *Xenopus* embryos that are responsible for Spemann organizer formation. Activation of many organizer-specific genes depends on functional TCF sites in their promoters (Brannon et al. 1997; Laurent et al. 1997; McKendry et al. 1997; Fan et al. 1998; Ryu et al. 2001; Leung et al. 2003). These early β-catenin targets include *Nodal* group genes that are essential for dorsal mesoderm formation in frogs, zebrafish, and mammals (Conlon et al. 1994; Feldman et al. 1998; Osada

and Wright 1999; Takahashi et al. 2000; Yang et al. 2002; Hilton et al. 2003; Zhang et al. 2003). Besides *Nodals*, other direct targets of β-catenin are *Siamois* and *Twin* in *Xenopus* (Lemaire et al. 1995; Brannon et al. 1997; Laurent et al. 1997; Fan et al. 1998), and *Bozozok/Dharma/Nieuwkoid* in zebrafish (Ryu et al. 2001), which are required for organizer formation (Fig. 2; Table 1) (Fan and Sokol 1997; Kessler 1997; Koos and Ho 1998; Yamanaka et al. 1998; Fekany et al. 1999). Taken together, available data establish a key role for Wnt signaling and its early targets in early dorsoventral axis specification (Moon and Kimelman 1998; Schroeder et al. 1999; Sokol 1999; Tao et al. 2005).

Although lack of effect of a dominant–negative form of Dishevelled (Dvl) on axis specification does not eliminate Dvl participation; it suggests that β-catenin is stabilized by a downstream Wnt pathway component that is translocated dorsally during the cortical rotation (Sokol 1996; Harland and Gerhart 1997). One of the key factors is GSK3-binding protein or Frat, which inhibits GSK3 and is essential for dorsal development in *Xenopus* embryos (Yost et al. 1998). However, the triple knockout of the existing Frat homologs in the mouse does not produce a morphological phenotype, suggesting that this mechanism is not conserved in mammals (van Amerongen et al. 2005). More recently, antisense oligonucleotide-mediated depletion of maternal Wnt5a, Wnt11, and Fz7 indicated their roles in dorsoventral axis specification (Sumanas et al. 2000; Tao et al. 2005; Cha et al. 2008). These observations suggest a different model, in which Wnt5a and Wnt11 influence β-catenin levels in the oocyte, raising the question how β-catenin becomes localized to the dorsal side of the embryo. Because maternal Wnt11 RNA is vegetally localized (Ku and Melton 1993), one possibility is that Wnt11 is delivered to its future site of action by the cortical rotation (Tao et al. 2005). So far, dorsoventral patterning defects have not been described in embryos with genetically reduced Wnt5a or Wnt11 function (Rauch et al. 1997; Yamaguchi et al. 1999; Heisenberg et al. 2000; Kilian et al. 2003; Majumdar et al. 2003; Ulrich

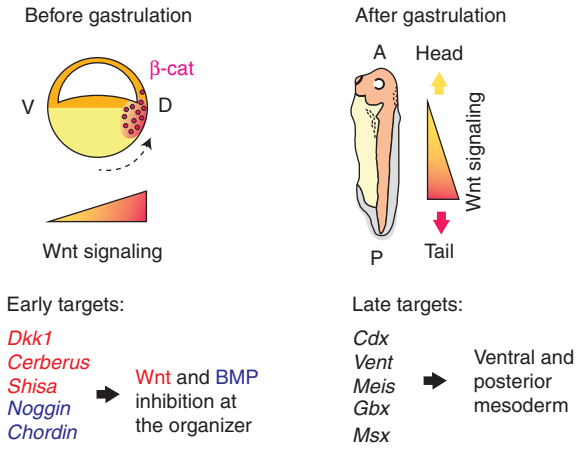

Figure 2. Axis specification by early and late Wnt signaling involves distinct targets. After cortical rotation (black dotted arrow), early β-catenin accumulation in the dorsal equatorial region activates gene targets to generate the Spemann organizer. β-Catenin up-regulates several Wnt and bone morphogenetic protein (BMP) antagonists, including *Dkk1*, *Cerberus*, *Shisa*, *Noggin*, and *Chordin*. The pathway is inhibited by Wnt antagonists in the anterior tissues, but the zygotic activation of Wnt8 causes ventral and posterior accumulation of β-catenin during gastrulation. The target genes of this late Wnt signaling, including *Cdx*, *Vent*, *Meis*, *Gbx*, and *Msx*, are critical for the specification of ventroposterior mesodermal fates and lead to tail formation.

Table 1. Putative Wnt targets during anteroposterior axis specification

Genes activated by Wnt3a	Genes inhibited by Dickkopf-1	References
Axin1/2	*Axin1/2*	Zeng et al. 1997; Jho et al. 2002
Kremen2	*Kremen2*	Mao et al. 2002
Cdx2, 4	*Cdx2, 4*	Isaacs et al. 1998
Gbx2	*Gbx2*	von Bubnoff et al. 1996
HoxA1/D1	*HoxA1/D1*	Kolm and Sive 1995
Esr9/10	*Esr9/10*	Li et al. 2003
Fz10	*Fz10*	Wheeler and Hoppler 1999; Garcia-Morales et al. 2009
Foxi1		Suri et al. 2005; Mir et al. 2007
Meis3	*Meis-like*	Elkouby et al. 2010
Msx1/2	*Msx1/2*	Maeda et al. 1997; Marazzi et al. 1997; Mathers et al. 1997; Suzuki et al. 1997; Willert et al. 2002
Nzl1	*Nzl1*	Andreazzoli et al. 2001; Nakamura et al. 2008
Nrh1		Bromley et al. 2004; Sasai et al. 2004
Riddle2		Shibata et al. 2008
Spr2		Weidinger et al. 2005
Vent/PV1	*Vent/PV1*	Onichtchouk et al. 1996
XARP	*XARP*	Itoh et al. 2000
Xmc		Frazzetto et al. 2002
Xpo	*Xpo*	Sato and Sargent 1991; Amaya et al. 1993; Hoppler et al. 1996

Only some of these genes have been shown to contain TCF/LEF sites in their promoters.

et al. 2003; Fossat et al. 2011). Potentially, these observations could be explained by functional redundancy or by the presence of maternally encoded proteins. On the other hand, overexpression of Wnt5a or Wnt11 does not lead to β-catenin stabilization (Torres et al. 1996; Mikels and Nusse 2006). Owing to these contradictory observations, the final conclusion regarding the involvement of these Wnt proteins in β-catenin stabilization and body-axis formation awaits additional experimental insights. Despite the variability in the existing models of vertebrate axis specification, there is a common outcome: the establishment of a signaling center that dorsalizes all three germ layers and mediates anteroposterior patterning.

ZYGOTIC WNT SIGNALING DURING ANTEROPOSTERIOR AXIS SPECIFICATION

Early studies revealed that the effect of Wnt proteins on embryogenesis is stage specific. When injected as mRNA, Wnt8 triggers secondary axis formation (Smith and Harland 1991; Sokol et al. 1991). However, when supplied as a plasmid, Wnt8 expression is initiated only at midblastula transition and the delayed stimulation leads to posteriorization, characterized by lack of head structures (Christian and Moon 1993). These observations correlated well with the time-sensitive effect of lithium chloride, a GSK3 inhibitor (Klein and Melton 1996), on anteroposterior patterning (Yamaguchi and Shinagawa 1989). Of note, Dkk1 RNA does not interfere with the early dorsal signaling, although being fully capable of inhibiting ectopic Wnt expression (Glinka et al. 1998; Brott and Sokol 2002), consistent with the idea that Wnt ligands are not accessible to Dkk-dependent inhibition at this early stage. These observations indicate that Wnt signaling plays different roles in axis specification at different developmental stages.

Several Wnt ligands, including Wnt3a, Wnt5a, Wnt8, and Wnt11, are expressed in the ventral or posterior region of the embryo (Christian et al. 1991a; Krauss et al. 1992; Moon et al. 1993; Kelly et al. 1995; Hong et al. 2008). On the other hand, multiple Wnt antagonists, such as Frzb/Sfrp3, Crescent, Shisa, and Dkk1, are expressed in the head region (Leyns et al. 1997; Wang et al. 1997; Glinka et al. 1998; Shibata et al. 2005; Yamamoto et al. 2005). This complementary expression pattern indicates a function for the Wnt pathway in anteroposterior axis specification. The involvement of Wnt signaling in anteroposterior patterning was first revealed in studies with overexpressed Wnt pathway modulators. Twofold titration of *Xenopus*

Dishevelled RNA caused graded appearance of region-specific positional markers, indicating a morphogenlike dose response with the highest point at the posterior of the embryo (Itoh and Sokol 1997). Wnt pathway antagonists, such as GSK3, Dkk1, and Shisa, cause anteriorization, in agreement with the proposed posteriorizing activity of Wnt ligands (Itoh et al. 1995; Glinka et al. 1998; Yamamoto et al. 2005). Further supporting this hypothesis, TCF-dependent reporter activity and the nuclear localization of endogenous β-catenin are elevated in the anterior-to-posterior gradient (Kiecker and Niehrs 2001; Dorsky et al. 2002).

The critical role of Wnt signaling in anteroposterior axis specification has been corroborated by depletion experiments for pathway components (Heasman et al. 2000; Erter et al. 2001; Lekven et al. 2001; Shimizu et al. 2005; Bellipanni et al. 2006; Hikasa et al. 2010) and the analysis of the corresponding zebrafish mutations. *Headless* (*hdl*) was identified as a point mutation in a *tcf3* gene homolog, which represses Wnt target genes in zebrafish embryos (Kim et al. 2000). Loss of forebrain and eyes and the modest expansion of ventral mesoderm in *hdl* embryos are consistent with up-regulated Wnt signaling. When both Headless and a closely related protein Tcf3b are down-regulated, this posteriorization is more severe (Dorsky et al. 2003). Similarly, *mastermind* zebrafish embryos with a mutation in *Axin1* are posteriorized with telencephalon and eyes transformed to diencephalon (Heisenberg et al. 2001). Thus, interference with Wnt pathway antagonists mimics Wnt protein overexpression and results in posteriorization. Phenotypes of mice lacking the genes for Dkk1, APC, TCF3, Wnt3, ICAT, and β-catenin also support the role of the Wnt pathway in posterior development (Takada et al. 1994; Liu et al. 1999b; Mukhopadhyay et al. 2001; Ishikawa et al. 2003; Merrill et al. 2004; Satoh et al. 2004). Combined with more recent studies of Cnidaria and Planaria (Holstein 2008; Martin and Kimelman 2009; Petersen and Reddien 2009), these findings establish a conserved key role for Wnt signaling in anteroposterior axis specification (Fig. 2).

PUTATIVE TRANSCRIPTIONAL TARGETS OF THE WNT PATHWAY IN AXIS SPECIFICATION

Molecular screens have identified transcriptional Wnt targets in early embryos and embryonic stem cells (Willert et al. 2002; Li et al. 2004; Hufton et al. 2006). A major obstacle to identifying Wnt gene targets is the stage and context dependence of cellular responses to Wnt ligands. Therefore, a special effort is needed to elucidate the Wnt targets that are relevant to anteroposterior axis specification.

Hybridization of RNA probes prepared from the control and Wnt3a-stimulated ectoderm cells with *Xenopus* copy DNA (cDNA) microarrays (Affymetrix) has identified a number of candidate Wnt targets (Table 1) (H Hikasa and SY Sokol, unpubl.). Among these are Wnt pathway components and modulators, such as Fz9, Fz10, Kremen 2, and the Axin family, highlighting the need for feedback regulation at this developmental stage. A second class contains many of the previously identified Wnt targets that are expressed ventrally or posteriorly. These genes are *Gbx2* (von Bubnoff et al. 1996), the *Cdx* group (Epstein et al. 1997; Haremaki et al. 2003; Shimizu et al. 2005; Keenan et al. 2006; Pilon et al. 2006), the *Vent/Vox/Ved/Xom* group (Gawantka et al. 1995; Ladher et al. 1996; Onichtchouk et al. 1996; Schmidt et al. 1996; Imai et al. 2001; Ramel and Lekven 2004; Thorpe and Moon 2004), and the *Meis* group (*Meis3*) (Choe et al. 2009; Elkouby et al. 2010). The remaining genes include some known bone morphogenetic protein (BMP)- and fibroblast growth factor (FGF)-inducible genes, reiterating the importance of the cross talk between these pathways, and novel putative Wnt targets (Table 1). Whereas some of these genes may be activated indirectly, *Meis3*, *Gbx2*, *Cdx4*, and *Vent1/2* are known to contain functional TCF-binding sites in their promoters that are required for Wnt responsiveness (Haremaki et al. 2003; Li et al. 2009; Elkouby et al. 2010; Hikasa et al. 2010). Supporting these findings, a complementary analysis revealed a similar list of genes that are inhibited in ventral mesoderm by Dkk1 (Table 1) (Hufton et al. 2006).

The *Cdx* genes, a group of four conserved vertebrate homologues of *Drosophila caudal*, have been widely implicated in posterior development (Northrop and Kimelman 1994; Isaacs et al. 1998; Pillemer et al. 1998; van den Akker et al. 2002; Shimizu et al. 2005, 2006; Faas and Isaacs 2009; Young and Deschamps 2009; Young et al. 2009; Beck and Stringer 2010). Loss of the *Cdx4* gene function in the zebrafish mutant *kugelig* shows its essential role in posterior development (Davidson et al. 2003). The promoter of the *Xenopus* and mammalian *Cdx4* gene is Wnt inducible and contains multiple TCF/LEF binding sites (Haremaki et al. 2003; Shimizu et al. 2005; Pilon et al. 2006). Up-regulation of *Cdx* genes causes the anterior shift of posterior markers in *Xenopus* and zebrafish (Isaacs et al. 1998; Shimizu et al. 2005; Faas and Isaacs 2009). Similar to *Cdx* genes, *Vent* family proteins (*Vent/Vox/Ved/Xom*) in *Xenopus* and zebrafish are homeodomain-containing transcriptional repressors that promote ventroposterior fates by suppressing organizer genes such as *bozozok* (in zebrafish), *chordin*, and *goosecoid* (Schmidt et al. 1996; Melby et al. 1999, 2000; Imai et al. 2001; Ramel and Lekven 2004; Sander et al. 2007). Inactivation of *Vent* genes results in a severe loss of ventroposterior structures and enhanced dorsoanterior fate, similar to the Wnt8 knockdown (Ramel and Lekven 2004; Sander et al. 2007). Together, these findings show that the Wnt pathway controls posterior development through multiple gene targets.

Putative Wnt target genes that are involved in anteroposterior patterning differ from the early β-catenin targets that mark the Spemann organizer region. These organizer-specific genes include *Xenopus Siamois* (Lemaire et al. 1995; Brannon et al. 1997; Fan et al. 1998) and *Twin* (Laurent et al. 1997), zebrafish *Bozozok/dharma/nieuwkoid* (Koos and Ho 1998; Yamanaka et al. 1998; Fekany et al. 1999), *Xnr3* (Smith et al. 1995), *Xnr5/6* (Takahashi et al. 2000; Yang et al. 2002) and several Wnt antagonists: *Frzb/Sfrp3* (Leyns et al. 1997), *Dkk1* (Glinka et al. 1998), *Crescent* (Shibata et al. 2000), *Cerberus* (Bouwmeester et al. 1996), *Shisa* (Yamamoto et al. 2005), as well as other genes listed in Table 2. The difference between the early and late targets for the Wnt pathway corresponds to the switch between stage-specific Wnt signaling mechanisms that are critical for vertebrate axis specification. For additional information about Wnt targets in different experimental systems, the reader is referred to Roel Nusse's Wnt Window (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

Table 2. Putative Wnt targets during dorsoventral axis specification

Genes activated by maternal β-catenin	References
Siamois	Lemaire et al. 1995; Brannon et al. 1997; Fan et al. 1998
Twin	Laurent et al. 1997
Xnr3	Smith et al. 1995
Goosecoid	Blumberg et al. 1991; Cho et al. 1991
Cerberus	Bouwmeester et al. 1996
Chordin	Sasai et al. 1994
Crescent	Shibata et al. 2000; Shibata et al. 2001
Noggin	Smith and Harland 1992
Xlim-1	Taira et al. 1994
Xnr5/6	Takahashi et al. 2000; Yang et al. 2002
Xnot1	von Dassow et al. 1993
Shisa	Yamamoto et al. 2005
Dkk1	Glinka et al. 1998
Frzb	Leyns et al. 1997
Frizzled 8	Deardorff et al. 1998; Itoh et al. 1998a
Bozozok	Koos and Ho 1998; Yamanaka et al. 1998; Fekany et al. 1999 (zebrafish-specific, also known as *Dharma* and *Nieuwkoid*)

Known antagonists of Wnt and BMP signaling are shown in bold.

REGULATION OF TCF PROTEIN FUNCTION DURING AXIS SPECIFICATION

How does Wnt signaling lead to target gene activation during anteroposterior patterning? A critical event seems to be TCF3 phosphorylation by HIPK2 allowing one to derepress the target genes (Hikasa et al. 2010; Sokol 2011), although

in some cases TCF3 is likely to function in a Wnt-independent manner (Cole et al. 2008; Gribble et al. 2009). Homeodomain-interacting protein kinases (HIPKs) have been shown to function in a context-dependent manner in response to Wnt signaling (Wei et al. 2007; Lee et al. 2009; Louie et al. 2009; Kim et al. 2010). Loss-of-function studies reveal that HIPK2 is required in vivo for Wnt8-dependent ventroposterior development and antagonizes the function of TCF3 in axis specification (Hikasa et al. 2010). Besides HIPK2, TCF3 phosphorylation requires β-catenin. TCF3 with a mutation that disrupts β-catenin binding is not phosphorylated (Hikasa et al. 2010). Thus, β-catenin appears to act by providing a scaffold for TCF3 phosphorylation by HIPK2. This is a novel function of β-catenin that differs from its commonly proposed role as transcriptional coactivator (Behrens et al. 1996; van de Wetering et al. 1997; Cavallo et al. 1998; Daniels and Weis 2005). Although TCF3 phosphorylation has been shown to require LRP6 activity and GSK3 inhibition (Hikasa and Sokol 2011), the mechanistic role of GSK3 in HIPK2 activation remains a mystery.

Nemolike kinase (Nlk) has been previously reported to phosphorylate TCF proteins (Ishitani et al. 1999; Meneghini et al. 1999) and is a positive regulator of Wnt8 signaling in anteroposterior patterning in zebrafish embryos (Thorpe and Moon 2004). In mammalian cells, Nlk and HIPK2 have been shown to regulate the stability of Myb oncoprotein in response to Wnt1 (Kanei-Ishii et al. 2004), indicating that these kinases might also function together during axis specification. The pathway leading to TCF regulation in vertebrates is reminiscent of Wnt signaling in *C. elegans*, because LIT-1, a homolog of Nlk, and WRM-1, a β-catenin paralog, phosphorylates POP-1/TCF to enhance its nuclear export, leading to transcriptional derepression (Rocheleau et al. 1997, 1999; Meneghini et al. 1999; Lo et al. 2004). Because POP-1 plays a dual role in gene target regulation, the reader is referred to other reviews for additional information (Phillips and Kimble 2009; Sokol 2011).

Chromatin precipitation analysis has shown that phosphorylated TCF3 is removed from target promoters, resulting in gene activation (Hikasa et al. 2010). Subsequent experiments showed that *Vent2* gene activation is accompanied by the removal of TCF3 from the promoter and its replacement by TCF1, an activator form of TCF that is resistant to HIPK2-mediated phosphorylation (Hikasa and Sokol 2011). This finding strongly supports the view that the four vertebrate homologs of TCF, expressed in a tissue-specific manner (Molenaar et al. 1998; Dorsky et al. 1999, 2003; Konig et al. 2000; Roel et al. 2003; Veien et al. 2005), play diverse roles in Wnt signaling and axis specification (Hamilton et al. 2001; Houston et al. 2002; Roel et al. 2002; Liu et al. 2005; Standley et al. 2006). Although both TCF3 and TCF1 can bind β-catenin and Groucho corepressors (Roose et al. 1998; Brantjes et al. 2001), the two proteins appear to function very differently. TCF3 serves exclusively as a transcriptional repressor, whereas TCF1 is a transcriptional activator (Kim et al. 2000; Gradl et al. 2002; Houston et al. 2002; Merrill et al. 2004; Liu et al. 2005; Nguyen et al. 2006; Gribble et al. 2009; Hikasa and Sokol 2011). Additional studies are needed to explain the difference in the activity of TCF1 and TCF3, which is likely caused by a distinct set of interacting partners.

PATHWAY SWITCH MECHANISMS

Whereas the early dorsal accumulation of β-catenin is essential for the activation of organizer genes, it is later followed by the stabilization of β-catenin ventrally (Kiecker and Niehrs 2001; Schohl and Fagotto 2002; Hikasa et al. 2010). This reversed distribution of β-catenin correlates with the ventroposterior expression of Wnt8 (Christian et al. 1991a,b; Kelly et al. 1995; Lekven et al. 2001) and dorsoanterior expression of several Wnt antagonists in both *Xenopus* and zebrafish (Fig. 2). Thus, a likely explanation for this change is that dorsal β-catenin stimulates the transcription of pathway antagonists, such as *Dkk1, Shisa, FrzB*, and, likely, *Axin* family proteins (Leyns et al. 1997; Glinka et al. 1998; Yamamoto et al. 2005). The negative feedback from Wnt antagonists decreases β-catenin in the dorsoanterior region, whereas

ventroposterior activity of Wnt8 up-regulates β-catenin ventrally. In agreement with this model, the increased level of β-catenin in ventroposterior tissues relies mostly on Wnt8 activity, rather than dorsally derived signals (Hikasa et al. 2010).

Although the molecular cause for the switch from early dorsal Wnt signaling to late ventroposterior Wnt signaling has not yet been identified, the gene targets for each round of signaling appear to be very different. A number of questions remain to be answered. (1) What prevents late Wnt targets from being activated early at the dorsal side? (2) Why are the early targets silent ventrally at gastrulation when β-catenin is stabilized in response to Wnt8 signals at later stages? A possible explanation of this context dependence is related to epigenetic changes in chromatin that underlie the developmental control of transcription (Li 2002; Kouzarides 2007; Ng and Gurdon 2008; Akkers et al. 2009; Xu et al. 2010). A plasmid reporter for the *Siamois* gene failed to respond to a constitutive TCF activator after gastrulation (Darken and Wilson 2001), suggesting that the stage-dependent regulation of target genes depends on some downstream components of the pathway. Moreover, β-catenin can modulate histone methylation of poised genes, indicating local epigenetic changes in chromatin (Blythe et al. 2010). Thus, ventroposterior Wnt targets, such as *Vent* or *Cdx*, may be poised for activation at a later time, predicting the existence of epigenetic differences in the chromatin state that are critical for axis specification.

Another explanation is that functionally diverse vertebrate TCF proteins are responsible for the early to late transition in Wnt target specificity. Besides the distinct roles of TCF proteins as discussed above, the switch from early to late Wnt signaling can be linked to the phosphorylation of TCF by Wnt8/HIPK2, attenuating target DNA binding. This takes place only after the beginning of gastrulation, likely depending on a factor that becomes available after the onset of zygotic transcription. Notably, Wnt8/HIPK2 signal can trigger the phosphorylation of TCF3, TCF4, and Lef1 (but not TCF1), owing to the conservation of the HIPK2 phosphorylation sites. Thus, context-dependent activation of early and late Wnt target genes may be explained by the opposing action of tissue-specific TCF proteins and their distinct response to Wnt8-mediated phosphorylation (Sokol 2011). Whether this explanation is correct remains to be experimentally tested.

CROSS TALK WITH OTHER PATHWAYS

Organizer-derived posteriorizing signals reach neuroectoderm during gastrulation to specify the anteroposterior axis (Nieuwkoop 1952; Doniach et al. 1992; Holowacz and Sokol 1999). Besides Wnt signaling, this process requires additional pathways, such as BMP and FGF signaling. The region- and stage-specific integration of distinct signals contributes to the differential selection of pathway targets that is crucial for the establishment of embryonic axes. The best known examples are the cross talk between the Wnt pathway and the BMP pathway in the ventroposterior region (Itasaki and Hoppler 2010) or FGF/IGF (insulinlike growth factor) receptor tyrosine kinase signaling dorsally and posteriorly (Schohl and Fagotto 2002; Pera et al. 2003; Kudoh et al. 2004; Marchal et al. 2009).

Both BMP and Wnt signaling are involved in setting up ventroposterior gene expression in vertebrate embryos (Hoppler and Moon 1998; De Robertis and Kuroda 2004; Schier and Talbot 2005). The synergistic function of Wnt8 and BMP4 in ventral mesoderm patterning has been first shown in *Xenopus* embryos (Hoppler and Moon 1998) and confirmed by genetic studies in zebrafish. Reduced *Vent* expression in zebrafish embryos depleted of β-catenin or containing a mutation in the *Wnt8* gene (Ramel and Lekven 2004; Bellipanni et al. 2006) indicates a requirement for the Wnt pathway in *Vent* activation. The *Wnt8* and *Swr* (*Bmp2*) double mutants display a progressive reduction of *Vent* gene expression and a concomitant expansion of the dorsal domain (Ramel and Lekven 2004; Ramel et al. 2005). The synergy between BMP and Wnt signaling has been documented in many other developmental processes, including bone, kidney, limb, and tooth development (Perantoni 2003; Tucker and Sharpe 2004;

Hartmann 2006; Yokoyama 2008; Itasaki and Hoppler 2010).

In contrast to the BMP pathway that is active in the ventral region of the blastula embryo, FGF signaling seems to be essential for dorsal posterior patterning (Amaya et al. 1991; Cox and Hemmati-Brivanlou 1995; Lamb and Harland 1995; Holowacz and Sokol 1999; Schohl and Fagotto 2002; Kudoh et al. 2004; Marchal et al. 2009; Martin and Kimelman 2009). Many Wnt-inducible genes, including *Cdx4, Xpo, Meis*, and *Marginal coil (Xmc)*, have been previously identified as FGF-responsive genes (Table 1) (Amaya et al. 1993; Frazzetto et al. 2002; Aamar and Frank 2004; Chung et al. 2004; Keenan et al. 2006). In studies using dominant–negative FGF receptor constructs and specific morpholino oligonucleotides, FGF signaling has been reported to be essential for the Wnt pathway to activate its targets during anteroposterior axis development (McGrew et al. 1997; Maegawa et al. 2006; Burks et al. 2009). The cross talk between the Wnt and the FGF pathway has been preserved in many other models, including brain patterning, limb formation, tail regeneration, and lung and kidney morphogenesis (Martinez et al. 1999; Schedl and Hastie 2000; Kawakami et al. 2001; Lupo et al. 2002; Villanueva et al. 2002; Lin and Slack 2008).

The mechanisms underlying these examples of pathway cross talk are being revealed. In some cases two pathways operate sequentially, with one pathway inducing the other. For example, FGF8a signaling transcriptionally activates Wnt8 to promote neural crest development (Fig. 3A) (Hong et al. 2008). Alternatively, two pathways may function in parallel, when a target promoter contains transcription factor binding sites that are specific for each pathway (Fig. 3B). Both Wnt and BMP signals promote ventral fate and directly activate the expression of *Vent* genes. The *Vent2* promoter contains a unique Wnt-responsive TCF-binding site and the Smad and OAZ sites needed for the BMP response (Candia et al. 1997; Hata et al. 2000; Karaulanov et al. 2004; Hikasa et al. 2010). Mutagenesis of individual promoter elements indicates that the Wnt and the BMP pathways separately control the *Vent2* gene (Hikasa et al. 2010).

Similarly, a combination of Wnt and FGF signals leads to a robust expression of *Cdx4* in *Xenopus* and zebrafish (Shimizu et al. 2005; Keenan et al. 2006). The regulatory region of the *Cdx4 gene* contains multiple TCF and Ets

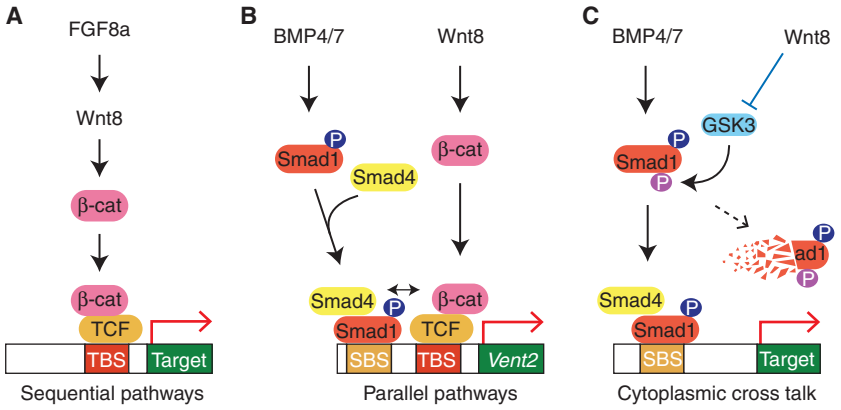

Figure 3. Cross talk between Wnt, BMP, and FGF pathways during axis specification. (*A*) Sequential interaction of two pathways. FGF8a induces the expression of Wnt8 transcripts to promote neural crest formation in *Xenopus* neurulae. (*B*) Parallel interaction of two pathways at a target promoter (*Vent2*). *Vent2* is up-regulated by BMP proteins acting through Smad1 interacting with the Smad binding site (SBS), whereas Wnt proteins act through TCF and the TCF-binding site (TBS). Both signals are responsible for the maximal activation of *Vent2*. (*C*) The cytoplasmic integration of two pathways. GSK3 phosphorylates the activated form of Smad1, marking it for degradation. Wnt proteins signal to inhibit or sequester GSK3, thereby promoting BMP target gene activation.

DNA-binding elements that are located in close proximity (Haremaki et al. 2003). This structure of the *Cdx4* regulatory region explains the observation that *Cdx4* is a direct target of both Wnt and FGF pathways, because Ets factors are known to regulate gene expression downstream from FGF (Nentwich et al. 2009; Znosko et al. 2010). This transcriptional cross talk does not preclude a more close interaction, in which two transcription factors interact physically, resulting in a synergistic response (Fig. 3B). The physical interaction of LEF1 with Smad3 and Smad4 may help explain the cross talk between Wnt and TGFβ signaling, relevant to the specification of the primary dorsoventral axis by early Wnt signaling (Sokol and Melton 1992; Sokol 1993; Labbe et al. 2000; Nishita et al. 2000).

A direct cytoplasmic mechanism has been proposed for Wnt-BMP and FGF-BMP pathway cross talk. Smad1 proteins are phosphorylated by MAPK in response to FGF and IGF, targeting Smad1 for degradation by the proteasome (Massague 2003; Pera et al. 2003; Sapkota et al. 2007). Similarly, GSK3 phosphorylates Smad1 and this phosphorylation can be inhibited by Wnt signaling in tissue culture cells (Fig. 3C) (Fuentealba et al. 2007; Sapkota et al. 2007). These phosphorylation events may provide specific interfaces for pathway cross talk. Nevertheless, this mode of regulation may not be the main mechanism for Wnt8-mediated anteroposterior axis determination, because no significant change in the amount of active Smad1 (phosphorylated at the carboxyl terminus) is observed in *Xenopus* embryos with altered Wnt8 activity (Hikasa et al. 2010). On the contrary, the requirement for the TCF-binding site in *Vent2* reporter activation and the regulation of TCF by Wnt signaling reiterates the importance of TCF regulation for Wnt signaling (Hikasa et al. 2010).

Besides FGF and BMP signaling, there are examples of Wnt pathway cross talk with other signaling pathways, especially those linked to proteasome-mediated protein degradation. The β-transducin repeat-containing protein (βTrCP) is an E3 ubiquitin ligase that represents a nodal point for down-regulation of β-catenin, Gli, and NF-κB levels (Liu et al. 1999a; Maniatis 1999). As Wnt signaling has been shown to up-regulate βTrCP activity in a negative-feedback loop (Spiegelman et al. 2000), the effect on the Hh and NF-κB signaling may be expected. Also, the Wnt pathway negatively regulates the activity of GSK3 (Cook et al. 1996; Itoh et al. 1998b; Taelman et al. 2010), an enzyme that has been implicated in the regulation (e.g., degradation) of a large number of proteins, in addition to β-catenin (Zhou et al. 2004; Xu et al. 2009). These findings suggest the interaction of the Wnt pathway with many signaling pathways, which is likely to be better understood in the course of future studies.

CONCLUDING REMARKS

Recent studies support the view that Wnt signaling plays a dual role in vertebrate axis specification. Initially, the maternally encoded components of the pathway help to establish the dorsoventral axis, whereas at a later stage the zygotic Wnt pathway is involved in anteroposterior axis specification. These two roles are mediated by two distinct sets of specific targets, yet the underlying mechanisms for target selection are unclear. Further work is needed to understand the role of maternally produced Wnt ligands in early axis determination in the context of the abundant evidence for the involvement of microtubule-dependent trafficking in the dorsal accumulation of β-catenin. Although HIPK2-mediated phosphorylation of TCF3 is a major route for anteroposterior target activation, this phosphorylation is undetectable before gastrulation, suggesting an alternative mechanism for early target derepression (Hikasa et al. 2010). Other possibilities should also be explored, given that β-catenin can use other cofactors, besides TCFs, for transcriptional control (Olson et al. 2006; Takao et al. 2007; Abu-Remaileh et al. 2010). Future research will be driven by the need to better understand pathway target selection and the contribution of Wnt5 and Wnt11 signaling to body-axis specification.

ACKNOWLEDGMENTS

We thank Stefan Hoppler, Keiji Itoh, and Jenya Grinblat for comments on the manuscript. We

apologize to those investigators, whose work has not been cited here because of limited space. The work in the Sokol laboratory is supported by National Institutes of Health grants.

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Cold Spring Harbor Perspectives in Biology

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Cold Spring Harb Perspect Biol 2013; doi: 10.1101/cshperspect.a007955 originally published online August 22, 2012