MECHANISMS OF WNT8 FUNCTION IN ZEBRAFISH MESODERM PATTERNING

A Dissertation

by

MARIE-CHRISTINE RAMEL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Biology

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ABSTRACT

Mechanisms of Wnt8 Function in Zebrafish

Mesoderm Patterning. (May 2005)

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Chair of Advisory Committee: Dr. Arne Lekven

In vertebrate embryonic development, correct specification of tissue fates along the dorsoventral (D/V) axis is known to require the secreted signaling ligand Wnt8. Wnt8 signaling promotes ventral fates and antagonizes the expansion of the dorsal domain known as the organizer. Maintenance of the organizer is critical for proper development as this tissue is known to produce inhibitors of Wnt and BMP (Bone Morphogenetic Protein) family ligands; BMPs are also known to play a major role in promoting ventral fates. In order to understand how Wnt8 antagonizes the organizer, we analyzed the epistatic relationship between Wnt8 and the transcriptional repressors Vent and Vox using zebrafish as a model organism. We found that Wnt8/β-catenin signaling directly regulates the transcriptional levels of *vent* and *vox* so that they can repress the transcription of dorsal genes on the ventral side of the embryo. To understand the contribution of Wnt8 towards ventral fate specification, we carefully analyzed its relationship with BMP signaling during gastrula stages. We found that *bmp* expression in the mesoderm is under the control of Wnt8 at mid-gastrulation and that regulation of

bmp explains many of the ventral defects observed in *wnt8* mutants. Antagonism of the expression of organizer-derived BMP inhibitors by Wnt8 also indirectly allows timely BMP signaling. Analysis of *wnt8*; *bmp* double mutants revealed an early unsuspected function of BMP in the antagonism of the organizer. Further, we uncovered a mechanism through which regulation of *vent*, *vox* and a related-gene *ved* expression by both Wnt8 and BMP antagonizes dorsal/axial mesoderm identity to preserve the integrity of ventral/non-axial tissues. In summary, we have revealed some of the mechanisms of Wnt8 function in D/V mesoderm patterning: it restricts the organizer domain by regulating *vent* and *vox*, it allows BMP induced differentiation through its inhibition of BMP antagonists derived from the organizer and it co-regulates *vent*, *vox*, and *ved* with BMP signaling to allow maintenance of the non-axial domain.

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LIST OF ABBREVIATIONS

Abbreviation	Description	Function
ActRIIB	Activin Receptor type II B	type II serine-threonine kinase receptor (for Nodal ligands)
A/P	Antero-Posterior	
APC	Adenomatous Polyposis Coli	part of the β-catenin destruction complex
BMP	Bone Mophogenetic Protein	secreted signaling ligand
BMPRIA	type I BMP Receptor-A (Alk3)	BMP receptor
BMPRIB	Type II BMP Receptor-B (Alk6)	BMP receptor
Boz	Bozozok	trancription factor
BrdU	BromodeoxyUridine	nitrogenous base analog, incorporates into DNA during synthesis
Cad1	Caudal-homeobox 1	trancription factor
CA	Consistitutively Active	
Chd	Chordin	secreted BMP inhibitor
CHX	Cycloheximide	protein synthesis inhibitor
DEX	Dexamethasone	hormone
dino	*chordino*	zebrafish *chordin* allele
Dkk1	Dickkopf-1	Wnt antagonist
DNBRIA	Dominant-Negative BMP Receptor type I A	inhibits signaling mediated by BMPRIA
DNBMPRIB	Dominant-Negative BMP Receptor type I B	inhibitits signaling mediated by BMPRIB
D/V	Dorso-Ventral	
Eve1	Even-skipped-1	transcription factor
Flh	Floating head	transcription factor
Frzb	Frizbee	Wnt antagonist
Fz	Frizzled	Wnt receptor
G1	Gap 1	Phase of the cell cycle prior to S phase
Gsc	Goosecoid	transcription factor
GSK3-β	Glycogen Synthase Kinase 3β	part of the β-catenin destruction complex
HPF	Hours Post Fertilization	
Lef	Lymphoid enhancing factor	transcription factor

Abbreviation	Description	Function
LiCl	Lithium Chloride	chemical that dorsalizes embryos by inhibiting GSK3-β function
LRP	Low-density Lipoprotein related protein	Wnt co-receptor
MO	Morpholino	antisense oligonucleotide
MZoep	*Maternal Zygotic one-eyed pinhead*	zebrafish mutant for maternal and zygotic *one-eyed pinhead* (Nodal co-receptor)
Ntl	No tail	transcription factor
Opl	Odd-paired-like	zinc ion binding protein
OPT	Opl-Pax2a-Tbx6	probe cocktail
ORF	Open Reading Frame	
Radar DN	Radar Dominant-Negative	dominant negative form of Radar that inhibits maternal BMP signaling upon overexpression
RT-PCR	Reverse Trancriptase Polymerase Chain Reaction	technique used to generate complementary DNA from total RNA
S	S phase	DNA replication phase of the cell cycle
sbn	*somitabun*	zebrafish *smad5* allele
snh	*snailhouse*	zebrafish *bmp7* allele
swr	*swirl*	zebrafish *bmp2b* allele
Tbx24	T-box 24	ATP binding protein
Tbx6	T-box gene 6	transcription factor
Tcf	T-cell factor	trancription factor
TUNEL	Tdt-mediated-dUTP Nick-End Labelling	technique used to detect apoptotic cells
UV	Ultra-Violet	
YSL	Yolk Syncytial Layer	Cell layer in the zebrafish embryo, located vegetally to the mesendoderm and source of signals that induce formation of the mesendoderm

CHAPTER I

INTRODUCTION

During the embryogenesis of bilaterian animals, the establishment of the primary axes (dorsoventral, anteroposterior, and left-right) is an essential early step that determines the body plan. This process coincides with the formation of the three primitive germ layers: ectoderm, mesoderm, and endoderm. The mesoderm is the layer that gives rise to structures such as heart, kidneys, and muscles. The precursor cells of these organs are laid out in a orderly fashion along the dorsoventral (D/V) axis of an early embryo. The creation of fate maps has allowed the determination of the fates of tissues located at different positions along the D/V axis. Thus, in vertebrates, blood is known to be a mesodermal ventral fate as blood precursor cells map to the ventral side of the embryo. Conversely, the brain and notochord are considered to be dorsal ectodermal- and mesodermal-derived structures respectively. An example of a fate map of the mesoderm in the vertebrate model system zebrafish (*Danio rerio*) is shown in Fig. 1.

This dissertation follows the style and format of *Development.*

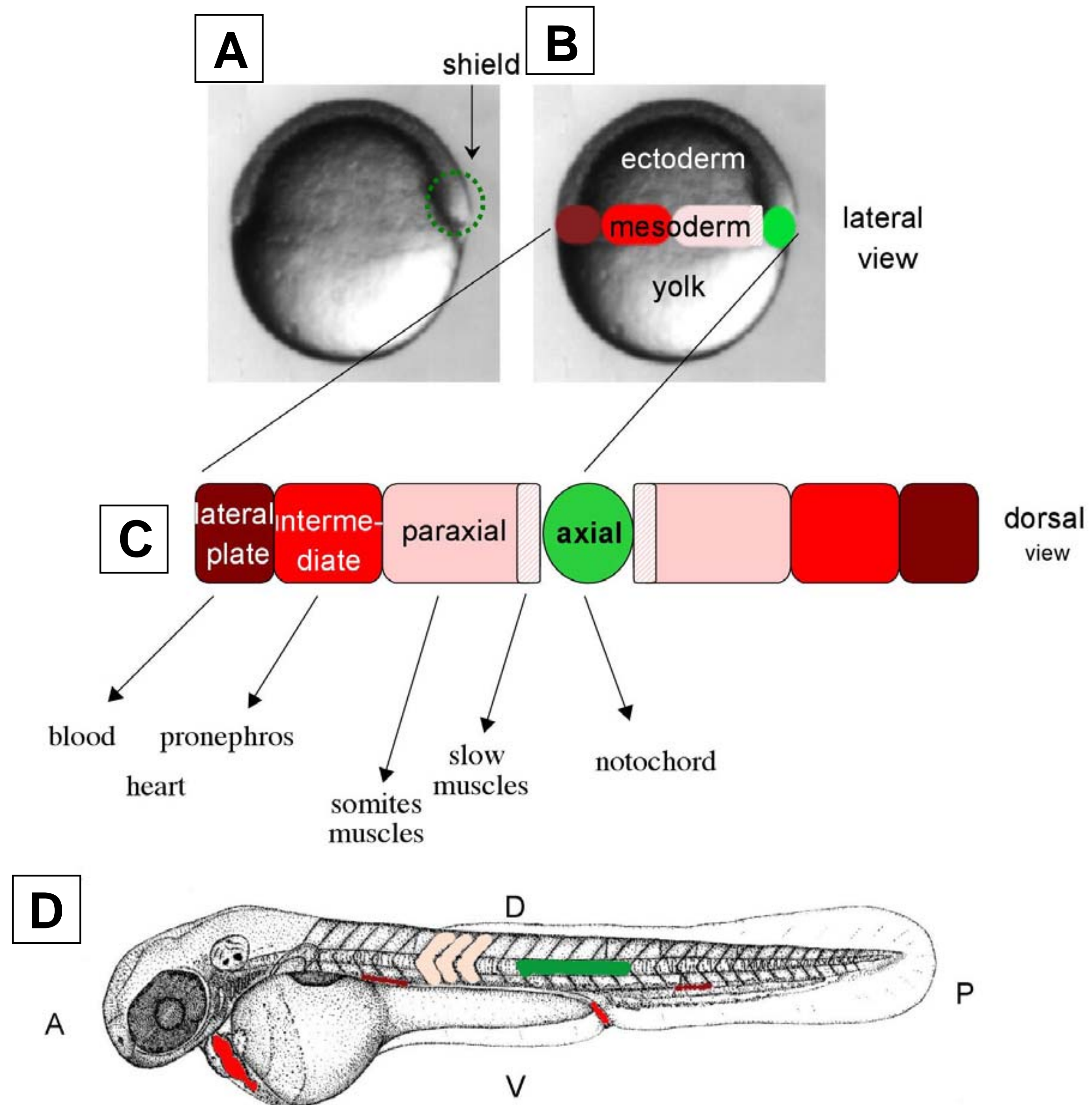

Fig.1. Overview of D/V fates in gastrulating zebrafish embryos and corresponding larval tissues. A: 6-hour-old embryo, lateral view. The first physical sign of asymmetry is a thickening of the blastoderm (embryo proper) dorsally that is called the shield. B: the embryo proper sits on a yolk cell and shows typical arrangement of the three germ layers: ectoderm, mesoderm, and endoderm (not shown, below mesoderm). C: detailed fate map of mesoderm and corresponding derived tissues. D: corresponding larval tissues relative to the mesodermal fate map. (adapted from Kimmel et al., 1995).

While the accuracy of most vertebrate fate maps has been widely accepted, recent research in the frog (*Xenopus laevis*) argues that the original designation of axes was erroneous (Lane and Sheets, 2002). For instance, the original position of the D/V axis in amphibian embryos would actually be the position of the A/P axis based on more recent lineage tracing experiment. Despite these findings, most of the research community still uses the original nomenclature and this will be the one used in this study when discussing amphibian development. As for axes designation in zebrafish embryos, we believe that the current model (shown in Fig. 1) is correct.

The molecular events that establish the primary axes have been the object of a large amount of research and it was shown that the molecules involved are conserved. In the invertebrate model system *Drosophila melanogaster,* the D/V axis is specified during early cleavage and is dependent on maternally provided localized determinants deposited in the egg prior to fertilization (Gilbert, 2003). In vertebrates like *Xenopus* and zebrafish, formation of the D/V axis results from a complex series of events that include regulated transport of maternally provided molecules, establishment of signaling centers, and subsequent cell-cell signaling (De Robertis et al., 2000; Schier, 2001).

The formation of the D/V axis in vertebrate embryos is the focus of this study. Historical experiments that initiated this field of research will first be described. The molecules and mechanisms involved will then be discussed, with particular focus on D/V axis maintenance as well as mesoderm formation. Lastly, the working hypotheses that were tested in the model system zebrafish will be presented.

THE D/V AXIS: PERSPECTIVE FROM HISTORICAL EMBRYOLOGY EXPERIMENTS

The first vertebrate studies of D/V axis formation were performed at the turn of the twentieth century by Hans Spemann and his colleagues (reviewed in Fäßler, 1996; Hamburger, 1988). Hans Spemann used amphibian eggs in most of his experiments because of their large size and because they were easily available.

In 1903, he performed a series of constriction experiments where he divided a newly fertilized salamander egg along different cleavage planes. He then allowed the isolated cells to develop. He found that when the embryo was divided along a certain cleavage plane, one cell would give rise to a normal embryo while the other one would give rise to a belly piece or "bauchstück" that lacked dorsal structures. This experiment was the first evidence that a dorsalizing factor is asymmetrically distributed in an amphibian embryo. While the cell that inherited this factor developed normally, the cell lacking it only developed ventral tissues. Thus, this experiment also suggests that 'ventral' may be a default state that is acquired in the absence of dorsalizing signal.

Further constriction experiments addressed the regulation ability of embryos. Cells that show regulative development adopt a fate that is dependent on their surrounding tissue. Spemann showed that the later the constriction was performed, the less the embryo was able to regulate and develop normally. In the 1920s, Spemann and his doctoral student Hilde Mangold, then performed a series of transplantation experiments to determine what accounted for the difference in the ability of early or late

embryos to perform regulation. They found that a transplanted tissue from a young gastrula donor usually develops and adopts the fate of his host tissue. This means that its prospective fate has not been irreversibly determined and that it can perform regulation. However, the same experiments using older gastrula showed that the transplanted tissue developed according to its original donor fate. Only one piece of transplanted tissue did not follow the rule. Spemann and Mangold found that transplantation of a young gastrula tissue that locates to what is now called the dorsal lip of the blastopore has the unique ability to induce neural differentiation (a dorsal fate) when transplanted in the epidermis of a host embryo. Further transplantation experiments performed by Hilde Mangold showed that a secondary axis, including a neural tube, notochord and somites, could be obtained and that this new axis was composed of both host and donor tissues. The transplanted dorsal lip of the blastopore that induces and organizes its surrounding tissues into neuroectoderm and dorsal mesoderm is now called the Spemann's organizer. Interestingly, not all transplantation experiments resulted in complete secondary embryos: some would show only anterior (head) structures but share a common trunk and tail with the host embryo, other secondary embryos would mostly display trunk and tail but share one head. Spemann therefore postulated that the organizer may actually display regional specificities, that is it could be divided into a head and a trunk-tail organizer. Later work by Otto Mangold tested this hypothesis by transplanting various parts of the dorsal mesoderm and he found that different parts did indeed show different abilities.

The importance of the dorsalizing signal first identified in the constriction experiments mentioned above was highlighted in more recent experiments in *Xenopus*. It was found that UV (ultra-violet) irradiation or cold temperature treatment of *Xenopus* eggs gives rise to ventralized embryos (Scharf and Gerhart, 1983). Indeed, "bauchstück" pieces are obtained upon these treatments. Thus, UV treatment and cold temperature must inhibit the dorsalizing signal first identified in the constriction experiments. Conversely, treatment of embryos with lithium chloride (LiCl) dorsalizes embryos (Kao and Elinson, 1989; Stachel et al., 1993). These various treatments not only affect the D/V axis but the A/P axis also appears disrupted. A ventralized embryo would also be posteriorized (lacking anterior structures) and a dorsalized embryo would be anteriorized (lacking posterior structures). Thus, these experimental treatments show multiple effects on the establishment of the body plan that may or may not be related to each other. It is a possibility that Spemann's hypothesis about the head and trunk-tail organizer may be at play in these observations. In a recent study, Agathon et al. (2003) found that zebrafish embryos appear to have a distinct tail organizer located ventrally. Transplantation of this presumptive tail organizer leads to embryos with an ectopic tail but no ectopic head. Thus, the fact that treatments that affect D/V axis formation also affect A/P axis formation could be explained by the two-organizer model. Treatments that dorsalize embryos may also dorsoanteriorize because they involve head organizer function. Conversely, treatments that ventralize embryos may also posteriorize because it affects the tail organizer. At a molecular level, it is possible that ventralization and posteriorization or dorsalization and anteriorization involve common factors.

FORMATION OF THE DORSAL SIGNALING CENTER

The following description of D/V axis formation is a summary of several decades of research in both amphibians and zebrafish that determined the mechanisms at work and the molecules involved in this process.

One of the first events during vertebrate embryogenesis is the specification of 'dorsal'. The dorsal side is established in two steps: a signaling center known as the Nieuwkoop center is first established in dorsal vegetal cells that subsequently induces the dorsal mesoderm above it to become the Spemann's organizer.

In *Xenopus*, fertilization of the egg by the sperm triggers a series of cytological events that lead to the movement of maternally provided molecules located in the vegetal pole towards the future dorsal side of the embryo. Microtubule transport is thought to be involved in this process. The outcome of this event is the accumulation of β-catenin molecules in dorsal nuclei (reviewed in Weaver and Kimelman, 2004). Thus, β-catenin is the putative asymmetrically distributed dorsalizing signal that was first identified during Spemann's constriction experiments. UV treatment was found to inhibit microtubule polymerization hence preventing cortical rotation and accumulation of β-

catenin dorsally. Conversely, LiCl was found to inhibit a protein called GSK3β (Glycogen Synthase Kinase 3β), which is known to negatively affect β-catenin function (Klein and Melton, 1996). Thus, LiCl treatment leads to hyperactivation of β-catenin function, hence dorsalization. Once it accumulates in the nucleus, β-catenin acts as a transcriptional activator that leads to the transcription of genes the expression of which defines the position of the dorsal signaling center known as the Nieuwkoop center. The Nieuwkoop center in *Xenopus* is characterized by the expression of genes such as Xnrs (nodal related molecules) and *siamois* (Gilbert, 2003).

In zebrafish, a similar system employing the accumulation of β-catenin in dorsal nuclei is required to establish the dorsal side of the embryo (Fig. 2; reviewed in Hibi et al., 2002). Genes expressed in the Nieuwkoop center include the transcriptional repressor *bozozok* (*boz*; Fig. 2) and the Nodal related molecule *squint* among others. *boz* mutants display axial structures defects and have reduced dorsal gene expression and expanded expression of ventral genes (Solnica-Krezel and Driever, 2001), suggesting that Boz function is an essential one in the Nieuwkoop center. Together with β-catenin, Squint and Boz are required for the expression of genes expressed in the dorsal mesoderm that becomes the organizer.

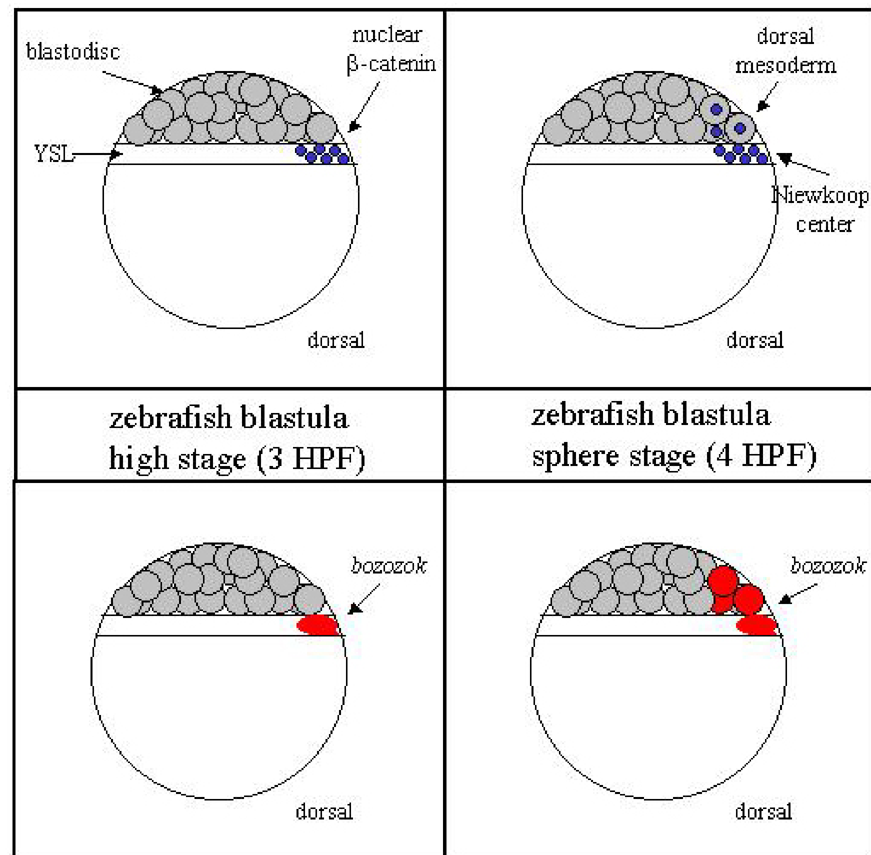

Fig. 2. Correspondence between nuclear β-catenin accumulation and *boz* gene expression. Accumulation of β-catenin in nuclei on one side of the embryo indicates that it is dorsal. β-catenin first accumulates in nuclei of the yolk syncytial layer (YSL) and turns on the expression of *bozozok* in the YSL. An hour later, β-catenin molecules are also observed in the nuclei of cells in the dorsal mesoderm (above the Nieuwkoop center). *bozozok* expression is also induced in this region, which will become the dorsal organizer (adapted from Hibi et al., 2002).

The next step of D/V axis formation is the formation of the organizer signaling center (reviewed in De Robertis et al., 2000; Hibi et al., 2002). It expresses transcription factors and secreted molecules. Transcription factors include *goosecoid* (*gsc*) and *floating head* (*flh*). Among the secreted molecules are Chordin (Chd), Noggin, and Follistatin, which inhibit the signaling molecules called BMPs (Bone Morphogenetic Proteins). The organizer also secretes Dickkopf1 (Dkk1) and a Frizzled Related Protein (Frzb), which are inhibitors of secreted molecules called Wnts as well as FGFs (Fibroblast Growth Factors). FGF activity was recently shown to be important in dorsoventral patterning as it downregulates *bmp* expression dorsally (Fürthauer et al., 2004).

The importance of the organizer has been highlighted in some zebrafish genetic studies. Mutants that lack functional organizer proteins display severe dorsal defects. For instance, the *chordino* (*dino*) mutant lacks a functional Chd protein and displays a ventralized phenotype (Hammerschmidt et al., 1996) and the *flh* mutant lacks a notochord, which is a dorsal-derived structure (Talbot et al., 1995). In the absence of Boz function, which is also expressed in the organizer, embryos display defects in dorsal structure formation (Solnica-Krezel and Driever, 2001). Thus, a smaller organizer or reduced organizer function strongly affects D/V patterning. Conversely, an increase in the amount of organizer derived factors such as Noggin or Dkk1 leads to a dorsalized phenotype (Fürthauer et al., 1999; Hashimoto et al., 2000). Therefore, the presence of the organizer is essential for proper D/V patterning and the maintenance of its size is critical to achieve normal development.

D/V MAINTENANCE AND PATTERNING

During and after the specification of the region that will be dorsal, molecules expressed ventrally are required to specify ventral fates. These molecules include BMPs, Wnt8, Vent and Vox.

BMPs are members of the TGFβ superfamily of secreted molecules that also include Nodals and Activin. BMP signaling is involved in many biological processes including differentiation, proliferation, and apoptosis. A schematic of the BMP signaling pathway is shown in Fig.3 (also reviewed in Shi and Massagué, 2003). BMP ligands are known to form dimers to bind a complex of serine-threonine kinase receptors (type I and type II). In zebrafish, type I BMP receptors include Alk3, 6, and 8 and they confer the binding specificity. Indeed, BMPs are known to show a higher affinity for type I receptors than for type II receptors. Activation of the receptors by BMP dimers leads to phosphorylation of the kinase domain of the type I receptor by the type II receptor. The kinase part of the type I receptor then phosphorylates an effector protein Smad (Smad 1, 5 or 8) associated with the receptor complex. The activated Smad further associates with a cytoplasmic co-Smad (Smad4). The Smad complex then translocates into the nucleus where it recruits other transcription factors at the promoter of BMP target genes. Depending on the cell types and the co-factors present (co-repressors or co-activators), BMP signaling may either inhibit or activate transcription of target genes.

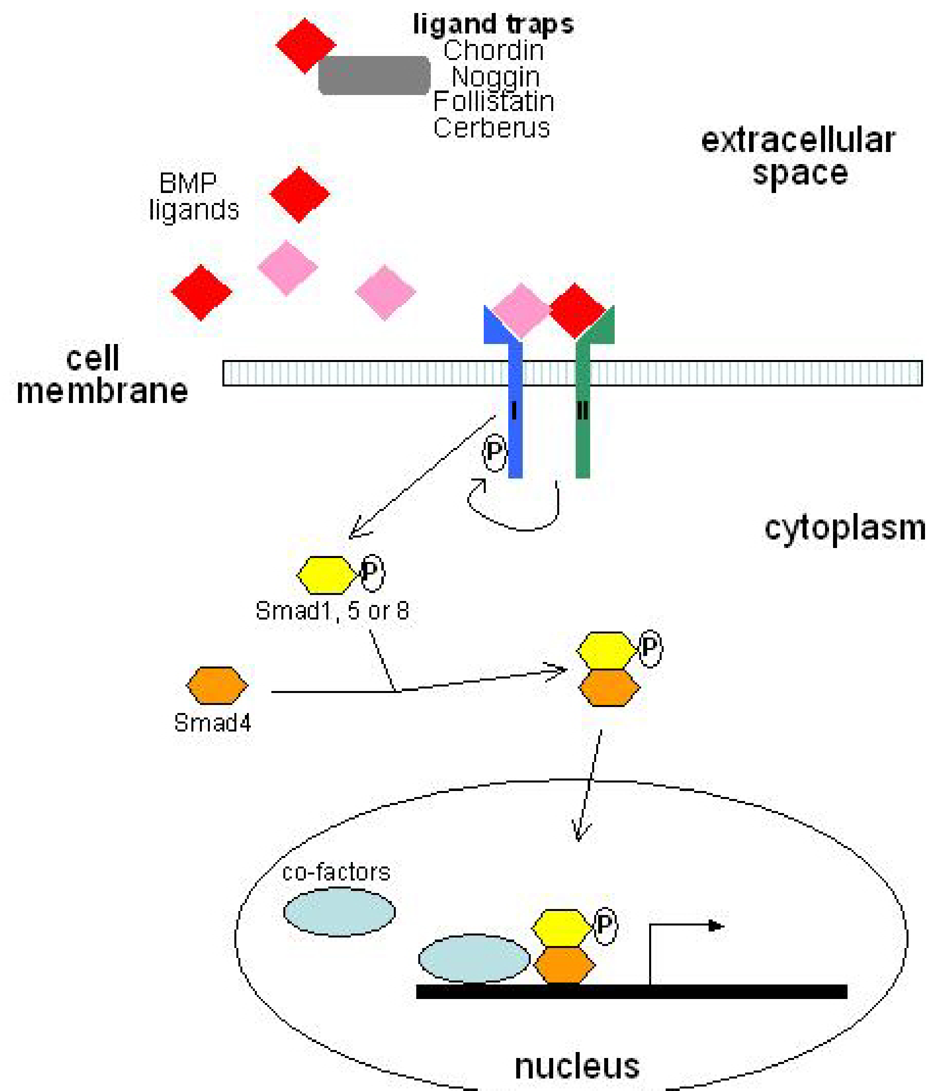

Fig. 3. Schematic representation of the BMP signaling pathway. In the extracellular space, BMP ligands form dimers and bind type I and II serine-threonine kinase receptors. Upon binding, the type II receptor phosphorylates the kinase domain of the type I receptor. This leads to the activation of receptor Smads, which later associate with Smad4 and translocate in the nucleus to modify gene transcription. In the extracellular space, ligand traps bind the BMP monomers and prevent binding of the ligands to their receptor.

Molecular and genetic studies have demonstrated that BMPs, expressed ventrally in the ectoderm and mesoderm during gastrulation in zebrafish, are essential for the specification of ventral fates (Hammerschmidt and Mullins, 2002). In zebrafish, there are four zygotic BMP signaling mutants: *swirl* (*swr*; *bmp2b*), *snailhouse* (*bmp7*), *somitabun* (*smad5*), and *lost-a-fin* (type I BMP receptor *alk8*). They all display a dorsalized phenotype, which is thought to result primarily from loss/reduction in the expression of essential ventral patterning proteins. In addition to BMP2b and 7, overexpression of BMP4 can induce the expression of ventral markers (Nikaido et al., 1997) but no zebrafish *bmp4* mutant has yet been identified. There is also evidence that a maternal BMP protein called Radar is required for ventral fates; presumably by inducing the expression of zygotic *bmps* (Sidi et al., 2003). Similarly, a maternal pathway involving Smad5 is also required for zygotic *bmp* induction (Kramer et al., 2002). The current model of BMP function during D/V patterning in zebrafish proposes that maternal BMP signaling by Radar and Smad5 is required to establish the ventral side of the embryo, thus arguing against the hypothesis that 'ventral' is a default state (Fig. 4; Wilm and Solnica-Krezel, 2003). Then, a gradient of zygotic BMP activity that is stronger ventrally is established. This gradient results from antagonism by BMP signaling inhibitors that diffuse from the organizer. Lastly, it is thought that patterning proper along the D/V axis occurs through the interpretation of the BMP morphogenetic gradient.

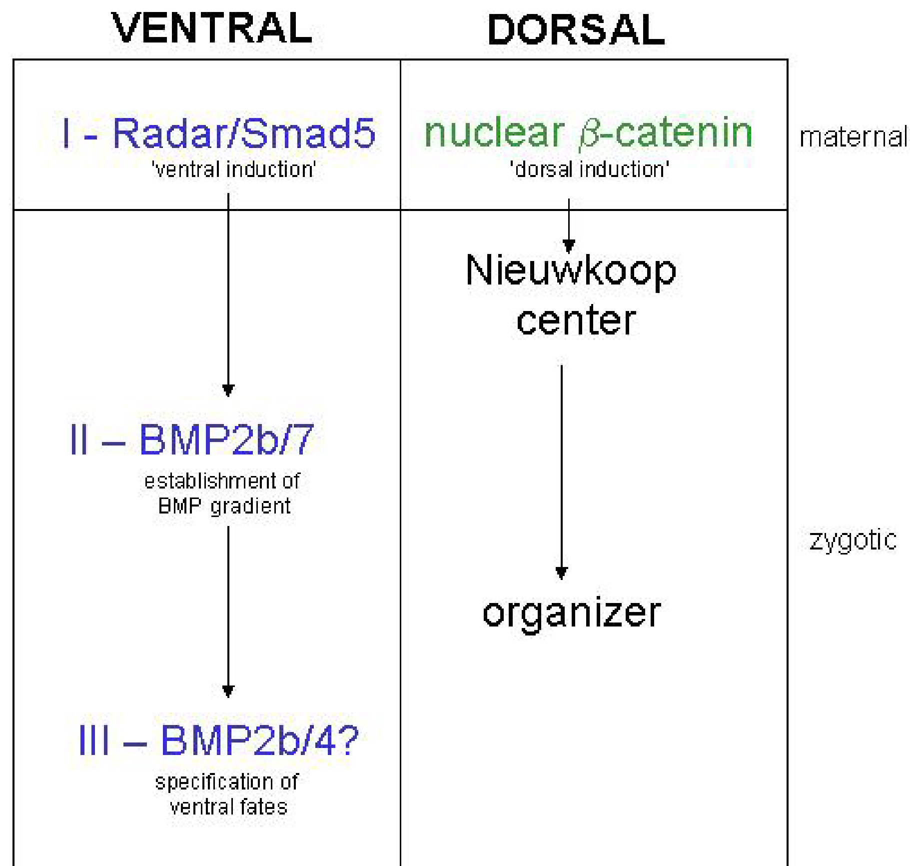

Fig. 4. BMP signaling and ventral specification in zebrafish. Left column: a three-phase model suggests that maternal BMP signaling specifies 'ventral' (step I). The BMP activity zone is then established by BMP2b/7 signaling (step II). Interpretation of the morphogenetic gradient is then thought to depend on BMP2b and maybe BMP4 (step III). Right column: specification steps of 'dorsal'. Nuclear β-catenin is the dorsal induction signal that establishes the Nieuwkoop center and the organizer.

In addition to BMPs, the Wnt8 secreted molecule is also required for ventral fates. Wnt8 is expressed in the non-axial mesoderm during gastrulation (Fig. 5; Christian et al., 1991; Kelly et al., 1995). Overexpression of Wnt8 in *Xenopus* and zebrafish leads to ectopic expression of ventral markers (Christian and Moon, 1993; Kelly et al., 1995). Genetic analysis of Wnt8 function in zebrafish has confirmed that it is necessary for proper D/V patterning as *wnt8* mutants are dorsalized. This phenotype is characterized by a ventral expansion in the expression of dorsal genes during gastrulation (expanded organizer phenotype; Lekven et al., 2001). Thus, it appears that Wnt8 is not only able to induce ventral genes but it is also necessary to limit dorsal gene expression. The expanded organizer phenotype is not present in zygotic *bmp* mutants where only ventral gene expression is reduced at similar stages (Mullins et al., 1996; Miller-Bertoglio et al., 1997). It is therefore unclear if the *wnt8-* dorsalized phenotype is solely due to a lack of organizer repression or if Wnt8, like BMP, is also essential for the expression of ventral patterning proteins.

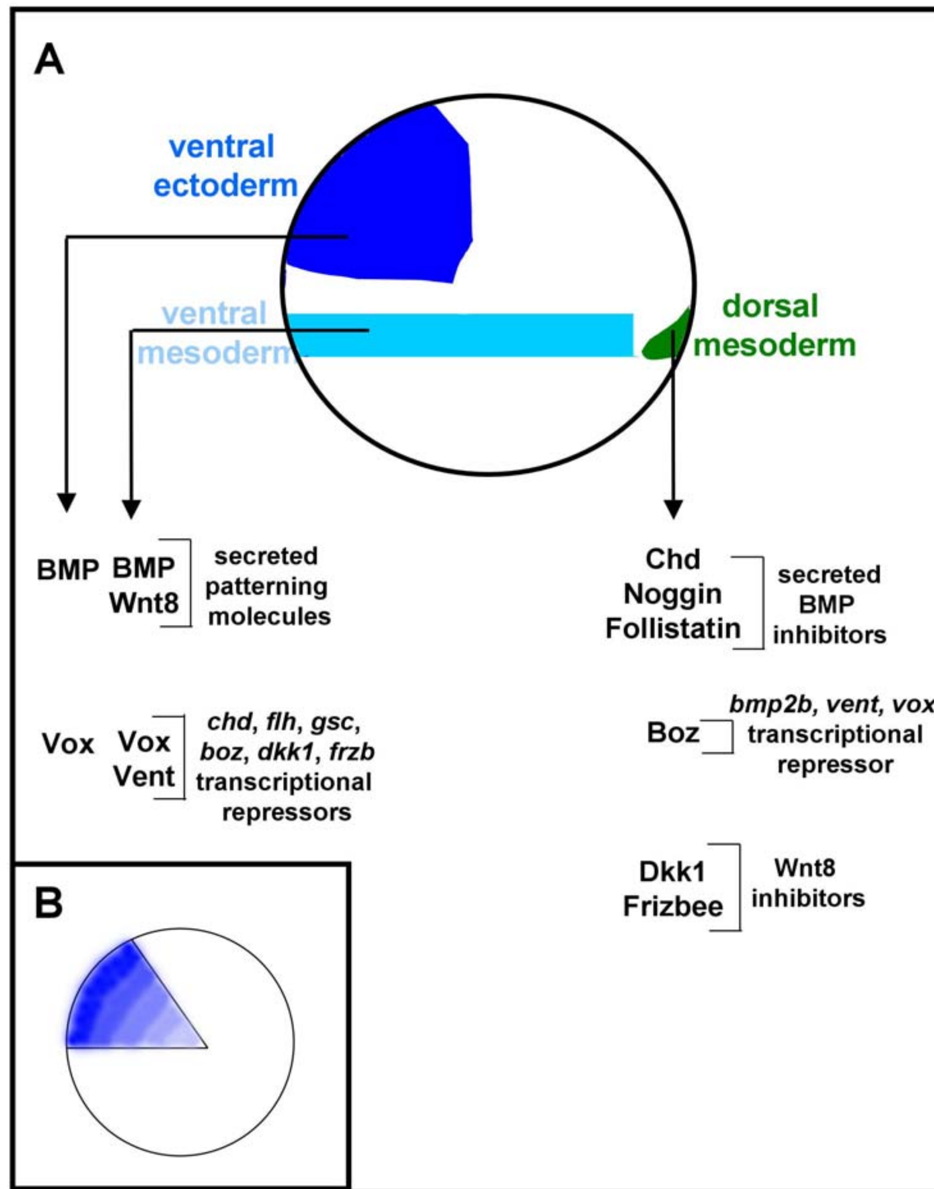

Fig. 5. Schematic representation of molecules involved in D/V maintenance and patterning in zebrafish and tissues where they are expressed. A: molecules expressed in each domain are indicated as well as their function. B: BMP activity gradient that results from antagonism by BMP inhibitors.

While BMP and Wnt8 are secreted factors, Vent and Vox are two transcriptional repressors. They are also essential for proper ventral patterning. They are expressed on the ventral side of the embryo during blastula and gastrula stages (Fig. 5; Melby et al., 2000). They are required to repress the transcription of dorsal genes such as *chd* and *gsc* (Onichtchouk et al., 1996; Melby et al., 1999; Trindade et al., 1999). In addition, genetic analyses have shown that they function redundantly: a stronger phenotype is observed when both proteins are lost (Imai et al., 2001). As we discussed previously, the maintenance of organizer size is critical for proper D/V patterning. Vent and Vox are thus very important to maintain this critical balance. To sum up, Vent and Vox contribute to organizer maintenance and this function is shared by Wnt8. A schematic representing the D/V interactions is shown in Fig. 5.

WNT8 FUNCTION IN D/V MAINTENANCE AND PATTERNING

Wnt8 is a secreted ligand that is part of the conserved family of Wnt molecules and, as described above, it is required for proper D/V patterning. The zebrafish genome contains at least 15 Wnts (see http://www.stanford.edu/~rnusse/wntgenes/zebrafwnt.html) some of which have been shown to function redundantly in some developmental processes. For instance, Wnt1 and 10b function redundantly during midbrain-hindbrain border formation (Lekven et al., 2003). Similarly, analysis of the *wnt8* locus in zebrafish showed that it contains two Open Reading Frames (ORFs; ORF1 and ORF2) coding for

two Wnt8 proteins that function redundantly in A/P and D/V patterning (Lekven et al., 2001).

Wnt molecules can be classified in two main groups, depending on what downstream signaling pathway they activate. The 'classical' pathway is called the canonical Wnt pathway and will be described further (Fig. 6; reviewed in He et al., 2004). In recent years however, a new pathway has emerged (non-canonical) that involves a different set of downstream players. In zebrafish, Wnt5 and 11 have been shown to function through this alternative pathway.

The canonical Wnt signaling pathway is present in all metazoans, from Hydra to humans. Canonical Wnts like Wnt8 are secreted in the extracellular space and bind a receptor complex made of the seven-pass transmembrane Frizzled (Fz) receptor and the transmembrane co-receptor LRP (low density Lipoprotein Related Protein). In the absence of ligand, a cytoplasmic complex made of Axin, GSK3β, and APC (Adenomatous Polyposis Coli) among others bind and phosphorylate β-catenin. β-catenin's stability and/or abundance is critical to achieve proper Wnt signaling. When phosphorylated, β-catenin is ubiquitinated and degraded. Binding of Wnt triggers a series of downstream events that inhibits the activity of the β-catenin phosphorylation complex. As a result, free β-catenin molecules accumulate in the cytoplasm and eventually translocate into the nucleus. There, β-catenin acts as a transcriptional co-activator of transcription factors from the Lef/Tcf family. Thus, in the presence of Wnt ligand, Wnt-responsive genes are being actively transcribed. Indeed, Tcf (T-cell factor) is thought to function as a transcriptional activator when bound to β-catenin, and usually

represses gene transcription in the absence of β-catenin (i.e. in the absence of Wnt signaling; reviewed in Eastman and Grosschedl, 1999). For instance, the zebrafish *headless* mutant (mutant for Tcf-3) lacks anterior neural structures, which is the same phenotype obtained upon Wnt overexpression (Kim et al., 2000). Lef (Lymphoid enhancer factor) is also thought to function in the same fashion except for some instances in cell culture systems where it can activate target genes independently of β-catenin (Eastman and Grosschedl, 1999). In *Xenopus*, Lef1 is thought to function downstream of Wnt8. Indeed, the injection of a constitutively repressive Lef-1 RNA construct gives rise to a phenotype similar to the one induced by inhibition of X-Wnt8 signaling (Roël et al., 2002). It is noteworthy that X-Tcf-3 and X-Lef-1 can mediate distinct tissue and stage-specific Wnt signaling (Roël et al, 2002). Thus, depending on the upstream Wnt ligand, Tcf and Lef can function non-redundantly.

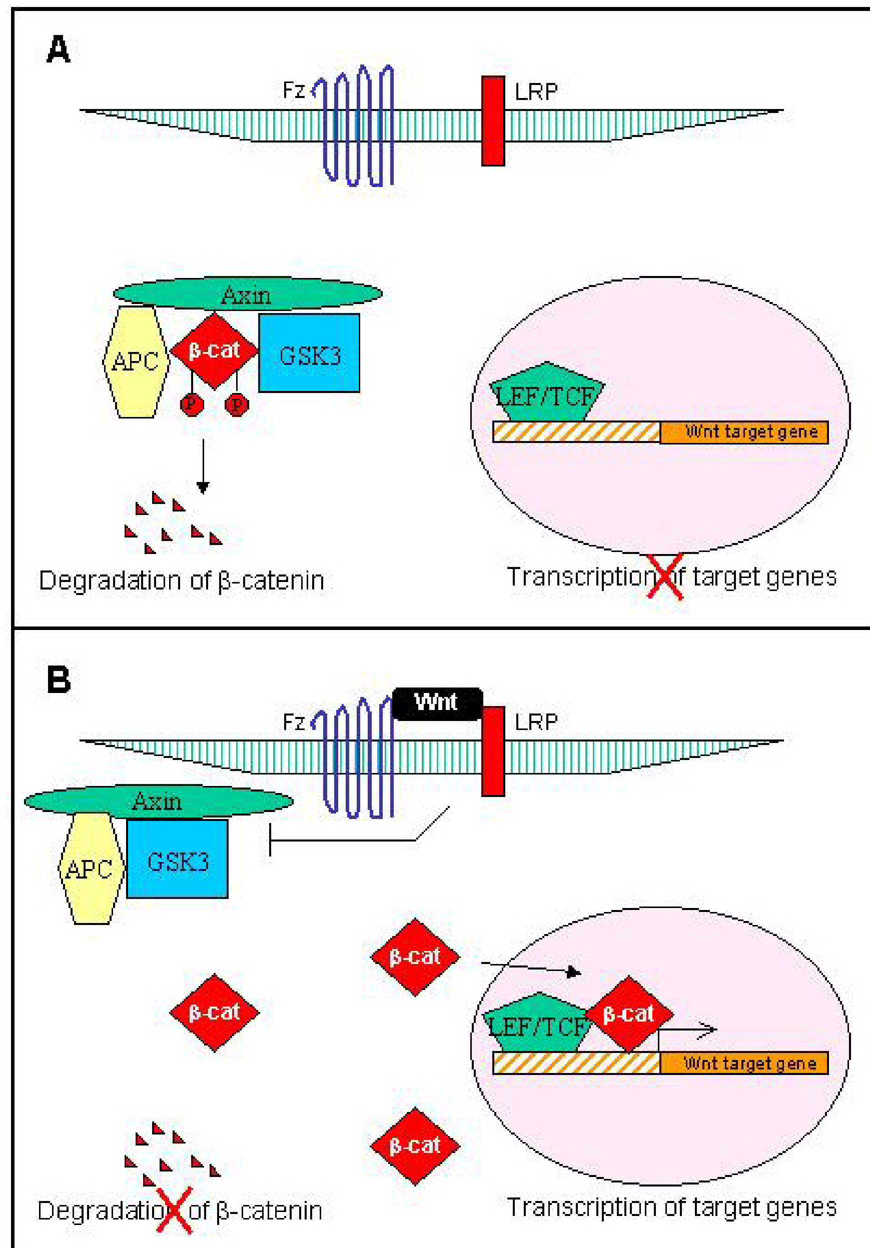

Fig. 6. Simplified representation of canonical Wnt signaling. A: in the absence of Wnt ligand, β-catenin is phosphorylated by GSK3β in the Axin-scaffolded complex. Phosphorylated β-catenin is recognized by the ubiquitination machinery and degraded. B: upon ligand binding, the Axin complex is inhibited leading to the accumulation of β-catenin cytoplasmically and translocation in the nucleus. There it activates the transcription of Wnt target genes by binding Lef/Tcf transcription factors and recruiting the transcriptional machinery. (adapted from He et al., 2004).

Interestingly, it has been demonstrated that disruption in the amount or activity of some of the signaling components of the canonical Wnt pathway correlates with the incidence of some cancerous tumors (reviewed in Kikuchi, 2003). For instance, mutations in the β-c*atenin* gene that remove the protein motifs recognized by the ubiquitination machinery have been found in a variety of human cancers including colon, ovarian and gastric cancer. Because of its ability to turn on gene transcription, β-catenin is thus considered to be an oncogene. Axin and APC, which are involved in the phosphorylation and inactivation of β-catenin, are tumor-suppressor genes. For example, a truncated form of Axin where the β-catenin binding site is eliminated has been observed in some human hepatocarcinomas. Thus, proper achievement of Wnt signaling is not only essential for normal embryonic development but also for normal cell division and growth in adult tissues.

MESODERM INDUCTION AND MAINTENANCE AND RELATION WITH D/V AXIS FORMATION

All of the events described above occur at the same time as mesoderm formation (reviewed in Weng and Stemple, 2003). It appears that mesoderm formation is divided in three phases: general mesoderm induction, specification of axial (dorsal) mesoderm, and maintenance of axial vs non axial (ventral) mesodermal domains.

In *Xenopus*, mesoderm induction depends on VgT (a transcription factor) and Vg1 (a TGFβ-related molecule), which are expressed maternally in the vegetal cells, and which signal through Nodal signaling (Gilbert, 2003). In zebrafish, it is known that the mesoderm inducing signal originates from the YSL (Yolk Syncytial Layer). This signal is believed to be activated soon after the start of zygotic transcription. It appears that, as in *Xenopus*, Nodals are required to mediate this YSL signal. Indeed, zebrafish double mutants for *squint* and *cyclops* (two Nodal ligands) do not form any mesoderm except for tail somites. The same phenotype is obtained in maternal zygotic *one eye pinhead* (MZ*oep*) mutants, which lack an essential co-receptor for the Nodal ligands.

β-catenin, by establishing the Nieuwkoop center and later the dorsal mesoderm, is responsible for the first division of the mesoderm into separate domains: axial vs non axial. After axial mesoderm and non-axial mesoderm are established, what allows these two domains to stay separated? Constriction experiments performed by Hans Spemann had suggested that 'ventral' may just be a default state that is acquired in the absence of dorsalizing signal. If 'ventral' mesoderm is a default state, then one might expect that ventral mesoderm needs to prevent 'dorsal' from expanding in order to preserve its identity. As discussed above, the balance between organizer and non-organizer is critical for proper D/V patterning and is achieved by Wnt8, Vent, and Vox ventrally and BMP and Wnt inhibitors dorsally. Thus, D/V patterning is tightly linked to the maintenance of non-axial and axial domains during mesoderm formation.

A STUDY LOOKING AT THE ROLE OF WNT8 IN D/V MAINTENANCE AND PATTERNING

The goal of this dissertation project is to understand how Wnt8 functions during D/V maintenance and patterning.

While it has been shown in both *Xenopus* and zebrafish that Wnt8 is required to limit the size of the organizer, no mechanism of action is known. More specifically, from what is known about Wnt signaling and its role in promoting active transcription of target genes, it is likely that Wnt8 signaling requires other molecules to perform its repressive function. Candidates that repress the transcription of organizer genes downstream of Wnt8 include the transcriptional repressors Vent and Vox. Previous studies in *Xenopus* have indeed suggested that *Xvent1b* is responsive to Wnt activity (Friedle and Knöchel, 2002) and that *vent* and *vox* can be induced by Xwnt8 overexpression (Hoppler and Moon, 1998). However, their relationship in zebrafish is unknown. Because of the similarity of the zebrafish *wnt8* and *vent*-; *vox*- expanded organizer phenotypes, we hypothesize that Wnt8 functions upstream of Vent and Vox in the organizer repressing process. Experiments that test this hypothesis are described in chapter II.

In addition, while both BMP and Wnt8 are classified as ventralizing agents, very little is known as to why Wnt8 is considered as such. Its cooperation with BMP signaling in the ventralizing process also appears to be complex and dynamic as suggested by *Xenopus* and zebrafish studies (Hoppler and Moon, 1998; Marom et al.,

1999; Mullins, 1999; Szeto and Kimelman, 2004). We hypothesized that repression of the vertebrate organizer by Wnt8 is essential to allow proper BMP function. To test our hypothesis, we analyzed in detail the epistatic relationship between the Wnt8 and BMP pathways during D/V patterning in zebrafish (see chapter III). In addition, to further understand the contribution of both pathways in this process, we carried out a detailed analysis of zebrafish *wnt8*; *bmp* double mutants (see chapter IV).

CHAPTER II

REPRESSION OF THE VERTEBRATE ORGANIZER BY WNT8 IS MEDIATED BY VENT AND VOX *

INTRODUCTION

Formation of the vertebrate embryonic axes requires Wnt signaling at two points: after fertilization to establish a dorsal signaling center and during gastrulation to pattern and specify ventral fates (for reviews see De Robertis et al., 2000; Schier, 2001). While canonical Wnt/β-catenin signaling is involved in both processes, it is triggered differently in each case. Specification of the dorsal signaling center appears to be a ligand-independent mechanism involving the accumulation of β-catenin, the nuclear effector of Wnt signaling, in dorsal nuclei (Larabell et al., 1997; Kelly et al., 2000; Schier, 2001). Accumulation of nuclear β-catenin leads to the formation of the Nieuwkoop center, which induces the dorsal mesodermal structure known as Spemann's Organizer (known as the "shield" in zebrafish or the "node" in the mouse; for review see Moon and Kimelman, 1998).

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* Reprinted from "Repression of the vertebrate organizer by Wnt8 is mediated by Vent and Vox"; by **Ramel, M-C., and Lekven, A.C.**, 2004, *Development*, **131**, 3991-4000.

After the establishment of the dorsoventral (D/V) axis, Wnt/β-catenin activity stimulated by the ligand Wnt8 is required to antagonize the organizer; thus, zebrafish *wnt8* mutants or *Xenopus* embryos expressing a dominant-negative Xwnt-8 display enlarged organizers and concomitant loss of posterior and ventral tissues (Hoppler et al., 1996; Lekven et al., 2001). Because proteins secreted by the organizer are known to be required for head formation and embryonic patterning (for review see De Robertis et al., 2000), understanding the mechanisms that limit organizer expansion is critical to understanding embryonic patterning.

The organizer influences D/V patterning through its secretion of BMP signaling inhibitors such as Chordin (Chd) or Noggin (De Robertis et al., 2000). However, BMP also exerts its own effect on the organizer. The *Xvent* ventral homeobox genes were identified as transcriptional targets of BMP in *Xenopus*, and were shown to repress organizer gene expression on the ventral side of the embryo (Gawantka et al., 1995; Onichtchouk et al., 1996; Onichtchouk et al., 1998; Melby et al., 1999; Lee et al., 2002). Indeed, Xvents repress the transcription of targets such as *chd* and *goosecoid* (*gsc*; Onichtchouk et al., 1996; Melby et al., 1999; Trindade et al., 1999). Analysis of the *Xvent-1B* and *Xvent-2B* promoters revealed the presence of consensus Lef/Tcf binding sites (Friedle and Knöchel, 2002). In addition, the *Xvent-1B* promoter is responsive to zygotic Wnt activity, suggesting that the expression of *Xvent* genes in general may be under the control of Wnt8 (Friedle and Knöchel, 2002). In support of this, Hoppler and Moon found that overexpression of dn-Xwnt-8 leads to the reduction of both *Xvent-1* and *Xvent-2* expression in *Xenopus* (Hoppler and Moon, 1998). Thus, these studies

suggest that the expression of transcriptional repressors required to restrict organizer gene expression may be under the concerted control of both the BMP and Wnt pathways.

Genetic analysis of zebrafish *vent* (also known as *vega2,* similar to *Xvent-1*) and *vox* (also known as *vega-1*, similar to *Xvent-2*) showed that these function as redundant transcriptional repressors (Kawahara et al., 2000; Melby et al., 2000; Imai et al., 2001). Zebrafish embryos homozygous for a chromosomal deficiency of the closely linked *vent* and *vox* loci show an expansion of organizer gene expression and severe D/V patterning defects (Imai et al., 2001). Further epistatic analysis suggested that the primary role of Vent and Vox is to modulate BMP inhibitors secreted by the organizer (Imai et al., 2001). *vent* and *vox* are known BMP transcriptional targets in zebrafish as well but their dependency on BMP signaling starts around 70-75% epiboly (Kawahara et al., 2000; Melby et al., 2000). As a result, zygotic BMP mutants do not have expanded organizers as in *vent/vox* mutants at shield stage (Mullins et al., 1996; Miller-Bertoglio, 1997; Imai et al., 2001). To date, only two zebrafish zygotic mutants are known to display significantly expanded organizers: *vent/vox* mutants and *wnt8* mutants. These data suggest that the relationship between BMP, Wnt8 and Vent/Vox is an important one for organizer regulation, the nature of which has been unclear but has been suggested to be complex (Hoppler and Moon, 1998; Marom et al., 1999).

We have utilized a loss-of-function approach in zebrafish to study the relationship between Wnt8, zygotic BMP and Vent/Vox regulation and activity in order to understand the mechanism by which Wnt8 antagonizes the organizer. Our results suggest that Wnt8 directly regulates the transcriptional levels of *vent* and *vox*, and that

the maintenance of high levels of *vent* or *vox* is required for repression of organizer genes on the ventral side of the embryo. Further, we provide evidence that Vent and Vox are absolutely essential to mediate the organizer repression activity of Wnt8. We also show that organizer repression and maintenance of ventrolateral mesoderm fates appear to be independent events. Finally, we show that the early regulation of both *vent* and *vox* is under Wnt8 and BMP control, but Wnt8 is the primary regulator; that is, at the onset of gastrulation, the requirement for BMP is only revealed in the absence of Wnt8. Zygotic BMP becomes the primary regulator of *vent* (but not *vox*) transcription during mid- to late gastrulation. Therefore, Wnt8 and BMP contribute to the repression of the organizer, which will, as a consequence, regulate the distribution of Wnt and BMP inhibitors.

MATERIALS AND METHODS

Fish maintenance and genetics

Animals were maintained as described (Westerfield, 2000). Embryos were staged according to Kimmel et al. (1995). Our wild-type strain is AB. Mutants used were *Df(LG14)wnt8*^{w8} (Lekven et al., 2001), *Df*$^{\mathrm{ST7}}$ (Imai et al., 2001), and *swr*$^{\mathrm{TC300}}$(Mullins et al., 1996). Results from *wnt8* or *vent;vox* deficiency mutants were confirmed with morpholinos (MOs).

In situ hybridization

In situ hybridizations were performed as described (Oxtoby and Jowett, 1993). Probes used were *gsc* (Stachel et al., 1993), *chd* (Miller-Bertoglio et al., 1997), *wnt8* ORF1 and *wnt8* ORF1+ORF2 (Lekven et al., 2001); *eve1* (Joly et al., 1993), *vent/vox* (Melby et al., 2000), *bmp2b* (Kishimoto et al., 1997), *opl* (Grinblat et al., 1998), *pax2a* (Krauss et al., 1991), and *tbx6* (Hug et al., 1997).

Genotyping of embryos

wnt8 mutants were genotyped as described (Lekven et al., 2001). *vent; vox* mutants were genotyped using *vox* R1 (5'-GATATTGCACACCAGCGTGA-3') and *vox* L1 (5'-GTTCCAGAACCGAAGGATGA-3') primers. *swr* mutants were genotyped as described (Wagner and Mullins, 2002). Embryos were classified according to their phenotype, photographed, and genotyped. For *wnt8*$^{-}$; *swr* double mutants, at least 85 embryos from an intercross were examined in the same fashion.

Embryo microinjection, morpholinos, constructs

MOs (Genetools, LLC), RNA or DNA, were injected into one to four-cell stage embryos. Approximately 3 nL was injected per embryo. Capped mRNAs were synthesized using mMESSAGE mMACHINE (Ambion) and diluted in water. MOs were diluted in Danieau's buffer as recommended (Genetools). *wnt8* MOs (targeting ORF1 and ORF2) and *vent* and *vox* MOs have been described (Lekven et al., 2001; Imai et al., 2001). GR-LEFΔN-βCTA RNA was injected at 300 ng/μL in one-cell stage embryos.

Embryos were dechorionated manually in fish water (Westerfield, 2000) prior to treatment. Dexamethasone (DEX; Sigma) treatments were performed for one hour at 1, 2, 3, 4 or 5 hours post-fertilization (HPF). DEX (100 mM stock solution in 100% ethanol) was used at a final concentration of 100 μM in 0.3 X Danieau's solution. Treated embryos were fixed at 6 HPF. For the Cycloheximide (CHX; Calbiochem) treatment, embryos were first injected with GR-LEFΔN-βCTA RNA then treated with CHX (10 μgs/mL) with or without DEX. For *vent* induction analysis, n(CHX)=37 and 55, n(DEX)=44, 37 and 11, n(CHX+DEX)=28, 34, and 28 where n=total number of embryos analyzed in each experiment. For *vox* induction, n(CHX)= 16, 17 and 12, n(DEX)=5, 12, and 20, n(CHX+DEX)= 9, 14 and 19 . As a control for CHX treatments, uninjected embryos were treated with CHX from 1.5 HPF to sphere stage, fixed, and stained for *gsc* (Leung et al., 2003). No treated embryos expressed *gsc* (n=34). The χ_2 test was used to determine statistical significance.

RESULTS

Zebrafish *wnt8* and *vent*; *vox* mutants have expanded organizers, *swr* mutants do not

Although BMP and Wnt8 both are described as "ventralizing agents" (i.e. overexpression leads to a shift in mesodermal fates), they play non-equivalent roles in

D/V patterning. To illustrate this, we compared the expression of D/V markers in *wnt8* (*Df*^{w8}; Lekven et al., 2001), *vent vox* (*Df*^{st7}; Imai et al., 2001), and *bmp2b* (*swr*^{tc300}; Mullins et al., 1996) mutants.

In zebrafish, *wnt8* contains two open reading frames (ORF1 and ORF2; Lekven et al., 2001). The two Wnt8 proteins were shown to function redundantly in anteroposterior (A/P) and D/V patterning, since the *Df*^{w8} phenotype is phenocopied only by coinjection of both ORF1 and ORF2 MOs (Lekven et al., 2001). Similarly, the *Df*^{st7} phenotype is phenocopied by coinjection of *vent* and *vox* MOs (Imai et al., 2001).

Expression analysis of the dorsal markers *chd*, *gsc*, *floating head (flh)*, and *dharma* (*bozozok*) at shield stage shows that they are expanded ventrally in *wnt8* mutants (Fig. 7B,F; Lekven et al., 2001, and data not shown) as well as *vent*;*vox* mutants (Fig. 7C, inset, and Fig. 7G; Imai et al., 2001). However, *swr* mutants do not exhibit a similar expansion at shield stage (Figs 7D and 7H; Mullins et al., 1996; Miller-Bertoglio et al., 1997). Importantly, the expansion of dorsal markers is stronger in *vent*;*vox* mutants compared to *wnt8* mutants. For instance, *gsc* encircles the margin of *vent*;*vox* mutants (Fig. 7C, inset) but extends over a ~ 90° arc in *wnt8*$^{-}$ embryos at the same stage (Fig. 7B). This comparative analysis shows that Wnt8 and Vent/Vox but not BMP are normally required ventrally during gastrulation to restrict the size of the organizer, in agreement with previous reports (Mullins et al., 1996; Miller-Bertoglio et al., 1997; Imai et al., 2001; Lekven et al., 2001).

The expanded organizer phenotype is first observable in *wnt8*$^{-}$ and *vent*$^{-}$; *vox*$^{-}$ embryos at 40% epiboly (data not shown; Imai et al., 2001), a developmental timepoint

when convergence movements have not yet started (Kimmel et al., 1995). Thus, the expansion of dorsal markers in these backgrounds must reflect a change in fate rather than an alteration of cell movements.

Wnt8 is also required to promote ventral fates: *eve1*, a ventral mesodermal marker, is reduced in *wnt8* mutants (Fig. 7B). It is similarly reduced in *swr* mutants (Fig. 7D; Mullins et al., 1996). In contrast, *eve1* is less reduced in *vent*;*vox* mutants (Fig. 7C) compared to *wnt8* and *swr* mutants (Fig. 7B,D), despite the fact that the dorsal markers *gsc* (Fig. 7C, inset) or *chd* (Fig. 7G) encircle the margin of the same embryos. Hence, Wnt8 and BMP are required in the ventral mesoderm for the maintenance of *eve1*, a ventral-specific gene, and this function is separable from repression of the organizer.

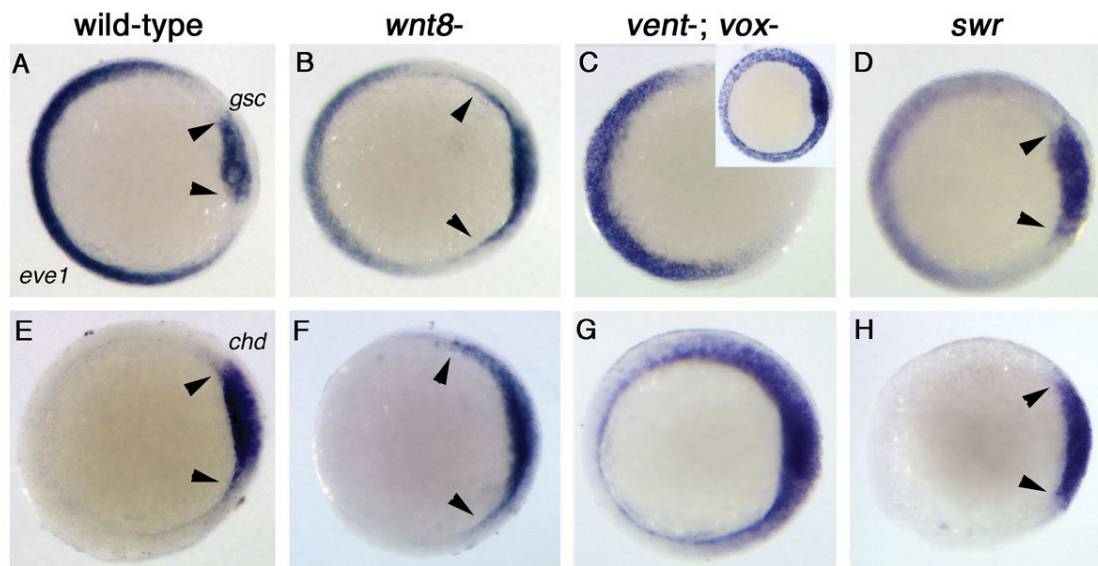

Fig. 7. The *wnt8*$^-$ phenotype is similar to the *vent*$^-$; *vox*$^-$ and *swr* phenotypes. (A,B,D) double in situ hybridization for *eve-1* and *gsc*. C: *eve-1*, inset shows *gsc*. Note strongly reduced *eve1* in *wnt8* and *swr* mutants but slightly reduced *eve1* in *vent;vox* mutants. Arrowheads indicate width of *gsc* expression (note circumferential *gsc* in C, inset). (E-H) in situ hybridization for *chd* (domain width indicated by arrowheads). Note expansion in both *wnt8* and *vent*;*vox* mutants but not *swr* mutants. All embryos are at shield stage. Animal view, dorsal right.

Wnt8 regulates *vent* and *vox* mRNA levels

Since Wnt8 and Vent/Vox share the function of repressing dorsal genes, we analyzed their epistatic relationship. We first examined *vent* and *vox* mRNA levels in wild-type versus *wnt8*$^{-}$ backgrounds (Fig. 8). In zebrafish, *vent* is expressed at the mesodermal margin during gastrulation while *vox* displays both ventral mesoderm and ectoderm expression (Melby et al., 2000).

Starting at 30% epiboly (late blastula), the accumulation of *vent* at the margin is visibly weaker in *wnt8* mutants or morphants compared to wild-type (Fig. 8A-C). We did not detect any differences in *vent* expression at earlier stages (data not shown). *vox* expression is not visibly different in *wnt8* mutants at 30% epiboly (data not shown), but is reduced in the margin of *wnt8* mutants/morphants at 40% epiboly (Fig. 8G-I).

To determine the correspondence between *vent* and *vox* reduction and the onset of an observable phenotype in *wnt8* mutants, we examined *chd* expression at these early stages. At 30% epiboly, no visible difference in the *chd* expression domain was observed in *wnt8* mutants (data not shown), but we did detect an expansion of *chd* expression at 40% epiboly, the timepoint at which both *vent* and *vox* are reduced in *wnt8*$^{-}$ embryos (Fig. 8M-O). Hence, our results suggest that a reduction in both *vent* and *vox* levels may be required to observe the expanded organizer phenotype at 40% epiboly, consistent with Vent and Vox functioning redundantly (Imai et al., 2001).

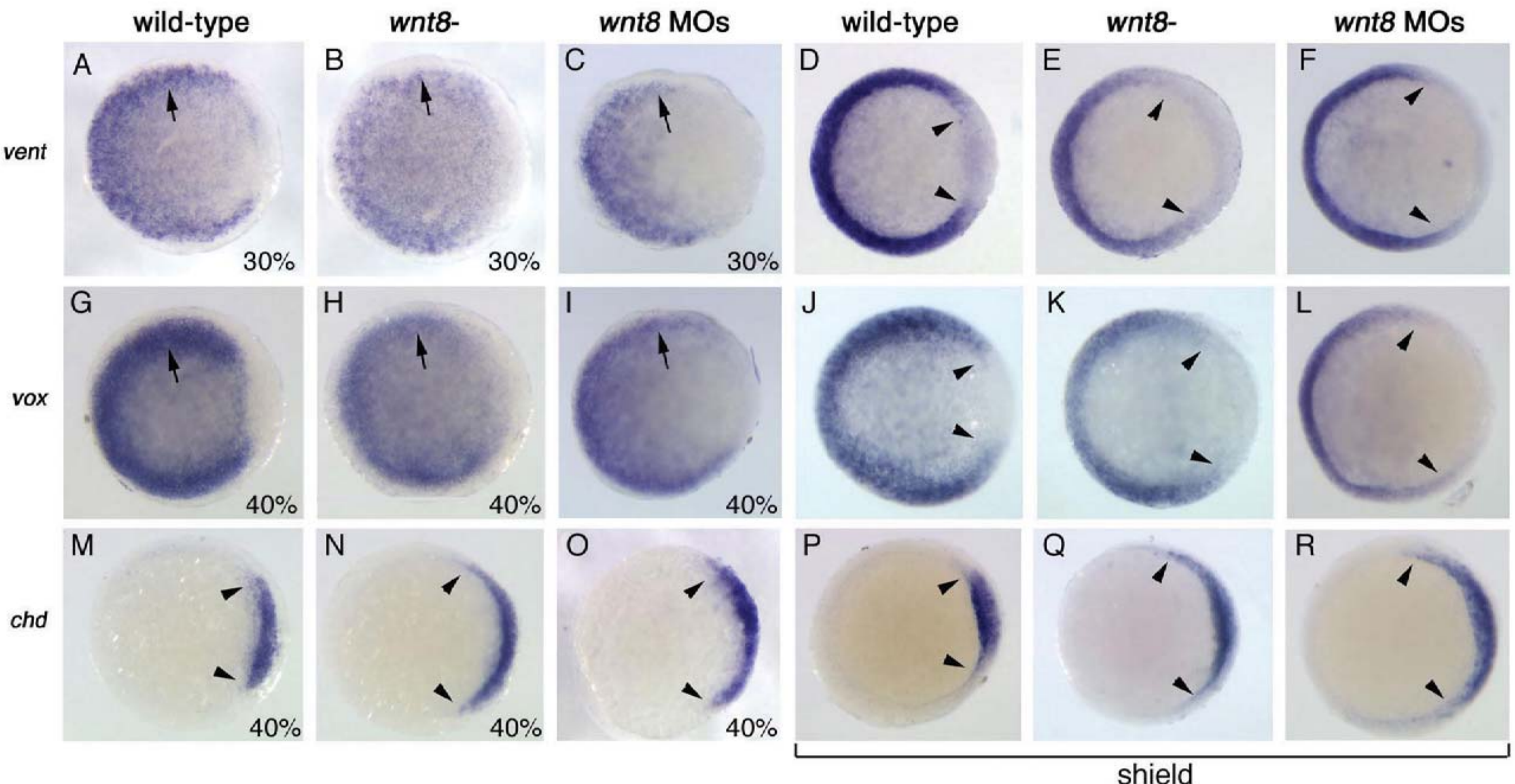

Fig. 8. *vent* and *vox* mRNA levels are reduced in *wnt8* mutants. In situ hybridization for *vent* (A-F), *vox* (G-L), or *chd* (M-R). Embryo genotypes are indicated above each column. Stages are indicated. At 30% epiboly, *vent* is reduced in *wnt8* mutants/morphants (arrow in B, C). *vox* is reduced at 40% epiboly (arrow in H, I), corresponding to increased *chd* (arrowheads in N, O). Both *vent* (E,F) and *vox* (K,L) are reduced in shield stage wnt8 mutants/morphants. Animal view, dorsal right.

During the rest of gastrulation, *vent* and *vox* mRNA levels stay reduced in *wnt8* mutants/morphants compared to wild-type (Fig. 8D-F, J-L; data not shown). In comparison, *vent* and *vox* levels are unchanged in *swr* mutants at shield stage (Kawahara et al., 2000; Melby et al., 2000), which explains the lack of an organizer phenotype (Mullins et al., 1996; Miller-Bertoglio et al., 1997). Indeed, BMP2b is only required at mid- to late gastrulation for the maintenance of *vent* and ectodermal *vox* expression (Melby et al., 2000). Therefore, Wnt8 regulation of *vent* and *vox* starts at the blastula/gastrula transition (30/40% epiboly) while BMP2b regulation of these genes occurs later (70% epiboly).

To test the reciprocal possibility of *wnt8* being regulated by Vent and Vox, we looked at the expression of *wnt8* in *vent*;*vox* mutants (Fig. 9). Since zebrafish *wnt8* produces transcripts for both protein coding regions, we used probes to detect either the ORF1/ORF2 bicistronic transcript (ORF1) or to detect both the bicistronic transcript and the ORF2 transcript (ORF1+ORF2; Lekven et al., 2001). No differences from wild-type expression were observed in 40% epiboly *vent*;*vox* mutants (Fig. 9A-B,G-H). Since 40% epiboly marks the onset of *vent*$^{-}$;*vox*$^{-}$ phenotype (Imai et al., 2001), this suggests that a change in *wnt8* expression is not responsible for the *vent*;*vox* mutant phenotype. The dorsal domain lacking ORF1 expression is slightly expanded in *vent*;*vox* mutants at shield stage (Fig. 9C-D; confirmed with MOs) and is more pronounced at 75% epiboly (Fig. 9F). While there is an observable difference dorsally, ORF1 levels ventrally seem to be unaffected in *vent;vox* mutants (Fig. 9C-F), suggesting that the reduction in dorsal *wnt8* ORF1 expression is an indirect consequence of an enlarged organizer.

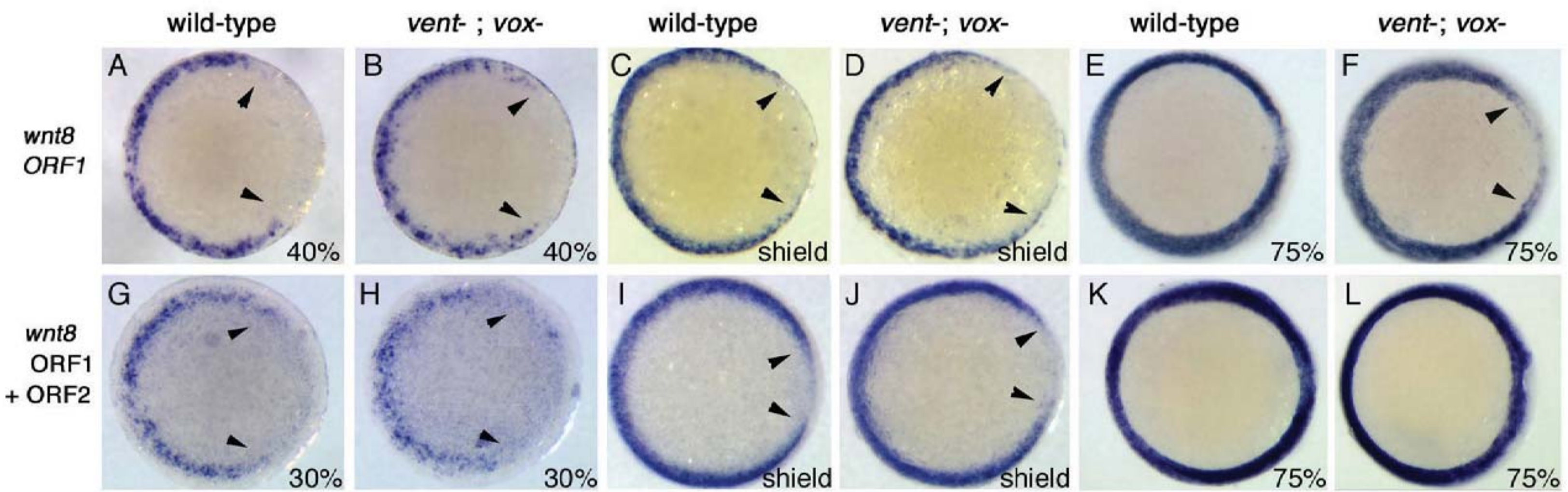

Fig. 9. *wnt8* ORF1 and ORF2 expression in *vent*;*vox* mutants.
In situ hybridization for *wnt8* ORF1 (A-F) and *wnt8* ORF1 + ORF2 (G-L). Genotypes are indicated above each column. Stages are indicated. Arrowheads indicate dorsal limit of *wnt8* expression. Note slight decrease in ORF1 dorsally in shield stage *vent*; *vox* mutants (C-D) and broadened dorsal clearing of *wnt8* ORF1 expression 75% epiboly (F). *wnt8* ORF2 expression is not affected. A-D and G-J: animal view, dorsal right. E-F and K-L: vegetal view, dorsal right.

Analysis of ORF2 expression at later stages revealed that it is not affected by the loss of Vent and Vox (Fig. 9I-L). This is not unexpected as *wnt8* ORF2 accumulates dorsally during gastrulation (Fig. 9K) and is therefore insensitive to molecules present in the organizer. Thus, only *wnt8* ORF1 expression depends on Vent and Vox but this dependency is restricted dorsally and may be indirect. In comparison, *wnt8* ORF2 expression does not depend on Vent and Vox.

Wnt8 functions through β-catenin to regulate *vent* and *vox* transcription

The above data show that Wnt8/β-catenin is necessary to maintain normal *vent* and *vox* expression. To test if Wnt8 is sufficient to induce *vent* and *vox*, we injected Wnt8 ORF1 or ORF2 expression plasmids in wild-type embryos and assayed *vent* and *vox* expression by in situ hybridization at shield stage. In both cases, ectopic domains were observed in the animal ectoderm region and/or dorsal mesoderm where *vent* and *vox* are normally absent (Table 1 and data not shown). To confirm that canonical Wnt signaling was involved in *vent* and *vox* regulation, we modulated β-catenin activity using a hormone inducible β-cat/Lef fusion protein (GR-LEFΔN-βCTA; Domingos et al., 2001). The GR-LEFΔN-βCTA protein contains the human glucocorticoid receptor domain fused to the DNA binding domain of murine LEF and the transactivation domain of murine β-catenin. Addition of the hormone dexamethasone (DEX) leads to the nuclear translocation of the fusion protein and to β-catenin/Lef induced transcription, thus allowing controlled induction of Wnt signaling (Domingos et al., 2001). Addition of DEX for a one-hour period at 1, 2, 3, 4 or 5 HPF led to ectopic *vent* and *vox* expression

in a proportion of injected embryos (~ 50 to 70% of embryos, Fig. 10A, panels b, d; data not shown). Consistent with the role of β-catenin in organizer induction, ectopic *gsc* was observed in a proportion of embryos treated at 1, 2, or 3 HPF but not at later timepoints (data not shown).

Table 1. Injection of either Wnt8 ORF induces ectopic *vent* and *vox*

injection	assay	% injected embryos showing ectopic expression	P value
wnt8 ORF1 DNA	*vent*	75 (n=56)	< 0.001
(40 ngs/μL)	*vox*	73 (n=52)	< 0.001
wnt8 ORF2 DNA	*vent*	91.8 (n=49)	< 0.001
(40 ngs/μL)	*vox*	89.1 (n=37)	< 0.001

While our results suggest that Wnt8/β-catenin regulates *vent* and *vox* transcription, it is unclear if this is direct (through β-catenin/Lef induced transcription) or indirect (through the synthesis of an intermediate transcriptional regulator). Interestingly, genomic region upstream of zebrafish *vox* contains consensus Lef/Tcf binding sites consistent with Wnt regulation of *vox* transcription (our own observations, and D. Kimelman, personal communication). To address this, we used cycloheximide (CHX) to test whether protein synthesis is required for induction of ectopic *vent* or *vox* by GR-LEFΔN-βCTA.

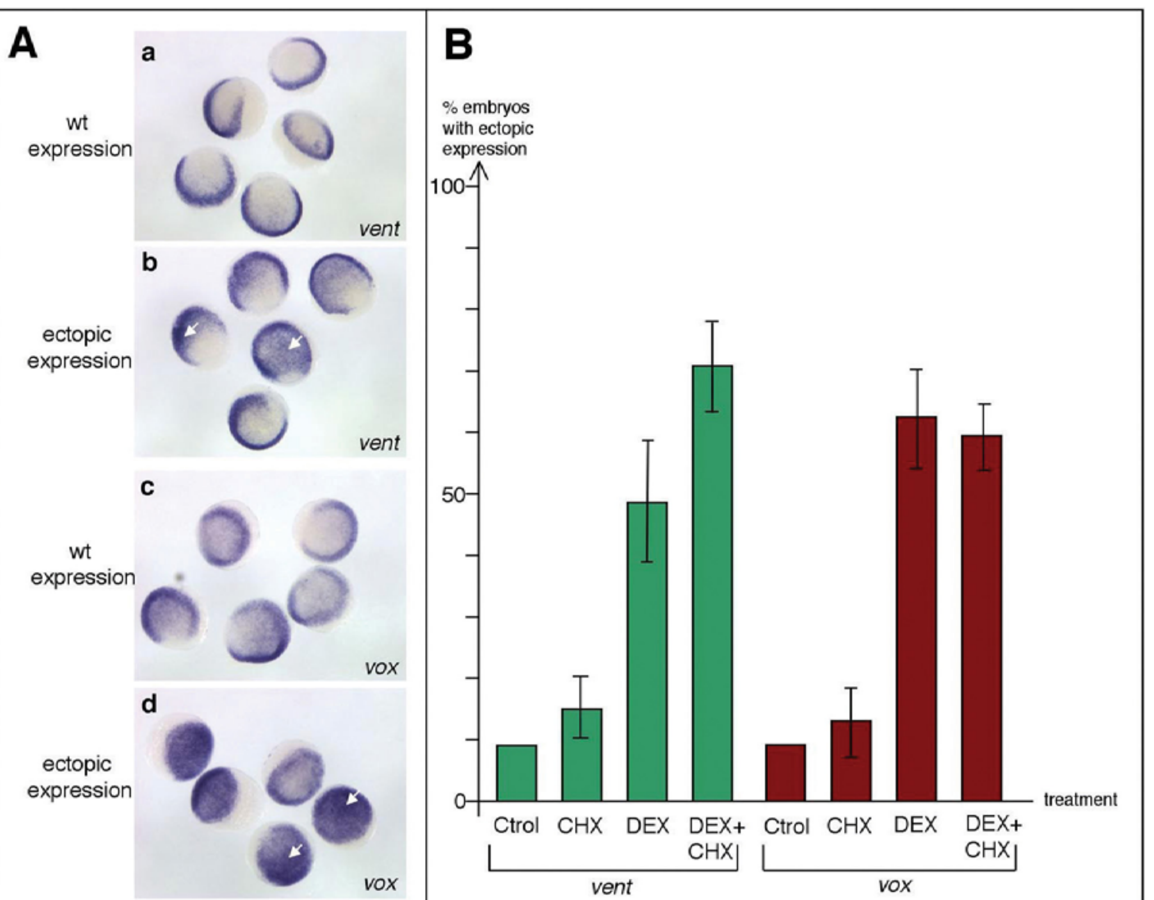

Fig. 10. *vent* and *vox* are direct transcriptional targets of Wnt8/β-catenin signaling. (A) *vent* and *vox* expression in control (a, c) or treated (b, d) embryos. Arrows in panels b and d indicate ectopic expression upon induction of GR-LEFΔN-βCTA with DEX. (B) graph of percent embryos displaying ectopic *vent* or *vox* domains (y-axis) upon treatment with CHX alone, DEX alone, or CHX + DEX (x-axis). The control bar represents embryos injected with GR-LEFΔN-βCTA and treated with ethanol (n=109 for *vent* and n=177 for *vox*). Error bars represent the standard error of the mean. When performing the $\chi 2$ test on DEX vs DEX+CHX means, $p>0.05$ for both *vent* and *vox*, meaning that the difference between the means is not statistically significant.

Treatment of GR-LEFΔN-βCTA injected embryos with DEX at 5 HPF results in ectopic *vent* or *vox* RNA expression in 49% and 62.1% of embryos, respectively (Fig. 10B). Addition of CHX simultaneously with DEX did not result in a statistically different number of embryos with ectopic *vent* and *vox* domains (72.2% and 59.5%; Fig. 10B), indicating that GR-LEFΔN-βCTA activation of *vent* and *vox* does not require de novo protein synthesis. Thus, our results suggest that *vent* and *vox* are direct transcriptional targets of Wnt8/β-catenin signaling.

Wnt8 repression of the organizer requires Vent/Vox

Since *vent* and *vox* transcription is regulated by Wnt8, we hypothesized that Vent and Vox function downstream of Wnt8 to repress dorsal genes and that the *wnt8*$^{-}$ organizer phenotype is due to reduced *vent* and *vox* levels. If so, injection of *vent* or *vox* RNA or DNA into *wnt8* mutants would suppress the expanded organizer phenotype. We first established amounts of injected Vox or Vent that are sufficient to reduce the expression of dorsal markers (*gsc*, *chd*, *flh*) in wild-type embryos (Fig. 11A, panels a and c; data not shown). When injected into *wnt8* mutants, Vox was able to reduce the expression of dorsal genes (Fig. 11A, compare panels b and d; Table 2). Similar results were obtained with either DNA or RNA injection for both *vent* and *vox* (Table 2 and data not shown). Thus, Vent and Vox expression can bypass *wnt8* loss-of-function in repressing organizer genes, supporting the placement of *vent* and *vox* genetically downstream of *wnt8*. These results suggest that the difference in severity of the *wnt8*$^{-}$ and *vent*$^{-}$; *vox*$^{-}$ organizer phenotypes (see Fig. 7) could be explained by residual Vent and Vox activity in *wnt8*

mutants. In agreement, further reduction of Vent and Vox in *wnt8* mutants by injection of sub-maximal concentrations of *vent* and *vox* MOs enhances the severity of the *wnt8*$^{-}$ phenotype (Fig. 11B).

While Vent and Vox can bypass Wnt8 to repress organizer genes, we wished to assess whether Wnt8 requires Vent and Vox to repress the organizer. If Vent and Vox are essential for this Wnt8 function, then Wnt8/β-catenin activity should be ineffective in their absence. In support of this, *vent*;*vox* mutants express nearly normal levels of *wnt8* mRNAs (see Fig. 9), hence the expansion of the organizer in *vent*;*vox* mutants occurs in the presence of *wnt8* transcripts.

Table 2. Increased Vent/Vox expression in *wnt8* mutants leads to the repression of dorsal genes

assay	injection	% rescued *wnt8* mutants *	P value
gsc	*vox* DNA (10 ngs/μL)	53.3 (n=15)	< 0.001
	vent RNA (10 ngs/ μL)	78.9 (n=19)	< 0.001
chd	*vox* RNA (10 ngs/μL)	68.7 (n=16)	< 0.001
flh	*vox* RNA (10 ngs/μL)	95.4 (n=22)	< 0.001

* rescue is defined as reduction in the dorsal markers assayed compared to uninjected *wnt8* mutants

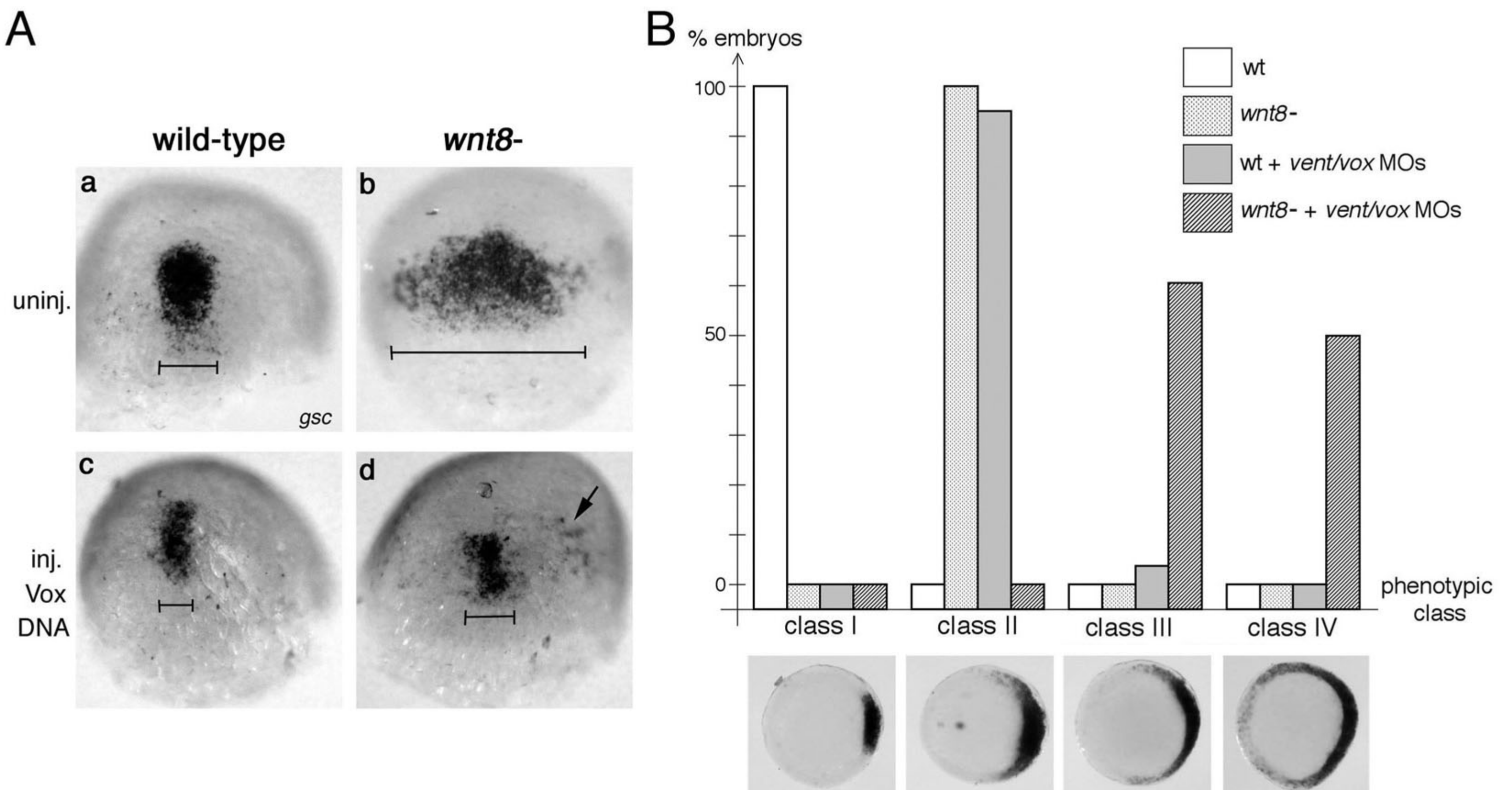

Fig. 11. The *wnt8⁻* expanded organizer phenotype is due to reduced *vent* and *vox.*
(A) Rescue of *wnt8* mutants by Vox. *gsc* expression in wild-type (a, c) or *wnt8⁻* (b, d) embryos uninjected (a, b) or injected (c, d) with a *vox* expression plasmid. Some isolated lateral cells still express *gsc* in injected *wnt8⁻* embryos due to the mosaic expression of Vox (panel d, arrow). 70% epiboly, dorsal view. (B) Reduction of Vent/Vox enhances the *wnt8⁻* organizer phenotype. Graph of percent of embryos belonging to a specific phenotypic class. Class I: wild-type *chd* expression; Class II to IV: increasingly expanded *chd*. 100% of wild-type and *wnt8⁻* embryos belong to class I and class II respectively. Upon injection of *vent+vox* MOs, most wild-type embryos belong to class II (96.5%, n=85) while *wnt8⁻* embryos belong to both classes III and IV (60.7% and 39.3% respectively, n=28). Embryos shown at the bottom of the graph are at shield stage, animal view, dorsal right.

To confirm that the *wnt8* transcripts in *vent*;*vox* mutants produce functional proteins, we used two assays of Wnt8 function. First, we examined the expression of the Wnt/β-catenin activity reporter TOPdGFP (Dorsky et al., 2002; Lewis et al., 2004). We analyzed the expression of TOPdGFP mRNA at 100% epiboly in embryos homozygous for the transgene after injection of *wnt8* or *vent* + *vox* MOs (Fig. 12A-D). As expected and confirming previous results (Phillips et al., 2004), *wnt8* MOs severely reduced TOPdGFP expression in 90% of injected embryos to almost undetectable levels (n=20; Fig 12B). In *vent/vox* morphants, three phenotypic classes were observed: the first class displayed wild-type TOPdGFP expression (50%, n=22; Fig. 12C), the second class showed moderate reduction in TOPdGFP (14%, not shown) and the third class displayed a stronger reduction in staining (36%; Fig. 12D) but this class had significantly more TOPdGFP expression than *wnt8* morphants (compare Fig. 12D to Fig. 12B). As a control for the strength of the *vent* + *vox* MO injections, a portion of injected embryos were examined at 24 HPF and all showed a strong *vent*/*vox* loss-of-function phenotype (n=23; Imai et al., 2001). Thus, TOPdGFP is a reporter of Wnt8 activity and is still expressed in *vent* + *vox* morphants. Reduced levels of TOPdGFP expression in some *vent* + *vox* morphants could reflect the fact that expression of the Wnt antagonists Dickkopf-1 and Frzb is significantly expanded (Imai et al., 2001 and our own observations).

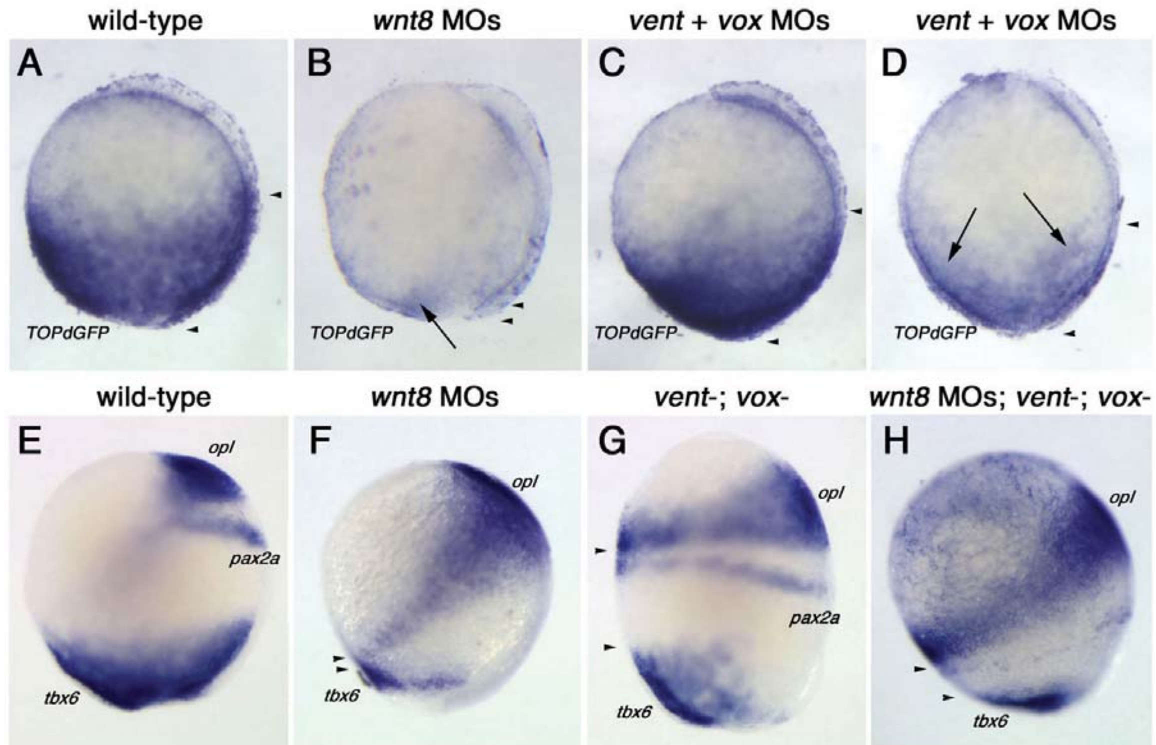

Fig. 12. Wnt8 requires Vent and Vox to repress dorsal genes. (A-D) GFP in situ hybridization to embryos homozygous for the TOPdGFP transgene. (E-H) *opl*, *pax2a* and *tbx6* in situ hybridization. Genotype/treatment is indicated above each panel. (A) TOPdGFP is expressed in the mesoderm. In *wnt8* morphants (B), TOPdGFP is barely detectable (arrow). *vent* + *vox* MO injected embryos display mostly wild-type TOPdGFP expression (C) but some display somewhat reduced expression (D, arrows). Arrowheads in A-D indicate the A/P extent of the TOPdGFP positive domain. (E) In wild-type, *opl* and *pax2a* in relation to *tbx6* indicate normal neural posteriorization. In *wnt8* morphants, *opl* is expanded posteriorly, *pax2a* is delayed and *tbx6* is reduced (F). *vent*;*vox* mutants (G) do not display a strong A/P defect and ventral *tbx6* staining is as strong as in wild-type embryos. Reducing Wnt8 in *vent*; *vox* mutants (H) results in decreased *tbx6* and *pax2a* expression. The distance between the arrowheads in F, G, and H show the degree of posteriorization.~100% epiboly, lateral view, dorsal right.

To confirm that expressed Wnt8 actively patterns *vent;vox* mutants, we analyzed A/P neural patterning, a function known to require Wnt8 (Lekven et al., 2001; Erter et al., 2001). To assess the A/P phenotype of *vent*; *vox* mutants, a combination of three probes was used: *opl* (anterior neuroectoderm), *pax2a* (midbrain-hindbrain border) and *tbx6* (posterior non-axial mesoderm). In *wnt8* mutants or morphants, A/P patterning is severely disrupted at 90%-100% epiboly: the *opl* domain is expanded along the AP axis, *pax2a* expression is delayed and *tbx6* is strongly reduced (Fig. 12F). In comparison, *vent*;*vox* mutants have only mildly affected A/P patterning illustrated by a slight posterior shift of the *opl* and *pax2a* domain away from the animal pole, but the distance between *opl* or *pax2a* and *tbx6* is significantly greater than in *wnt8* morphants (Fig. 12G, compare with Fig. 12F). As expected, the expanded organizer of *vent*;*vox* mutants results in an enlarged dorsal clearing of *tbx6* expression, while the levels of *tbx6* ventrally are relatively unaffected (Fig. 12G, compare with Fig. 12E). Since *tbx6* expression depends on Wnt8, our results argue against an absence of Wnt8/β-catenin activity in *vent*;*vox* mutants. Furthermore, reducing Wnt8 translation in *vent*;*vox* mutants results in an additive phenotype. *opl* extends ventrally, as in *vent*;*vox* mutants, while *pax2a* and *tbx6* expression is severely reduced as in *wnt8* mutants (Fig. 12H). Taken together, these results show that Wnt8 expression and patterning activity does not depend on Vent and Vox, with the significant exception that Wnt8 is unable to repress organizer genes when Vent and Vox are absent.

To further show that Wnt8 requires Vent and Vox in organizer repression, we tested whether exogenous Wnt8 can repress organizer genes in *vent*;*vox* mutants. We injected a *wnt8* ORF1 expression plasmid (20 ngs/μL) in 1-cell stage *vent*;*vox* mutants and assayed *gsc* expression at shield stage. No injected *vent*;*vox* mutant embryos (n=25; genotyped by PCR) displayed reduced *gsc* expression, although this treatment did result in decreased *gsc* expression in wild-type siblings (n=54). As a control, we checked that the injected *wnt8* DNA was sufficient to induce ectopic *ven*t and *vox* expression in wild-type embryos (64% ectopic expression for *vent*, n=25; 42.8% ectopic expression for *vox*, n=35). Thus, repression of the organizer by exogenous Wnt8 requires Vent or Vox.

Our results show that in the absence of Vent and Vox, *wnt8* is expressed and active as assayed by TOPdGFP reporter expression, *tbx6* expression and embryonic A/P patterning. Further, ectopic Wnt8 cannot repress *gsc* in *vent*;*vox* mutants . These data strongly support a linear model where Wnt8 acts directly upstream of Vent and Vox to repress the organizer.

Both Wnt8 and BMP2b are required at different timepoints for the maintenance of *vent* and *vox*

Two pathways are required for the maintenance of *vent* and *vox* expression in zebrafish: the zygotic BMP pathway (Melby et al., 2000; Imai et al., 2001) and the Wnt pathway (this work). To understand the combined regulation of *vent* and *vox* during gastrulation by the Wnt8 and BMP pathways, we analyzed the phenotype of *wnt8;swr* double mutants (Fig. 13). Using *swr* (*bmp2b*) mutants is sufficient to assess the influence of

zygotic BMP signaling as it was previously shown that loss of BMP2b produces a zygotic *bmp*$^{-}$ null phenotype (Schmid et al., 2000). The requirement for both BMP and Wnt8 inputs towards *vent* and *vox* expression would be revealed if *wnt8*;*swr* double mutants exhibit a phenotype similar to the *vent*$^{-}$;*vox*$^{-}$ phenotype. We found that *gsc* and *chd* are expressed in a broader domain around the mesodermal margin in shield stage *wnt8;swr* double mutants compared to either single mutant (Fig 13B, compare with Fig. 7, and data not shown), and thus they phenocopy *vent;vox* mutants (Fig. 13A). The same results were obtained when using the *wnt8* deficiency or *wnt8* MO knockdown (Fig. 13G), confirming the specificity of the interaction.

Since *wnt8;swr* double mutants display the same expanded organizer phenotype as *vent;vox* mutants at shield stage, we expected *vent* and *vox* mRNAs to be absent or strongly reduced. We found both *vent* and mesodermal *vox* to be strongly reduced but not completely absent in shield stage *wnt8; swr* double mutants (Fig. 13E,J). Both *vent* and *vox* transcripts are not detectable in the mesoderm of later stage *wnt8;swr* double mutants (data not shown).

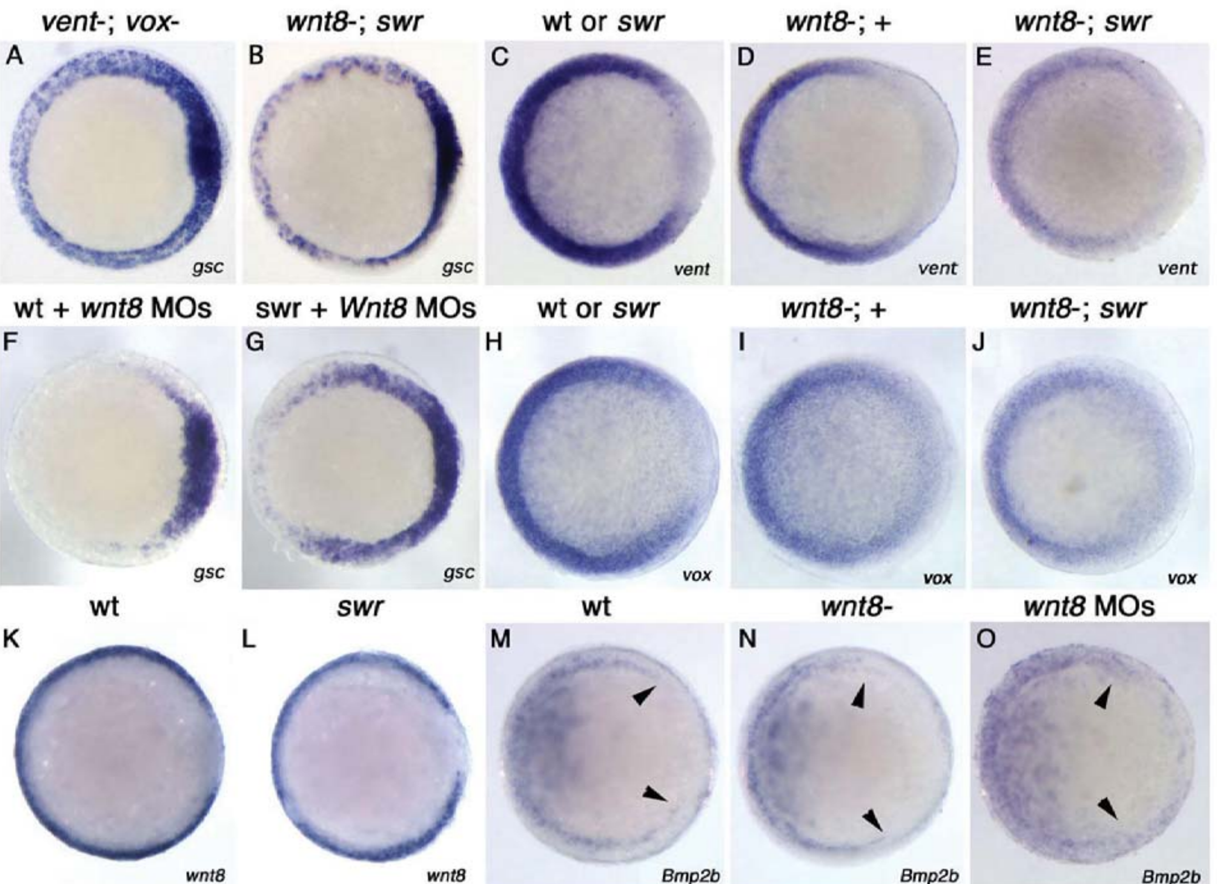

Fig. 13. Wnt8 and zygotic BMP both regulate *vent* and *vox* but differently. In situ hybridization for *gsc* (A-B,F-G), *vent* (C-E), *vox* (H-J), *wnt8* (K, L), and *bmp2b* (M-O). Genotypes/treatments are indicated above each panel. Note circumferential *gsc* in *vent*;*vox* (A) and *wnt8*;*swr* double mutants/morphants (B,G) and strong reduction of *vent* (E) and *vox* (J) in *wnt8*$^-$;*swr*$^-$. Wnt8 is still expressed in *swr* mutants (L), and *bmp2b* is still expressed in *wnt8* mutants/morphants (N, O). Arrowheads in M-O indicate dorsal limits of mesodermal *bmp2b*, which is shifted slightly ventrally in *wnt8* mutants/morphants (N, O). Shield stage, animal view, dorsal right.

The fact that double mutants look worse than *wnt8* or *swr* single mutants suggests that Wnt8 and BMP function in parallel to regulate *vent* and *vox*. Consistent with this, *bmp2b* expression in *wnt8* mutants/morphants is close to wild-type (Fig. 13M-O) and *wnt8* expression in *swr* mutants is normal at shield stage (Fig. 13K,L). Hence, both Wnt8 and BMP2b are early regulators of *vent* and *vox*, but Wnt8 has a more prominent role until mid-gastrula stages.

DISCUSSION

To understand the D/V phenotype of *wnt8* mutants, we have analyzed the interaction of Wnt8, BMP, Vent and Vox. We found that levels of both repressors are lower in *wnt8*$^{-}$ embryos at 40% epiboly when the expanded organizer phenotype initiates (Fig. 14). Consistent with a direct role for Wnt8 in *vent/vox* regulation, an inducible Lef/β-catenin fusion protein induces ectopic *vent* and *vox* transcription in the absence of new protein synthesis. Vent and Vox can repress organizer genes in the absence of Wnt8, arguing that a simple linear pathway connects Wnt8/β-catenin with Vent/Vox-dependent organizer repression. In support of this, Wnt8 is unable to repress the organizer in the absence of Vent and Vox, although it is able to induce a Wnt reporter gene and to function in A/P patterning. In addition, exogenous Wnt8 cannot repress *gsc* in *vent*;*vox* mutants. Finally, *vent* and *vox* regulation is under the control of both Wnt8 and zygotic BMP (Fig. 14), although Wnt8 is the primary regulator during early-mid gastrula stages.

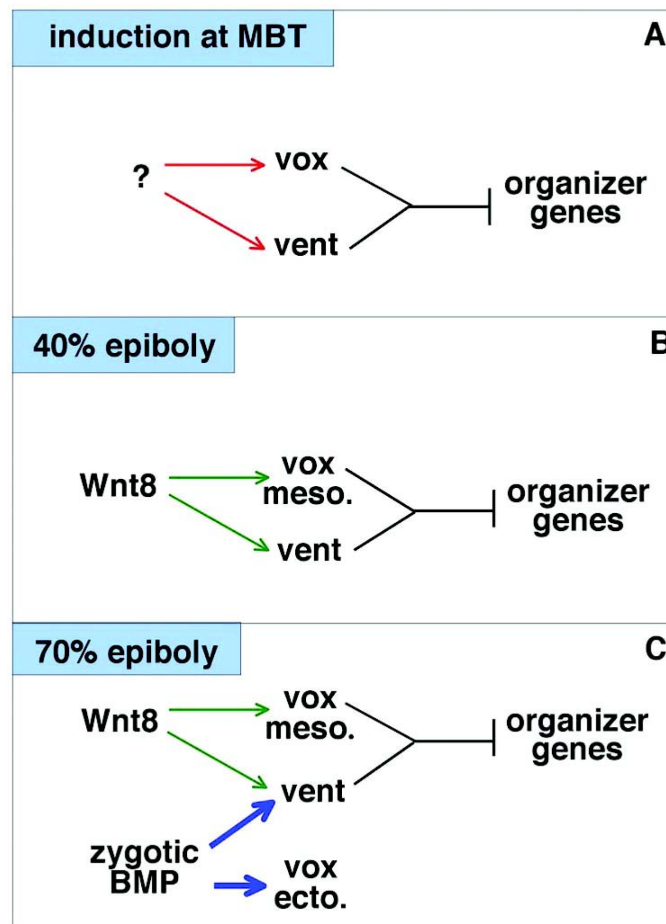

Fig. 14. Regulation of *vent* and *vox* by Wnt8 and zygotic BMP. (A) *vent* and *vox* are induced around MBT by an unknown factor. (B) At 40% epiboly, Wnt8 is required to maintain high levels of *vent* and mesodermal *vox* expression. (C) At 70% epiboly, in addition to Wnt8, zygotic BMP is required to maintain *vent* expression. BMP is also required for ectodermal *vox* expression. Thicker arrows represent stronger regulatory connections, as *vent* and ectodermal *vox* expression is absent in zygotic BMP mutants at this stage, while *vent* and mesodermal *vox* expression are only reduced in *wnt8* mutants.

vent and *vox* are transcriptional targets of Wnt8/β-catenin signaling

While it is not known what induces *vent* and *vox*, our data show that Wnt8 regulates their early transcriptional maintenance. What is unclear is which Lef or Tcf proteins are involved in Wnt8-mediated transcriptional regulation. Studies in *Xenopus* suggest that Lef1 and not Tcf3 may mediate Xwnt-8 function (Roël et al., 2002) but this has not yet been addressed in zebrafish.

Interestingly, it has recently been observed that overexpression of a conditional dominant repressor form of Tcf (hs-ΔTcf) leads to a more severe phenotype than the loss of Wnt8 (Lewis et al., 2004). Lewis et al. found that *gsc* expression encircles the margin of transgenic hs-ΔTcf embryos heat-shocked at 4 HPF, a phenotype similar to *vent;vox* or *wnt8;swr* double mutants. Why would overexpression of a dominant-negative Tcf produce a more severe phenotype than loss of Wnt8 signaling? This could be explained if ΔTcf not only abolishes Wnt8 function but also prevents other factors from positively regulating *vent* and *vox*. One such factor could be the Smads that mediate Bmp2b function, since we have shown that zygotic Bmp signaling is essential for maintaining *vent* and *vox* expression in the absence of Wnt8. In other words, ΔTcf may prevent Smad-dependent regulation of *vent* and *vox*.

Regulation of *vent* and *vox* by Wnt8: comparison between zebrafish and *Xenopus*

The transcriptional regulation of *Xvent* genes has been studied quite extensively in *Xenopus* where most were found to be direct targets of BMP4 signaling (Rastegar et al., 1999, Henningfeld et al., 2000; Henningfeld et al., 2002; Lee et al., 2002). The analysis

of their regulation by Xwnt-8 is, however, less complete. It was found that zygotic Wnt signaling is necessary and sufficient for *Xvent-1* and *Xvent-2* expression (Hoppler and Moon, 1998; Marom et al., 1999) in agreement with our findings for zebrafish Wnt8. Analysis of *Xenopus* embryos overexpressing dominant-negative Xvent-1 and Xvent-2 revealed that *Xwnt-8* expression is not affected by the loss of Xvent activity (Onichtchouk et al., 1998). Again, our data agree as *wnt8* is expressed in *vent*;*vox* mutants. The inability of Xwnt-8 to rescue the dominant-negative *Xvent* phenotype was interpreted to mean that Xwnt-8 functions in a different pathway than BMP4/Xvent (Onichtchouk et al., 1998). However, we propose that, as in zebrafish, Xwnt-8 functions upstream of *Xvent* genes and that apparent differences between our model and *Xenopus* models may be due to the different experimental approaches. For example, concomitant reduction of Xwnt-8 and Xvent-1 and –2 activities using dominant-negative proteins results in a more severe phenotype than reducing Xvent-1 and –2 alone (Onichtchouk et al. 1998). This is also what we observed when injecting *vent* and *vox* MOs in a *wnt8*$^{-}$ background. Thus, our results agree with data obtained in *Xenopus*, although our interpretation of the Wnt8/Vent/Vox relationship is somewhat different.

Wnt8 and zygotic BMP are required during gastrulation to maintain *vent* and *vox* expression at different timepoints

Our results show that both Wnt8 and BMP2b (hence zygotic BMP) are required to maintain *vent* and *vox* levels during gastrulation but that Wnt8 regulation of those genes occurs earlier at the blastula/gastrula transition (Fig. 14). The lack of an expanded

organizer in *swr* mutants can be explained by the late regulation of *vent* and *vox* by zygotic BMP after the organizer has been formed. In addition, mesodermal *vox* levels are unchanged in *swr* mutants (only ectodermal *vox* levels are reduced at 70%; Melby et al., 2000). Hence, mesodermal Vox can repress dorsal genes in *swr* mutants. Consistent with this, injection of *vox* MO in *swr* mutants results in expanded *gsc* expression at 70% epiboly (our unpublished observations).

There are two known BMP signaling pathways in *Xenopus* and zebrafish (Dale and Jones, 1999; Wilm and Solnica-Krezel, 2003). In zebrafish, the maternal BMP pathway is thought to establish ventral identity in a manner analogous to the establishment of a dorsal axis by maternal β–catenin activity (Kramer et al., 2002; Sidi et al., 2003). Understanding the regulation of Wnt8 by maternal and zygotic BMP may explain apparently contradictory results from *Xenopus* and zebrafish. For instance, while it was found that regulation of zebrafish *vent* and *vox* by zygotic BMP occurs at mid- to late gastrulation (Melby et al., 2000), *Xenopus Xvent-2* regulation by BMP signaling occurs during early gastrulation (stage 10.5; Ladher et al., 1996). *Xvent-2* regulation was observed in embryos overexpressing a truncated BMP2/4 receptor that does not distinguish between BMP2 or BMP4 ligands (Suzuki et al., 1994). However, BMP2 is both maternally provided and zygotically expressed (Dale and Jones, 1999). It has therefore been suggested that *Xvent-2* expression may be under the influence of a maternal BMP signal (Ladher et al., 1996). Interestingly, the use of the same BMP knockdown approach also results in decreased *X-wnt8* expression (Schmidt et al., 1995, Hoppler and Moon, 1998). In zebrafish, it has been reported that loss of maternal BMP

(Radar) signaling does not interfere with the induction of *vent* and *vox* at MBT (Sidi et al., 2003) although embryos homozygous for maternal *smad5* display slightly expanded *gsc* and *chd* expression (Kramer et al., 2002). Thus, the elucidation of the relationship between Wnt8 and maternal or zygotic BMP in zebrafish using a loss-of-function approach may address whether the regulation of *vent* and *vox* is fundamentally different between zebrafish and *Xenopus*.

CHAPTER III

MULTIPLE LEVELS OF INTERACTION BETWEEN WNT8 AND BMP DURING DORSOVENTRAL PATTERNING IN ZEBRAFISH

INTRODUCTION

Dorsoventral (D/V) patterning in vertebrates requires the input of both the BMP (Bone Morphogenetic Protein) and Wnt signaling pathways to specify ventral fates (reviewed in De Robertis et al. 2000; Schier, 2001). Both pathways can activate ventral genes independently of each other, but they also share some common targets (Hoppler and Moon, 1998; Marom et al., 1999; Szeto and Kimelman, 2004; Ramel and Lekven, 2004). While studies in zebrafish have highlighted a dynamic regulation of target genes by both pathways (Ramel and Lekven, 2004), it is still unclear what their relationship is, although work in *Xenopus* suggests that their relationship is complex (Hoppler and Moon, 1998; Marom et al., 1999).

Wnt8 is a known ventralizing agent in *Xenopus* and zebrafish (Christian and Moon, 1993; Hoppler et al., 1996; Kelly et al., 1995; Lekven et al., 2001). It has two main functions during D/V patterning. First, it is required to maintain the size of the dorsal organizer during gastrulation (Lekven et al., 2001) and our recent study of its relationship with the ventrally expressed transcriptional repressors Vent and Vox showed that the repression of the organizer by Wnt8 is mediated by Vent and Vox

(Ramel and Lekven, 2004). Second, Wnt8 is also required to maintain the expression of ventral genes in the mesodermal margin such as *even-skipped-1* (*eve1)*, *vent* or *vox*, which are also known BMP targets (Ramel and Lekven, 2004; Mullins et al., 1996; Melby et al., 2000).

BMPs are members of the TGF-β family of proteins and, like Wnt8, they are required for the specification of ventral fates (reviewed in Hammerschmidt and Mullins, 2002). BMP overexpression or knockdown in *Xenopus* leads to an increase or decrease in the expression of ventral markers, respectively (reviewed in Dale and Jones, 1999). Analysis of *bmp* mutant phenotypes in zebrafish has revealed that their ventralizing function is conserved in vertebrates (Mullins et al., 1996; Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). Four BMP ligands have been identified in zebrafish that are required during D/V axis formation and patterning: Radar, BMP2b, BMP4 and BMP7 (reviewed in Wilm and Solnica-Krezel, 2003). Radar is a maternally contributed BMP ligand and is required for BMP2b and BMP4 induction (Sidi et al., 2003). BMP2b and BMP7 are expressed starting at the Mid-Blastula Transition (MBT) and are also required for ventral patterning. Indeed, zebrafish mutants for *bmp2b* (*swirl*) and *bmp7* (*snailhouse*) are dorsalized and lack ventrally derived structures (Mullins et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998; Dick et al., 2000; Schmid et al., 2000). BMP7 is also maternally contributed but is not thought to play an active ventralizing role before MBT (Dick et al., 2000; Schmid et al., 2000). In addition, BMP2b and 7 are thought to function as heterodimers during ventral patterning (Schmid et al., 2000). In contrast with *bmp2b* and *7*, *bmp4* zygotic expression starts at 30% epiboly (late

blastula/early gastrula; Nikaido et al., 1997). No *bmp4* mutant has yet been identified in zebrafish but overexpression experiments suggest that it is also involved in promoting ventral fates (Nikaido et al., 1997).

Processed BMP ligands form homo- or hetero-dimers and activate a complex made of type II (BMPRII) and type I (BMPRI) serine-threonine kinase receptors to activate downstream components of the BMP signaling pathway (Shi and Massagué, 2003). Upon binding, the type II receptor phosphorylates the type I receptor and in this complex, the type I receptor determines the specificity of the signal. In zebrafish, three BMPRI receptors (also called Alks) have been identified downstream of ventralizing BMPs. *alk8*, *alk3* (BMPRIA) and *alk6* (BMPRIB) are all maternally contributed and zygotically expressed in zebrafish embryos (Nikaido et al., 1999a; Nikaido et al., 1999b; Bauer et al., 2001). *alk8* is disrupted in the dorsalized *lost-a-fin* mutant (Bauer et al., 2001). Genetic and morpholino (MO) knockdown analysis suggest that Alk8 functions zygotically downstream of BMP2b and BMP7 (Bauer et al., 2001). A constitutively active form of Alk3 (CA-BMPRIA) ventralizes the mesoderm of injected embryos, while injection of a dominant negative version (DN-BRIA) results in a dorsalized phenotype similar to *bmp* mutants (Nikaido et al., 1999a). Similarly, injection of a dominant negative version of Alk6 (DN-BMPRIB) leads to strong dorsalization (Goutel et al., 2000). Thus, there is evidence that Alk3, 6, and 8 are involved in the ventralizing process during zebrafish embryogenesis.

Activation of BMP receptors leads to the activation of Smads (Smad1, 5, or 8), which, together with a co-Smad (Smad4), regulate the expression of target genes by

binding to promoters and recruiting other transcription factors (reviewed in Zwijsen et al., 2003). In zebrafish, the zygotic *smad5* mutant *somitabun* (*sbn*) displays a dorsalized phenotype, consistent with Smad5 being required in the BMP signaling network to specify ventral fates (Mullins et al., 1996; Hild et al., 1999). Data suggest that Smad5 is the receptor Smad that functions downstream of BMP2b/BMP7 signaling (Bauer et al., 2001; Dick et al., 2000; Hild et al., 1999). In addition, analysis of a maternal *smad5* mutation revealed that it has an additional early function and turns on *bmp7* expression at MBT (Kramer et al., 2002). Unlike *smad5*, which is both maternally and zygotically expressed, *smad1* expression starts at 30% epiboly and its expression is thought to depend on earlier activation of the BMP2b/BMP7/Smad5 pathway (Dick et al., 1999). Interestingly, while Smad5 overexpression can rescue *snailhouse* (*snh)* mutants, only Smad1 overexpression can rescue both *swirl* (*swr*) and *snh* mutants (Dick et al., 1999), suggesting that BMP2b has a later function independent of Smad5 and BMP7 and mediated by Smad1.

A current model suggests that there are three phases of BMP function during DV patterning in zebrafish (Dick et al., 1999; Hammerschmidt and Mullins, 2002; Wilm and Solnica-Krezel, 2003). During the first phase, one or two maternal BMP pathways involving Radar and Smad5 are required to turn on BMP2b and BMP7 expression at MBT and thus specify the ventral region of the zebrafish blastula (Kramer et al., 2002; Sidi et al., 2003). The second phase of BMP signaling involves BMP2b and BMP7 possibly functioning as a heterodimer, acting through Alk8 and Smad5, to turn on ventrally expressed genes (Schmid et al., 2000; Bauer et al., 2001). At the same time,

BMP antagonists including Chordin (Chd) and Noggin secreted by the dorsal organizer (or shield in zebrafish) shape the gradient of BMP activity, leading to stronger BMP signaling ventrally and weaker signaling dorsally (De Robertis et al., 2000). The third phase of BMP signaling probably involves BMP2b, BMP4 and Smad1. This third signal may be responsible for patterning proper of the ventral region previously defined by maternal BMP signaling and refined by BMP2b/7. Thus, in zebrafish, maternal vs zygotic BMP pathways are relatively well defined and loss-of-function tools are available to study the activity of each one. Such an approach is important for understanding these pathways since a dominant-negative construct, used to generate a *bmp* loss-of-function phenotype in *Xenopus*, is known to disrupt both maternal and zygotic BMP activity (Dale and Jones, 1999; Suzuki et al., 1994).

The relationship between Wnt8 and BMP is an essential one for proper ventral patterning but still remains poorly characterized. In *Xenopus*, it was shown that Wnt8 is downstream of BMP signaling, but it is unclear if this reflects a regulation by maternal or zygotic BMP (Hoppler and Moon, 1998). Additionally, it has been shown that overexpression of BMP at moderate levels induces *wnt8* expression but high level of BMP represses it (Marom et al., 1999). Studies have also shown that *bmp4* expression in *Xenopus* does not depend on Wnt8 (Hoppler and Moon, 1998). In zebrafish, it has been reported that Wnt8 overexpression can induce ectopic *bmp2b* expression (Agathon et al., 2003) but BMP is not necessary for normal *wnt8* expression (Mullins, 1999; Ramel and Lekven, 2004; Szeto and Kimelman., 2004), although analysis of *swr* mutants at the end

of gastrulation suggests that high BMP activity can negatively regulate *wnt8* (Mullins, 1999). Thus, the interdependency of BMP and Wnt8 remains unclear.

In this study, we have analyzed the epistatic relationship between BMP and Wnt8 during D/V patterning of the zebrafish embryo using both loss-of-function and overexpression assays. We found that, starting at 75% epiboly (mid-gastrulation), the mesodermal domain of zygotic *bmps* is dependent on Wnt8. However, the expression of other components of the BMP signaling pathway such as *alks* and *smads* are not affected in the absence of Wnt8 with the exception of *alk6,* which displays ventrally expanded expression in *wnt8* mutants. The domain-specific loss of *bmp* expression appears to be an indirect consequence of the enlarged organizer of *wnt8* mutants. Our results also suggest that *bmp2b* expression depends on direct regulation by Wnt8. Conversely, we found that the expression of Wnt8 is not induced nor positively maintained by BMP signaling. However, BMP was found to be sufficient for *wnt8* expression in gain-of-function assays. To understand how Wnt8 functions in the BMP ventralizing process, we overexpressed BMP signaling components in a *wnt8*- background and it revealed that Wnt8 function is essential between the second and third phase of BMP signaling. Thus, we propose that Wnt8 function is essential for BMP signaling, mainly by allowing proper generation of the BMP gradient through the repression of organizer genes.

MATERIALS AND METHODS

Fish maintenance and genetics

Zebrafish adults were maintained as described (Westerfield, 2000). Embryos were staged as described (Kimmel et al., 1995). Wild-type fish were of the AB background. Alleles used in this study were *Df(LG14)wnt8*^{w8} /+ (*Df*^{w8}/+; *wnt8*-/+ in this study; Lekven et al., 2001) and *swr*^{TC300}/+ (*swr/*+; Mullins et al., 1996). Results obtained using *Df*^{w8} were confirmed using *wnt8* ORF1 + ORF2 morpholinos (MOs). *wnt8*- and *swr* mutant embryos were genotyped as described previously (Lekven et al., 2001; Wagner and Mullins, 2002).

In situ hybridization

In situ hybridizations were performed essentially as described (Jowett, 2001). The probes used in this study were *wnt8* ORF1 and *wnt8* ORF1+ORF2 (Lekven et al., 2001), *even-skipped-1* (*eve1*; Joly et al., 1993), *bmp2b* (Kishimoto et al., 1997), *bmp7* (Schmid et al., 2000), *odd-paired like* (*opl*; Grinblat et al., 1998), *pax2a* (Krauss et al., 1991), and *tbx6* (Hug et al., 1997).

Embryo microinjection, morpholinos, constructs

MOs (Genetools, LLC), RNA or DNA, were injected into one to four-cell stage embryos. A volume of approximately 3 nL was injected per embryo. Capped mRNAs were synthesized using mMESSAGE mMACHINE (Ambion) and diluted in RNase-free

water for injection. MOs were diluted in Danieau's buffer as indicated (Genetools, LLC). The *wnt8* splice blocking MOs targeting ORF1 and ORF2 are described elsewhere (see chapter IV) as well as the *chd* MO (Nasevicius and Ekker, 2000).

DNA constructs

The *bmp2b* and *smad1* cDNAs were amplified by RT-PCR and cloned into the pCS2P+ vector. *alk3* and *6* cDNAs were amplified by RT-PCR and cloned into the pGEM-T Easy vector (Promega) to make probes. pSP64T-*radar* (Goutel et al., 2000) and pSP64T-*bmp4* (Nikaido et al., 1997) were gifts from Frederic Rosa and Mary Mullins respectively. To generate pCS2P+-*radar* and *bmp4*, pSP64T-*radar* and *bmp4* were digested with BamHI and HindIII, and ligated into pCS2P+. pCS2+-*bmp7* (Schmid et al, 2000) was a gift from Mary Mullins. The pCS2P+-*radar* DN construct was generated as described by removing 52 amino acids at the C-terminus of *radar* from the pCS2P+-*radar* plasmid (Sidi et al., 2003). CA-*alk8* and pSP64T-*smad5* were gifts from Matthias Hammerschmidt (Bauer et al., 2001; Hild et al., 1999). PSP64T-CA-*alk3* (CA-BRIA) and pSP64T-CA-*alk6* (CA-BRIB) were gifts from Naoto Ueno (Nikaido et al., 1999a; unpublished).

RESULTS

Wnt8 is required to maintain mesodermal *bmp2b/4/7* expression

To start deciphering the relationship between Wnt8 and BMP signaling in D/V patterning, we asked if zygotic Wnt8 function is essential for zygotic *bmp* expression. Previous work in zebrafish had shown that Wnt8 was sufficient to induce *bmp2b* (Agathon et al., 2003), but it is unclear if Wnt8 is also necessary for *bmp* expression. The zebrafish *wnt8* locus contains two Open Reading Frames (ORFs), coding for two Wnt8 proteins (ORF1 and ORF2) that can substitute for each other in the ventralizing or posteriorizing process (Lekven et al., 2001). The *wnt8*- phenotype can be obtained by injecting *wnt8* ORF1 and ORF2 MOs or by using a deficiency line that removes the *wnt8* locus (*Df(LG14)wnt8*^{w8} /+ or *Df*^{w8}/+; called *wnt8*-/+ in this study, Lekven et al., 2001).

No significant difference in expression between wild-type and *wnt8* mutants was observed prior to 75% epiboly for all zygotic *bmps* tested (data not shown; Ramel and Lekven, 2004). However, at 75% epiboly, *bmp2b/4/7* expression in the embryonic margin was strongly reduced or absent in *wnt8* mutants/morphants (Fig. 15B,C,G,H,L,M). In *wnt8* mutants/morphants, some of the ectodermal bmp2b domain was preserved (asterisk Fig. 15B,C) and dorsal *bmp2b* was expanded ventrally (red arrow in Fig. 15A-C), consistent with the expanded organizer present in *wnt8* mutants (Lekven et al., 2001), while mesodermal *bmp2b* expression was strongly reduced (black arrow in Fig. 15A-C). Most of the *bmp4* expression in the mesoderm and vegetal ectoderm was reduced in the *wnt8*- background (Fig. 15F,G). A similar result was

observed for *bmp7* expression (Fig. 15K,L). Thus, Wnt8 function is required to maintain mesodermal *bmp* expression at mid-gastrulation.

To obtain a comprehensive knowledge about the status of zygotic BMP signaling components in *wnt8* mutants at 75% epiboly, we also looked at the expression of BMP type I receptors and Smads. *alk3* (BMPRIA) and *alk8* are expressed ubiquitously in wild-type embryos at 75% epiboly (Fig. 15D,N; Nikaido et al., 1999a; Bauer et al., 2001). They are expressed at similar levels in *wnt8* mutants (Fig. 15E,O). In contrast with *alk3* and *8* expression, *alk6* (BMPRIB) is weakly expressed dorsally in the future anterior neuroectoderm at 75% epiboly (Fig. 15I; Nikaido et al., 1999b). In *wnt8* mutants, *alk6* is weakly expressed and expanded ventrally, consistent with the expanded organizer phenotype of these mutants (Fig. 15J; Ramel and Lekven, 2004). *smad1* is expressed ventrally at mid-gastrulation while *smad5* is expressed ubiquitously (Fig. 15P,R; Hild et al., 1999; Dick et al., 1999). We found that neither *smad1* nor *smad5* expression was reduced in *wnt8* mutants at mid-gastrulation compared to wild-type (Fig. 15Q,S). Thus, Wnt8 is necessary for mesodermal *bmp* expression starting at mid-gastrulation but not for the expression of other members of the BMP signaling pathway. Also, loss of *bmp* in *wnt8* mutants is not merely due to downregulation of other BMP pathway components.

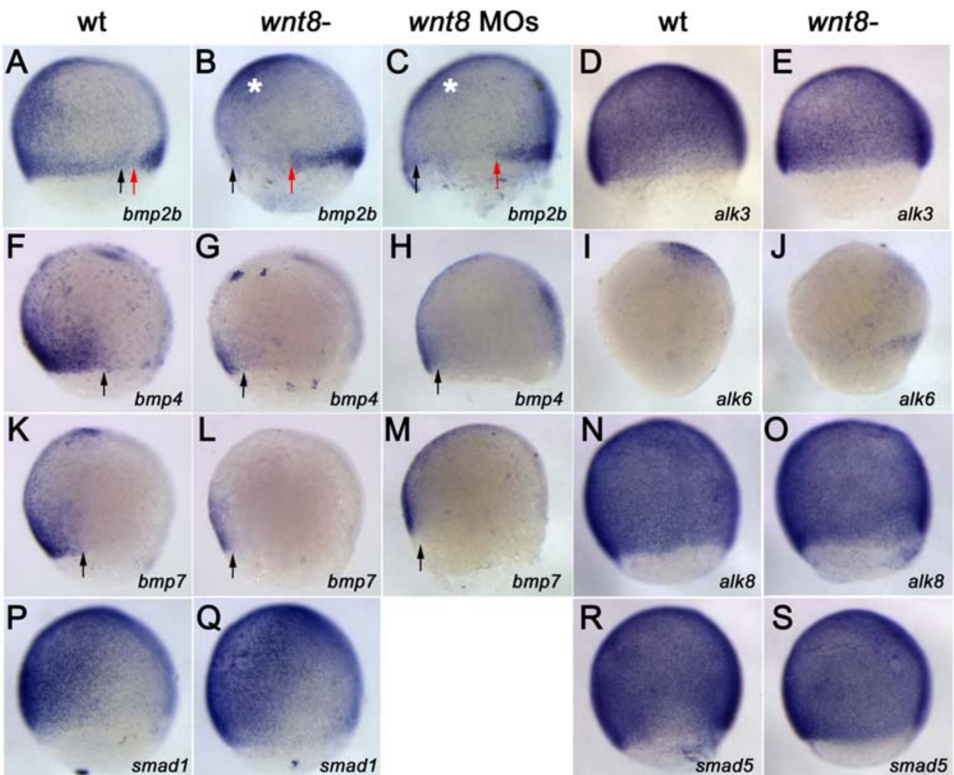

Fig. 15. Wnt8 maintains zygotic *bmp* expression in the mesoderm. In situ hybridizations for *bmp2b* (A-C), *bmp4* (F-H), *bmp7* (K-M), *alk3* (D,E), *alk6* (I,J), *alk8* (N,O), *smad1* (P,Q), and smad5 (R,S). Genotypes are indicated above each column. Expression of all zygotic bmps is strongly reduced in a *wnt8*- background (black arrow in B,C,G,H,L,M). B,C: some ectodermal *bmp2b* expression is preserved (asterisk) in wnt8 mutants/morphants. The dorsal domain of *bmp2b* expression (red arrow in A-C) is expanded in *wnt8* mutants/morphants reflecting the expanded organizer. *alk3, 8, smad1,* and *5* are expressed at wild-type levels in *wnt8* mutants (E,O,Q,S). *alk6* is weakly expressed in *wnt8* mutants and expanded ventrally (J). All panels show 75% epiboly embryos, lateral view, dorsal right.

Wnt8 maintains mesodermal *bmp* expression mainly through organizer repression

bmp2b expression is known to be at least partly under autoregulatory control. For instance, *bmp2b* expression is reduced in the ectoderm of *swr* (*bmp2b*) mutants at mid-gastrulation (Kishimoto et al., 1997). The dorsal domain of *bmp2b* expression is independent of BMP2b as it is still present in *swr* mutants (Kishimoto et al., 1997). *bmp2b* expression is strongly reduced in *vent*; *vox* mutants which have enlarged organizers and correspondingly expanded domains of BMP inhibitors (Imai et al., 2001). Since *wnt8* is still expressed in *vent*; *vox* mutants (Ramel and Lekven, 2004), this implies that *bmp2b* is not a direct transcriptional target of Wnt8 but may be reduced when the organizer is expanded. To test whether the expanded organizer of *wnt8* mutants is responsible for the observed loss of *bmp2b* expression, we generated embryos lacking Wnt8 and the BMP inhibitor Chd by injecting *wnt8* mutants with *chd* MO and examined *bmp2b* expression at 75% epiboly (Fig. 16). The dose of *chd* MO that was injected was sufficient to completely ventralize the ectoderm of *wnt8*- embryos when looking at *gata2* expression (not shown). Some mesodermal *bmp2b* expression was restored in 67% of *wnt8* mutants injected with *chd* MO (n=12; arrow in Fig. 16C). However, no complete rescue of mesodermal *bmp2b* expression was ever observed. Thus, the loss of *bmp2b* in the margin of *wnt8* mutants is partly due to an increase in the Chd expression domain. In contrast with the effects that we observed with *bmp2b*, *bmp4* and *7* expression was restored and expressed at high levels in *wnt8* mutants injected with *chd* MO (100% for *bmp4*, n=7, Fig. 16E,F; 71.4% for *bmp7*, n=7, Fig. 16H,I). Therefore, Wnt8 appears necessary for some levels of *bmp2b* expression in the mesoderm, even in the absence of

a BMP inhibitor like Chd. This does not appear to be the case for *bmp4* and 7 as their expression is restored upon Chd knockdown.

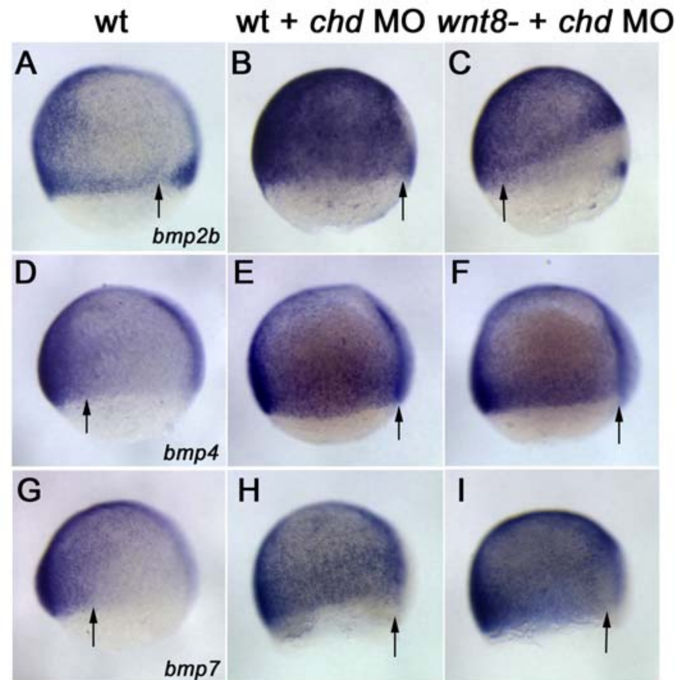

Fig. 16. Repression of the organizer by Wnt8 is essential for maintenance of zygotic *bmp* expression. In situ hybridization for *bmp2b* (A-C,), *bmp4* (D-F), and *bmp7* (G-I). Genotypes and treatments are indicated above each column. *chd* MO injection in *wnt8* mutants only restores some *bmp2b* expression (arrow in C) but leads to strong expression of *bmp4* (F) and *bmp7* (I). All panels show 75% epiboly embryos, lateral view, dorsal right.

Our results point to an essential role of Wnt8 for *bmp2b* transcription that is not only due to reduced autoregulation. As mentioned above, *bmp2b* expression is strongly reduced in the ectoderm of *swr* mutants but mesoderm expression persists (Kishimoto et al., 1997). This argues that *bmp2b* expression in the mesoderm does not depend entirely on the BMP2b autoregulatory loop and that another regulatory factor present in *swr* mutants maintains mesodermal *bmp2b*. Consistent with the critical requirement for Wnt8 towards *bmp2b* expression, we found *wnt8* to be expressed at wild-type levels in the mesoderm of *swr* mutants at 75% epiboly (not shown; Mullins, 1999). Wnt8 is also expressed and active in *vent*; *vox* mutants at this stage (Ramel and Lekven, 2004) while these mutants do not maintain any *bmp2b* expression (Imai et al., 2001). Since this loss of *bmp2b* expression has been attributed to increased *chd* expression (Imai et al., 2001), we hypothesized that knockdown of Chd should rescue mesodermal *bmp2b* expression in *vent*; *vox* mutants (where Wnt8 is active) better than in *wnt8* mutants. This experiment is in progress.

Thus, Wnt8 is essential to maintain zygotic *bmp* expression starting at mid-gastrulation. Wnt8 achieves this at least partly by maintaining *chd* expression in the dorsal organizer (for all *bmps* tested) but also affects *bmp2b* differently (see discussion).

BMP activity is sufficient but not necessary for *wnt8* expression

While Wnt8 activity is essential to maintain *bmp* expression, it is unclear if the reverse relationship holds true. In zebrafish, BMP2b has been reported not to be able to induce *wnt8* expression (Agathon et al., 2003) and it was shown that high BMP activity

negatively regulates *wnt8* at the end of gastrulation (Mullins, 1999). We chose to further investigate this relationship by testing the input of both maternal and zygotic BMP pathways in *wnt8* regulation, and we re-evaluated the sufficiency of BMP towards *wnt8* induction.

To assess whether *wnt8* is regulated by BMP signaling in zebrafish, we analyzed its expression in various *bmp* loss-of-function backgrounds. First, to determine if maternal BMP signaling is required for *wnt8* expression, we injected RNA for a dominant-negative form of *radar* (*radar*-DN) that is thought to disrupt maternal BMP pathways (Radar and maternal Smad5; Sidi et al., 2003). As reported previously, only a portion of injected embryos display a dorsalized phenotype ranging from C1 to C5 upon injection (70%, n=187; Sidi et al., 2003). None of the injected embryos showed a significant reduction in *wnt8* expression at 30% epiboly or shield stage when using a probe against *wnt8* ORF1 (n=36 for 30% and 39 for shield; data not shown) or when using a probe that recognizes both ORF1 and ORF2 messages (n=65 for 30% and 36 for shield; Fig. 17A,B). These results are consistent with the fact that neither *vent* nor *vox* expression is affected in *radar*-DN injected embryos, while both genes are known Wnt8 transcriptional targets (Sidi et al., 2003; Ramel and Lekven, 2004).

While these results show that *wnt8* transcription is not dependent on maternal BMP activity, it has also previously been shown that *wnt8* ORF1 expression is not diminished in *swr* mutants (Mullins, 1999; Ramel and Lekven, 2004). Identical results were obtained using a probe that binds both ORF1 and ORF2 transcripts (not shown). Further, we confirmed these results in embryos lacking Alk8 function (not shown).

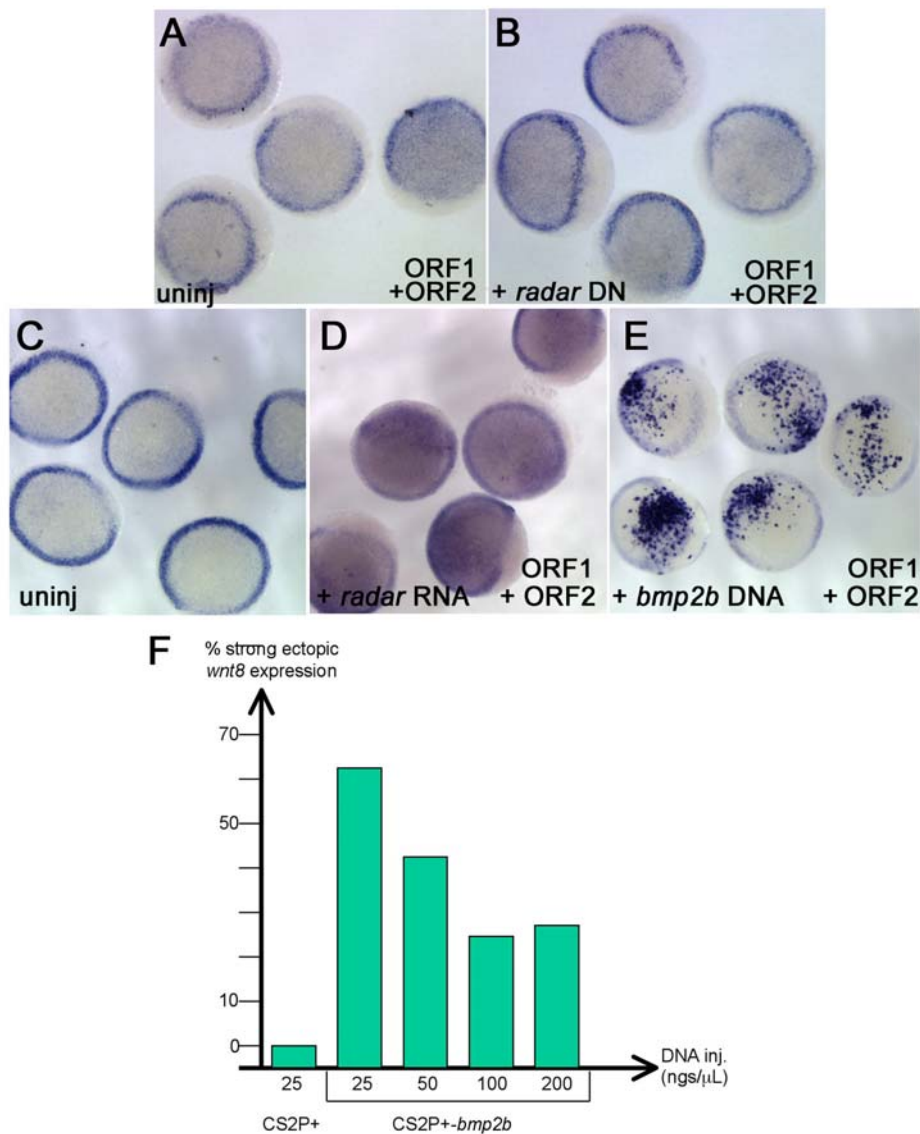

Fig. 17. BMP is not necessary but sufficient for *wnt8* expression.
A-E: in situ hybridization for *wnt8* ORF1 + ORF2 upon injection of *radar* DN RNA (B) and upon BMP overexpression (D-E). B: injection of *radar* DN RNA does not affect *wnt8* expression. D: upon injection of *radar* RNA, ectopic *wnt8* expression is observed in the animal ectoderm and dorsal mesoderm. E: *bmp2b* DNA injection can also induce ectopic *wnt8* expression. Note the difference between the RNA or DNA injection. Injection of *bmp2b* DNA leads to punctate ectopic expression (E), due to mosaic distribution of injected DNA. A,B: 30% epiboly, animal view. C,E: shield stage, animal view.
F: diagram representing effects of increasing *bmp2b* concentration on the % of embryos displaying ectopic *wnt8* expression. As *bmp2b* concentration increases, a smaller percentage of embryos show ectopic *wnt8*.

The only aspect of *wnt8* expression that appears dependent on zygotic BMP is a reduction in expression in the ventral margin that begins during late gastrulation (>80% epiboly; Mullins, 1999; confirmed with ORF1 + ORF2 probe). These results show that BMP activity is not necessary for *wnt8* expression but BMP is required for normal expression of *wnt8* during late gastrulation.

These observations suggest that BMP activity may repress but not activate *wnt8*. This in in contrast to *Xenopus* where BMP activation is sufficient to induce *wnt8* (Hoppler and Moon, 1998). We sought to test this relationship further in overexpression assays. Intriguingly, we observed that injection of *radar*, *bmp2b* and *bmp7* RNA or DNA can induce ectopic expression of *wnt8* in both dorsal mesoderm and ectoderm (Fig. 17C-E). *radar* RNA injection induced ectopic *wnt8* expression in 85% of embryos injected (n=34; Fig. 17D) . *bmp2b* DNA injection induced ectopic *wnt8* in 90% of embryos (n=30; Fig. 17E) while the efficiency of *bmp7* was lower compared to *radar* and *bmp2b*: 64 % of embryos with ectopic *wnt8* (n=39; not shown). Since our findings differ from that of Agathon et al. (Agathon et al., 2003), we hypothesized that, as in *Xenopus*, high or low levels of BMP can have different effects on *wnt8* expression. To test this, we injected increasing concentrations of *bmp2b* plasmid in wild-type embryos and counted the number of embryos displaying strong ectopic *wnt8* expression (covering at least a third of the animal ectoderm area as shown in Fig. 17E). We found that increasing the amount of BMP2b resulted in fewer embryos displaying strong ectopic *wnt8* expression (Fig. 17F) and thus confirmed that *wnt8* induction by BMP2b is dose-dependent.

To sum up, our data show that *wnt8* induction does not depend on maternal or zygotic BMP activity, although BMP does act to repress *wnt8* in the ventral margin during late gastrulation. Moreover, moderate levels of BMP are sufficient to induce ectopic *wnt8*.

BMP2b, Alk8, and Smad1 can ventralize *wnt8* morphants

Since *wnt8* is ectopically induced upon BMP overexpression, this raises the question whether BMP overexpression effects, which have been described in zebrafish (Dick et al., 2000; Goutel et al., 2000; Nikaido et al., 1997; Schmid et al., 2000) involve Wnt8. To test this, we overexpressed zebrafish BMP ligands and members of the BMP signaling pathway in a Wnt8-knockdown background. RNA for each zebrafish BMP ligand, BMP receptor, or Smad was injected in one-cell stage wild-type embryos with or without *wnt8* MOs. Resulting 24 hour phenotypes were scored and classified according to their degree of ventralization or dorsalization where V1 to V4 signify an increasing ventralization while C1 to C5 signify an increasing dorsalization (Kishimoto et al., 1997).

All BMP ligands were found to ventralize injected embryos as expected (Table 3; Fig. 18C,E). While *radar* RNA injection ventralized wild-type embryos when injected alone, co-injection with *wnt8* MOs resulted in dorsalized phenotypes such as the ones obtained upon *wnt8* MOs injection alone (Table 3). This result suggests that Wnt8 function is required for Radar-induced ventralization. In contrast, co-injection of *bmp2b* RNA with *wnt8* MOs resulted in strongly morphologically ventralized embryos similar

to embryos injected with *bmp2b* RNA alone (Table 3, Fig. 18C,D). Thus, BMP2b-induced ventralization does not appear to require Wnt8 function. Injection of *bmp7* RNA in *wnt8* morphants resulted in dorsalized embryos (Table 3). However, most of the embryos were less strongly dorsalized than *wnt8* morphants alone, suggesting that ectopic BMP7 does suppress some of the *wnt8* MOs effects. *bmp4* RNA injection in *wnt8* morphants gave mixed results: a large proportion of embryos were dorsalized, but some were also ventralized (Table 3). In addition, we observed a new phenotypic class that we judged intermediate between dorsalized and ventralized embryos (Fig. 18G,H). The frequency of this phenotype was higher in the *bmp4* + *wnt8* MOs injection than in the *bmp2b* or *bmp7* + *wnt8* MOs injection (Table 3). The affected embryos displayed a short axis like dorsalized embryos (Fig. 18H) but they also showed a moderate loss of head structures like ventralized embryos (Fig. 18G). In addition, the presence of the dorsally derived notochord was random (Fig. 18G,H). This phenotype probably reflects some degree of ventralization of *wnt8* morphants by BMP4. As a control, we also injected expression plasmids of the same zebrafish BMP ligands in combination with *wnt8* MOs and obtained similar results (Table 3, Fig. 18E,F). These results suggest that BMP ligands have differential requirements for Wnt8 in order to induce morphological ventralization and that BMP2b is uniquely able to affect *wnt8*-depleted embryos.

Table 3. Wnt8 function can discriminate various BMP signaling components

Injection	n	% ventralized				% interm ediate	% dorsalized					% wt	D/V index
		-4	-3	-2	-1	0	+1	+2	+3	+4	+5	0	
		V4	V3	V2	V1		C1	C2	C3	C4	C5		
wnt8 MOs	400	0	0	0	0	0	0	10.3	76	12.7	1	0	**+3.05**
radar RNA	33	0	21.2	30.3	48.5	0	0	0	0	0	0	0	**-1.73**
radar RNA + *wnt8* MOs	56	0	0	0	0	0	0	7.1	80.4	12.5	0	0	**+3.05**
CS2P+-*radar*	21	5	9.5	42.8	38	0	0	0	0	0	0	4.7	**-1.72**
CS2P+-*radar* + *wnt8* MOs	58	0	0	0	0	0	0	13.8	60.3	19	6.9	0	**+3.19**
bmp2b RNA	48	68.7	18.8	6.2	6.3	0	0	0	0	0	0	0	**-3.5**
bmp2b RNA + *wnt8* MOs	47	40.4	51.1	0	0	8.5	0	0	0	0	0	0	**-3.15**
CS2P+-*bmp2b*	78	67.9	29.5	2.6	0	0	0	0	0	0	0	0	**-3.65**
CS2P+-*bmp2b* + *wnt8* MOs	44	68.1	27.3	4.6	0	0	0	0	0	0	0	0	**-3.63**
bmp4 RNA	30	0	16.7	30	53.3	0	0	0	0	0	0	0	**-1.63**
bmp4 RNA + *wnt8* MOs	51	0	5.9	3.9	0	29.4	3.9	17.6	33.4	5.9	0	0	**1.37**
CS2P+-*bmp4*	46	4.3	10.9	19.6	60.9	0	0	0	0	0	0	4.3	**-1.5**
CS2P+-*bmp4*+ *wnt8* MOs	48	2.1	25	12.5	22.9	16.7	0	10.4	8.3	2.1	0	0	**-0.77**
bmp7 RNA	71	0	5.6	11.3	83.1	0	0	0	0	0	0	0	**-1.22**
bmp7 RNA + *wnt8* MOs	45	0	0	0	0	2.2	11.1	22.2	53.3	11.2	0	0	**+2.6**
CS2+-*bmp7*	44	0	9.1	20.4	59.1	0	0	0	0	0	0	11.4	**-1.27**
CS2+-*bmp7* + *wnt8* MOs	103	0	0	0	0	0	5.9	28.1	34	28.1	3.9	0	**+2.96**

Table 3 (continued)

Injection	n	% ventralized				% intermediate	% dorsalized					% wt	D/V index
		-4	**-3**	**-2**	**-1**	**0**	**+1**	**+2**	**+3**	**+4**	**+5**	**0**	
		V4	**V3**	**V2**	**V1**		**C1**	**C2**	**C3**	**C4**	**C5**		
wnt8 MOs	400	0	0	0	0	0	0	10.3	76	12.7	1	0	**+3.05**
CA-alk3 RNA	58	0	12.1	36.2	51.7	0	0	0	0	0	0	0	**-1.6**
CA-alk3 RNA + *wnt8* MOs	57	0	0	0	0	26.3	3.5	5.3	43.8	7	3.5	10.6	**+1.91**
CA-alk6 RNA	48	10.4	20.8	68.8	0	0	0	0	0	0	0	0	**-2.42**
CA-alk6 RNA + *wnt8* MOs	41	0	0	0	0	9.7	0	0	22	65.8	2.5	0	**+3.42**
CA-alk8 RNA	42	9.5	66.7	11.9	11.9	0	0	0	0	0	0	0	**-2.86**
CA-alk8 RNA + *wnt8* MOs	67	11.9	46.3	17.9	6	17.9	0	0	0	0	0	0	**-2.28**
smad1 RNA	46	2.2	8.7	15.2	73.9	0	0	0	0	0	0	0	**-1.39**
smad1 RNA + *wnt8* MOs	46	0	0	4.3	2.2	21.8	0	13	54.3	2.2	2.2	0	**+1.98**
smad5 RNA	43	7	41.9	23.2	4.6	0	0	0	0	0	0	23.3	**-2.05**
smad5 RNA + *wnt8* MOs	134	0	0	0	0	1.6	0	35.8	38.8	21.6	0.7	1.5	**+2.78**

V4 to V1: decreasing ventralized phenotype, C1 to C5: increasing dorsalized phenotype

C (*radar* RNA) & (CS2P+-*radar* DNA) = 5 ngs/µL, C (*bmp2b* RNA) & (CS2P+-*bmp2b* DNA) = 25 ngs/µL

C (*bmp4* RNA) = 50 ngs/µL, C (CS2P+-*bmp4* DNA) = 25 ngs/µL, C (*bmp7* RNA) = 380 ngs/µL, C (CS2+-*bmp7* DNA) = 50 ngs/µL, C (*CA-alk3* RNA) = 120 ngs/µL, C (*CA-alk6*) RNA = 2 ngs/µL, C (*CA-alk8* RNA) = 5 ngs/µL, C (*smad1* RNA) = 661 ngs/µL, C (*smad5* RNA) = 627 ngs/µL

- dead embryos and embryos with gastrulation defects were not tabulated -

- *radar* was injected at a low concentration because high amounts cause gastrulation defects as reported (Goutel et al., 2000)-

- CA-*alk3* was not a potent ventralizer compared to CA-*alk6* and *8*, so a high concentration of CA-*alk3* was injected-

-at similar concentrations, we did not observe that Smad1 was a more potent ventralizer compared to Smad5 as opposed to a previous report (Dick et al., 1999)-

- the D/V index (right column, blue) was obtained by assigning a numerical value to each degree of ventralization or dorsalization (second row, blue) and by calculating the average for each treatment -

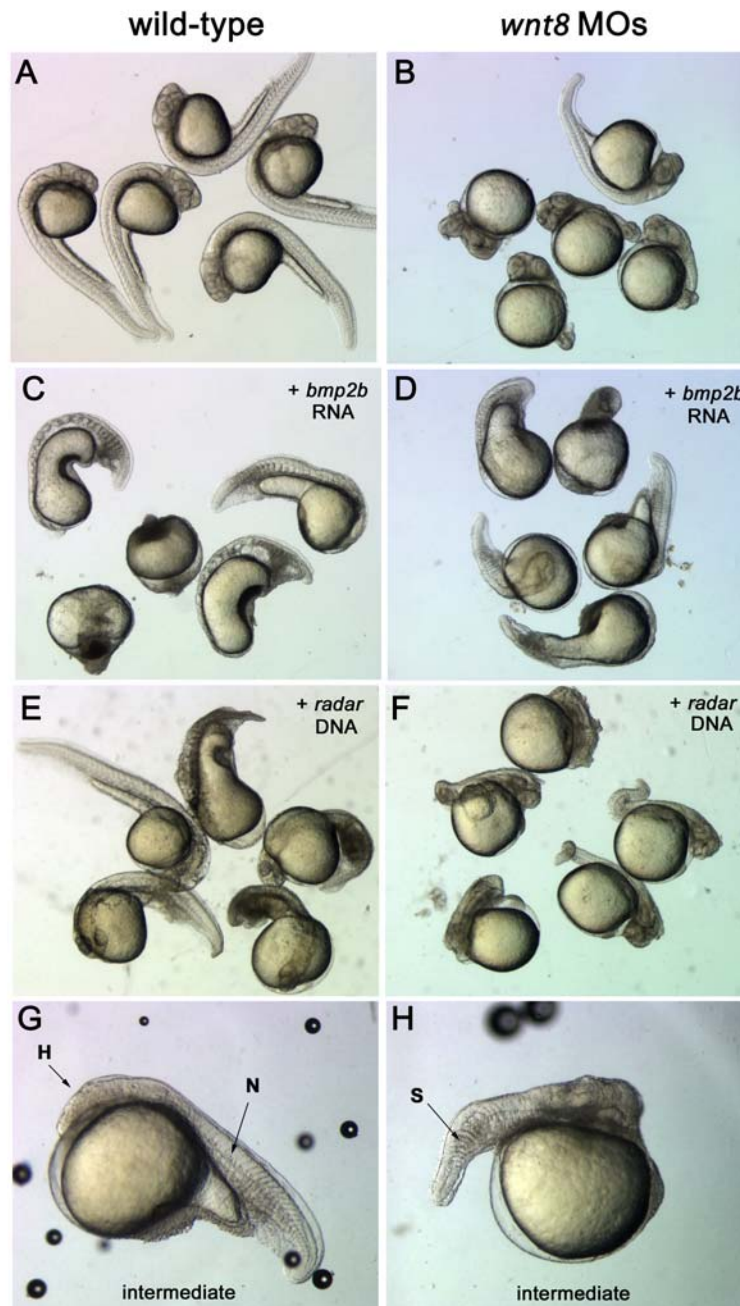

Fig. 18. Differential effects of BMP injection in *wnt8* morphants. 24 hour phenotype of zebrafish wild-type (A,C,E) and *wnt8* morphants (B,D,F) injected with *bmp2b* RNA (C,D) or *radar* DNA (E, F). Both BMP2b and Radar injection ventralize wild-type embryos (C,E) but only BMP2b ventralizes *wnt8* morphants (D). G,H: intermediate phenotype of *wnt8* morphants injected with *bmp4* DNA. In panel G, H = small head lacking eyes as in ventralized embryos and N=notochord. In panel H, S=somites but no notochord is present.

The difference in BMP activity in *wnt8* morphants suggests that downstream BMP components should behave similarly. To test this, constitutively active forms of Alk3, 6 or 8 (Bauer et al., 2001; Nikaido et al., 1999a; Goutel et al., 2000) were injected in wild-type or *wnt8* morphant embryos (Table 3). CA-Alk3 and 6 showed a similar requirement for Wnt8 function: they were unable to ventralize *wnt8* morphants or if so it was inefficient (see intermediate phenotype, Table 3). In contrast, overexpression of CA-Alk8 ventralized *wnt8* morphants suggesting that Alk8 can bypass the requirement for Wnt8. Since Alk8 was shown to be a BMP2b receptor (Bauer et al., 2001), these results fit with the ability of BMP2b to ventralize *wnt8* morphants.

To further explore the Wnt8-BMP epistatic relationship, we tested the activity of Smad1 and 5 in *wnt8* morphants. Since Smad1 is able to rescue *swr* mutants while Smad5 is not, thus placing Smad1 downstream of BMP2b (Dick et al., 1999), we asked whether these Smads would likewise differ in their ability to affect patterning in *wnt8* mutants. As described previously, injection of large amounts of *smad1* and *5* RNA was able to ventralize wild-type embryos (Dick et al., 1999; Table 3). Smad5 was unable to ventralize *wnt8* morphants and most embryos displayed a dorsalized phenotype (Table 3). In contrast, injection of *smad1* RNA did result in a range of phenotypes: ventralized, intermediate, and dorsalized (Table 3). These phenotypes were obtained at a concentration where Smad1 does not dramatically ventralize wild-type embryos compared to Smad5 (Table 3). Thus, it appears that Smad1 does relieve *wnt8* MOs mediated dorsalization better than Smad5 does. For instance, the percentage of

intermediate embryos in *smad1* + *wnt8* MOs injected embryos is higher than in *smad5* + *wnt8* MOs (21.8% vs 1.6%).

To further address the ventralizing potentials of Smad1 and 5 in *wnt8* mutants, we assessed ventralization using a probe cocktail (OPT) consisting of *odd-paired-like* (*opl*; anterior neuroectoderm marker), *pax2a* (midbrain-hindbrain border) and *tbx6* (posterior mesoderm) at bud stage (10 hours post-fertilization). Analysis revealed that Smad1 was indeed a more potent ventralizer of *wnt8* deficiency mutants compared to Smad5 (Fig. 19). Injection of *smad5* RNA ventralized 45% of wild-type embryos (n=44; Fig. 19C) but only slightly ventralized 27% of *wnt8* mutants (n=15; Fig. 19D). In contrast, Smad1 was able to ventralize 58% of wild-type embryos and 52.6% of *wnt8* mutants (n=62 and 19 respectively; Figs. 19E,F). The ventralization of *wnt8* mutants by Smad1 was characterized by a reduction in the width of the neural plate but *tbx6* expression was not rescued (arrow in Fig. 19F), suggesting that Smad1 cannot rescue all aspects of the *wnt8*- phenotype and that Smad1 cannot completely ‘reverse” the *wnt8*-dorsalized phenotype.

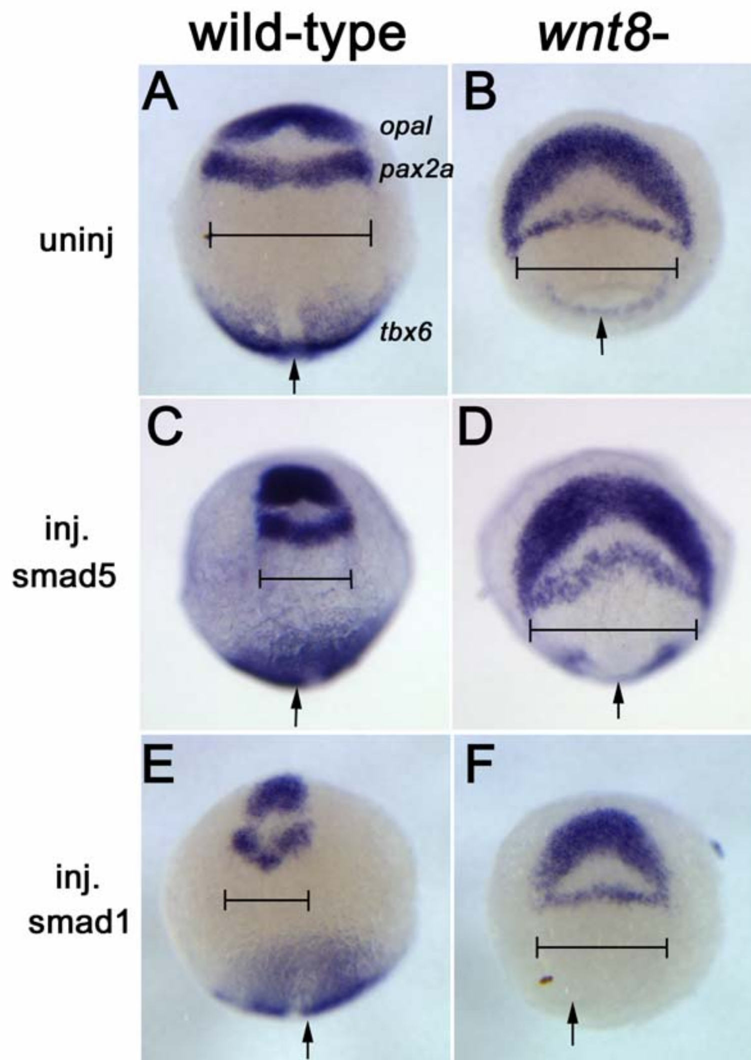

Fig. 19. Smad1 can rescue some of the dorsalized phenotype of *wnt8* mutants.
In situ hybridizations for *opl*, *pax2a* and *tbx6* (OPT) at bud stage in wild-type (A,C,E) or *wnt8* deficiency mutants (B,D,F). Horizontal lines indicate the width of the neural plate while the vertical arrow points to *tbx6* expression. Both Smad5 and 1 injection can ventralize wild-type embryos (C,E) but only Smad1 efficiently ventralizes the ectoderm of *wnt8* mutants (narrow neural plate in F). Note that *tbx6* expression is not rescued by Smad1 injection (arrow in F). All panels are bud stage embryos, dorsal view, anterior up.

Radar-induced ventralization requires Wnt8 function during gastrula stages

While embryos injected with any of the tested BMP ligands display a similar ventralized phenotype at 24 hours and can be classified according to the 'universal' ventralized/dorsalized nomenclature (Kishimoto et al., 1997), removal of Wnt8 is able to distinguish their differing activities. For instance, injection of Radar or BMP2b results in opposite phenotypes upon co-injection with *wnt8* MOs (Table 3). To further characterize the differential requirements for Wnt8 function by Radar and BMP2b, we looked at early patterning markers in manipulated embryos (Table 4). For this series of experiments, we used the *wnt8* deficiency mutant as it provides a null genetic background (Lekven et al., 2001).

First, we assessed ventralization using the OPT probe cocktail in situ hybridization at bud stage. In wild-type embryos, BMP overexpression leads to a strong ventralization at bud stage: the *opl* and *pax2a* domains are much narrower in width or absent, reflecting an increase in non neural ectoderm, while the ventral posterior marker *tbx6* is expanded dorsally (Fig. 20B,C). *wnt8* mutants lack posterior structures and their *opl* and *pax2a* domains expand posteriorly and ventrally (Fig. 20D). The *tbx6* expression in *wnt8* mutants is strongly reduced compared to wild-type embryos (inset Fig. 20D, compare to inset Fig. 20A) and this pattern easily enables the identification of mutants in addition to PCR genotyping. The domain of *tbx6* expression however can indicate whether embryos are ventralized or not. Overexpression of Radar ventralized 41% of wild-type embryos (Fig. 20B; Table 4). However, it could not efficiently ventralize *wnt8* mutants (Fig. 20E; Table 4). BMP2b overexpression in wild-type

embryos strongly ventralizes wild-type embryos (Fig. 20C) and *wnt8* mutants (Fig. 20F; Table 4). Thus, at the end of gastrulation, the presence of Wnt8 discriminates ventralizing BMPs: BMP2b is active in *wnt8* mutants but Radar is not.

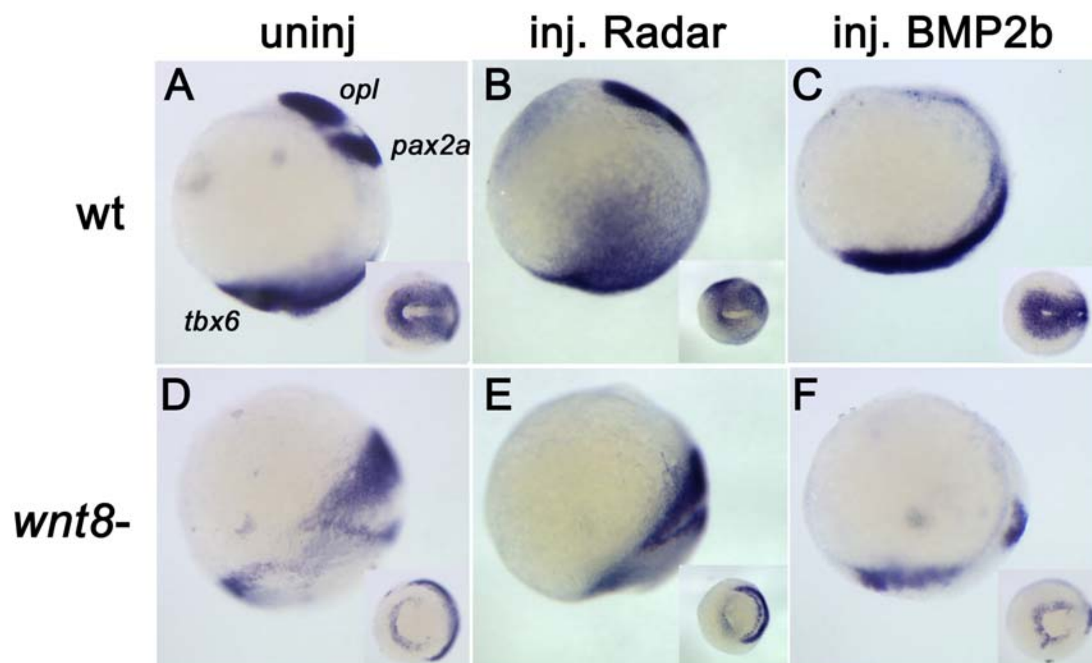

Fig. 20. BMP2b but not Radar can ventralize the ectoderm of *wnt8* mutants at bud stage. In situ hybridization for *opl*, *pax2a*, and *tbx6* (OPT) in embryos that were uninjected (A,D), injected with *radar* (B,E), or *bmp2b* DNA (C,F). Genotypes are indicated on the left. Radar and BMP2b strongly ventralize wild-type embryos (narrowing of neural plate and expansion of *tbx6*). Only BMP2b is able to ventralize the ectoderm of *wnt8* mutants. Note that *tbx6* expression is not restored to wild-type levels upon BMP overexpression (E,F). Bud stage embryos, lateral view, anterior up.

Table 4. Radar and BMP2b display different ventralizing abilities in *wnt8* mutants during gastrula stages

assay	injection	% wild-type	% *wnt8* mutants	P value
ventralized OPT	*CS2P+-radar*	41 (n=34)	0 (n=9)	< 0.001
	CS2P+-bmp2b	89 (n=36)	100 (n=17)	
increased eve1	*CS2P+-radar*	75 (n=24)	77 (n=9)	> 0.05
	CS2P+-bmp2b	52.8 (n=36)	100 (n=11)	
reduced gsc	*CS2P+-radar*	34.4 (n=32)	6.25 (n=16)	< 0.005
	CS2P+-bmp2b	57 (n=30)	54.5 (n=11)	

The P-value was calculated when performing the $\chi 2$ test of independence. We tested if the difference observed between the % of *wnt8* mutants displaying the phenotypes in the two treatments performed (*radar* or *bmp2b* injection) was statistically significant.

To further assess ventralization by Radar and BMP2b, we examined *eve1* expression at early gastrulation. *eve1* is a mesodermal marker expressed in the ventrolateral mesoderm at shield stage and has been used as a ventral marker in overexpression experiments (Joly et al., 1993, Nikaido et al., 1997). Both Radar and BMP2b were able to induce ectopic *eve1* expression in wild-type and *wnt8* mutants (Table 2). Thus, with regard to *eve1* induction, both Radar and BMP2b are active in *wnt8* mutants, although BMP2b appears more efficient at inducing it (see Table 4).

If all BMPs tested are able to induce a ventral marker in the absence of Wnt8, can they also repress organizer genes, a function known to require Wnt8 function? We injected *wnt8* mutants with *radar* or *bmp2b* DNA and looked at *gsc* expression at shield stage. Both BMPs, when overexpressed, were able to reduce *gsc* expression expression in wild-type embryos (Table 4). Radar injection was able to reduce *gsc* expression in about 34% of wild-type embryos (n=32). However, it could only repress *gsc* in a small

proportion of *wnt8* mutants (6.25%, n=16). Injection of *bmp2b* DNA resulted in reduced *gsc* expression in 57% of wild-type embryos (n=30) and in 54.5% of *wnt8* mutants (n=11). The difference between the ability of Radar and BMP2b to repress *gsc* in *wnt8* mutants was found to be statistically significant (Table 4; $P<0.005$). Thus, when it comes to the repression of *gsc* in *wnt8* mutants, BMP2b and Radar show different activities.

The reduced ability of Radar to repress *gsc* expression must reflect a reduced ability to induce *vent* and *vox* expression since it was shown that Vent and Vox are essential to repress the expression of *gsc* (Trindale et al., 1999; Imai et al., 2001). Preliminary analysis suggest that both Radar and BMP2b can induce ectopic *vent* and *vox* expression in *wnt8* mutants, but the location of ectopic domains appears different as BMP2b is more efficient than Radar at inducing ectopic *vent*/*vox* in the dorsal mesoderm (not shown).

Thus, our results show that the requirement for Wnt8 towards Radar-induced ventralization starts during gastrula stages, prior to the end of gastrulation. Further, the ability of BMP2b to prevent organizer expansion appears critical for its unique ability to bypass the requirement for Wnt8.

DISCUSSION

In this study, we have shown that Wnt8 function is essential for the expression of zygotic *bmps* starting at mid-gastrulation. It appears that Wnt8 accomplishes this through repression of Chd and additional mechanisms. While we demonstrated that BMP is not necessary to induce or positively regulate zygotic *wnt8* expression, we found that overexpression of BMP ligands is sufficient to induce ectopic *wnt8*. In addition, injection of various BMP signaling pathway components with *wnt8* MOs suggests that Wnt8 function is required upstream of the third BMP signaling phase (BMP2b/Alk8/Smad1). The difference in the ability of various BMP ligands to ventralize *wnt8* mutants reflects molecular events that occur during gastrula stages. It may also reflect the ability of some BMPs to repress organizer genes in the absence of Wnt8 while others cannot. Therefore, our results pinpoint to a critical role of Wnt8 during ventral patterning because it affects BMP signaling. Proper Wnt8 signaling allows the maintenance of the organizer-derived activity, which include BMP antagonists like Chd. This function allows the maintenance of most *bmp* expression in the mesoderm at mid-gastrulation so that proper patterning by third phase BMP signaling can occur.

Importance of Wnt8 function for *bmp* expression

Our results show that Wnt8 is necessary to maintain the expression of zygotic *bmps* in the mesoderm starting at mid-gastrulation. Reducing Chd function in *wnt8* mutants is

sufficient to restore *bmp4* and *7* expression. However, *bmp2b* regulation appears more complex since not all of mesodermal *bmp2b* can be restored upon Chd knockdown. Not all of *bmp2b* transcription in the mesoderm depends on its own autoregulation as *bmp2b* is still expressed in *swr* mutants. It appears that Wnt8 is a necessary factor to maintain *bmp2b* expression and that this regulation is not solely due to its repression of Chd. If Wnt8, through canonical Wnt signaling, directly affects *bmp2b* transcription, one would expect *bmp2b* in the mesoderm to still be reduced in a context where all of organizer derived activity (and not only Chd) is abolished. The fact that the *bmp2b* promoter contains Smads binding sites (A.C. Lekven, unpublished observations) would be consistent with this hypothesis. If Wnt8 solely regulates *bmp2b* expression in the mesoderm through its organizer repression function (indirect), one would expect *bmp2b* in the mesoderm to be restored to at least wild-type levels upon ablation of all organizer activity. These two models are not exclusive and require further investigation and it is likely that Wnt8 displays both functions (direct and indirect regulation of *bmp2b* expression in the mesoderm).

The BMP overexpression/Wnt8 connection: a cautionary note

Our results show that BMP overexpression-induced ventralization requires Wnt8 except for BMP2b that can overcome the lack of Wnt8. The effects of BMP overexpression upon patterning have been well characterized in *Xenopus* and zebrafish (Dale and Jones, 1999; Kishimoto et al., 1997). It has been shown that it affects both A/P and D/V patterning: ventralized embryos are also posteriorized (see Fig. 18). Interestingly

however, it is known that BMP signaling is only required for proper D/V patterning in vivo as *bmp* mutants display relatively normal A/P patterning (Mullins et al., 1996). Thus, BMP overexpression must stimulate a pathway that posteriorizes embryos. There is a large amount of genetic evidence that shows that Wnt8 is a posteriorizing factor (Lekven et al., 2001; Erter et al., 2001). Hence, since BMP overexpression does induce ectopic *wnt8*, we postulate that BMP overexpression affects both axes by stimulating Wnt8. The exception of BMP2b not requiring Wnt8 function to posteriorize embryos is however puzzling and does not fit with the model proposed above. It is therefore possible that another posteriorizing parallel pathway can compensate for Wnt8 absence but can only be stimulated by BMP2b. Noteworthy however, BMP2b cannot rescue all aspects of the *wnt8*- phenotype since *tbx6* expression is still reduced in *wnt8* mutants injected with BMP2b. Analysis of molecular markers at 24 hours may reveal actual differences in the A/P patterning of *bmp2b* or *bmp2b* + *wnt8* MOs injected embryos that are not obvious when looking at gross morphology.

BMP and Wnt8 epistasis: comparison between zebrafish and *Xenopus*

In *Xenopus*, it was postulated that BMP is upstream of Wnt8 as dnBMPR injection and XBMP4 overexpression lead to reduction and ectopic *wnt8* expression respectively (Hoppler and Moon, 1998). However, this approach did not discriminate between maternal and zygotic BMPs. In zebrafish, tools are available to discriminate maternal and zygotic BMPs. Zygotic *wnt8* expression is turned on at mid-blastula transition (MBT; Kelly et al., 1995) and we have now shown that *wnt8* is not induced by a

maternal BMP pathway nor is positively regulated by zygotic BMP signaling. However, as in *Xenopus*, BMP overexpression is able to induce ectopic *wnt8* expression. In zebrafish, it has been suggested that Nodals (other members of the TGFβ superfamily) are necessary for *wnt8* expression (Erter et al., 2001; Ragland and Raible, 2004). It is possible that BMP overexpression does stimulate a Nodal like response, thus leading to ectopic *wnt8* expression. There is indeed evidence that BMP signaling can also act through activin type II receptor (ActRIIB), a receptor that also mediates activin/nodal signaling (Nagaso et al., 1999), thus supporting our hypothesis.

CHAPTER IV

WNT8 AND BMP2b CO-MAINTAIN NON-AXIAL MESODERM IDENTITY IN ZEBRAFISH

INTRODUCTION

During embryogenesis, a complex series of regulated events leads to the formation of the three germ layers: ectoderm, mesoderm and endoderm. The mesoderm gives rise to structures such as notochord, muscles, kidneys and blood and these structures are arranged in a distinguishable pattern along the dorso-ventral (D/V) axis (reviewed in Schier, 2001; Kimelman and Griffin, 2000; Weng and Stemple, 2003). While many aspects of mesoderm induction and development are well understood, less is known about the molecular events leading to the subdivision of the mesoderm into D/V domains.

In zebrafish, it is believed that maternal signals emanating from the yolk syncytial layer (YSL) and relayed by Nodal signaling induce cells in the blastoderm to acquire a mesodermal fate (reviewed in Kimelman and Schier, 2002). The role of Nodal signaling in mesoderm induction has been revealed through the analysis of double mutants for the Nodal ligands *squint* and *cyclops* (Feldman et al., 1998). These double mutants lack most of the mesoderm except the most ventral mesoderm derivatives and, as a result, *cyc*; *sqt* embryos still form tail somites. Maternal zygotic *One-eyed pinhead*

(MZ*Oep*) mutants, which lack an essential co-factor for Nodal signaling, also show strong reduction in mesoderm formation (Gritsman et al., 1999). Embryos injected with *antivin*/*lefty*, which is a feedback inhibitor of Nodal signaling, also display the same phenotype (Thisse and Thisse, 1999). Thus, most of the mesoderm is induced through the action of Nodal signaling with the exception of the mesoderm that gives rise to the tail (the 'tail organizer') that is known to also require Wnt8 and BMP signaling (Agathon et al., 2003)

The first step in the differentiation of the mesoderm is its subdivision into two domains: axial, which constitutes the dorsal organizer and gives rise to the prechordal plate and notochord, and non-axial (ventral), which is the remainder. Specification of axial mesoderm requires β-catenin signaling on the dorsal side of the embryo as well as high Nodal activity (reviewed in Hibi et al., 2002). Identification of a maternal BMP pathway that specifies ventral fates in zebrafish embryos supports the hypothesis that active signaling also specifies the non-axial mesoderm (Sidi et al., 2003).

Once established by Nodal and β-catenin activity, the axial and non-axial mesoderm domains are further delineated and maintained through mutual repression systems. For instance, the dorsally expressed protein Bozozok (Boz) prevents the transcription of the ventrally expressed genes *bmp2b* and *vox* (Leung et al., 2003; Shimizu et al., 2002). Ventrally, Vent, Vox and Ved prevent the transcription of *boz* and other dorsal genes such as *goosecoid* (*gsc*), and *chordin* (*chd*) (Imai et al., 2001, Shimizu et al., 2002). Although the mechanisms of defining axial mesoderm identity are becoming well defined, it is less clear how the non-axial mesoderm becomes

progressively subdivided into paraxial, intermediate, and lateral plate domains. Most evidence points to a gradient of BMP activity, highest on the ventral side of the embryo, being critical for differentially determining D/V domains at late gastrula stages (Hammerschmidt and Mullins, 2002). Loss-of-function data in zebrafish suggests that BMP in the mesoderm is required only for intermediate and lateral plate fates as *swr* (*bmp2b*) mutants lack blood and pronephric precursor cells but still retain some paraxial mesoderm derivatives such as anterior somites (Hammerschmid and Mullins, 2002). What determines paraxial identity is still unknown although recent evidence suggests that, in the mouse, *foxc1* and *foxc2* promote paraxial identity by antagonizing intermediate mesoderm identity (Wilm et al., 2004).

We have recently shown that zebrafish Wnt8 prevents the expansion of axial mesoderm into the non-axial domain though the direct transcriptional regulation of *vent* and *vox* (Ramel and Lekven, 2004). Our analysis further showed that BMP2b has a supporting role in co-regulating *vent* and *vox* during early gastrula stages. In other words, *wnt8*-; *swr* double mutants fail to repress the axial marker *gsc* anywhere in the nascent mesoderm. This finding raises the possibility that Wnt8 and BMP2b might sit at the top of a genetic hierarchy required for establishing non-axial mesoderm fates. To test this possibility, we have characterized the *wnt8*-; *swr* phenotype. Our results show that Wnt8 and BMP2b function in parallel throughout gastrulation to establish the non-axial mesoderm and its early subdivisions. Wnt8 and BMP2b perform this function through the combined regulation of the transcriptional repressors Vent, Vox and Ved. We placed Wnt8 and BMP2b function in promoting non-axial identity downstream of Nodal

activity. We also showed that tail-organizing activity present in Nodal deficient embryos depends on the residual activity of Wnt8 and BMP2b.

MATERIALS AND METHODS

Fish maintenance and strains

Animals were maintained as described previously (Westerfield, 2000). Wild-type fish were AB. The strains used were: *DfLG14wnt8*^{w8}/+ (also called *Df*^{w8}/+ or *wnt8*-/+ in this study; Lekven et al., 2001), *swr*^{TC300}/+ (*swr*/+; Mullins et al., 1996), *Df*$^{\delta T7}$/+ (*vent*-/+; *vox*-/+ in this study; Imai et al., 2001). All mutants are considered to be null or strong loss-of-function (Lekven et al., 2001; Nguyen et al., 1998; Imai et al., 2001). To generate double *wnt8*-/+; *swr/*+ animals, a *wnt8*- heterozygote was crossed to a *swr* heterozygote. Progeny were individually screened for *wnt8*-/+ and *swr/*+. Double mutants were confirmed by PCR genotyping as previously described (Ramel and Lekven, 2004).

In situ hybridization and probes

In situ hybridizations were performed essentially as described (Jowett, 2001). The probes used were: *even-skipped-1* (*eve1*; Joly et al., 1993), *caudal homeobox 1* (*cad1*; Joly et al., 1992), *tbx6* (Hug et al., 1997), *T-box24* (*tbx24*; Nikaido et al., 2002), *ved* (Shimizu et al., 2002), *chd* (Miller-Bertoglio et al., 1997), *floating head* (*flh*; Talbot et

al., 1995), *boz* (also called *dharma*, Yamanaka et al., 1998), *gsc* (Stachel et al., 1993), *myoD* (Weinberg et al., 1996), *no tail* (*ntl*; Schulte-Merker et al., 1992).

Injection and morpholinos

Because previously reported translation blocking morpholinos (MOs) targeted against *wnt8* produce *wnt8*- phenotypes of variable penetrance and expressivity (Lekven et al., 2001), MOs designed to block the splicing of *wnt8* pre-mRNAs were utilized. Sequences are as follows (5' to 3'):

orf1 E1i1 MO: AATATGACTGTACCATGCTGTTGAC

orf1 exon3 MO: ATATTTAACTTACCACTCCGCAGGC

orf2 E4i4 MO: AACTGTTCTTACCAAGTCTGCCGTT

orf2 exon3 MO: CTTATGAATATCTTACCACTTCTCA

Simultaneous injection of the four splice blocking MOs (2.5 ngs/nL each) gave results comparable to the translation blocking MOs but with higher penetrance and expressivity as well as lower lethality. Further, the phenotypic effects of the splice

blocking MOs were rescued by RNA injection (Buckles et al., manuscript in preparation). The *ved* MO has previously been described (Shimizu et al., 2002). MOs were diluted as described in Danieau's buffer (Genetools, LLC) and injected into one to four-cell stage embryos. To replicate the *wnt8*-; *swr* phenotype, the progeny from a cross between *swr* heterozygotes were injected with *wnt8* MOs to obtain *wnt8* ^{MO}; +/+, *wnt8* ^{MO}; *swr*/+, and *wnt8* ^{MO}; *swr*/*swr* embryos. To achieve a *vent*/*vox*/*vent* knockdown, embryos obtained from a cross between *Df*^{ST7} heterozygotes were injected with *ved* MO (10 ngs/nL). For the rescue of *tbx24* expression in *wnt8*; *swr* double mutants, the progeny from 3 to 4 *wnt8*/+; *swr*/+ intercrosses were injected with *vent* RNA (7 ngs/μL; higher concentrations can be toxic to embryos and produce gastrulation defects, our unpublished observations). After in situ hybridization, the embryos were individually photographed and genotyped by PCR to identify the double mutants. *antivin* RNA was injected at a concentration of 25 ngs/μL. In all injections, a volume of approximately 3 nL was injected per embryo.

RESULTS

Wnt8 and BMP2b act in parallel to control non-axial mesoderm identity

Previous studies in *Xenopus* and zebrafish have suggested that Wnt8 and BMP signaling interact during D/V patterning and that they share common transcriptional targets (Ramel and Lekven, 2004; Agathon et al., 2003, Hoppler and Moon, 1998; Marom et al., 1999, Szeto and Kimelman, 2004). However, there is also evidence that Wnt8 and BMP regulate independent transcriptional targets. For instance, *myf5* expression in *Xenopus* is dependent on Xwnt8 but not on BMP (Marom et al., 1999). If both pathways are required for unique as well as combined patterning functions, then one would expect to detect this when comparing single and double mutant phenotypes. To this aim, we analyzed the phenotype of zebrafish embryos that lack functional Wnt8 and BMP2b (see materials and methods).

We first compared the bud stage morphology of embryos resulting from *wnt8* MO injection in the progeny of *swr* heterozygotes (Fig. 21). *swr* mutant embryos are distinguished morphologically at bud stage by their characteristic elongated shape (Fig. 21B,G; Mullins et al., 1996), which can be attributed to altered convergence-extension movements of non-axial cells (Myers et al., 2002). In contrast, *wnt8* morphants have widened and poorly defined notochords combined with deficiencies in trunk and tail mesoderm (Figs 21C,H; Lekven et al., 2001). When *wnt8* MOs are injected into embryos derived from a *swr*/+ intercross, two new morphological classes emerge (Figs 21D,E,I,J).

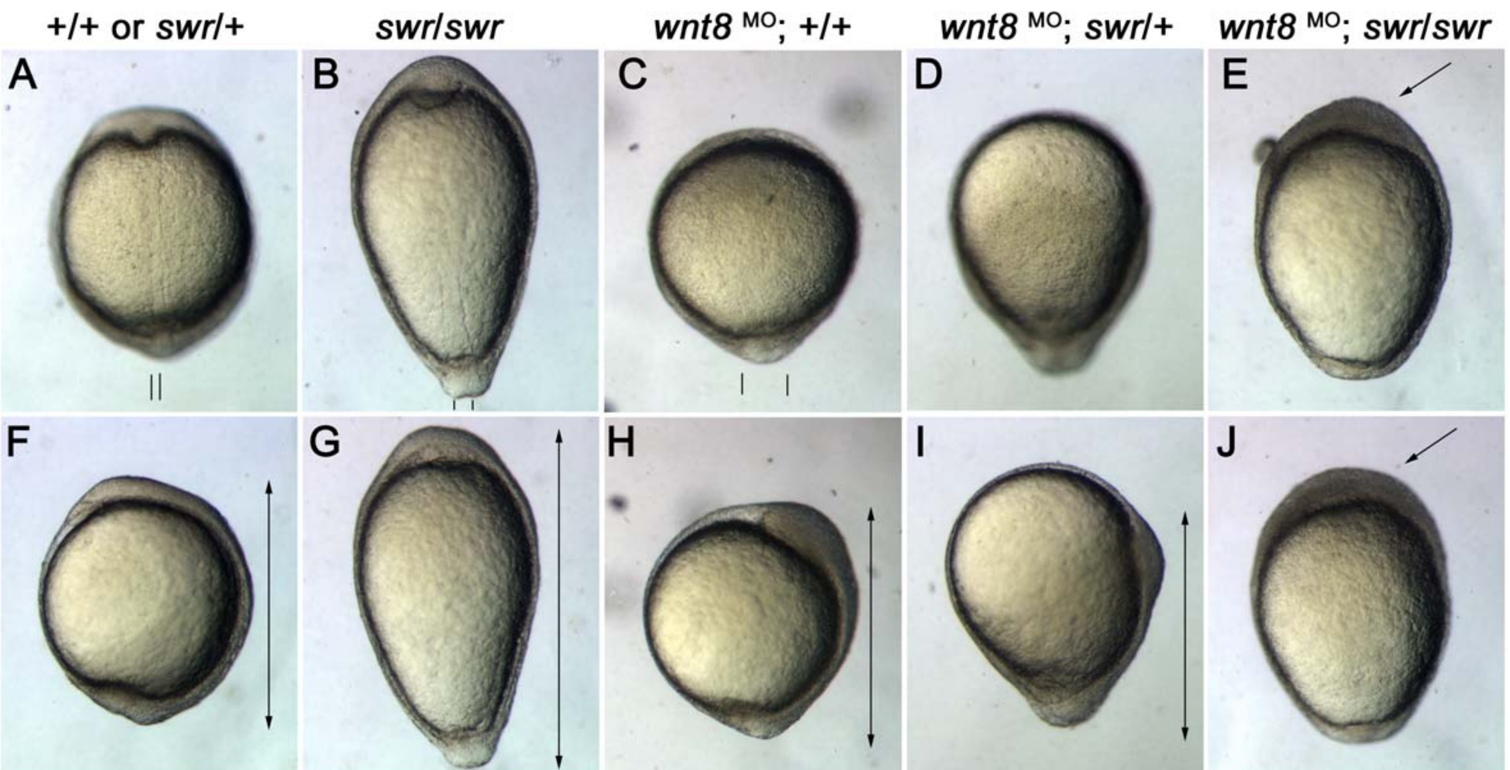

Fig. 21. Wnt8 and BMP2b are required to maintain axial/non-axial polarity.
Morphology of bud stage embryos. Genotypes are indicated above each column. A-D: dorsal views. F-I: lateral views, dorsal right. Bars in A-C indicate the width of the notochord when visible. Double arrows in F-I indicate the length of the A/P axis. Note the shortened axis of *wnt8* ^{MO} embryos (C,H), and the posterior displacement of the axis in *wnt8* ^{MO}; *swr*/+ embryos (D,I). *wnt8* ^{MO}; *swr*/*swr* embryos display a large mass of cells at the animal pole (arrows in E and J) and do not have a recognizable D/V polarity.

wnt8 ^{MO}; *swr*/+ embryos (genotyped by PCR, see materials and methods) display a shortened axis and more significant mesoderm convergence defects compared to *wnt8* morphants or *swr*/*swr* embryos (Figs 21D,I). Additionally, the notochord is not easily distinguished in *wnt8* ^{MO}; *swr*/+. Despite this phenotype, D/V polarity is recognizable (Fig. 21I). *wnt8* ^{MO}; *swr*/*swr* embryos, in contrast, display a striking morphology in which it is impossible to distinguish D/V polarity (Figs 21E,J). In addition, these embryos display a large mass of cells at the animal pole (arrows in Fig. 21E,J). Thus, reducing the gene dosage of *bmp2b* enhances the *wnt8* ^{MO} phenotype significantly.

To understand the morphology of the embryos described in the experiment above, we analyzed the expression of various molecular markers by in situ hybridization at bud stage. *gsc* expression at this stage marks the prechordal plate mesoderm (anterior axial mesoderm; Fig. 22A). We found *gsc* expression to encircle the circumference of both *swr* heterozygotes and homozygotes that were injected with *wnt8* MOs (Fig. 22D,E). However, the expansion of *gsc* ventrally was much stronger in *wnt8* ^{MO}; *swr/swr* embryos (Fig. 22E). Interestingly, while it is difficult to morphologically determine dorsal from ventral in these embryos, *gsc* staining revealed that these embryos still preserve some D/V polarity in the anterior mesoderm as *gsc* is more strongly expressed

on one side of the embryo (putative dorsal side; see arrow in Fig. 22E). This observation was confirmed with the anterior neuroectodermal marker *odd paired like* (not shown). In the posterior part of the embryo, *myoD* expression marks the adaxial cells (part of the paraxial mesoderm) that flank the developing notochord at bud stage (Fig. 22F). *swr* homozygotes have a slightly widened adaxial domain (Fig. 22G; Mullins et al., 1996). Loss of Wnt8 results in a widened notochord (Fig. 22H) and this widening is accentuated in *swr* heterozygotes (Fig. 22I), confirming the dominant enhancement of *wnt8* morphants by the *swr* allele. In *wnt8* ^{MO}; *swr/swr* embryos, *myoD* expression was completely abolished (Fig. 22J). The expansion of axial mesoderm observed with *gsc* staining (Fig. 22E) was also illustrated by the analysis of *flh*, which marks the notochord (posterior axial mesoderm). While *flh* is expanded in *wnt8* ^{MO}; +/+ and *wnt8* ^{MO}; *swr*/+ embryos (Fig 22M,N), it encircles the entire margin of *wnt8* ^{MO}; *swr/swr* embryos (Fig. 22O). Thus, in the absence of Wnt8 and BMP2b function, markers of axial mesoderm fates expand around the circumference of the embryo and there is a corresponding loss of non-axial mesoderm.

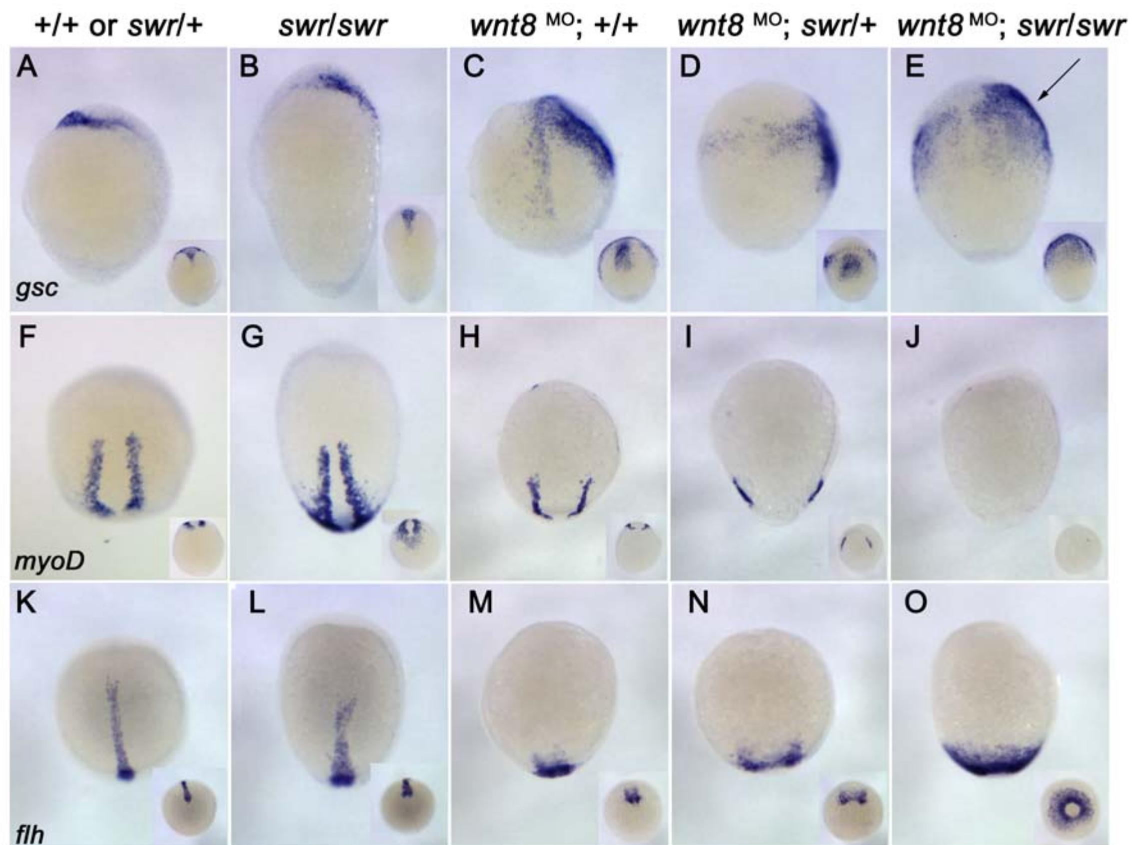

Fig. 22. Wnt8 and BMP2b are necessary to repress anterior and posterior axial mesoderm and to maintain non-axial mesoderm. In situ hybridization for *gsc* (anterior axial mesoderm, A-E)), *myoD* (adaxial mesoderm, F-J), and *flh* (prospective notochord, K-O) at bud stage. Genotypes are indicated above each column. Main panels A-E: lateral views, dorsal right. Insets: dorsal view, anterior up. F-O: dorsal views, anterior up. Insets: posterior view, dorsal up. Note the slightly widened adaxial domain in *swr* mutants (G) and the progressive widening of the axial domain as *wnt8* ^{MO} embryos have a reduced dosage of *bmp2b* (C-E, H-J, M-O).

Loss of non axial fates occurs during early gastrulation in *wnt8*; *swr* double mutants

To more clearly understand the progression of the *wnt8* ^{MO}; *swr*/*swr* phenotype, we looked at various non-axial and axial mesoderm markers at gastrula stages. Since *vent* and *vox* encode known repressors of dorsal fates that are regulated by Wnt8 and BMP2b, we included *vent*; *vox* mutants (Imai et al., 2001) in our analysis for comparison.

At shield stage, *chd* and *flh* expression indicates the dorsal axial mesoderm (Fig. 23A,G; Miller-Bertoglio et al., 1997; Talbot et al., 1995). In agreement with previous results, both genes are expressed in ventrally expanded domains in *wnt8* mutants/morphants at this stage (Figs 23B,H; Ramel and Lekven, 2004)), but not in *swr* mutants (Fig. 23C,I; Miller-Bertoglio et al., 1997). In contrast, both *chd* and *flh* expression domains encircle the margin of shield stage embryos lacking both Wnt8 and BMP2b function (Figs 23D,E,J,K). This effect is almost identical to that seen in shield stage *vent; vox* mutants (Fig. 23F,L; Imai et al., 2001). One significant though subtle difference between the *wnt8*-; *swr* and *vent*-; *vox*- phenotypes observed is that the ventral expansion of *flh* is not as robust in *vent*; *vox* mutants as it is in embryos lacking Wnt8 and BMP2b function (compare Fig 23L with Fig. 23J). As described below, this difference reflects the persistent specification of non-axial mesoderm in the absence of Vent and Vox.

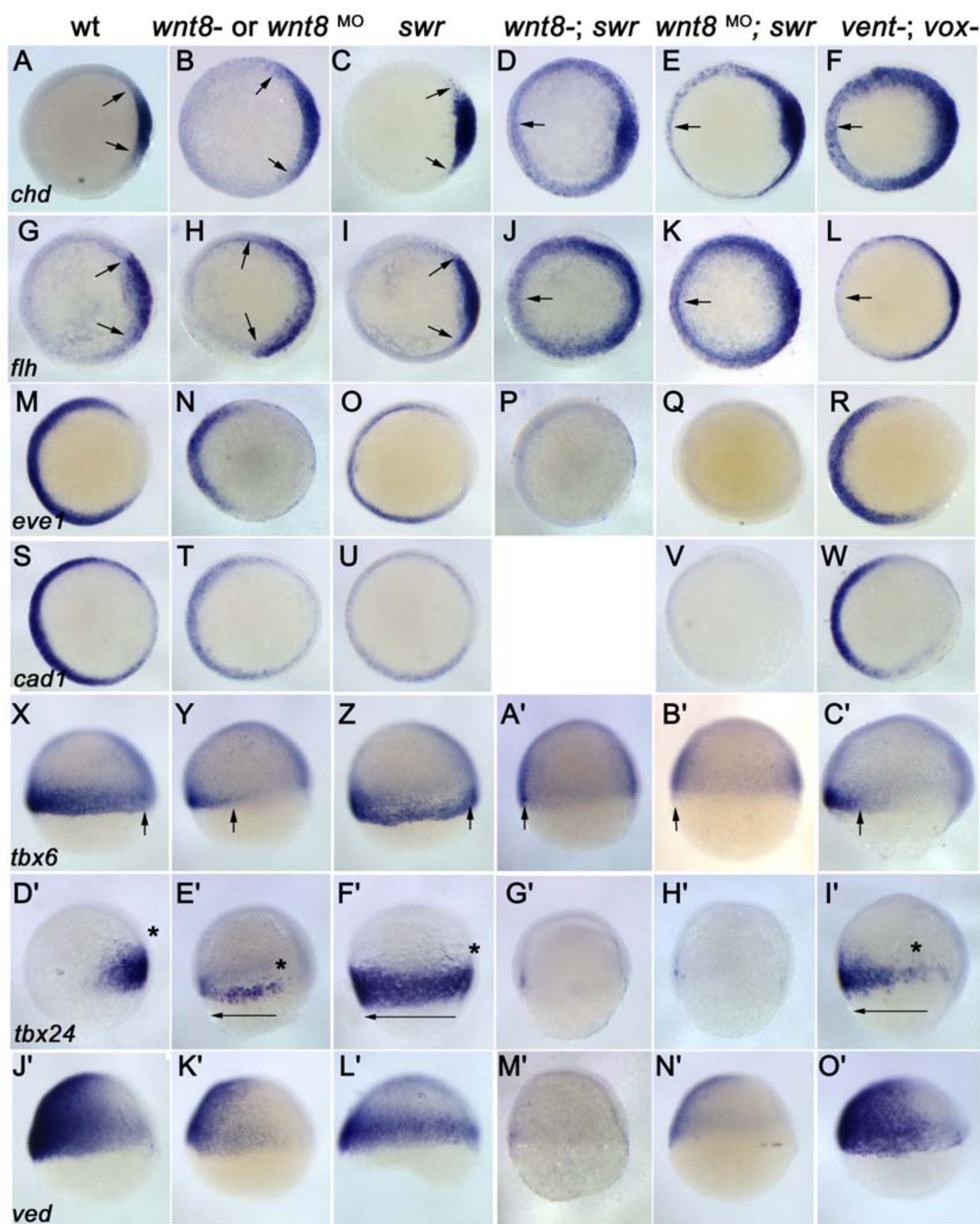

Fig. 23. Both *wnt8*; *swr* and *vent*; *vox* double mutants display expansion of axial but only *wnt8*-; *swr* embryos show loss of non-axial mesoderm. In situ hybridizations for *chd* (A-F), *flh* (G-L), *eve1* (M-R), *cad1* (S-W), *tbx6* X-C'), *tbx24* (D'-I'), and *ved* (J'-O'). Genotypes are indicated above each column. All embryos are at shield stages except X-C' (60% epiboly) and D'-I' (70% epiboly). Both *wnt8*; *swr* (D,J) and *vent*; *vox* (F,L) double mutants show a loss of axial mesoderm repression, although *flh* expansion is less severe in *vent*; *vox* mutants than in *wnt8*; *swr* mutants (arrow in L, compare to J). All non axial-mesoderm markers (*eve1*, *cad1*, *tbx6*, *tbx24* and *ved*) are strongly reduced or absent in *wnt8*; *swr* double mutants (P,V,A',G',M') but are still expressed in *vent*; *vox* mutants (R,W,C',I',O'). Asterisks in D'-I' represent the dorsal limit of *tbx24* staining.

To determine whether the expansion of axial mesoderm markers at shield stage is accompanied by the loss of non-axial mesoderm markers, we examined the expression of *eve1* and *cad1* (Fig. 23M-W). *eve1* is expressed in most of the non-axial mesoderm at shield stage (Fig. 23M; Joly et al., 1993). It is reduced in both *wnt8* and *swr* mutants (Fig. 23N,O; Ramel and Lekven, 2004) and is completely absent in the double mutants (Fig. 23P,Q). Similarly, *cad1*, the expression of which overlaps with *eve1*, is also reduced in *wnt8* morphants and *swr* mutants (Fig. 23T,U; the *cad1* gene is deleted in the *wnt8*- deficiency so MOs were used to generate the *wnt8*- phenotype). *cad1* expression is absent in *wnt8* ^{MO}; *swr/swr* embryos (Fig. 23V). Surprisingly, both *eve1* and *cad1* are expressed at robust levels in *vent; vox* mutant embryos at shield stage (Fig. 23R,W) which indicates that some ventral mesoderm is produced in *vent; vox* mutants and also highlights the fact that Wnt8 and BMP2b must have additional roles in non-axial mesoderm specification besides regulation of *vent* and *vox*. *eve1* and *cad1* regulation by Wnt8 and BMP2b is also paralleled by *tbx6*, a T-box gene expressed in involuting non-axial mesoderm (Fig. 23X-C'; Hug et al., 1997).

To address the status of dorsolateral mesoderm domains in *wnt8*; *swr* mutants, we examined *tbx24* expression (Figs 23D'-I'). The bilateral domains that give rise to paraxial mesoderm are first indicated by *tbx24* expression at shield stage/60% epiboly (Nikaido et al., 2002). In wild-type 70% epiboly embryos, *tbx24* is expressed in two domains that each extend over a ~90° arc (Fig. 23D'). The loss of Wnt8 results in a strong reduction in the observable number of *tbx24* positive cells, and the bilateral domains are shifted in position ventrally such that the axial domain lacking *tbx24* is

expanded and the ventral limits of the bilateral domains meet at the ventral midline (Fig. 23E'). *tbx24* expression in *swr* mutants shows the same expansion toward the ventral midline but the axial domain is not different from wild-type (Fig. 23F'). The number of cells expressing *tbx24* is not diminished in *swr* as it is in *wnt8* mutants/morphants (compare Figs 23E' and F'). In contrast to the single mutants, *wnt8*; *swr* double mutants or *wnt8* ^{MO}; *swr/swr* embryos have no observable *tbx24* expression (Fig. 23G',H'), which is consistent with the expansion of axial fate markers. Interestingly, *vent*; *vox* mutants retain both *tbx6* and *tbx24* expression, suggesting that the non-axial mesoderm that forms in the absence of Vent and Vox adopts a paraxial fate.

Recently, a new member of the *vent/vox* family of transcriptional repressors called *ved* was identified in zebrafish (Shimizu et al., 2002). We found that its expression correlates with the fate changes seen in the mutant backgrounds examined (Fig. 23J'-O'). In *wnt8* mutants, the mesodermal expression of *ved* is reduced in intensity while most of the epiblast staining is retained (Fig. 23K'). In contrast, *swr* mutants display a strong reduction in epiblast expression but normal marginal expression (Fig. 23L'). *wnt8* ^{MO}; *swr/swr* and *wnt8-;swr* embryos, in which all mesoderm expresses axial markers, do not express detectable levels of *ved* (Fig. 23M',N'). Importantly, *vent*; *vox* mutants, which do retain expression of non-axial markers, express *ved* in both epiblast and marginal regions, consistent with previous reports (Gilardelli et al., 2004). Taken together, these results suggest that a fate shift occurs in the mesoderm of early gastrula stage *wnt8*; *swr* mutants such that non-axial fates are not expressed while axial fates are expanded throughout the entire D/V mesoderm. Further, this major fate shift is

not due solely to the loss of *vent* and *vox* expression but also correlates with the regulation of *ved*.

Loss of non axial mesoderm in *wnt8*; *swr* mutants is replicated in *vent*, *vox* and *ved* loss-of-function

Ved has been shown to function redundantly with Vent and Vox (Shimizu et al., 2002) although the relationships between Vent, Vox and Ved may be complex (Gilardelli et al., 2004). To test whether *ved* expression is responsible for the phenotypic differences between *wnt8*; *swr* and *vent*; *vox* mutants, we examined the phenotype of embryos lacking Vent, Vox and Ved function (see materials and methods). *ved* MO injection in wild-type embryos consistently produced moderate dorsalization as assayed by the expression of *eve1*, *tbx24*, *myoD* and *flh* (Fig. 24C,G,K,O,S). Injection of *ved* MO in *vent; vox* mutants, however, resulted in a phenotype very similar to *wnt8*; *swr* mutants. For instance, in the *vent*/*vox*/*ved* triple knockdown, the axial mesoderm marker *flh* is strongly expressed around the circumference (and very strongly in the most ventral region) of the embryonic margin at shield stage (Fig. 24D), a stronger phenotype than that observed in *vent*; *vox* mutants (Fig. 24B). The expression of the non-axial markers *eve1*, *tbx24*, *and myoD* was found to be almost undetectable in the triple loss of function (Fig. 24H,L,P). The enhanced loss of non-axial markers in the triple knockdown exactly correlates with the circumferential expansion of axial markers such as *flh* (Fig. 24D,T). We also found that *ved* expression persists in the margin of *vent*; *vox* mutants until at least bud stage (Fig. 24V). Thus, reducing levels of Ved in a *vent*/*vox* loss of function

background results in the loss of non-axial mesoderm fates and the concomitant expansion of axial mesoderm identity.

Our analysis of *myoD* also points out a significant relationship between Vent, Vox and Ved in patterning non-axial mesoderm. In *swr* mutants, *vent* expression is not maintained and this correlates with a mild widening of the adaxial *myoD*$^+$ domain at bud stage (Melby et al., 2000; Mullins et al., 1996). We confirmed this association by knockdown of *vent* in wild-type embryos (data not shown). Injection of *vox* MO in wild-type embryos also gives a similar phenotype (not shown). In the absence of Vent and Vox, the mesoderm appears to express one of two identities: axial or adaxial as indicated by the complementary patterns of *flh* and *myoD* (Fig. 24N,R). This implies that residual *ved* expression in *vent*; *vox* mutants is able to repress axial mesoderm markers in a limited portion of the mesoderm but is not able to restrict adaxial *myoD* expression to the cells immediately adjacent to the notochord. These observations are consistent with the proposal (Onichtchouk et al., 1998) that levels of Vent/Vox/Ved mediated repression are necessary to differentially specify D/V mesoderm domains.

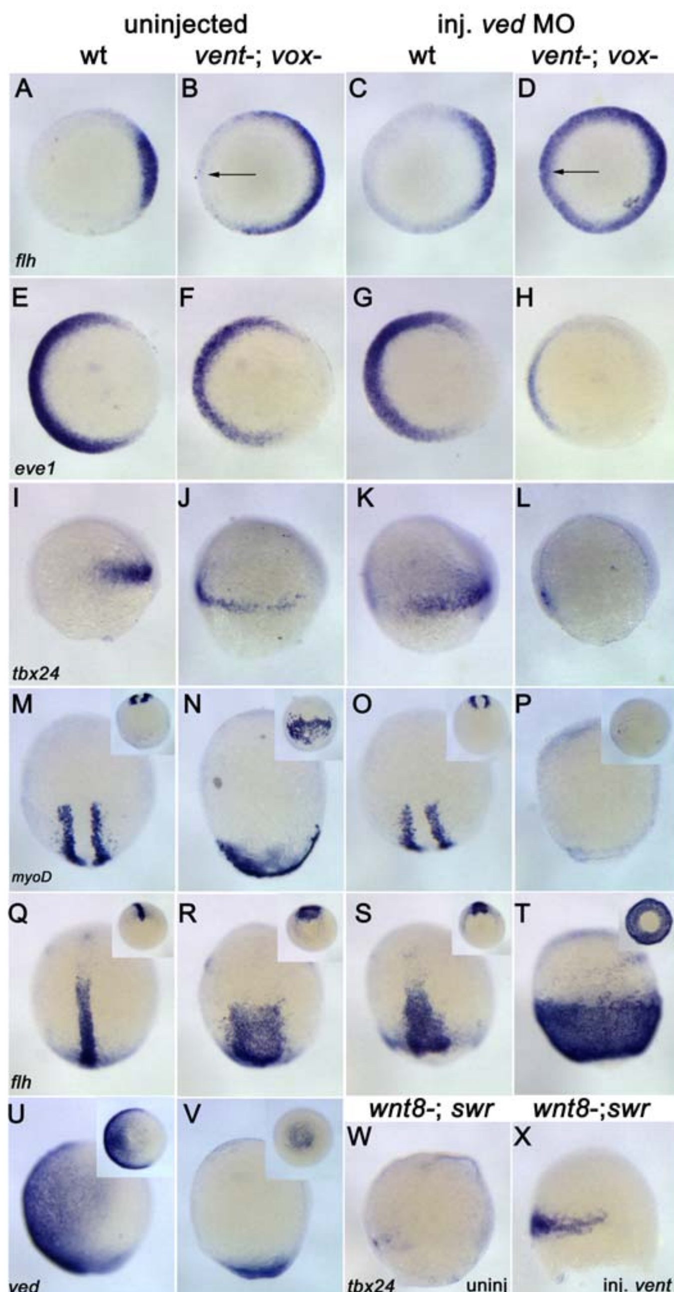

Fig. 24. Knockdown of Ved in *vent*; *vox* mutants replicates the phenotype of *wnt8*; *swr* mutants. In situ hybridization for *flh* (A-D, Q-T), *eve1* (E-H), *tbx24* (I-L,W-X), *myoD* (M-P), and *ved* (U-V). Genotypes are indicated. A-H: shield stage, animal views, dorsal right. I-L, W-X: 70% epiboly, lateral views, dorsal right. M-V: bud stage, dorsal view (M-T) or lateral views, dorsal right (U-V). Insets M-T: vegetal views, dorsal up. Insets U-V: vegetal view, dorsal right. *flh* expression in *vent*; *vox* mutants is expanded ventrally but not in a robust fashion (arrow B, inset R). Injection of *ved* MO in *vent*; *vox* mutants results in a stronger expansion of *flh* (arrow D, inset T). The expression of the non-axial mesoderm markers *eve1*, *tbx24*, and *myoD* is strongly reduced in the *vent*/*vox*/*ved* triple knockdown (H,L,P). At bud stage, *vent*; *vox* mutants still display *ved* expression at the margin but not in the future epidermis (U,V). Injection of *vent* RNA in *wnt8*; *swr* double mutants does restore some *tbx24* expression but does not rescue the phenotype to wild-type (W, X, compare to I).

If the regulation of non-axial mesoderm patterning by Wnt8 and BMP2b is mediated primarily through the regulation of *vent*, *vox* and *ved*, then ectopic expression of any of these transcriptional repressors in *wnt8*; *swr* mutants should rescue the mesoderm patterning phenotype since they are thought to antagonize axial mesoderm in a redundant fashion (Shimizu et al., 2002). To test this hypothesis, we misexpressed Vent in *wnt8*; *swr* double mutants and assayed the expression of *tbx24* at 70% epiboly (see materials and methods). While uninjected *wnt8-*; *swr* embryos consistently display strongly reduced or absent *tbx24* expression (Fig. 24W), 94% of *wnt8*; *swr* mutants injected with *vent* RNA showed robust *tbx24* expression (n=16; Fig. 24X). In most of the rescued embryos, *tbx24* was expressed in the ventral half of the mesoderm but was not observed in a wild-type pattern. Thus, the ability of Vent to rescue some of the *wnt8-*; *swr* phenotype suggests that Vent/Vox/Ved function is indeed critical downstream of Wnt8 and BMP2b to maintain non-axial mesoderm identity.

Wnt8 and BMP2b are required downstream of mesoderm induction for the maintenance of non-axial mesoderm fates

While non-axial mesoderm shifts to an axial fate in *wnt8*; *swr* double mutants, it is unclear if this reflects a defect in mesoderm induction or maintenance. To address this, we identified when the *wnt8-*; *swr* phenotype is first visible.

no tail (*ntl*) is considered a pan-mesoderm marker and its induction is dependent on Nodal signaling (Sakaguchi et al., 2002). We found *ntl* to be properly induced in *wnt8*; *swr* double mutants or in *wnt8* ^{MO}; *swr/swr* embryos at 30% epiboly (Fig. 25F;

data not shown). However, since *ntl* expression does not distinguish axial and non-axial domains at this stage, we also examined *gsc* and *boz* expression at blastula stages (dome stage, 30%, and 40% epiboly). Neither *gsc* or *boz* was significantly expanded in the double mutants compared to wild-type or *wnt8* single mutants (Fig. 25A-C, data not shown). Consistent with this, *ved* is still present in the non-axial mesoderm of double mutants at 30 and 40% epiboly and was not observably different from its expression in *wnt8* mutants (data not shown). Since the *vent*-; *vox*- phenotype was reported to start at dome stage for *gsc* and 30% for *boz* (Imai et al., 2001), our data suggest that *vent* and *vox* must also still be expressed at 40% in *wnt8*; *swr* double mutants. At 50% epiboly, *gsc* is more expanded in *wnt8*; *swr* mutants than in *wnt8* single mutants (Fig. 25I) and this difference becomes more pronounced by shield stage (Ramel and Lekven, 2004). Thus, mesoderm induction occurs normally in the absence of zygotic Wnt8 and BMP2b and the *wnt8*-; *swr* phenotype reflects a defect in the maintenance or reinforcement of non-axial mesoderm identity at the onset of gastrulation (50% epiboly).

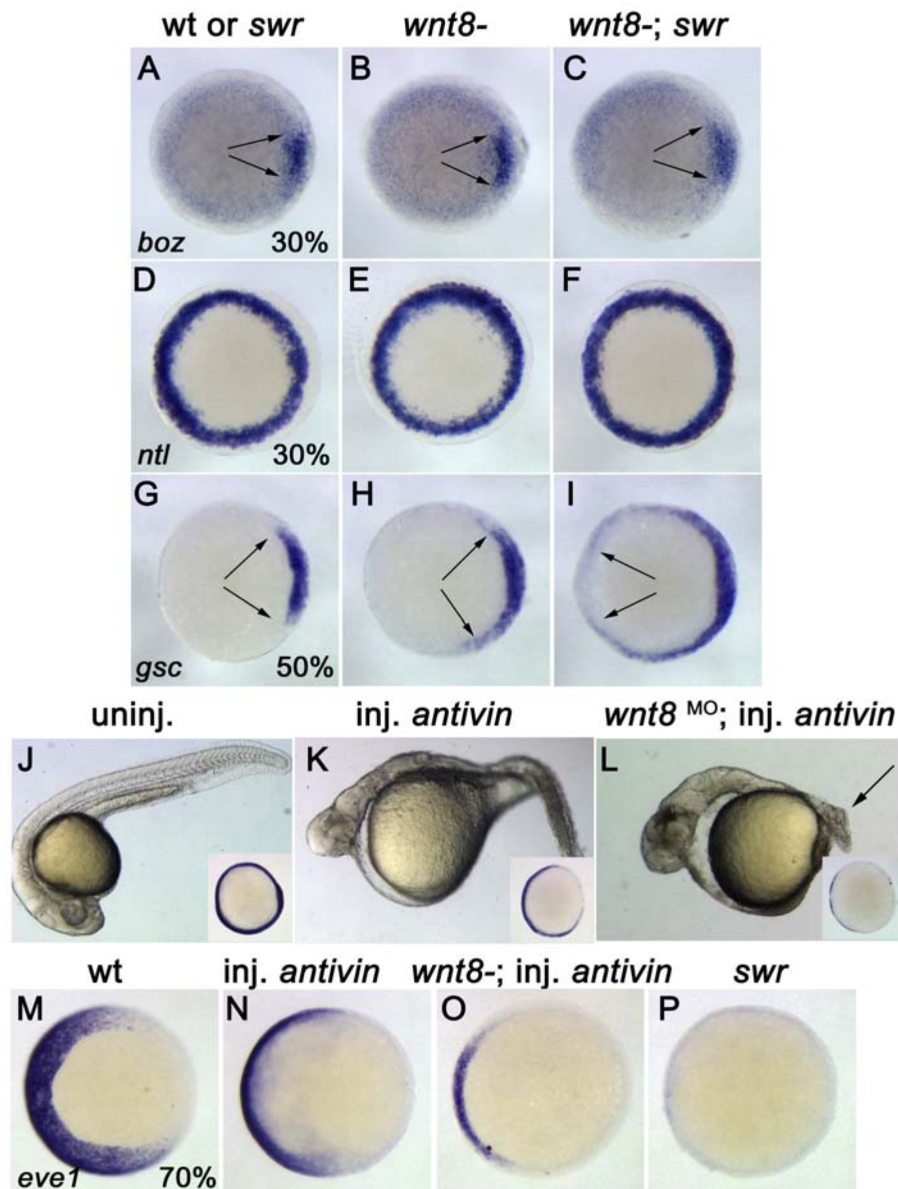

Fig. 25. Wnt8 and BMP2b are required to maintain non-axial identity and both contribute to tail formation in *nodal*- embryos. A-I: the *wnt8*; *swr* phenotype starts at 50% epiboly. In situ hybridizations for *boz* (A-C), *ntl* (D-E) and *gsc* (G-I). Genotypes and stages are indicated. The dorsal marker *boz* is expressed in a wild-type pattern in *wnt8*- (B) and *wnt8*-; *swr* embryos (C) at 30% epiboly. The pan-mesoderm marker *ntl* is also induced normally in *wnt8*; *swr* double mutants (F). Note the enhanced expanded *gsc* phenotype of *wnt8*; *swr* compared to *wnt8* mutants that begins at 50% epiboly (H,I).

J-P: formation of tail somites in *nodal*- embryos is due to residual Wnt8 and BMP2b activity. 24-hour phenotypes of wild-type embryos uninjected (J), injected with *antivin* RNA (K), or injected with *antivin* RNA and *wnt8* MOs (L). Insets in J-L show corresponding *ntl* staining at shield stage, animal view, dorsal right. Knockdown of Wnt8 function is insufficient to prevent tail formation (arrow in L; n=26). M-P: in situ hybridization for *eve1* at 70% epiboly, vegetal view, dorsal right. *eve1* is still expressed in the tail organizer in the absence of Nodal and Wnt8 function (O; n=8) but is not expressed in *swr* mutants (P).

Wnt8 and Bmp2b contribute to tail formation in the absence of Nodal function

Our results suggest that a major function of Wnt8 and BMP2b is to co-maintain the expression of *vent*/*vox*/*ved* after the initial induction of mesoderm. In turn, these transcriptional repressors promote the maintenance of non-axial mesoderm identity. The *wnt8*-; *swr* phenotype clearly differs from the *nodal* mutant phenotype in which tail somites (non-axial fate) are the only mesoderm derivatives that form (Feldman et al., 1998; Gristman et al., 1999). Since no non-axial mesoderm forms in *wnt8*; *swr* mutants, these observations suggest that *wnt8* and *bmp2b* must still be expressed in *nodal*-embryos. Indeed, *wnt8* expression is reduced but not absent in Nodal deficient embryos (Erter et al., 2001; Ragland and Raible, 2004) and there is evidence that *bmp2b* is expressed in the mesoderm of *MZOep* mutants or embryos overexpressing the Nodal inhibitor Antivin (Ragland and Raible, 2004). Thus, the presence of a tail and somites in Nodal deficient embryos is likely due to residual Wnt8 and BMP2b activity. This conclusion is consistent with the findings of Agathon et al. (2003), who, based on overexpression experiments, have proposed that Nodal, Wnt8 and BMP signaling all contribute to the formation of the vertebrate tail organizer with BMP signaling being the one irreplaceable component of the network. Considering our results and the model proposed by Agathon et al., we predicted that elimination of Wnt8 and Nodal should not eliminate tail formation if *bmp2b* expression is maintained.

To test our prediction, we examined the morphological phenotype and expression of *eve1*, an indicator of the prospective tail organizer (Agathon et al., 2003), in embryos in which both Nodal and Wnt8 activity are compromised. To achieve a *nodal*-

phenotype, we injected *antivin* RNA, an inhibitor of Nodal signaling in wild-type embryos (Thisse and Thisse, 1999), at a concentration that is sufficient to phenocopy the *MZOep* phenotype morphologically (Fig. 25K) or as assayed by *ntl* expression (Fig. 25K inset; Sakaguchi et al., 2002). Upon co-injection of *antivin* RNA and *wnt8* MOs, embryos were still able to form a tail and some tail somites (Fig. 25L arrow; n=26) arguing that tail organizer function is preserved in these embryos. We further found that *eve1* expression, which is only slightly reduced in *nodal*- embryos (Fig. 25N; n=23) and moderately reduced in *wnt8* mutants at 70% epiboly (not shown), is severely reduced but still detectable in *wnt8*-; *nodal*- embryos, at least through 70% epiboly (Fig. 25O; n=8 *wnt8* mutants; confirmed using *wnt8* MOs). Since *eve1* expression is not detectable in *swr* mutants at this stage (Fig. 5P), our results argue that *bmp2b* expression and activity must persist in *wnt8*-; *nodal*- embryos, at least through 70% epiboly. However, the fact that the tails of *wnt8*-; *nodal*- embryos are significantly smaller and less organized than in wild-type (Fig. 25L and data not shown) suggests that Wnt8 and Nodal signaling have required roles in the expansion of the tail precursors, which is consistent with finding that the expression of *tbx6* in the involuting non-axial mesoderm is severely reduced in *wnt8* mutants (Lekven et al., 2001; Ramel and Lekven, 2004).

DISCUSSION

In this study, we have shown that Wnt8 and BMP2b have an essential role during zebrafish gastrulation in the maintenance of non-axial mesoderm. The loss of Wnt8 and BMP2b activity results in the progressive expansion of axial mesoderm markers into the entire mesodermal domain beginning at 50% epiboly. The phenotype of *wnt8*; *swr* mutants appears to be attributable to the loss of *vent*, *vox*, and *ved* expression. Consistent with this, *vent*/*vox*/*ved* triple knockdown replicates the *wnt8*; *swr* phenotype and ectopic expression of Vent is able to partially rescue non-axial mesoderm development in *wnt8*; *swr* mutants. Further highlighting the importance of Wnt8 and BMP2b in regulating non-axial and posterior mesoderm development, the formation of a tail in Nodal-deficient embryos can be attributed to persistent *wnt8* and *bmp2b* expression despite impaired Nodal-dependent mesoderm induction.

Wnt8 and BMP2b act downstream of mesoderm induction to impart non-axial identity during gastrulation

The relative roles of Wnt8 and BMP signaling in mesoderm patterning have been difficult to decipher, likely due to the fact that they function in parallel and can affect each other's expression (Hoppler and Moon, 1998; Marom et al., 1999; Mullins, 1999; Ramel and Lekven, 2004; Ramel et al., manuscript in preparation). Our results show that Wnt8 and BMP both regulate D/V patterning but differently; their differential activities are revealed in loss-of-function contexts and when examined with respect to the

temporal control of patterning. For example, *vent* expression in the embryonic margin becomes primarily dependent on BMP signaling but only after 70% epiboly (Melby et al., 2000); prior to this stage, *vent* is primarily dependent on Wnt8 (Ramel and Lekven, 2004). *vox* expression in the margin does not rely significantly on BMP activity, although its expression in the ectoderm does (Melby et al., 2000). *ved* appears to be regulated similarly to *vox* (this study), although the expression of *ved* has been reported to be under both positive and negative regulation by BMP, Vent, and Vox in a temporally dynamic fashion (Gilardelli et al., 2004). These results also raise the question of whether regulating *vent*, *vox*, and *ved* explains all of Wnt8 and BMP function. This may be a difficult question to answer since Wnt8 and BMP activity is required first to establish the mesodermal domain that they subsequently act upon during gastrulation. Nonetheless, several pieces of data suggest that each pathway has unique functions in mesoderm development. For example, *swr* mutants have a characteristic dorsalized phenotype that develops in the presence of Wnt8 activity (Ramel and Lekven, 2004). Further, *wnt8* mutants have a characteristic loss of posterior mesoderm that cannot be attributed to modulation of BMP activity since this phenotype is not observed in gastrula stage *swr* mutants. Understanding this relationship will significantly enhance our understanding of mesoderm patterning and development.

Regardless of the precise control mechanisms, Wnt8 and BMP signaling are essential for establishing the non-axial mesoderm domain, which forms as a consequence of the repression of axial mesoderm gene expression. We observed that the phenotype of *wnt8*; *swr* mutants develops between 40 and 50% epiboly, corresponding to the temporal loss of *vent/vox/ved* expression. These findings suggest that the establishment of ventral fates may be viewed as a permissive event, similar to the view of neural induction resulting from the inhibition of ectodermal BMP activity. Based on the results presented here, we propose a model for non-axial mesoderm establishment and patterning that views this process as a temporally regulated establishment of transcriptional repression domains. After mesoderm induction and specification of the Nieuwkoop center (Fig. 26A), Wnt8/BMP2b dependent regulation of *vent*, *vox*, and *ved* functions to antagonize the acquisition of axial identity within a non-axial domain (Fig. 26B). Because of several mutually repressive interactions, axial and non-axial domains within the margin are defined.

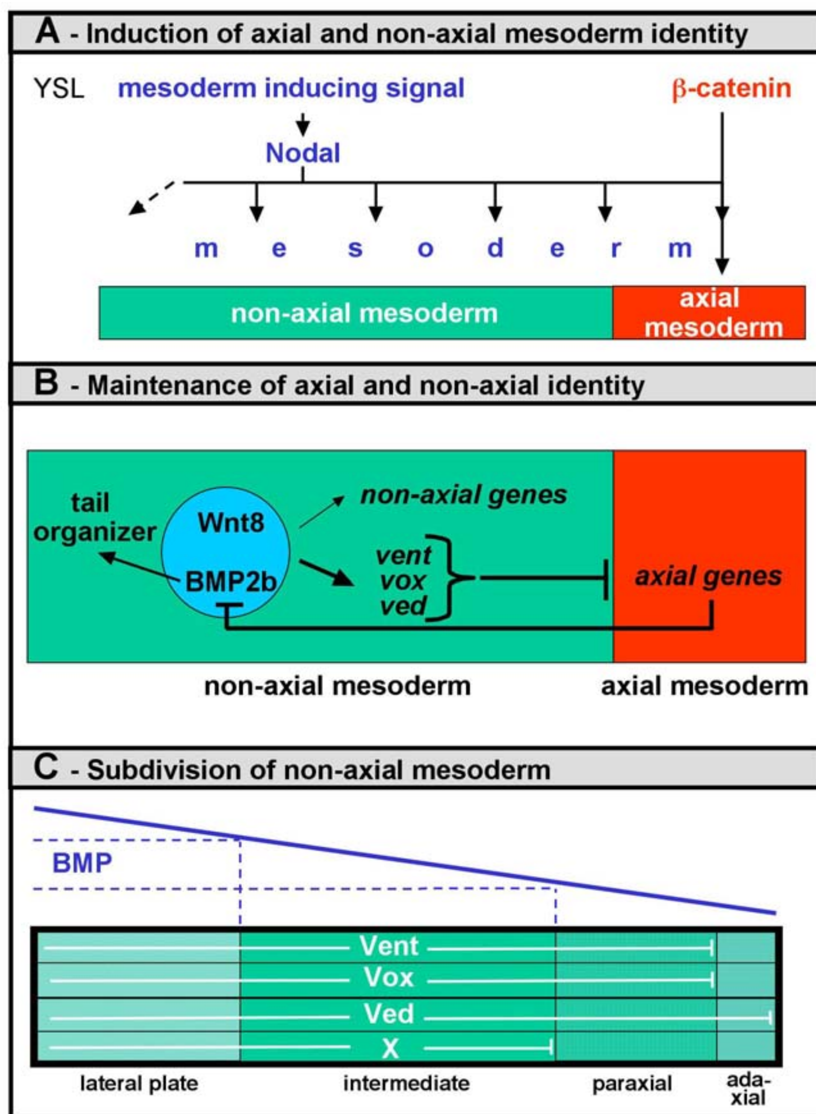

Fig. 26. Progressive specification of mesoderm domains in zebrafish. A: Nodal signaling relays a maternal signal to induce most of the mesoderm with the exception of the most ventral mesoderm (indicated as dashed arrow). Dorsally, β-catenin signaling acts as an axial mesoderm-inducing factor. B: axial vs non-axial mesoderm domains are maintained through two repression systems. In the non-axial mesoderm, Wnt8 and BMP2b co-regulate *vent*, *vox* and *ved* expression (thick arrow), which in turn repress the expansion of axial gene expression Wnt8 and BMP2b may also maintain non-axial mesoderm though direct co-regulation of other non-axial genes (thin arrow). The axial mesoderm does produce factors that inhibit BMP2b expression (Imai et al., 2001) but *wnt8* is still expressed in *vent/vox/ved* triple knockdown (data not shown). C: subdivision of non-axial mesoderm depends on a gradient of BMP signaling and differential effects of Vent, Vox and Ved. Vent and Vox function redundantly towards the repression of adaxial mesoderm while Ved appears to have no significant effect on adaxial repression by itself. Repression of paraxial mesoderm is likely due to an unknown factor (or factors indicated by “X”) that is absent in *wnt8* or *swr* mutants, thus resulting in ventral expansion of *tbx24*.

Mesodermal domains as zones of differential transcriptional repression

Our studies further suggest that throughout gastrulation the coordinated control of *vent*, *vox* and *ved* expression within the non-axial mesodermal domain is critical for establishing multiple mesodermal subdomains (Fig. 26C). This is illustrated by the regulation of the adaxial expression of *myoD*: lowering the expression of *vent*, *vox* and *ved* shifts non-axial domains in specific ways. In other words, embryos lacking Wnt8 activity and having only a single copy of *bmp2b* experience a global shift in mesodermal domains and *myoD* expression (see Fig. 21), which may be attributable to a simultaneous decrease of *vent*, *vox* and *ved*. In contrast, completely removing *vent* and *vox* results in the expansion of both the axial and adaxial domains (see Fig. 23), an effect that can be attributed to remaining *ved* expression.

Wnt8 and BMP2b are also essential to limit the expansion of *tbx24*, as *tbx24* expands ventrally in *wnt8* and *swr* mutants. This loss of *tbx24* repression occurs in the presence of some *vent*, *vox*, and *ved* expression in both mutant backgrounds. One can thus invoke additional factors regulated by Wnt8 and BM2b that are able to repress *tbx24* expression in the lateral plate and intermediate mesoderm. Further experiments are necessary to determine the identity of these factors.

Vent overexpression can rescue some *tbx24* expression in *wnt8*; *swr* mutants (see Fig. 4) but is unable to restore *tbx24* in its normal bilateral domains of expression in the paraxial mesoderm. It is likely that simultaneous overexpression of all transcriptional repressors will not be able to achieve a normal *tbx24* expression pattern in *wnt8*; *bmp*

double mutants as these embryos may still lack the ability to repress *tbx24* expression ventrally.

Thus, specific combinations or expression levels of these repressors (and possibly others) may be required for the establishment of different mesodermal domains, not just the regulation of dorsal gene expression. Understanding this relationship will require a more thorough understanding of *vent*, *vox*, and *ved* regulation, a careful analysis of their expression domains, as well as their impact on non-axial mesoderm fates. A global understanding of how this mode of mesoderm specification fits with the known functions of BMP and Nodal/FGF (Fibroblast Growth Factor) activity in this process is also necessary.

Support for a revised view of the primary axes

A recurring observation in these studies is that non-axial mesoderm patterning is a temporally dynamic process: the regulation of downstream targets by Wnt8 and BMP2b signaling (and the relationship between Wnt8 and BMP2b; manuscript in preparation) changes during gastrulation. It has recently been proposed that the traditional means of designating the D/V axis in *Xenopus* does not accurately reflect the progression of mesoderm differentiation (Lane and Sheets, 2002; Kumano and Smith, 2002). This may have profound implications upon the interpretation of D/V patterning interactions in zebrafish since the same molecular mechanisms are at play in both organisms. A reanalysis of the results of classic organizer transplants in *Xenopus* shows that grafted organizer tissue results in the precocious differentiation of prospective posterior

mesoderm surrounding the graft into axial structures, not in an alteration to D/V patterning specifically (Lane et al., 2004). In other words, regulation of BMP activity did not alter the D/V patterning of surrounding tissue, but rather resulted in a disruption of the temporal sequence of differentiation: inhibiting BMP resulted in earlier differentiation of prospective posterior regions (Lane et al., 2004). One interpretation of these data is that BMP activity is required to prevent responding cells from adopting a program of differentiation at a specific time. We, in zebrafish, found that the primary activity of BMP, in conjunction with Wnt8, is to prevent premature axial differentiation by maintaining the non-axial mesoderm identity at early gastrula stages and thus allowing BMP-induced differentiation at later stages. Thus, we find that there is a remarkable parallel between this proposed temporal function of BMP and our observations of BMP/Wnt8 regulation of *vent*, *vox* and *ved* and their control of mesoderm fate specification through transcriptional repression.

CHAPTER V

SUMMARY OF EXPERIMENTS AND DISCUSSION

SUMMARY OF FINDINGS

The purpose of this study was to uncover the mechanisms of Wnt8 function during D/V axis maintenance and patterning using the zebrafish as a model system. In this process, Wnt8 appears to have two main functions. First, it allows the maintenance of the ventral (non-axial) mesodermal domain through its regulation of the transcriptional repressors Vent and Vox which inhibit the transcription of dorsal (axial) genes. Wnt8 was shown to be necessary to maintain high levels of *vent* and *vox* transcription. Further, *vent* and *vox* were shown to be direct transcriptional targets of Wnt8/β-catenin signaling. Wnt8 is unable to repress dorsal genes in the absence of Vent and Vox and Vent/Vox overexpression rescues the expanded organizer phenotype of *wnt8* mutants, thus supporting the hypothesis of a linear pathway where Wnt8 is upstream of Vent and Vox. Second, Wnt8 was shown not only to regulate *vent* and *vox* transcription but all non-axial mesodermal genes looked at. Wnt8 is not required for their induction, but rather for their maintenance at wild-type levels during gastrulation. Thus, Wnt8 can be considered

as a general input of non-axial mesoderm fates, a function that is shared by BMP signaling. Epistatic analysis between Wnt8 and BMP showed that Wnt8 is required to maintain zygotic *bmp* expression at mid-gastrulation while BMP is not necessary for *wnt8* expression. Loss of zygotic *bmp* expression in *wnt8* mutants appears to result from increased organizer activity by Wnt8 signaling. Interestingly, simultaneous removal of zygotic Wnt8 and BMP2b function leads to the striking phenotype of complete loss of non-axial mesodermal fates at the onset of gastrulation. This phenotype is not only due to the loss of Vent and Vox function but to the additional loss of Ved, another transcriptional repressor closely related to Vent and Vox. In addition, we have shown that the requirement for Wnt8 and/or BMP function in the non-axial mesoderm is dynamic during zebrafish embryogenesis (see Fig. 27). To summarize, our ability to genetically dissect Wnt8 function in zebrafish was instrumental in expanding our knowledge of the role of Wnt8 in D/V axis maintenance and also of mesoderm patterning in general.

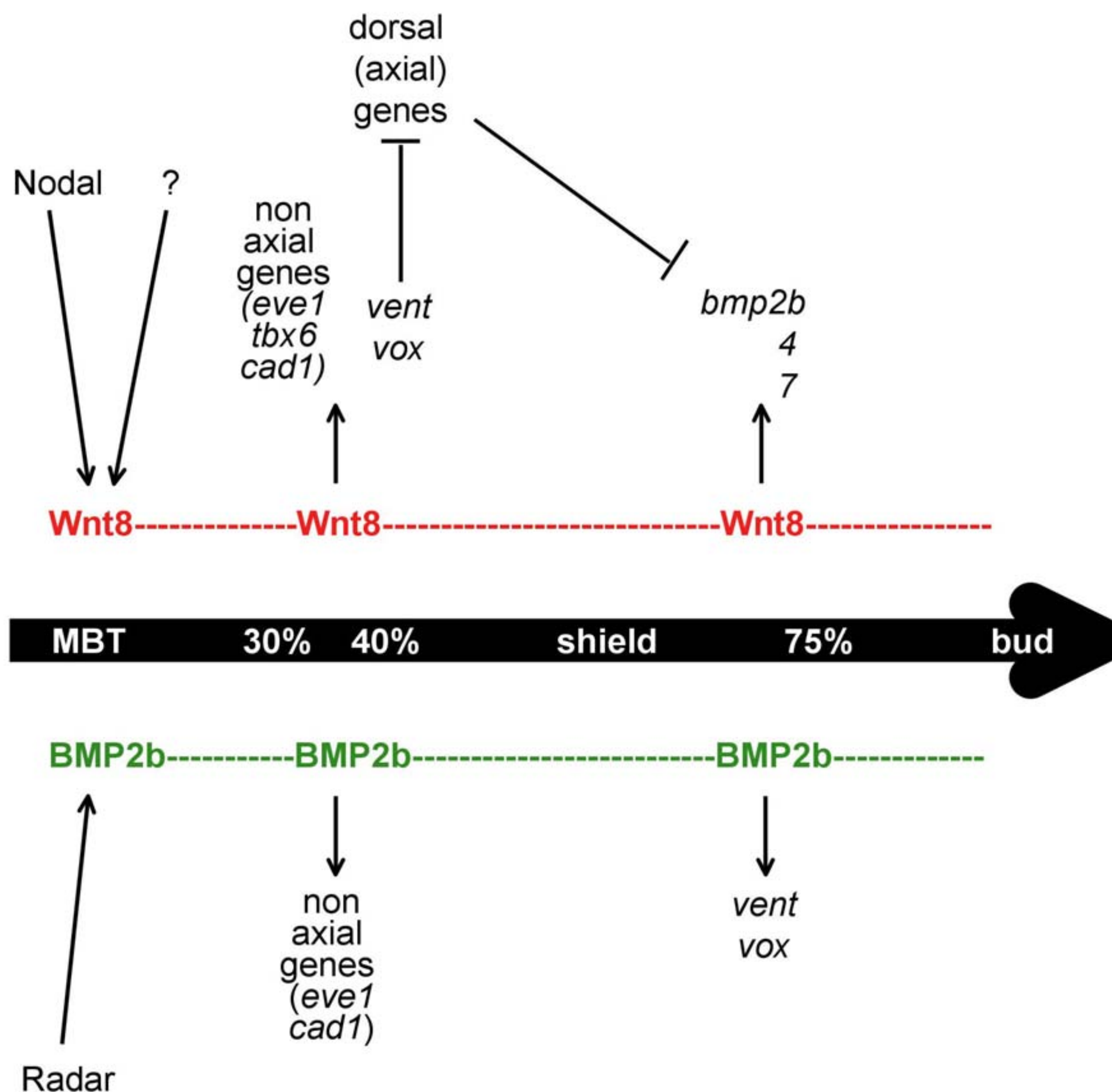

Fig. 27. In vivo model of Wnt8 and BMP2b function in the mesoderm during zebrafish development. *wnt8* and *bmp2b* expresssion at MBT is induced by maternal factors. Wnt8 requirement in D/V maintenance starts at early gastrulation through its regulation of *vent* and *vox*. In contrast, BMP2b is only necessary starting at mid-gastrulation for *vent*/*vox* expression. Wnt8 and BMP2b are required for the maintenance of other non-axial genes besides *vent* and *vox*. Wnt8 is required for *bmp2b/4/7* expression at mid-gastrulation by maintaining the expression of BMP antagonists (dorsal) and transcriptionally regulates *bmp2b*.

GENERAL FUNCTION OF WNT8: MODULATOR OF TRANSCRIPTION AND PROLIFERATION

For all ventral/non-axial markers tested, we found that Wnt8 is not required for their induction but rather for their transcriptional maintenance at the onset of gastrulation. In addition, we found that Wnt8 is not the sole input towards the transcription of all the markers tested. Indeed, the expression of these markers was reduced compared to wild-type levels but not absent (see chapter IV). These results suggest that Wnt8 signaling modulates the transcription of target genes from basal to normal levels. Since LEF/TCF proteins cannot act as transcriptional activators by themselves (Eastman and Grosschedl, 1999), other inputs must allow for basal transcription of Wnt8 target genes. Smad mediated transcription (downstream of BMP signaling) could allow basal transcription in the absence of Wnt8 signaling. A promoter region of *tbx6* that recapitulates endogenous expression has been analyzed in detail and it was shown that it contains both TCF/LEF and Smads binding sites that allow input of both Wnt8 and BMP pathways (Szeto and Kimelman, 2004). Our findings that *vent* and *vox* transcription at shield stage is almost completely abolished in the absence of both Wnt8 and BMP signaling while it is normal in *swr* mutants and reduced in *wnt8* mutants reinforce the hypothesis that BMP signaling allows an on/off transcription if Wnt8 signal is absent while Wnt8 acts as an amplifying signal in the presence of functional BMP signaling. Alternatively, BMP, like Wnt8, could act as an amplifying signal with the difference that the transcription of target genes may be more sensitive to BMP than to Wnt8 input. The sensitivity of our

assay (in sity hybridization) does not allow us to clearly understand the mode of transcriptional regulation of target genes by Wnt8 and BMP. Nonetheless, these observations apply to the expression of most of the non-axial mesodermal markers tested at shield stage: *vent*, *vox*, *ved*, *eve1*, *cad1*, and *tbx6*. Interestingly, the role of Wnt signaling as a modulator of BMP target genes was also illustrated in a recent study by Nishanian et al. in which induction of a *msx2* luciferase reporter by BMP4 was found to be attenuated in the absence of functional TCF/LEF binding sites (and thus the absence of Wnt responsiveness) in the *msx2* promoter (Nishanian et al., 2004).

In addition to the reduction in levels of transcription, we also made the observation that Wnt8 may be required for cell proliferation. This is most evident when looking at *tbx6* or *tbx24* expression in *wnt8* mutants compared to wild-type or *swr* mutants (see chapter IV). Not only is the expression intensity of these markers reduced but there actually seems to be fewer positive cells compared to wild-type. Further experiments are necessary to quantify *tbx6*- and *tbx24*-expressing cells in *wnt8* mutants compared to wild-type.

The requirement for Wnt signaling towards cell proliferation has been observed previously. For instance, *cyclin D1*, a master regulator of cell cycle progression that promotes the G1 to S transition in the cell cycle and a protein that is often upregulated in cancerous cells, was identified as a Wnt target gene (Rimerman et al., 2000). During development of the spinal cord in the chick, Wnt1 and Wnt3a were shown to promote cell proliferation (Megason and McMahon, 2002). Additionally, Wnt signaling was found to promote G1/S phase transition of neural crest cells in chick, confirming the in

vivo function of Wnt signaling towards proliferation (Burstyn-Cohen et al., 2004). Puzzlingly, analysis of proliferation in *wnt1*/*wnt3a*/*wnt10b* triple knockdown in zebrafish did not reveal any convincing differences in proliferation compared to wild-type (Buckles et al., 2004). However, increased apoptosis was observed in the absence of all three genes. Thus, it has been postulated that Wnt signaling may serve as an anti-apoptosis factor rather than a proliferation factor. Whether this is the case for Wnt8 function in zebrafish could be addressed through the analysis of BrdU incorporation (proliferation) or TUNEL assay (apoptosis) in the mesodermal cell population during gastrulation.

Thus, Wnt8 may maintain the expression of non-axial markers through modulation of transcription and by maintaining proper cell proliferation/preventing apoptosis and it is likely that both mechanisms are simultaneously at play.

REGULATION OF WNT8 TRANSCRIPTION

In zebrafish, RT-PCR analysis of *wnt8* transcripts showed that they are both maternally distributed and zygotically expressed (Kelly et al., 1995). The function of maternal Wnt8 was not addressed in this study. Through our epistatic analysis of zygotic Wnt8 and BMP, we have shown that maternal BMP signaling is not necessary for inducing *wnt8* transcription at MBT nor is zygotic BMP signaling required to positively maintain *wnt8* transcription (see chapter III). Some studies have implicated Nodal signaling as a

regulator of *wnt8* transcription since *antivin* RNA injected embryos or *cyc*; *sqt* double mutants display reduced *wnt8* expression (Erter et al., 2001; Ragland and Raible, 2004). The fact that BMP overexpression can induce ectopic *wnt8* may be explained if BMP overexpression leads to a Nodal-like response. This hypothesis could be tested by overexpressing BMP in a MZoep background. Since Oep is an essential co-factor for Nodal signaling (Gritsman et al., 1999), ectopic *wnt8* should not be observed upon BMP overexpression in this mutant background if our hypothesis is true. Because *wnt8* expression is reduced in the absence of Nodal signaling but not absent, it also suggests that multiple signaling pathways may be responsible to turn on *wnt8* expression at MBT. Nodal and maternal BMP pathways may both contribute towards *wnt8* induction. This hypothesis could be tested by injecting *radar* DN RNA in a *MZoep* mutant. Detailed promoter analysis of the *wnt8* locus could help reveal the regulatory inputs of *wnt8* transcription. Preliminary analysis has shown that the upstream region of the *wnt8* genes does contain some BMP response elements as well as TCF/LEF binding sites, suggesting that maternal Wnt8 may also be involved in *wnt8* induction (Fig. 28; A.C. Lekven, unpublished observations). Additionally, once *wnt8* expression is turned on at MBT, it is possible that autoregulation allows continuous expression during the rest of development.

A NEW MODEL OF AXIAL VS NON-AXIAL MAINTENANCE

In zebrafish, initial mesoderm induction by Nodal signaling and Nieuwkoop center activity leads to two distinct mesodermal domains: axial (characterized by *boz* or *gsc* expression) and non-axial (characterized by *ved* expression). The previous model of axial vs non-axial maintenance implicated Wnt8, Vent, Vox and Ved as being necessary to maintain the non-axial mesoderm while Boz was required to maintain axial mesoderm (Lekven et al., 2001; Imai et al., 2001; Solnica-Krezel and Driever, 2001; Leung et al., 2003). Partly due to its late regulation of *vent* and *vox* (Melby et al., 2000), BMP was not thought to be essential to maintain the non-axial mesoderm (Ramel and Lekven, 2004).

In the course of this study, we demonstrated a critical role for BMP towards non-axial mesoderm maintenance that is only revealed in the absence of Wnt8 since *wnt8*; *bmp* double mutants display a complete loss of non-axial mesodermal fates (see chapter IV). Indeed, BMP2b activity is essential for *vent*, *vox* and *ved* transcription at early stages in the absence of Wnt8. These results demonstrate the critical importance of multiple levels of regulation of developmentally essential genes. A new model showing the role of Wnt8 and BMP2b towards non-axial mesoderm maintenance is shown in Fig. 28.

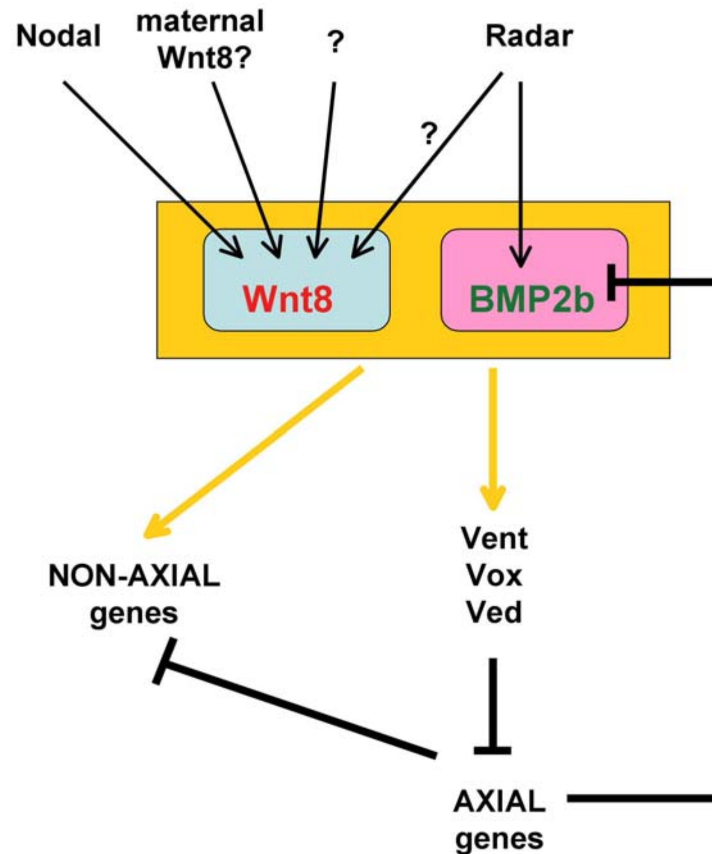

Fig. 28. Schematic representation showing induction of Wnt8 and BMP2b and their role towards non-axial mesoderm maintenance. Induction of *wnt8* transcription is known to depend on Nodal and it has not been tested yet if maternal Wnt8 or an unknown factor is responsible for *wnt8* induction. Radar is not thought to induce *wnt8* expression by itself but may do so in combination with other *wnt8* inducers. Non-axial mesoderm maintenance by Wnt8 and BMP2b does occur through both the regulation of Vent, Vox and Ved and possibly in a more direct regulatory fashion. Increased expression of axial genes does negatively affect *bmp2b* expression but not *wnt8* (Imai et al., 2001; data not shown).

ARE ALL WNT8 FUNCTIONS IN D/V AXIS MAINTENANCE AND PATTERNING BMP-DEPENDENT?

Historically, Wnt8 and BMP functions have been thought to be equivalent as both factors were classified as ventralizing agents. In zebrafish, *wnt8* and *bmp* mutants/morphants have some similarities with regard to the D/V phenotype (Lekven et al., 2001; Hammerschmidt and Mullins, 2002). *wnt8* morphants are dorsalized like *bmp* mutants with the exception that *wnt8* morphants display the additional phenotype of altered A/P polarity while *bmp* mutants do not (Lekven et al., 20001; Mullins et al., 1996). Thus, in A/P patterning, Wnt8 seems to function independently of BMP. Our epistatic analysis of Wnt8 and BMP in D/V patterning revealed that zygotic *bmp* expression is reduced in the mesoderm of *wnt8* mutants starting at mid-gastrulation (see chapter III). This regulation appears to be both direct and indirect (due to increase in BMP antagonists in *wnt8* mutants). Thus, reduced BMP activity in the absence of Wnt8 may explain the dorsalized phenotype of *wnt8* mutants. Consistent with this hypothesis, dorsalized *wnt8* morphants as strong as C5 (as strong as *bmpb2b* null) can be observed. However, analysis of early markers reveals some differences between *wnt8* and *bmp* mutants. For instance, *tbx6* expression at the beginning of gastrulation is reduced in *wnt8* mutants but it is not in *bmp* mutants (see chapter IV and unpublished observations). This phenotype persists until bud stage arguing that there is indeed a difference between *wnt8* and *swr* mutants. If the dorsalized phenotype of *wnt8* mutants is solely due to reduced BMP activity, overexpressing BMP should be able to rescue or ventralize *wnt8* mutants.

Of all BMPs tested, BMP2b was the most potent ventralizer of *wnt8* mutants as it was able to repress the ventral expansion of neural ectoderm present in *wnt8* mutants (see chapter III). Since *wnt8* is not expressed in the ectoderm, these results argue that the expansion of neural ectoderm is an indirect consequence of the expanded organizer of *wnt8* mutants. However, strong *tbx6* expression cannot be restored by BMP2b overexpression, suggesting that BMP2b cannot rescue all aspects of the *wnt8*- dorsalized phenotype. These results also suggest that indeed Wnt8 has some BMP-independent functions in D/V patterning but that it is restricted to the mesoderm. These observations also show the limit of the 'universal' nomenclature describing the ventralized and dorsalized phenotypes (Kishimoto et al., 1997). It would probably be more accurate to design a different classification system specific to each signaling pathway that would describe D/V phenotypes.

CORRELATION OF FINDINGS WITH CANCER RESEARCH

Cancer progression involves some of the same processes that happen during embryogenesis, such as cell proliferation and cell migration. Disruptions in Wnt signaling have been observed in a variety of cancerous tumors (reviewed in Giles et al., 2003). β-catenin is described as an oncogene since it actively promotes transcription when activated and translocated into the nucleus upon Wnt stimulation. β-catenin also localizes at the adherens junctions of epithelial cells. Upon Wnt signaling, it is thought

that the pool of available β-catenin increases as a result of inhibited degradation as well as recruitment of β-catenin from adherens junctions. Thus, Wnt signaling results in both increased transcriptional activity and decreased cell adhesion, two hallmarks of cancerous cells. Despite the ubiquitous nature of β-catenin, specific tissues are more prone to tumor formation upon disruption of the Wnt signaling pathway. As a way to understand this specificity, several studies have used microarrays to identify target genes in various cell lines. The targets identified include known oncogenes such as *c-myc*. In this study, we have identified *vent* and *vox* as two new direct transcriptional targets of Wnt8 in zebrafish (see chapter II). Homologs of *vent* and *vox* are present in humans where at least two *vent*-like genes have been identified: *ventx1* (also known as *hpx42*) and *ventx2* (Moretti et al., 1994; Moretti et al., 2001). Three additional *vent*-like pseudogenes have been sequenced in the human genome. Of all these genes, only *ventx2* was identified as a bona fide member of the *vent* class of homeobox genes and Ventx2 protein was found to be highly expressed in immature hemopoietic cells (Moretti et al., 2001). Moreover, human *ventx2* RNA injection in zebrafish embryos gave rise to ventralized embryos, confirming the conserved function of human Ventx2 (Moretti et al., 2001). However, no loss-of-function analysis of Ventx2 function has yet been performed in a mammalian model system. Thus, correlating our findings in zebrafish with putative roles of the Wnt8/Vent/Vox pathway in mammalian embryonic growth and cancer requires further study.

BMP signaling has also been implicated in the etiology of certain tumors. BMP, as opposed to Wnt signaling, is thought to have a general tumor-suppressing activity.

Towards the regulation of non-axial mesodermal genes, however, BMP signaling appears to promote transcription rather than repress it. Our epistatic analysis of Wnt8 and BMP suggested that Wnt8 is required for BMP expression and BMP signaling (see chapter III). In a human colorectal cancer cell line, it was also found that BMP4 is downregulated in the absence of Wnt signaling (van de Wetering et al., 2002). Further, microarray analysis of a NTERA2 undifferentiated cancer cell line treated with BMP4 (a treatment that leads to differentiation) revealed that BMP4 signaling results in the transcriptional upregulation of members of the Wnt signaling pathway such as LEF1, *wnt2b*, and *wnt5B* (Nishanian et al., 2004). This result is similar to our observation that BMP misexpression can induce ectopic *wnt8* expression (see chapter III). In addition, the same authors found that BMP4 treatment of a cell line NCCIT that does not undergo differentiation but whose transcriptome is regulated by BMP4 did not induce the expression of Wnt pathway components. Thus, it has been suggested that activation of Wnt signaling may be a prerequisite for BMP4-induced differentiation. Our analysis of *wnt8*; *bmp* double mutants, which revealed that most BMP target genes are also co-regulated by Wnt8, does support the hypothesis that Wnt signaling is absolutely required for BMP signaling to be able to act as a differentiation factor. Since differentiation can be considered as the opposite of clonal expansion of undifferentiated cells observed in cancer, these studies highlight the critical requirement for tightly regulated Wnt8 and BMP signaling.

Of all cancers known to correlate with disruption of Wnt signaling, colon cancer is the most prevalent (Giles et al., 2003). Interestingly, it was found that colon cancer

lines are resistant to the tumor suppressive properties of BMP4 (Nishanian et al., 2004). Thus, these results also argue that normal Wnt signaling (disrupted in colorectal cancer) is also necessary for BMP4 to exert its effects on growth. All our results are therefore consistent with observations in the cancer research field and they do reinforce the idea that delicately combined and regulated inputs from multiple signaling pathways are critical not only for development but for proper tissue homeostasis.

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PEER-REVIEWED PUBLICATIONS

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