THE ROLE OF WNT8 IN VENTROLATERAL MESODERM PATTERNING AND POSTERIOR GROWTH

A Dissertation

by

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ABSTRACT

Vertebrate dorsoventral patterning requires both Wnt8 and BMP signaling. Because of their multiple interactions, discerning roles attributable specifically to Wnt8 independent of BMP has been a challenge. For example, Wnt8 represses the dorsal organizer that negatively regulates ventral BMP signals, thus Wnt8 loss-of-function phenotypes may reflect the combined effects of reduced Wnt8 and BMP signaling. We have taken a loss-of-function approach in the zebrafish to generate embryos lacking expression of both Wnt8 and the BMP antagonist Chordin. wnt8;chordin loss-of-function embryos show rescued BMP signaling, thereby allowing us to identify Wnt8-specific requirements. Our analysis shows that Wnt8 is uniquely required to repress prechordal plate specification but not notochord, and that Wnt8 signaling is not essential for specification of tailbud progenitors but is required for normal expansion of posterior mesoderm cell populations. Further, we find that Wnt8 is required for the normal expression of *cdx4* and *ntl,* but not *wnt3a.* Finally, we find that Wnt8 is required for cell proliferation in the tailbud. Thus, Wnt8 and BMP signaling have independent roles during vertebrate ventrolateral mesoderm development that can be identified through loss-of-function analysis.

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CHAPTER I

INTRODUCTION

Mesoderm Induction and Maintenance

Vertebrate mesoderm gives rise to several different tissue types, among them are heart, blood, vasculature, muscle, and kidney. Our earliest understanding of the way this germ layer is induced comes from experiments in *Xenopus* performed by Nieuwkoop. Utilizing the classic “animal cap assay”, he showed that prospective ectodermal tissue removed from the top animal pole of the embryo and grafted onto the bottom vegetal part of the embryo could induce the formation of mesoderm in the ectodermal caps. (Nieuwkoop, 1973). This was the first evidence that mesoderm is induced by signals that emanate from the vegetal pole of *Xenopus* embryos.

The mesoderm inducing signal was initially thought to be Activin, a member of the TGFβ superfamily of signaling molecules. Smith et al. were able to purify a mesoderm inducing signal from cultured *Xenopus* animal caps which later proved to be Activin (Smith et al., 1990). Importantly, this signal was found to induce different mesodermal tissues in a concentration dependent manner and had the capacity to act at a distance. However, the discovery that Activin mutant mice were able to specify mesoderm and exhibited only a mild phenotype

caused researchers to continue the search for a mesoderm inducing signal (reviewed in Weng and Stemple, 2003).

Later, an insertional mutagenesis project in mice discovered another signaling molecule in the TGFβ superfamily that appeared to be capable of inducing mesoderm. Mice mutant for this gene demonstrate an overproliferation of ectoderm at the expense of mesoderm, which is absent. This gene was named *nodal* due to its abundant expression in the murine node (Zhou et al., 1993).

In *Xenopus,* a maternally derived signal and member of the TGFβ family was found to be critical for mesoderm induction and gastrulation. Loss of this gene Vg1, causes the misspecification of ectoderm, mesoderm and endoderm and prevents vegetal cells from releasing mesoderm inducing signals (Zhang and King, 1996*).* Further discovery revealed that *Xenopus* has six nodal related genes and that Vg1 is indispensable for their proper activation (reviewed in Zorn and Wells, 2007). A gene orthologous to Vg1 has been identified in *D. rerio*, but it is not maternally contributed (reviewed in Kimelman and Griffin, 2000). It is unclear what the activating agent for *nodals* is in zebrafish, but it has been shown that the injection of RNAse into the vegetal pole of a zebrafish embryo prevents mesoderm specification, so it is assumed that this agent is maternally contributed (Chen and Kimelman, 2000). In conclusion, zebrafish shares the common vertebrate mode of mesoderm induction; an activating agent that turns

on zygotic *nodal* genes which are then responsible for the induction of mesoderm.

The mechanisms of mesoderm formation are highly conserved and a large measure of what we know about this complex process has been gained from investigations involving zebrafish. Zebrafish mesoderm is induced in a layer of cells that is formed by the involution of the leading edge, or margin, of the gastrulating embryo. These cells are adjacent to the yolk syncytial layer (YSL), which is formed when the cells closest to the vegetal pole collapse into the yolk (Kimmel and Law, 1985). Mesoderm gives rise to several tissue types, including muscles, blood, notochord, and pronephros. The nodal genes responsible for mesoderm induction in zebrafish are *squint (sqt)* and *cyclops (cyc).* Embryos deficient for both of these genes form small tails, but lack trunk mesoderm and all endoderm (Feldman et al., 2002). In support of this evidence, the loss of the EGF-CFC protein *one-eyed-pinhead* (*oep*), an essential membrane bound co-receptor for Nodal ligands, phenocopies double mutations in these genes (Zhang et al., 1998).

Organizer Formation and DV Patterning

The process of mesoderm induction is followed by dorsoventral patterning of this population of cells. The mechanism for the establishment of the dorsoventral (D/V) axis is conserved between invertebrates and vertebrates. In both cases,

the dorsal side of the embryo is established by maternally contributed factors which induce the production of genes that promote dorsal fates and repress ventral genes. The ventral genes in turn promote ventral fates and repress dorsal factors. This mutual antagonism is the basis for the establishment of a gradient of signaling by genes of the Bone Morphogenetic Protein (BMP) family, a subgroup of the TGF-β superfamily.

The process of an initial establishment of the dorsal side of the embryo followed by the creation of a dose dependent BMP gradient on the dorsal side has been well studied in fruit flies. In *Drosophila,* the D/V axis is established in the earliest stages of development by *dorsal,* a maternally deposited factor (Gilbert, 2010). *gurken* RNA is localized to a position near the presumptive dorsal region by the translocation of the oocyte nucleus. This message diffuses locally and inhibits the ubiquitous Pipe in neighboring cells. The default fate of the follicle cells is ventral and is induced by Dorsal which is localized to the nucleus by the Pipe/Cactus cascade. Dorsal promotes different ventral fates in a dose dependent fashion, *gurken* dosage decreases further from the oocyte nucleus and in turn nuclear Dorsal increases (Gilbert, 2010).

In vertebrates, some of the earliest investigations into D/V axis establishment were done by Hans Spemann using *Xenopus* embryos. He found that embryos sectioned along different planes gave rise to different tissue types. Of particular

importance, he found that one such division gave rise to both a normal embryo and an embryo that lacked dorsal tissues. This was the first evidence of the molecular asymmetry of the early embryo. This evidence led Spemann to perform a series of transplantation experiments wherein he found that the dorsal-most part of the embryo, the blastopore lip, was able to induce dorsal fates (reviewed in Fassler et al., 1996). Later, his graduate student Hilde Mangold found that transplantation of the blastopore lip was sufficient to induce a secondary axis. Because of this unique ability to induce dorsal fates and organize the embryo along a D/V axis, the blastopore lip is now referred to as Spemann's organizer.

The mechanism for formation of the blastopore lip was revealed in a series of classic embryology experiments also involving *Xenopus* embryos. This research found that rotation of the egg cortex, driven along a scaffold of microtubules, uncovers a crescent of tissue and moves a dorsal determinant to the side of the embryo opposite the sperm entry point. This cortical rotation is required for the formation of the Nieuwkoop Center which is in turn required for the induction of the Spemann Organizer. Later, it was found that induction of the Spemann Organizer required the accumulation of β-catenin (Schneider et al., 1996).
In zebrafish the accumulation of β-catenin is necessary for the establishment of the dorsal side of the embryo. However, the nature of the "dorsal determinants" that induce the accumulation of β-catenin in zebrafish is not clear. Evidence

suggests they originate in the vegetal pole and are transported by microtubules to a single point along the embryonic margin (reviewed in Hibi et al., 2002).

β-catenin is an effector of the Wnt Signal transduction pathway. *wnt8* is a secreted glycoprotein that binds to the co-receptors Frizzled and LRP5/6. Binding of the ligand inhibits the formation of a complex of proteins including Axin, GSK3β, APC, Ck1α, and β-catenin (Tamai et al., 2000). This complex phosphorylates and targets β-catenin for destruction. Binding of the Wnt ligand prevents formation of this complex, allowing β-catenin to enter the nucleus and along with the co-activators Lef/Tcf, activate transcription of downstream genes (Figure 1). In zebrafish, the *wnt8* locus exists as a bicistronic gene comprised of two open reading frames (Lekven et al., 2001). These open reading frames give rise to two functionally redundant proteins; knockdown of one or the other alone yields embryos indistinguishable from wild type.

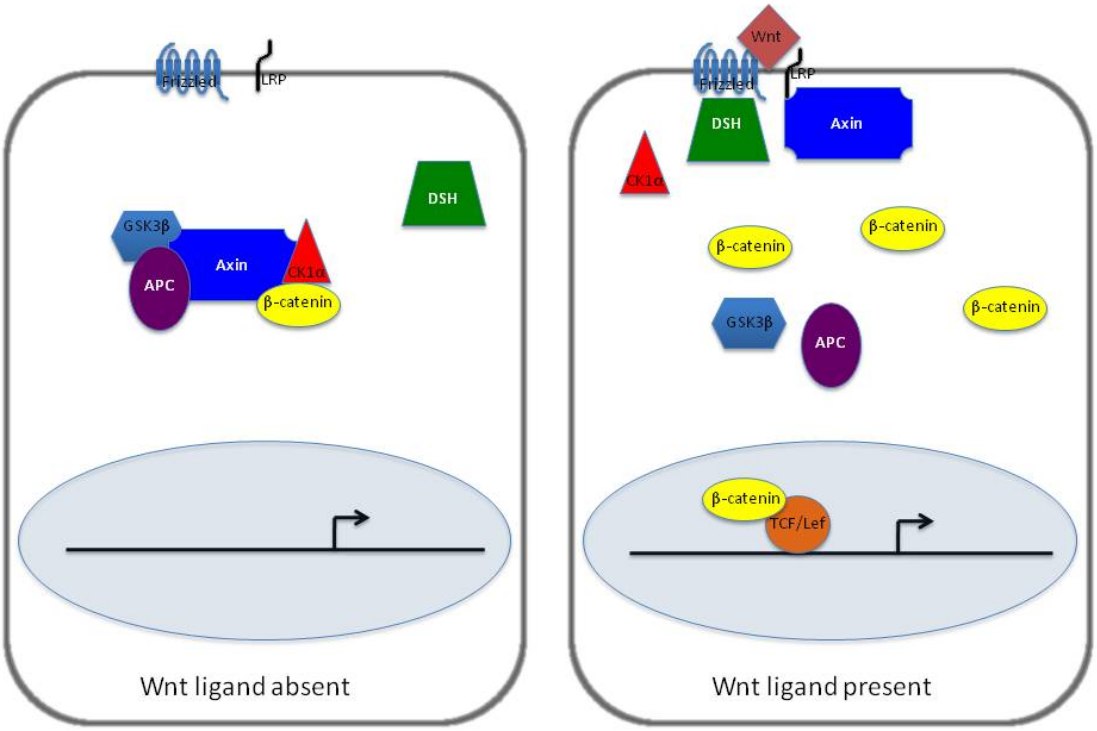

Figure 1. The Wnt/β-catenin signaling pathway. In the absence of the Wnt ligand, β-catenin is phosphorylated by GSK3β in the Axin scaffold complex. β-catenin is then ubiquinated and degraded. When Wnt ligand binds to the Frizzled receptor in the presence of LRP, the Axin complex is inhibited allowing β-catenin to translocate to the nucleus where it binds with Lef/Tcf transcription factors to turn on transcription of downstream genes.

β-catenin acts as a transcriptional activator to turn on the expression of the organizer genes *bozozok* (*boz*) and *squint* (*sqt*). *boz* has been shown to be necessary for the expression of dorsal genes, and *boz* mutants have reduced head and other dorsal tissues (Solnica-Krezel and Driever, 2001). *sqt* mutants

exhibit severely reduced dorsal gene expression in the mesendoderm before gastrulation (Dougan et al., 2003).

Critical to the formation and maintenance of the D/V axis are the organizer genes *chordin* (*chd*) and *noggin* (*nog*). Zebrafish *chd* was found to be responsible for the ventralized mutant *dino,* and this secreted molecule binds and inhibits the activity of extracellular ligands in the BMP pathway (Schulte-Merker et al., 1992). This antagonism is highly conserved and has been found between *Drosophila* homologues *short gastrulation* (*sog,* homologue of zebrafish *chd*) and *decapentapalegic* (*dpp*, homologue of zebrafish *bmp4*) (Biehs et al., 1996). Because they are expressed in the organizer, their ability to inhibit BMP's diminishes as the distance from the organizer increases. Thus, a gradient of BMP activity is produced. This gradient is made more complex and robust by the addition of the ventrally expressed metalloprotease *tolloid* (*tld*) which binds and cleaves *chd* in the extracellular space (Blader et al., 1997).

Like Nodals and Activin, BMPs are members of the TGFβ superfamily of secreted molecules. They bind to a complex formed by dimers of type I and II serine/threonine kinase receptors. Binding is mediated by the presence of the type I receptors Alk3, 6, and 8. Upon activation, the kinase domain of the type II receptor phosphorylates the type I, which in turn phosphorylates cytoplasmic transduction proteins known as Smads. In zebrafish, BMP signal transduction is

mediated by *smads1, 5,* and *8*, along with the co-factor *smad4.* The activated Smad complex is then transported to the nucleus where, depending on the context, it activates or represses transcription (reviewed in Hoffmann et al., 2001).

The gradient of BMP signaling molecules is essential for specification of ventral fates (Hammerschmidt and Mullins, 2002). Three BMP ligands are expressed in zebrafish ventral mesoderm, *bmp2b, bmp4,* and *bmp7.* Of these, dorsalized mutants have been identified in *swirl* (*swr; bmp2b*), *snailhouse* (*bmp7*), the BMP receptor *lost-a-fin* (*alk8),* and a component of the BMP transduction cascade, *somitabun* (*smad5*) (Mullins et al., 1996; Solnica-Krezel et al., 1996). The three ligands are expressed in the ectoderm and ventral mesoderm, and *swr* and *snh* mutants show a progressive loss of transcript indicating that they are auto-regulatory (Kishimoto et al., 1997; Dick et al., 1999). The most severe phenotype is produced by the *swr* mutant, and *swr/snh* double mutants do not appear more acute than *swr* alone, indicating that *bmp2b* and *bmp7* may function as heterodimers (Dick et al., 2000; Schmid et al., 2000). Overexpression of all three BMP ligands produce strongly ventralized embryos at 24 hpf and are able to induce ventral markers at earlier stages (Nikaido et al., 1999). Radar, a maternally contributed protein is also part of the BMP family and evidence suggests that it along with the maternal protein Smad5 are necessary for the establishment of the ventral side of the embryo (Wilm and Solnica-Krezel, 2003).

Also necessary for the proper specification of ventral fates and the maintenance of the embryonic axis is the ventral, mesodermally expressed gene *wnt8.* In *Xenopus,* ectopic expression of *Xwnt8* is capable of ventralizing organizer cells and over expression produces a ventralized embryo lacking eyes and cement gland (Christian and Moon, 1993). In zebrafish, our current understanding is that *wnt8* acts in two phases. The first phase, from early to mid-gastrulation it is essential for limiting the size of the organizer and thus, dorsal fates (1). During late gastrulation, *wnt8* is necessary for posterior growth (2). Loss of *wnt8* signaling results in expanded forebrain and dorsal fates at the expense of ventral, as well as a severe loss in posterior tissue.

wnt8 limits the size of the organizer by activating the transcriptional repressors *vent* and *vox* (Ramel and Lekven, 2004). These two genes act by repressing the transcription of *chd* and *gsc*. Importantly, BMP's have also been shown to activate these repressors as well, providing an additional layer of organizer inhibition (Ramel and Lekven, 2004).

Proper patterning and maintenance of ventrolateral mesoderm at shield stage is critical for formation of the tailbud and posterior growth. Formation of the tail and other posterior structures depends on these crucial events that take place during gastrulation.

Tailbud Formation and Activity

Posterior growth originates in the tailbud, which forms from a population of mesodermal cells on the ventral-most margin. This group of cells requires high levels of BMP activity throughout gastrulation (Tucker et al., 2008). In vertebrates, the tailbud forms at the end of gastrulation when these cells converge at the apex of the vegetal pole.

Discerning the nature and makeup of the tailbud, and the posterior growth that arises from it, has been a subject of scrutiny for over one hundred years. Researchers in the late nineteenth century were already aware of the unique character of this population of cells, but technological limits confined early studies to histology, transplantations and extirpations. The earliest attempts to understand the nature of the tailbud were undertaken by Holmdahl in 1925 (Holmdahl, 1925). He proposed that the embryo had two separate programs for development. The first was described as being “indirect” because the organs and other tissue types were derived from germ layers which were formed as a consequence of gastrulation. The second was described as being direct because tissues such as the posterior neural tube and posterior somites derive from the homogenous population of mesenchymal cells that make up the tailbud (Holmdahl, 1925).

As research progressed, the hypothesis that the tailbud was a homogenous population began to come into criticism. Fate mapping in *Xenopus laevis* indicated that, much as the germ ring before it, the cells within the tailbud differentiate according to their position when the tailbud forms (Tucker and Slack, 1995).

In addition to being a heterogeneous population of cells with a predictable fate map, it became clear that the tailbud mirrored the dorsal organizer in its inductive ability as well. In 1945 Spofford showed that grafts taken from the posterior chordo-mesoderm region of an *Amblystoma punctatum* (salamander) embryo were able to induce different fates in recipient embryos depending on where on the anterior-posterior axis the grafts were removed from the donor. The more posterior regions were found to induce mesodermally derived tissues such as somites while anterior cells gave rise to spinal cord and other tissues of the neural tube (Spofford, 1945). A similar set of experiments performed in chick revealed that tailbud cells induce and contribute to the somites, mesenchyme, caudal arteries, and neural tube but not the notochord (Schoenwolf, 1977). Schoenwolf followed his transplantations in chick with extirpations of the tailbud and found that in addition to having a strong ability to regenerate after excision (including limb buds) the notochord does not derive from the tailbud at all and can extend through a partially regenerated tail without showing any signs of discontinuity (Schoenwolf, 1978).

More recently, several lines of investigation have produced a clearer picture of the origin of the tailbud and movement and differentiation of the cells therein. Catala et. al. used the quail-chick chimaera system to reinforce the idea that the tailbud is "pre-organized", and cell movements such as invagination and divergence* characteristic of germ ring gastrulation continue beyond the 25 somite stage (Catala et al., 1995). In zebrafish, Kanki and Ho used fluorescent lineage tracing to establish a detailed fate map showing significant mixing of cells fated to become spinal cord, notochord, and muscle. Further, they discovered a cell movement unique to the tailbud wherein superficial posterior cells move anteriorly and deeper into the hypoblast in a process they term "subduction" (Kanki and Ho, 1997). In *Xenopus*, the apparent similarities between gastrulation and tailbud formation were reinforced using molecular techniques to show that the tailbud forms from the dorsal lip of the gastrula. This tissue retains some of its organizer properties as evidenced by its ability to induce notochord formation, providing a molecular basis for previous histological observations (Gont et al., 1996).

The Molecular Basis for Posterior Growth

Posterior growth involves a complex array of interacting signaling pathways. Wnts, FGF's, the transcription factors Cdx, Tbx, Hox, and retinoic acid have all

* The authors use "divergence" to describe the same movement as convergence during gastrulation, from the opposite point of reference. Viewed this way, cells in the ventral margin would be said to diverge towards the dorsal pole.

been shown to play a role in both zebrafish and mice. In mice, *wnt3a* is indispensable for trunk and tail development. Mice deficient for this gene are severely truncated, lacking somites and notochord caudal to the forelimbs (Takada, et. al. 1994). Additionally, *wnt3a* has been shown to be necessary for both somite formation and marking the boundary of presomitic mesoderm (Dunty et al., 2008).

Both *wnt8* and *wnt3a* are required for posterior growth in zebrafish. *wnt3a* expression starts during mid to late gastrulation, and from that point these two share partially redundant function (Shimizu et al., 2005). These genes are also share a partially redundant function in maintaining presomitic mesoderm and tailbud markers (Thorpe et al., 2005).

Cdx1 and *4* are functionally redundant but required for posterior development. In mice, *cdx2* has been shown to be required for normal posterior elongation, and *cdx2* has been shown to directly regulate *wnt3a* (Savory et al., 2009). *cdx1,* expressed in the posterior of elongating zebrafish embryos, is lost in *wnt3a/wnt8* partial loss of function, and *cdx4* is partially lost in this context as well (Shimizu et al., 2005).

Fibroblast growth factors (Fgf) are also required for posterior development. Mice lacking a functional *Fgfr1* gene, which produces an Fgf receptor, have reduced

posterior mesoderm (Yamaguchi et al., 1994), and expression of a dominant negative Fgf receptor blocks the formation of posterior tissues (Amaya et al., 1991) In zebrafish, injection of *fgf24* morpholino into the *fgf8* mutant *acerebellar* (*ace*) produces a severely truncated embryo, lacking tail somites (Fischer et al., 2003).

The activity of retinoic acid (RA) is critical for elongation and somitogenesis. It has been shown that soaking embryos in RA induces a truncated embryo. RA promotes differentiation and premature exposure to this chemical may deplete the number of undifferentiated tailbud cells. Antagonizing the activity of RA is the enzyme Cyp26. In zebrafish the domains of *cyp26* and the RA synthesizing *aldh1a2* abut one another in the posterior embryo, where *cyp26* is in the tailbud and *aldh1a2* is immediately posterior (Skromne et al., 2007).

Wnts and Cell Proliferation

Wnt/β-catenin signaling is implicated in cell cycle regulation in several different ways. One of the most direct effects has been observed in zebrafish, where Wnts have been shown to regulate cell proliferation in regenerating the tail fin. Overexpression of *wnt8* is sufficient to increase the rate at which cell proliferation occurs in ablated tissue, and fish heterozygous for a mutation in *axin-1*, a negative regulator of Wnt signaling, undergo fin regeneration faster than wildtype controls (Stoick-Cooper et al., 2007). In SW480, 293T, Neuro 2A,

and HeLa tissue culture cells, cyclin D1 has been shown to be directly regulated by the transcription factors β-catenin and Lef-1, members of the Wnt transduction pathway. The cyclin D1 promoter contains a consensus Lef binding site and activation of the Wnt pathway produce strong induction of a luciferase reporter (Shtutman et al., 1999; Tetsu and McCormick, 1999). Further, one of the key facets of cyclin D1 activity is sub-cellular localization. GSK-3β, another member of the Wnt transduction pathway has been shown when overexpressed to cause cyclin D1 to translocate from the nucleus to the cytoplasm (Diehl et al., 1998). Finally, Wnt signaling has been implicated in the regulation of the tumor suppressor p53. Homeodomain-interacting protein kinase-2 (HIPK2) is an important regulator of p53 and it has recently been shown in *Xenopus* that HIPK2 is capable of activating, in a context dependent fashion, the transcriptional repressor TCF3, as well as the transcriptional activator LEF1 (Hikasa and Sokol, 2011).

Zebrafish As Model System For This Study

The model system used in this study, *Danio rerio*, has proven ideal. Indeed, zebrafish may be the only vertebrate system with the flexibility to perform this entire study at reasonable cost and in relatively short time.

In general, *D. rerio* makes for an excellent model system due to the ease that one can maintain large stocks of fish and the high fecundity seen in this species.

They require a clean aquarium system and daily feedings, but they do well in high densities allowing for significant economy of scale. Additionally, a single zebrafish female can spawn over a hundred embryos. This allows the researcher to obtain large numbers of replicates in a single experiment and lessens the need for multiple trials and intensive statistical analysis. Zebrafish embryos develop outside the mother, and development is rapid, taking less than three days to go from a single cell to free swimming larva. The embryos are transparent allowing internal structures to be easily studied, and they maintain a relatively constant size up to 14 hpf which simplifies staining. Lastly, the genome of zebrafish is fully sequenced, allowing for rapid cloning and promoter analysis among other benefits.

An additional advantage is that the ability to use morpholino injections for gene knockdown makes the analysis of double loss-of-function practical. Morpholinos are a gene specific synthetic oligonucleotide that allows for the rapid and simple reduction gene product (Summerton 1998). The conclusions of this study are based on the knockdown of two recessive genes that are embryonic lethal. Ordinarily, obtaining vertebrate embryos that have loss-of-function for two critical genes requires the maintenance of stocks of adults with each mutation or the creation of a line that has both. Morpholino injection allows these embryos to be created from plentiful wild type lines and importantly, without any loss of replicates due to mendellian segregation that would be encountered using

mutant lines. Further, the ease of morpholino knockdown allows for practical and rapid proof-of-principle experiments.

The transparency of zebrafish embryos has made fate mapping a relatively simple process. Any cell can be labeled initially, and labeled cells are easily visualized later even when the cell is deep within a tissue. The ability to label cells in many embryos simultaneously allows fate mapping data to be collected rapidly across multiple genetic backgrounds.

The ability to easily create and maintain transgenics has been beneficial to this study. The existence of the Fucci line, which enables the researcher to distinguish between mitotic and non-mitoic cells in real time (Sugiyama et al., 2009) has been critical. This transgenic line, as well as the transparent nature of zebrafish has greatly simplified our ability to test for cell proliferation. The potential to produce many embryos simultaneously has enabled the statistically significant calculation of this index in multiple genetic backgrounds and in a time frame, just a few weeks, that would be impossible in any other vertebrate model.

It was possible to recapitulate the data obtained from the Fucci line by the use of flow cytometry. Obtaining the large number of cells required from embryos of different genetic backgrounds without the use of cell cultures would have been a significantly larger undertaking in a mammalian model. The fecundity of

zebrafish will allow this experiment to continue in real time at different developmental time points and genetic backgrounds.

CHAPTER II

A DIRECT ROLE FOR WNT8 IN VENTROLATERAL MESODERM PATTERNING*

Introduction

Dorsoventral (D/V) patterning in vertebrates requires the input of both the BMP (Bone Morphogenetic Protein) and Wnt signaling pathways to specify ventral and posterior fates (reviewed in De Robertis et al., 2001; Schier and Talbot, 2001; De Robertis and Kuroda, 2004; Kimelman and Szeto, 2006; Itasaki and Hoppler, 2010). The events downstream of Wnt and BMP signaling that lead to ventral specification are not fully understood, but transcriptional regulation is a major output for both pathways. Wnt and BMP activities can activate ventral genes independently of each other, but they also share some common targets (Hoppler and Moon, 1998; Marom et al., 1999; Ramel et al., 2004; Szeto and Kimelman, 2004; Fuentealba et al., 2007). While studies in zebrafish have highlighted a dynamic regulation of target genes by both pathways (Ramel et al., 2004), it is still unclear what their relationship is: work in several model systems has shown that their relationship is complex and may involve cross-regulatory interactions at multiple levels (Hoppler and Moon, 1998; Marom et al., 1999;

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Fuentealba et al., 2007). Understanding the individual roles of Wnt and BMP signaling is critical for deciphering the regulation of vertebrate D/V patterning.

In anamniotes, Wnt8 is essential for D/V patterning, but its role is not yet fully understood. Studies in frogs and fish have shown that Wnt8 signaling is responsible for repressing the organizer at the onset of gastrulation (Hoppler et al., 1996; Lekven et al., 2001). In zebrafish, Wnt8 signaling limits the size of the organizer through transcriptional regulation of *vent, vox* and *ved* (Ramel and Lekven, 2004; Ramel et al., 2005), which encode transcriptional repressors of dorsal genes (Imai et al., 2001). In the absence of Wnt8, zebrafish embryos display greatly expanded dorsal fates and the concomitant loss of ventrolaterally derived cell fates (Ramel and Lekven, 2004). In the ectoderm, this is manifested in the expansion of neurectoderm at the expense of non-neural ectoderm. In the mesoderm, this is reflected in the expansion of axial mesoderm (i.e. prechordal plate and notochord domains) at the expense of ventrolateral mesoderm fates (e.g. posterior somites, blood). Consequently, embryos lacking Wnt8 display a severely dorsalized phenotype at 24 hours post fertilization characterized by the absence of trunk and tail (Lekven et al., 2001). Additionally, embryonic dorsalization is also accompanied by expansion of anterior tissues at the expense of posterior tissues in *wnt8* mutants (Lekven et al., 2001). The mesodermal phenotype of *wnt8* mutants could be explained by two alternative hypotheses: on the one hand, mesoderm dorsalization may result in

specification of fewer ventrolateral/posterior mesoderm progenitors thereby restricting the progenitor pool that contributes to ventral and posterior fates. On the other hand, Wnt signaling may have a role in promoting the maintenance or expansion of posterior mesoderm progenitors, for example through *cdx* or *no tail/brachyury* gene regulation (Shimizu et al., 2005; Thorpe et al., 2005; Martin and Kimelman, 2009).

BMP signaling plays a critical role in D/V patterning and the formation of posterior and ventral tissues. D/V axis patterning is modulated by a gradient of BMP activity with the highest BMP signaling levels specifying the most ventral and posterior fates (De Robertis and Kuroda, 2004; Kondo, 2007). BMP mutants define the classic dorsalized phenotype characterized by expanded neurectoderm at the expense of epidermis as well as loss of posterior and ventral mesoderm cell types (Little and Mullins, 2006). In the mesoderm, high BMP levels during gastrulation direct progenitor cells to contribute to the tail (Agathon et al., 2003; Szeto and Kimelman, 2006; Tucker et al., 2008; Harvey et al., 2010), possibly by preventing their premature differentiation, and also influence cell convergence (Myers et al., 2002; von der Hardt et al., 2007). In zebrafish, Bmp2b co-regulates the Wnt8 target genes *vent, vox,* and *ved* during early gastrulation (Melby et al., 2000; Imai et al., 2001; Ramel et al., 2005), although the fact that BMP loss-of-function does not result in an expanded organizer (Mullins et al., 1996; Ramel and Lekven, 2004) or defective

anterioposterior patterning in the gastrula (Barth et al., 1999) illustrates differences between their regulatory roles. Nonetheless, mutations in *bmp2b* (*swr*) and *bmp7* (*snailhouse*) exhibit dramatic reductions in ventral and posterior tissues, producing a dorsalized phenotype that appears at least morphologically similar to that seen in *wnt8* mutants (Mullins et al., 1996; Kishimoto et al., 1997; Schmid et al., 2000; Ramel et al., 2005).

The apparent similarity of the Wnt8 and BMP mutant phenotypes, as well as the identification of common target genes, supports the hypothesis that Wnt and BMP signaling act cooperatively to regulate D/V patterning. However, the relationship between Wnt8 and BMP remains poorly characterized, despite its importance to the mechanism of D/V patterning. For instance, Wnt8 and BMP may have direct interactions in addition to common downstream transcriptional targets. In *Xenopus*, Wnt8 was shown to be downstream of BMP signaling, but it is unclear if this reflects a regulation by maternal or zygotic BMP (Hoppler and Moon, 1998). Additionally, while over-expression of BMP at moderate levels was found to induce *wnt8* expression, high levels of BMP repress *wnt8,* suggesting the induction of *wnt8* in response to BMP is under complex regulation (Marom et al., 1999). In zebrafish, it has been reported that Wnt8 over-expression can induce ectopic *bmp2b*, but BMP is not necessary for normal *wnt8* expression (Mullins, 1999; Ramel and Lekven, 2004; Szeto and Kimelman, 2004), suggesting an alternate transcriptional relationship in zebrafish. However,

analysis of *swr* mutants suggests that high BMP activity can negatively regulate *wnt8* at the end of gastrulation (Hammerschmidt and Mullins, 2002), similar to the relationship observed in amphibians. Taken together, data suggest interdependent and potential cross-regulatory interactions between Wnt8 and BMP signaling that may obscure the analysis of potential unique roles in axis patterning for each.

One important interaction between Wnt8 and BMP activities is known to be indirect. The vertebrate organizer is the source of several secreted antagonists of BMP ligands, including Chordin and Noggin (De Robertis and Kuroda, 2004); therefore, any increase in the number of these axial cells will result in a simultaneous increase in the levels of BMP antagonists produced by the organizer and a corresponding decrease in BMP signaling activity. As a consequence of this relationship, expanded organizers will produce a greater level of BMP antagonism in *wnt8* morphant embryos. The outcome is that the dorsalized *wnt8* mutant phenotype may reflect the combined effects of reduced Wnt and BMP signaling, thus obscuring the phenotypic effects that can be attributed directly to Wnt8.

To disentangle the role for Wnt signaling from that of BMP signaling in patterning the vertebrate axes, we have undertaken a gene knockdown approach in the zebrafish. We generated embryos lacking the activity of both Wnt8 and the BMP

antagonist Chordin, and show that this results in the restoration of BMP activity in a Wnt8 morphant background. Our analysis shows that Wnt8 signaling has different BMP-independent functions within different mesodermal domains: Wnt8 is uniquely needed to prevent the expansion of the prechordal mesoderm domain but not the prospective notochord domain, and Wnt8 is essential for the expansion or proliferation of ventrolateral mesoderm derived tissues. Thus, Wnt8 has several roles in vertebrate axis patterning that can be experimentally separated from those of BMP.

Results

Increased BMP antagonism leads to reduced BMP signaling in *wnt8* mutants. To assess the possibility that the *wnt8* knockdown phenotype is due partially to the loss of BMP signaling, we looked at the expression pattern of the BMP antagonists *chordin (chd)* and *sizzled* (*szl)*. *chd* expression is limited to the organizer in early gastrula wild type embryos, but expression is expanded in broad arcs along the ventrolateral mesoderm in *wnt8* morphants (Ramel and Lekven, 2004). At bud stage, *chd* is expressed in the forebrain, midbrain, hindbrain, the tailbud and surrounding cells (Figure 2A; (Miller-Bertoglio et al., 1997). In *wnt8* morphants, the anterior neural expression domains expand laterally and posteriorly in a band that completely encircles the embryo (Figure 2B), consistent with the suggestion that Wnt8 loss-of-function embryos do not provide a BMP-permissive environment. *szl* encodes a feedback inhibitor of

BMP, thus its expression is a readout of BMP signaling activity (Martyn and Schulte-Merker, 2003; Yabe et al., 2003). *szl* is expressed in the ventral embryo before and during gastrulation and in the tailbud and ventral posterior epidermis at bud stage (Yabe et al., 2003). In *wnt8* morphants, *szl* expression is completely lost by bud stage (Figure 2D, 92% of embryos, n=49). Thus, the expansion of Chordin-dependent BMP antagonism in *wnt8* morphants is associated with an embryonic environment that does not permit active BMP signaling and also suggests that reducing Chordin expression may allow BMP signaling to be restored in *wnt8* loss-of-function embryos.

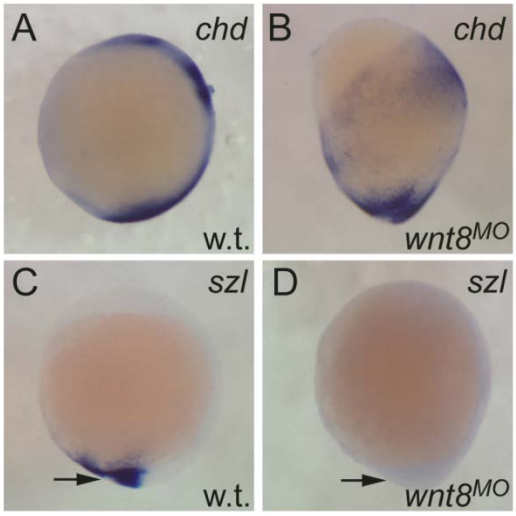


Fig. 2. *wnt8* morphants have reduced BMP signaling. A–D: In situ hybridizations on bud stage embryos, lateral views, anterior up. A: chordin expression marks several domains in the wild-type that are expanded in wnt8 loss of function embryos (B). C: sizzled expression is a readout for BMP signaling in the tail at bud stage (arrow). D: This expression is absent in wnt8 morphants.

BMP Signaling is Rescued in wnt8;chordin *Double Loss-of-Function Embryos*

As an initial step in determining whether BMP signaling can be restored in *wnt8* loss-of-function embryos, we examined the expression of BMP ligands. BMP signaling in gastrulating zebrafish is provided by three ligands, Bmp2b, Bmp4 and Bmp7, that are under positive autoregulation (Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Stickney et al., 2007); thus, BMP ligand expression is also an indicator for BMP signaling activity. *bmp2b, bmp4* and *bmp7* have roughly similar expression domains in the ventral embryo during gastrulation (Fig. 3A, E, I; also see (Schmid et al., 2000; Kondo, 2007). One notable difference between them is that *bmp2b* has a discrete expression domain in the ventrolateral embryonic margin that, in contrast to its ectodermal expression domain, is not regulated by BMP signaling (see (Kishimoto et al., 1997), and unpublished results). In *wnt8* loss-of-function embryos, ventral expression of all three BMP genes is significantly reduced, including the ventrolateral margin expression of *bmp2b* (Fig. 3B,F,J). In contrast, BMP ligand expression expands dorsally in *chordin* morphants (Fig. 3C,G,K), consistent with BMP transcriptional autoregulation. In *wnt8;chordin* double loss-of-function embryos, BMP ligand expression expands dorsally, suggesting BMP signaling activity recovers in *wnt8* mutants when Chordin levels are reduced (Fig. 3D,H,L). We did observe one difference in BMP ligand response in the double loss-of-function embryos, which is that *bmp2b* does not appear to recover in the ventrolateral margin (Fig. 3D) suggesting that this expression domain of *bmp2b*

may be Wnt signaling-dependent. Nonetheless, the dorsal expansion of BMP ligand expression is consistent with elevated BMP signaling in *wnt8;chordin* double mutant embryos.

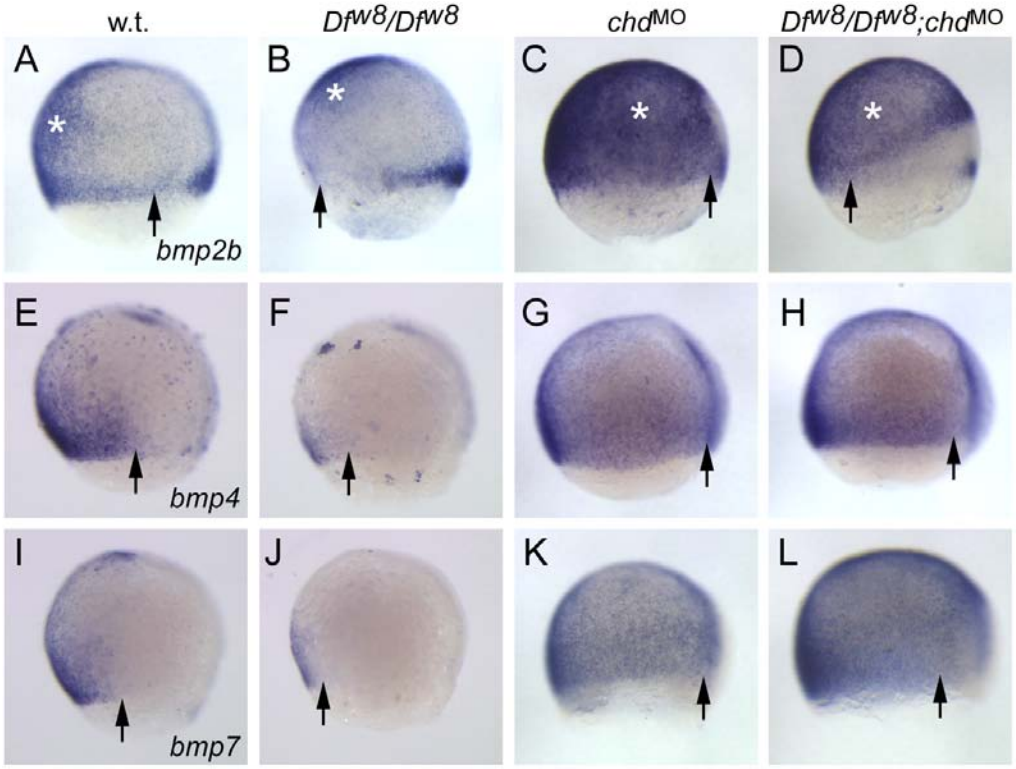


Fig. 3. Wnt8 is not required for BMP expression. In situ hybridizations at $70% epiboly to detect expression of bmp2b (A–D), bmp4 (E–H), and bmp7 (I–L). Shown are lateral views, anterior up, dorsal right. Genotypes are indicated above each row. A,E,I: bmp2b, bmp4, and bmp7 are expressed in the ventral embryo. Arrows indicate the dorsal limit of the observable signal for each ligand. B,F,J: Ventral BMP expression is significantly reduced in wnt8 mutant embryos, indicated by ventral shift in position of arrow. Ventral ectoderm bmp2b expression is not as significantly affected (white asterisk). C,G,K: Chordin knockdown leads to dorsal expansion of BMP expres- sion. Note dorsal shift of arrow positions. D,H,L: BMP ligand expression is dorsally expanded in wnt8 mutants that have Chordin knocked down. Note the expanded ventral ectoderm domain of bmp2b in wnt8;chordin double loss-of-function (asterisk in D), compared to wnt8 mutant (asterisk in B). While bmp2b in the margin does not appear to recover in the double loss-of-function (arrow in D), both bmp4 and bmp7 show dorsally expanded expression in the double loss-of-function.

In order to directly determine whether the changes to BMP ligand expression reflect similar changes in BMP signaling, we assayed the expression of phospho-Smad1/5 (p-Smad1/5). In the wild-type early gastrula, p-Smad1/5 is localized to nuclei distributed around the ventrolateral margin and the ventral ectoderm, with the greatest level of staining most ventrally, consistent with the generation of a ventral to dorsal BMP signaling gradient (Fig. 4A,E; (Tucker et al., 2008). In the late gastrula, p-Smad1/5 nuclear localization is found mainly adjacent to the ventral embryonic margin (Fig. 4I; see also (Tucker et al., 2008). In *wnt8* morphants, p-Smad1/5 staining is significantly reduced both in level and spatial distribution at shield stage (Fig. 4B,F), and is not detected in the late gastrula (Fig. 4J) or 5-6 somite stage embryo (Supplemental Fig. 1). In *chordin* morphants, p-Smad1/5 staining is significantly expanded toward the dorsal midline (Fig. 4C,G,K; Supplemental Fig. 1). Consistent with our observations of BMP ligand expression, *wnt8;chordin* double morphants show an expanded distribution of p-Smad1/5 similar to that observed in *chordin* morphants (Fig. 4D,H,L; Supplemental Fig. 1). However, one difference observed between *chordin* and *wnt8;chordin* morphants is that the staining intensity is slightly lower in the double knockdown (compare panels D and C, L and K), perhaps reflecting a role for Wnt signaling in stabilizing phosphorylated Smad (Fuentealba et al., 2007)(10). From these results, we deduce that reducing Chordin expression results in elevated BMP signaling in a *wnt8* loss-of-function background. We

next used *wnt8;chordin* double loss of function to determine the relative inputs of Wnt8 and BMP signaling into mesodermal patterning.

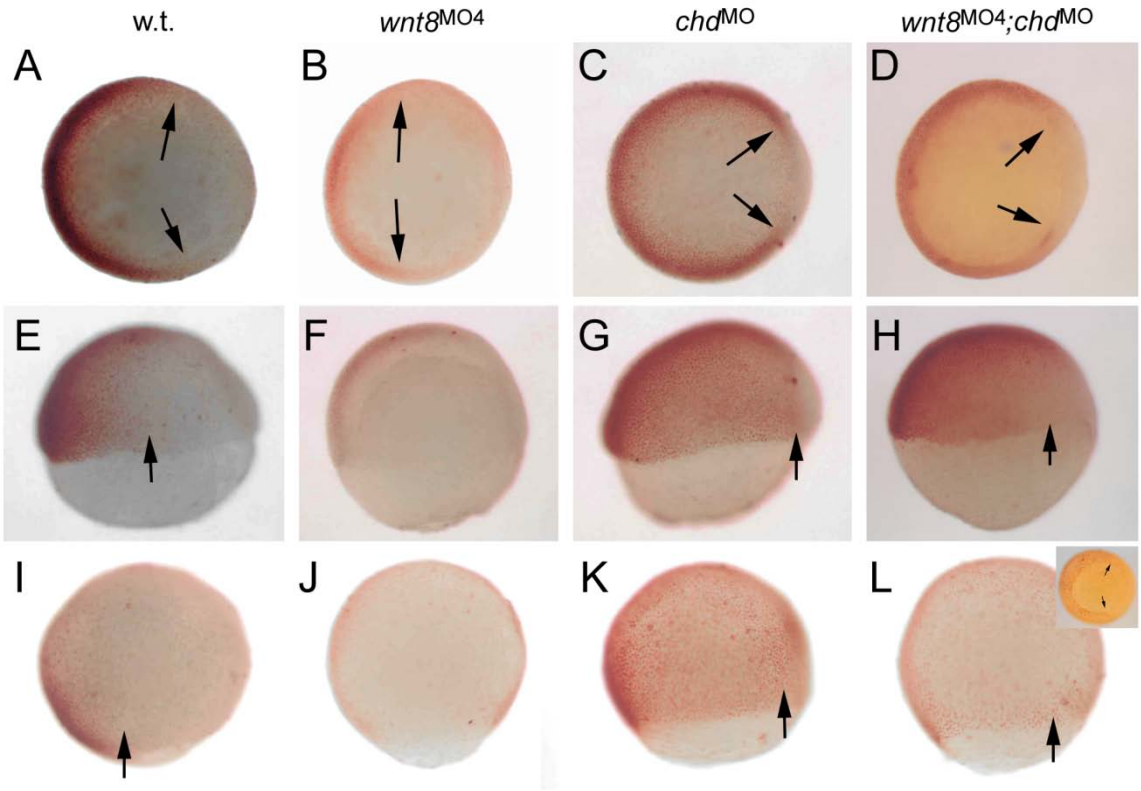


Fig. 4. Wnt8 is not required for BMP signaling activity. Images of anti-phosphorylated Smad1/5 staining. Genotypes are indicated above each row. Arrows indicate dorsal extent of visible labeling. A–D: Animal pole views of shield stage embryos, dorsal right. E–H: Lateral views, shield stage, dor- sal right. I–L: Lateral views, $80% epiboly, anterior up, dorsal right. P-Smad1/5 is confined to the ventral embryo at shield stage (A,E), and expression is significantly reduced in wnt8 morphants (B,F). P-Smad1/5 expands dorsally (arrows) in chordin morphants (C,G) and in wnt8;chordin mor- phants (D,H). P-Smad1/5 can still be detected in a dorsally expanded domain of chordin (K) and wnt8;chordin (L) morphants in late gastrulation. Inset in L: vegetal view, dorsal right; arrows indicate dorsal extent of staining. Note absence of staining in wnt8 morphant at 80% epiboly (J).

The Organizer is Regulated Differentially by Wnt8 and BMP Signaling

Previous studies showed that the organizer is expanded in *wnt8* mutants due to reduced *vent*, *vox* and *ved* expression (Ramel and Lekven, 2004; Ramel et al., 2005). However, *vent*, *vox* and *ved* are also BMP target genes (Kawahara et al., 2000b; Kawahara et al., 2000a; Imai et al., 2001; Shimizu et al., 2002), raising the question of whether the *wnt8* organizer phenotype is due to compound effects of both Wnt and BMP loss of function. To address this question, we examined these genes as well as markers of the organizer in *wnt8;chordin* double loss of function embryos (Fig. 5).

Consistent with previous reports, *vent* and *ved* are reduced in *wnt8* morphants (Fig. 5A,B,E,F) and show elevated expression that expands toward the organizer in *chordin* morphants (Fig. 5C,G). In *wnt8;chordin* double loss of function embryos, both *vent* and *ved* expand toward the dorsal midline, indicating that these genes can be positively regulated by BMP independently of Wnt8 at the onset of gastrulation (Fig. 5D,H).

We next examined the expression of the organizer genes, *goosecoid* (*gsc*) and *floating head* (*flh*), to determine if BMP signaling is sufficient to repress the organizer in the absence of Wnt8 signaling. Consistent with previous findings, we observed a significant expansion of the expression domains of *gsc* and *flh* in *wnt8* morphants (Fig. 5J,N), but nearly wild-type expression patterns in *chordin*

morphants (Fig. 5K,O). Surprisingly, *gsc* expression remains expanded in *wnt8;chordin* double loss of function embryos (Fig. 5L, 95%, n=40) but *flh* is rescued to the wild-type pattern in double morphants (Fig. 5P; 97%, n=31). These observations are also made on embryos at the end of gastrulation (Supplemental Fig. 2). This indicates that Wnt8 and BMP have different effects on gene expression in different organizer cell populations: *gsc* expression in prechordal plate progenitors (anterior axial mesoderm) is insensitive to BMP signaling, but *flh* expression in prospective notochord progenitors (posterior axial mesoderm) is sensitive to BMP-dependent repression.

Ventral and Posterior Mesoderm Expansion Requires Wnt8

The above results establish that BMP activity can partially compensate for the loss of Wnt8 signaling in organizer regulation, and leads to the question of whether the differentiation of ventrolateral mesoderm progenitors can be

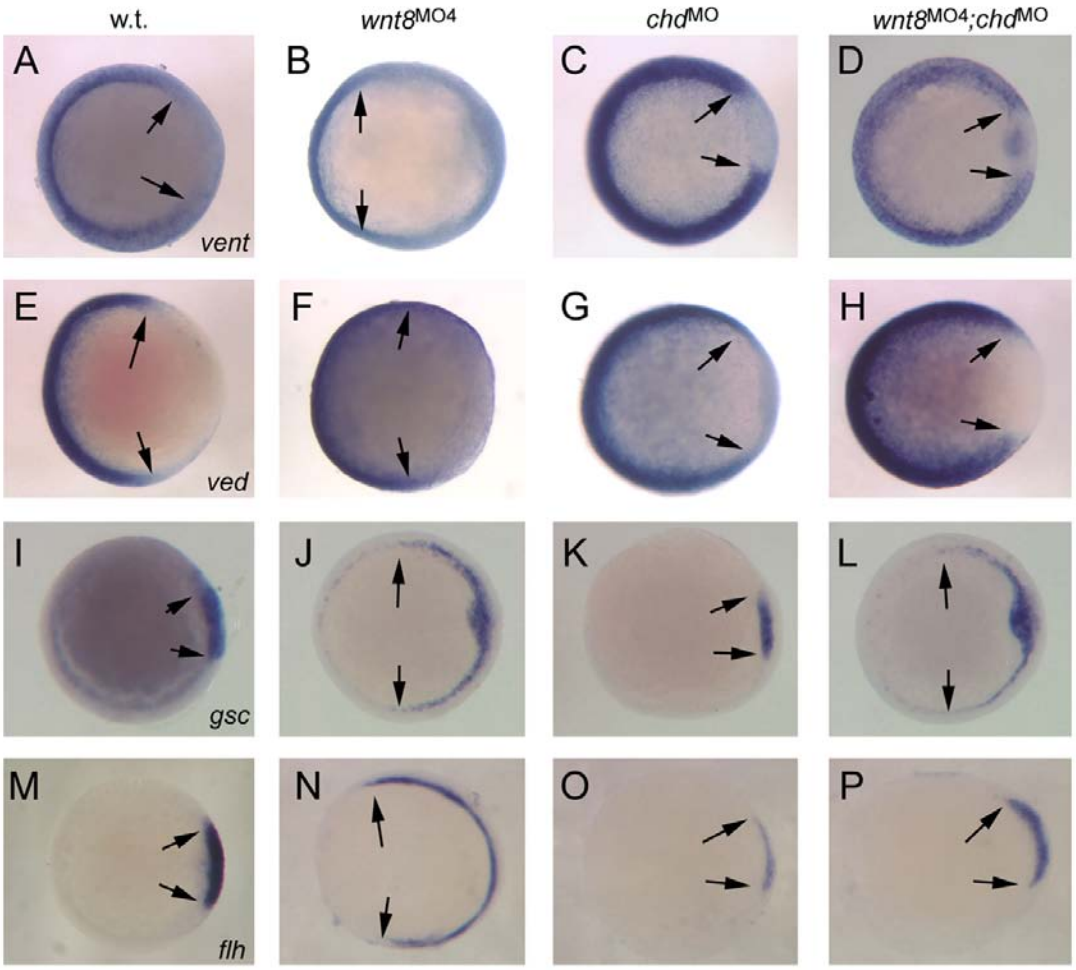


Fig. 5. Differential organizer regulation by Wnt8 and BMP signaling. In situ hybridizations for vent (A–D), ved (E–H), gsc (I–L), and flh (M–P). All images show animal pole views, shield stage, dorsal right. Arrows indicate detectable limits of corresponding expression domain. Knockdown condition is indicated above each column. Note expansion of vent and ved in double knock- down (D,H), which correlates with rescued flh expression (P) but not gsc (L).

similarly rescued. The zebrafish ventrolateral embryonic margin comprises a complex pool of progenitors for all non-axial mesodermal derivatives (Schier and Talbot, 2005). To address the specification and patterning of progenitors from

the ventrolateral margin in our knockdown conditions, we assayed the expression of *follistatin a* (*fsta*) and *eve1. fsta* marks presumptive cephalic mesendoderm, which arises at the embryonic margin adjacent to the organizer (Dal-Pra et al., 2006). In the late gastrula, *fsta* expression marks bilateral fields of cells located midway along the anteroposterior axis (Fig. 6A). In *wnt8* morphants, the *fsta* expression domain is slightly expanded toward the ventral midline but occupies a relatively normal position in the anteroposterior axis (Fig. 6B). In contrast, *fsta* expression is almost absent in both *chordin* morphants and *wnt8;chordin* double morphants (Fig. 6C,D; almost absent in 94% of double morphants, n=52). Thus, BMP signaling is able to suppress cephalic mesoderm fate independently of Wnt8.

Ectopic BMP can cause mesoderm progenitors to delay their differentiation and as a consequence contribute to tail rather than trunk tissues (Szeto and Kimelman, 2006). The suppression of *fsta* expression in *wnt8;chordin* double morphants is therefore consistent with the possibility that BMP signaling in the double morphant redirects cephalic mesoderm progenitors from their normal anterior body fate toward a posterior fate, i.e. they contribute to tail tissues rather than head. If so, we expected to observe an expanded tailbud in *wnt8;chordin* double knockdown embryos. To determine whether tailbud specification is affected as predicted in *wnt8;chordin* double loss of function embryos, we examined *eve1*, a common marker for ventral mesoderm and tailbud progenitors

and a known downstream target of both BMP and Wnt8 signaling (Hammerschmidt et al., 1996; Nikaido et al., 1997; Agathon et al., 2003; Ramel and Lekven, 2004). *eve1* expression marks the ventral tailbud at bud stage and mesoderm progenitors at the tip of the tail through 27 hours post fertilization (Fig. 6E,I, arrow). In *wnt8* morphants, *eve1* expression is significantly reduced at bud stage (Fig. 6F) and is not detectable at 27 hpf (Fig. 6J, asterisk). In *chordin* morphants, *eve1* expression is significantly expanded at bud stage and 27 hpf (Fig. 6G,K). In the *wnt8;chordin* double knockdown, *eve1* expression is rescued at both bud stage and 27 hpf (Fig. 6H,L; 96% with rescue, n=54). This result further confirms that BMP activity is maintained in the double knockdown, and shows that mesoderm progenitors maintain tailbud identity.

Interestingly, *wnt8;chordin* double knockdown embryos show a dramatic loss of trunk and tail structures despite the presence of tailbud progenitors (Fig. 6L). We confirmed the general truncation of posterior mesoderm in *wnt8;chordin* double knockdown embryos by assaying *pax2a* expression (Fig. 6M-P). At the 5 somite stage *pax2a* is expressed in the optic stalk, midbrain-hindbrain boundary (MHB), otic vesicle and prospective pronephros. *wnt8* morphants do not display optic stalk or otic placode *pax2a* expression at this stage, the MHB domain is expanded ventrally, and the prospective pronephros is significantly diminished (Fig. 6N). *chordin* morphants show a slightly narrowed neural plate and significantly increased *pax2a* expression in the prospective pronephros (Fig.

6O). In the *wnt8;chordin* double knockdown, optic stalk expression is expanded posteriorly, the MHB domain is present but slightly widened and otic placode expression is diminished but present. The prospective pronephros is rescued but occupies a significantly shortened length of the axis (Fig. 6P; 94% of embryos rescued, n=34). We find similar effects on heart, blood and body musculature (*cmlc2*, *gata1* and *myoD* expression at 24 hpf, Supplemental Fig. 3). Taken together, these results suggest that BMP signaling is sufficient to increase the population of posterior mesoderm progenitors, but this effect is not sufficient for normal posterior development in the absence of Wnt8 signaling.

To further assess the possibility that BMP and Wnt8 have different roles in the specification of posterior progenitors, we assayed *myf5* and *mesogenin* expression, markers for different populations of presomitic and somitic mesoderm. *myf5* is expressed in the segmental plate, adaxial cells and

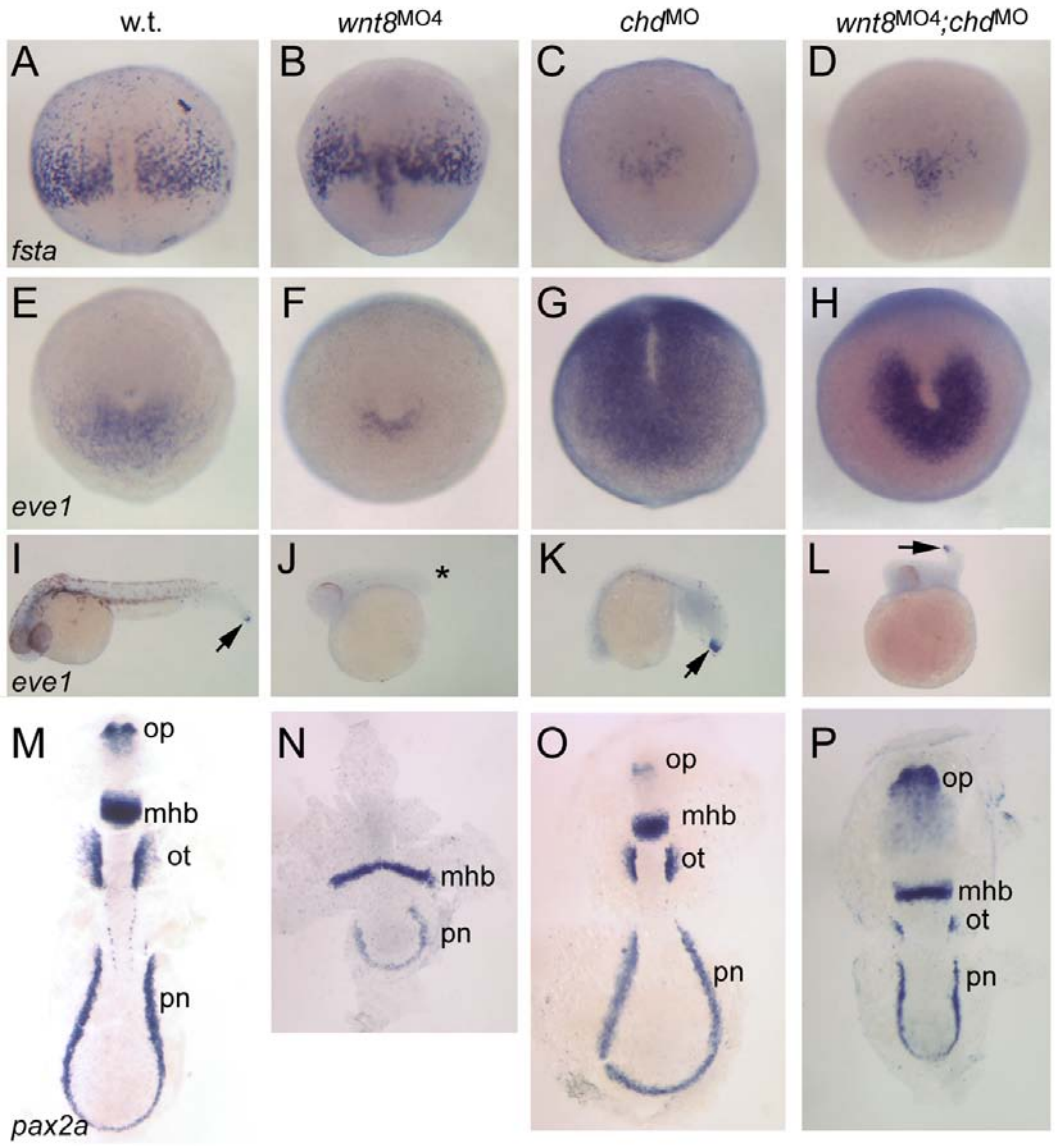


Fig. 6. Differential response of ventrolateral mesoderm to Wnt8 and BMP regulation. In situ hybridizations to fsta (A–D), eve1 (E–L), and pax2a (M–P). A–D: 80% epiboly, dorsal view, anterior up. fsta is slightly expanded in wnt8 morphants, but is suppressed in chordin morphants (C) and wnt8;chordin double mor- phants (D). E–H: Tailbud stage, posterior view, dorsal up. eve1 marks tailbud progenitors. Note reduced expression in wnt8 morphant (F), but strongly expanded expression in chor- din morphant (G) and somewhat expanded expression in wnt8;chordin morphants (H). I–L: $30 hpf, lateral views, anterior left. eve1 con- tinues to mark tailbud progenitors (I, arrow). Note absence in wnt8 morphant (J, asterisk) but presence in chordin morphant (K) and wnt8;chordin double morphant (L, arrow). M–P: Five- to six-somite stage, flat mount, anterior up. pax2a labels optic stalk (op), midbrain- hindbrain boundary (mhb), otic vesicle (ot), and pronephros (pn). Note absence of optic stalk and otic vesicle staining in wnt8 morphant (N) and slight narrowing of neural plate in chordin morphant (O). In the double morphant (P), note posterior expansion of optic stalks, slightly widened MHB, reduced otic vesicles, and shortened pronephros domain.

posterior somites, but is excluded from a small region of the ventral posterior tailbud at the five somite stage (Fig. 7A). In *wnt8* morphants, expression is reduced and occupies a morphologically altered domain. The tailbud and segmental plate of *wnt8* knockdown embryos form a small nub-like projection at this stage. *myf5* is still expressed in this projection in domains that are ostensibly the segmental plate and adaxial cells, but the tailbud itself and surrounding tissues are greatly reduced in size and somites are malformed (Fig. 7B). Additionally, *myf5* expression in wild type embryos occupies a significant portion of the anteroposterior length of the embryo, but in *wnt8* loss of function embryos the domain is greatly truncated and occupies a region extending only slightly anterior to the tailbud projection (Fig. 7B). In *chordin* morphants, *myf5* reveals anterior somites to be reduced, and the presomitic domain is also altered. Most notably, the region of the tailbud from which *myf5* expression is excluded is significantly expanded and occupies the majority of the posterior embryo (Fig. 7C). Importantly, *wnt8;chordin* double morphants also display an expanded *myf5*-free region of the tailbud (Fig. 7D, outlined by arrowheads; 90% of embryos as indicated, n=31), indicating that BMP signaling is sufficient to promote progenitor recruitment to this region in the absence of Wnt8 signaling. However, this domain consistently appears smaller in size compared to the *chordin* morphant (compare outlined regions in Fig. 7D to 7C; see also Supplemental Fig. 4), suggesting that Wnt8 may either act in parallel to BMP in

specifying tailbud progenitors, or that Wnt8 is required for tailbud progenitor proliferation.

To confirm this result, we examined *mesogenin* (*msgn*), a marker for presomitic mesoderm and the tailbud. In the *wnt8* knockdown, *msgn* expression is reduced significantly, nearly the opposite effect of that observed in *chordin* morphants (Fig. 7F,G). Consistent with *myf5* expression, *wnt8;chordin* double morphants show an expanded *msgn* expression domain compared to that of the *wnt8* morphant, but the domain is not equal to that observed in *chordin* morphants (Fig. 7H; 88% of embryos as indicated, n=34).

Discussion

In this report, we used a loss-of-function approach in the zebrafish to understand essential patterning functions of Wnt8 outside of its role in organizer regulation and the associated indirect effects on BMP activity. By knocking down the BMP

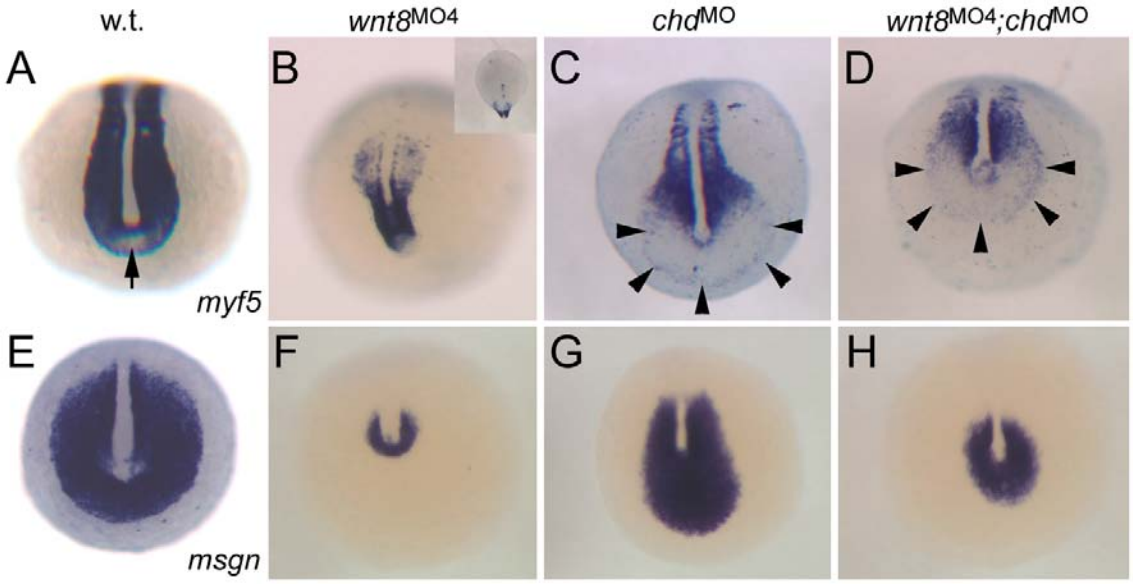


Fig. 7. Wnt8 and BMP input on tailbud progenitor specification. All images: posterior views, dorsal up, 5–6 somite stage. A–D: *myf5* expression marks presomitic mesoderm and somites, but is excluded from a small region of the ventral tailbud (arrow in A). Expression is significantly reduced in wnt8 morphant (B; inset: dorsal view). Note expansion of *myf5*- free progenitor zone in chordin morphant (C, outlined by arrowheads), and smaller expansion in *wnt8;chordin* morphants (D). E–H: msgn expression mirrors the behavior of the *myf5*- free zone of the tailbud.

antagonist Chordin in *wnt8* loss-of-function embryos, we created conditions wherein high levels of BMP are induced in embryos lacking Wnt8. We found that BMP expression is rescued in these double knockdown embryos, which is further reflected by a corresponding increase in the domain of phosphorylated Smad1/5. The expression patterns of *vent, ved,* and *flh* in the double knockdown provide further evidence that shield stage D/V patterning is largely consistent with wild type, although prechordal plate expansion is not suppressed by elevated BMP. Interestingly, we have found that ventro-posterior mesoderm

and tailbud specification occurs in *wnt8;chordin* morphants, but the size of posterior mesoderm domains is reduced compared to wild-type or *chordin* knockdown. Thus, BMP signaling can increase the size of the tailbud and specify posterior cell fates in *wnt8* loss of function embryos, suggesting that posterior mesoderm growth, but not fate specification per se, is an outcome of Wnt8 signaling.

An interesting observation is that BMP activity is elevated above wild type levels to some degree in *wnt8;chordin* double morphants. However, our data indicate that there is a temporal aspect to this observed increase: although p-Smad1/5 appears considerably elevated at shield stage, it appears only slightly elevated at 80% epiboly in the double knockdown. While the BMP signaling domain is dorsally expanded in *wnt8;chordin* double morphants, lower levels of p-Smad1/5 staining at 80% epiboly stand in contrast to consistently elevated p-Smad1/5 staining in *chordin* morphants. Decreased p-Smad1/5 staining in the double morphant is consistent with a recently proposed role for Wnt signaling in stabilization of activated Smads (Fuentealba et al., 2007), but it also correlates with the absence of *bmp2b* expression in the embryonic margin. However, *bmp2b* absence from the margin may not be relevant as *bmp4* and *bmp7* expression recover in the double morphant. Regardless, our data argue that BMP signaling is rescued in *wnt8;chordin* double morphants, and these embryos

therefore provide the opportunity for deciphering essential Wnt8 functions that are independent of organizer-dependent BMP regulation.

Wnt8, BMP and Axial Versus Non-Axial Mesoderm Specification

Wnt8 and BMP have a complex relationship to each other and to mesoderm patterning (Itasaki and Hoppler, 2010). In the zebrafish, Wnt8 and BMP signaling cooperatively regulate the *vent*/*vox*/*ved* transcription factor family to suppress organizer gene expression in the ventrolateral mesoderm and thereby promote non-organizer (i.e. non-axial) mesoderm specification (Ramel et al., 2005). Wnt8 and BMP are not equivalent, however. In the pre-gastrula zebrafish, Wnt8 is the primary regulator of mesodermal *vent*, *vox* and *ved* expression (Ramel and Lekven, 2004; Ramel et al., 2005). By 70% epiboly mesodermal *vent* is primarily under BMP regulation, while mesodermal *vox* and *ved* expression continues to depend predominantly on Wnt8 (Hammerschmidt et al., 1996; Kawahara et al., 2000b; Kawahara et al., 2000a; Melby et al., 2000; Imai et al., 2001; Shimizu et al., 2002; Ramel et al., 2005). In the absence of both Wnt8 and BMP signaling, all nascent mesoderm expresses an axial mesoderm fate. Considering Wnt8 expression is unaffected in BMP mutants (Hammerschmidt and Mullins, 2002; Ramel and Lekven, 2004), this suggests that Wnt8 and BMP function in parallel to suppress organizer gene expression, with a critical role for Wnt8 in maintaining *vox* and *ved* in the nascent mesoderm.

The relationship of Wnt and BMP signaling to each other and to *vent/vox/ved* expression may differ between zebrafish and *Xenopus*. In frogs, BMP signaling lies upstream of Wnt8 transcription and, while *Xenopus* Wnt8 is essential to mesoderm patterning, BMP expression is sufficient to activate ventral gene expression when Wnt8 activity is blocked (Hoppler and Moon, 1998). Our data are consistent with this *Xenopus* study, as *vent* and *ved* expression is expanded dorsally in *wnt8;chordin* morphants compared to wild type. However, we also find that expanded *vent* and *ved* does not correlate with restricted *goosecoid* expression. This may be due to differing levels of *vent, vox,* and *ved* that Wnt8 and BMP induce, although expression of *ved* and *vent* in *wnt8;chordin* morphants does not appear appreciably lower than in the wild-type. Alternatively, this result may reflect a temporal difference in Wnt8 and BMP regulation of *vent/vox/ved*, consistent with our previous finding that Wnt8 fulfills an earlier requirement than BMP for limiting the organizer (Ramel and Lekven, 2004). Another possibility is that Vent, Vox and Ved are different in their ability to repress organizer genes (Gilardelli et al., 2004).

Wnt8, BMP and Ventrolateral Mesoderm Patterning

The interaction of Wnt and BMP signaling in ventral and posterior mesoderm development has been the subject of intense investigation (reviewed in (Kimelman, 2006). Because of the difficulty in separating Wnt8 from BMP function, teasing apart their individual influences on ventrolateral mesoderm

development has been challenging. Current models suggest that high levels of Wnt and BMP signaling specify tail progenitors (Agathon et al., 2003). Consistent with this, previous results show that Wnt8 and BMP establish a pool of non-axial mesoderm progenitors in the ventrolateral margin (Ramel et al., 2005), a portion of which will contribute to the tailbud (Kanki and Ho, 1997; Agathon et al., 2003). Our current results suggest Wnt8 is dispensable for the specification of tail progenitors, since tailbud progenitors, defined by *eve1* expression, are observed in *wnt8;chordin* morphants. Therefore, elevated BMP respecifies ventrolateral mesoderm progenitors to contribute to the tailbud of *wnt8;chordin* morphants and the lack of tail progenitors in *wnt8* mutants is due to the expansion of BMP antagonists secreted by the organizer. Indeed, the expanded *eve1* domain of *wnt8;chordin* morphants correlates with the suppression of cephalic mesoderm, a cell fate that arises in the ventrolateral mesoderm adjacent to the organizer. Our observation is thus consistent with the hypothesis that in the early gastrula, BMP signaling instructs progenitors to contribute to the tail (Szeto and Kimelman, 2006), while Wnt8 only indirectly affects this process by restricting organizer expansion.

Interestingly, we have found that *wnt8;chordin* double morphant embryos maintain a tailbud but have severely reduced trunk and tail. Other studies have suggested that Wnt signaling prevents posterior mesoderm progenitor differentiation, thereby maintaining a posterior mesoderm progenitor pool

(Shimizu et al., 2005; Thorpe et al., 2005; Martin and Kimelman, 2008; Row and Kimelman, 2009). This suggests an additional role for *wnt8* wherein BMPs are necessary for the maintenance of the tailbud, but the release of progenitors into presomitic mesoderm is a Wnt regulated process. In addition to a smaller tailbud than *chd* knockdown alone, double knockdown embryos also have severely reduced presomitic mesoderm, suggesting that cells that occupy the tailbud are not being released to form somites at wild type levels. Thus, our results suggest Wnt8 signaling may affect mesoderm patterning in distinct ways during early and late gastrulation. It is not yet understood whether the mechanism of mesoderm patterning during tail growth is similar to the mechanism of mesoderm patterning during gastrulation, perhaps because of the complex signaling interactions involved. The ability to identify the unique and shared roles of Wnt and BMP signaling during mesoderm may help shed new light on the process.

Experimental Procedures

Fish Maintenance and Strains

Fish were maintained as described (Westerfield, 2000). Morpholino injections were performed into wild type embryos derived from an intercross of AB and TL wild type lines. Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). The *Df(LG14wnt8)*^{w8} line was described previously (Lekven et al., 2001).

MO Injection

MOs were diluted in Danieau's buffer as described (Genetools, LLC). 2ng/nL of each splice blocking *wnt8* MO and 6 ng/nL translation blocking *chd* MO were used as previously described (Nasevicius and Ekker, 2000; Ramel et al., 2005). For the coinjection experiments, both morpholinos were injected consecutively into the same clutch of one to two cell stage embryos. Each morpholino was injected into two thirds of *wnt8* MOs and one third received the *chd* MO.

In Situ Hybridization and Immunostaining

Whole-mount in situ hybridization was performed as previously described (Jowett, 2001). Embryos were mounted in glycerol and photographed using a Spot RT color camera (Diagnostic Instruments).

For immunohistochemistry, embryos were prepared essentially as described (Tucker et al., 2008). Following fixation and blocking, embryos were probed with a 1:100 dilution of anti-phospho-Smad 1/5/8 antibody (Cell Signaling Technology). This was followed by a 1:500 dilution of HRP-conjugated horse anti-mouse secondary antibody (Vector Labs) and DAB staining according to the manufacturer's protocol. Embryos were mounted in glycerol and photographed. Images for Figure 3 were adjusted in Adobe Photoshop by reducing the saturation of red hues to remove the background non-specific coloration of the embryo and reveal the specific signal.

CHAPTER III

THE ROLE OF WNT8 IN POSTERIOR GROWTH AND CELL PROLIFERATION

Introduction

In the previous chapter, I described experiments that showed the direct role that *wnt8* plays in patterning ventrolateral mesoderm. We found that *wnt8* is not necessary for specification of mesodermal progenitors *per se*, but is instead required to ensure that these domains are of appropriate size and cell number. Furthermore, we have found that *wnt8;chd* double morphants fail to show posterior elongation, despite the presence of a tailbud. This pool of progenitors is reduced in double loss of function embryos compared to wild type, but instead of a proportionally reduced tail, we have found a severe lack of posterior growth.

For the second part of our study, we have once again used morpholinos to create *wnt8;chd* double loss of function embryos as a way of producing *wnt8* loss-of-function while maintaining BMP signaling. As previously reported, this protocol allows us to create a strong knockdown of Wnt8 signaling, without concomitant loss of ventrolateral mesoderm resulting from *wnt8* knockdown alone (Baker et al., 2010).

The first part of our study was an attempt to discover if certain genes known to be involved in tail formation are regulated by *wnt8*. Specifically, *cdx4* is known to be required to for posterior growth and previous research has found that it is regulated by Wnts (Shimizu et al., 2005), but this discovery involved a partial loss of function of *wnt8;wnt3a*. Accordingly, we attempt to explicate the roll of *wnt8* alone in this process. Similarly, Shimizu et al. (2005) found that FGF expression is reduced in *wnt8;wnt3a* morphants, and we again attempted to parse out the role of *wnt8* in this process. We examine the expression of *ntl*, which is involved in a feedback loop with Wnts and *brachyury* in the development of the tail (Martin and Kimelman, 2008). Finally, in an attempt to parse out the relative contributions of *wnt8* and *wnt3a* to mesodermal patterning and posterior growth, we assay the expression of *wnt3a* and compare the *wnt8;chd* double morphant with a general knockdown of Wnt signaling.

Our evidence suggests that *wnt8* may be necessary for mesodermal cell proliferation. Importantly, Wnt pathway components have been shown to regulate the cell cycle (Shtutman et al., 1999). *wnt8;chd* morphants show a lack of posterior growth and plausible hypothesis is that this truncation is due to lack of cell proliferation. We test this by examining the tailbud for mitotic activity using the transgenic Fucci line (Sugiyama et al., 2009) and by performing flow cytometry on loss of function embryos.

It has been established that a cell's position at shield stage predicts the fate it will take on at later stages (Kimmel et al., 1990). Therefore, it is possible that the reduction In posterior progenitors we see in *wnt8;chd* embryos is due to changes in patterning at shield stage. We used fate mapping to determine if the loss of posterior tissues reflects changes in patterning at shield stage.

Results

wnt8 *Regulation of Genes Responsible for Posterior Growth*

Our previous work on this topic was concerned with the role *wnt8* plays in directly patterning ventrolateral mesoderm. In our current investigation, our first goal was to attempt to discover if any known promoters of ventral mesoderm posterior fates were downstream of *wnt8*. Our first candidate was *cdx4*. *cdx4* knockdown embryos exhibit a truncated tail, suggesting a possible mechanism for *wnt8* mediated posterior growth (Davidson et al., 2003).The expression of this transcription factor is reduced by knockdown of both *wnt8* and *wnt3a*, but the individual contribution of *wnt8* for its expression is not clear (Shimizu et al. 2005).

At bud stage, *cdx4* is expressed in a broad domain that comprises the entire posterior and extends dorsally nearly to the dorsal/ventral midline (Figure 8, A). When *wnt8* activity is knocked down, this large domain is greatly reduced (Figure 8, B). When *chd* function is reduced, very robust *cdx4* expression can be

seen in an expanded domain extending to over half the embryo (Figure 8, C). Mimicking a pattern observed in our previous findings (Figure 7H) the *wnt8/chd* double knockdown reveals a *cdx4* domain that recovers significantly from the single *wnt8* loss of function, but fails to reach wild type levels (Figure 8, D). It is possible to interpret this data by concluding that *wnt8* positively regulates *cdx4*, however based on our previous results a more parsimonious explanation is that the size of the tailbud domain and surrounding cells has decreased in size and number and *cdx4*, expressed within those cells, has decreased with them. Also important for posterior growth are ligands in the Fgf pathway. Double knockdowns of *fgf8/fgf24* have severely truncated tails and reduced expression of posterior markers, and expression of a dominant-negative Fgf receptor results in complete loss of ventral mesoderm (Draper et al, 2003). In order to assess whether Fgf signaling is affected when *wnt8* is knocked down, we utilized our double loss of function method and assayed the expression of *erm* and *spry4*. Both of these genes are regulated by Fgfs (reviewed in Thisse and Thisse, 2005) and we use them here as an output to measure Fgf activity in the posterior. Both *erm* and *spry4* are expressed in the tailbud at 5-6 somite stage (Figure 8, E, I). When *chd* is knocked down and BMP signaling is elevated, the expression domain of each of these genes is expanded within the enlarged posterior tissue (Figure 8, G,K). In embryos that have been injected with *wnt8* morpholino, the expression of both genes is severely reduced (Figuure 8, F, J). *erm* expression persists in a small patch at the ventral base of the nub that

projects from these embryos, while *spry4* expression appears to be lost completely. However, in both cases expression recovers in the double loss of function embryos in an area that corresponds in size to what we have previously established to be the norm for the tailbud at this stage and for this treatment. Taken together, our data show that Fgf signaling is maintained in the posterior in the absence of *wnt8.* These data agree with previously published reports; however, *wnt8;wnt3a* morphants show Fgf signaling in posteror somites whereas in *wnt8;chd* morphants Fgf activity is limited to the tailbud (Shimizu et al., 2005; Thorpe et al., 2005).

Several published data indicate that *no tail* (*ntl*) is regulated by Wnt signaling. *ntl* is necessary for the formation of the notochord and for the formation of tail somites (Martin and Kimelman, 2008). Wnt signaling is an important mediator of these processes, but the relative roles of *wnt8* and *wnt3a* are not clear. We sought to address this by looking at the expression of *ntl* when *wnt8* has been knocked down.

At the 5-6 somite stage, *ntl* is expressed in the tailbud and notochord (Figure 8, M, Q). The notochord domain is unaffected in *chd* knockdown embryos, but the tailbud is enlarged as expected (Figure 8, O, S). In the *wnt8* knockdown, the tailbud domain is completely lost but the notochord persists in a shortened stripe that is confined to the projection of posterior tissue typical of these embryos

(Figure 8, N, R). When both *chd* and *wnt8* are knocked down, the tailbud domain is partially rescued but the notochord does not lengthen significantly compared to the *wnt8* single loss of function. We have seen previously that the double knockdown, while showing generally rescued patterning at shield stage, exhibits a shortened axis, which likely accounts for the difference.

The Differential Role of wnt8 *and* wnt3a *in Tailbud Specification*

It has been previously established that *wnt3a* is involved in posterior development, but it is not clear if it acts in parallel or is downstream of *wnt8* signaling (Shimizu et al., 2005; Thorpe et al., 2005). In addition to showing reduced expression of posterior molecular markers, embryos deficient for *wnt3a* show reduced somites and other posterior structures. Further, the phenotype induced by reduced *wnt8* expression produced by a low dose of morpholino can be enhanced by an additional low dose of morpholino against the *wnt3a* message (Shimizu et al., 2005; Thorpe et al., 2005). In order to determine the contribution of both of these signaling factors on posterior formation, we tested the expression of *wnt3a* in the context of a *wnt8* knockdown.

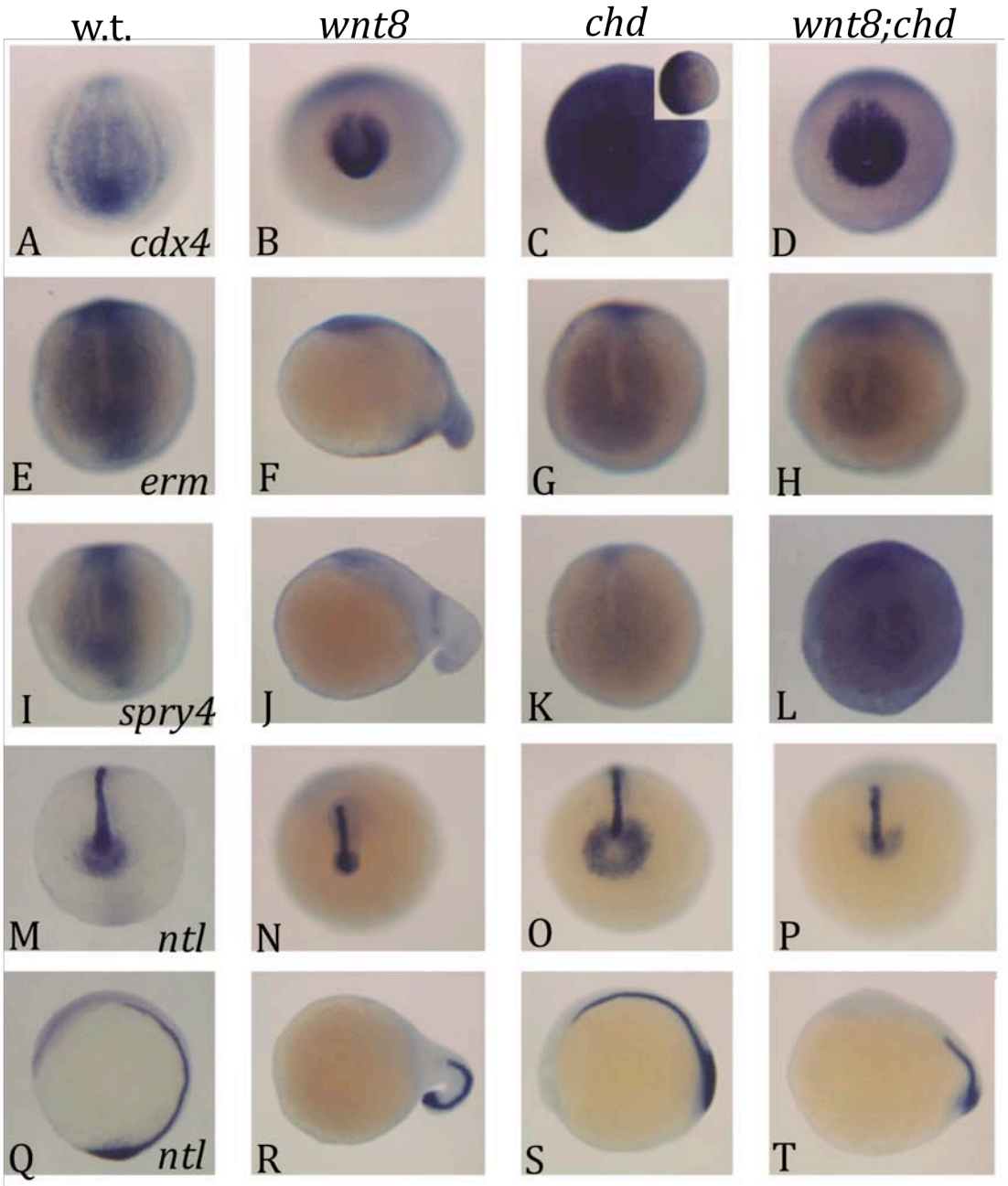


Figure 8. Regulation of posterior genes by *wnt8.* In situ hybridization at 5-6 somite stage to detect the expression of *cdx4* (A-D), *erm* (E-H), *spry4* (I-L), and *ntl* (M-T). Genotypes are listed at the top of each column. Posterior view with dorsal up in A-P with the exception of F and J which are in lateral orientation for clarity. Lateral view with dorsal oriented up in Q-T. *cdx4* expression is maintained in the tailbud in *wnt8;chd* embryos but does not extend into the somites (D). Both *erm* and *spry4* are nearly lost in *wnt8* morphants (F,J), but tailbud expression is rescued in *wnt8;chd* double loss-of-function (H,L). Note that expression is the somites is not rescued (arrow). *ntl* expression in the tailbud is reduced in *wnt8;chd* morphants (P, T), however, notochord expression persists.

From the beginning of somitogenesis, wild type *wnt3a* expression is present in the forebrain, hindbrain and the tailbud (Figure 9, A, and data not shown). As expected, the tailbud expression domain is somewhat larger in *chd* morphants, due most likely to the enlargement of the population of cells occupying the tailbud (Figure 9, C). This domain is reduced but persists in the tailbud of *wnt8* knockdown embryos (Figure 9, B). However, this reduction may in fact be due to loss of tailbud tissue generally rather than an indication that *wnt3a* expression is specifically regulated by *wnt8.* Embryos with both *wnt8* and *chd* knocked down show nearly complete rescue of tailbud expression, supporting the alternate hypothesis that *wnt3a* initiation and maintenance is not dependent on Wnt8 but that they act in parallel and potential crosstalk between these two genes occurs downstream of both (Figure 9, D).

If *wnt3a* does indeed act in parallel with *wnt8* we should be able to assess this by looking at the expression of the posterior marker *mesogenin* when Wnt signaling has been disrupted during late gastrulation in embryos that already have *wnt8* knocked down. We performed this test by injecting morpholinos against *chd, wnt8,* and *wnt8/chd,* into transgenic embryos that contain the Wnt inhibitor *dkk1* downstream of a heat shock (hs) promoter.

mesogenin is expressed in the tailbud and presomitic mesoderm and both of these tissues are reduced in size when the hs:*dkk* promoter is activated at 90%

epiboly (Figure 9; I). In the *chd* knockdown, the enlarged tailbud gives rise to an expanded *mesogenin* domain but this population of cells is reduced down to below wild type levels after heat shock (Figure 9, K). The tailbud is nearly lost in *wnt8* loss of function embryos and this effect is compounded when Wnt signaling is further reduced by *dkk* (compare Figure 9, F and J). As previously reported, the tailbud in *wnt8/chd* double loss of function embryos recovers substantially in size and form as compared to embryos where *wnt8* is singly reduced, but remains smaller than that of wild type (Figure 9, H). This effect is compounded when *dkk* is induced in these embryos. The *mesogenin* tailbud domain retains the shape of that seen in the double loss of function, but is smaller and considerably less robust after heat shock (Figure 9, L).

These data reveal that *wnt8* is not a significant regulator of *wnt3a*. Further, the reduction of the tailbud when Wnt signaling is reduced globally compared to that of *wnt8* alone suggests that Wnt mediated posterior development is not completely ablated when *wnt8* function is reduced. As we have demonstrated, *wnt3a* expression is maintained in *wnt8* MO embryos and it is therefore the most likely candidate for this additional Wnt input in posterior growth. Taken together, these observations reveal that the *wnt8* phenotype as it reflects posterior development is unique and independent of *wnt3a.*

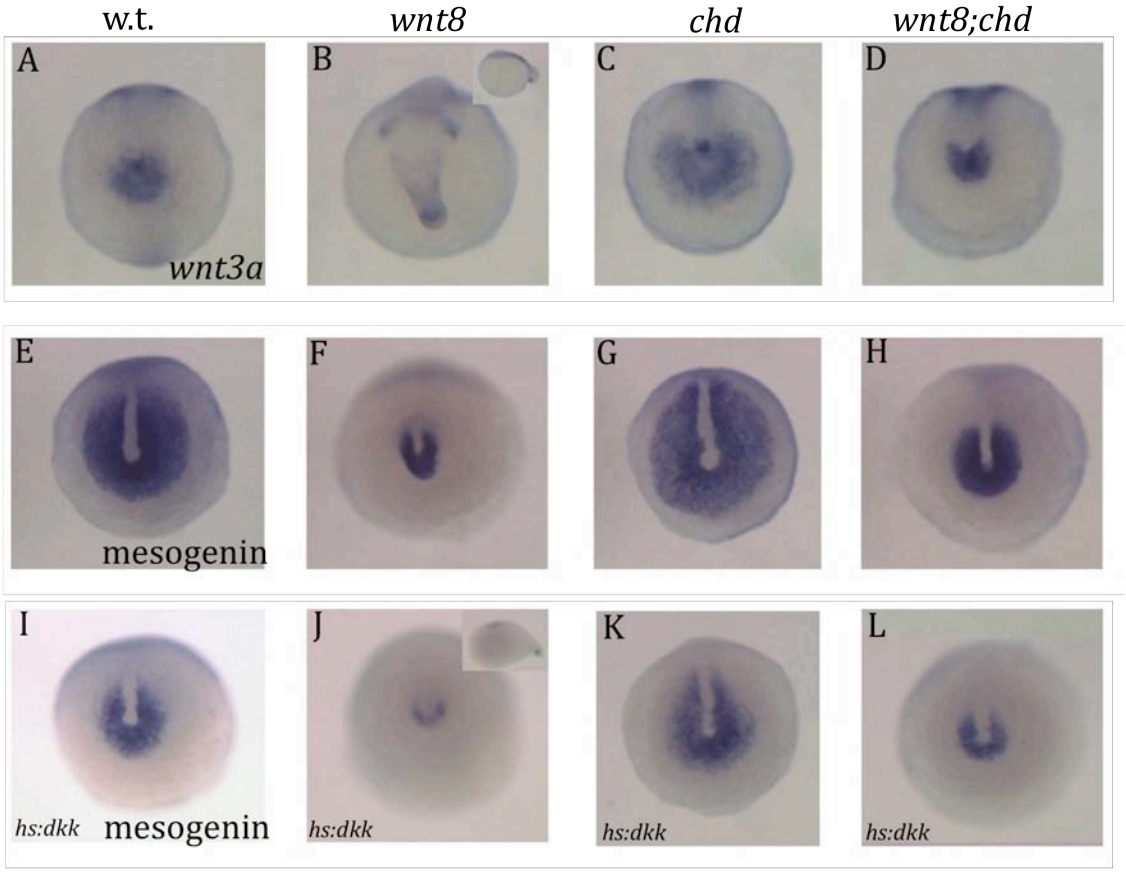


Figure 9. Differing roles for *wnt8* and *wnt3a* in tailbud formation. In situ hybridization at 5-6 somite stage to detect the expression of *wnt3a* (A-D), and *mesogenin* (E-L). Genotypes are listed at the top of each column. Embryos are in posterior orientation with dorsal toward the top. Expression of *wnt3a* is lost in *wnt8* knockdown but recovers to nearly wild type levels in *wnt8;chd* morphants (D). Embryos from heterozygous *hs:dkk* transgenic fish were injected with morpholinos at single cell stage and heat shocked at 90% epiboly (E-L). Embryos from the same clutch were segregated according to those that lack the transgene (E-H) and those that have it (I-L). Note the reduction in expression in J compared to F when only *wnt8* is knocked down and L compared to H in *wnt8;chd* double morphants.

wnt8 *As a Proliferative Agent*

One possible reason for the truncated phenotype seen in *chd;wnt8* loss of function embryos is the differentiation factor retinoic acid (RA). RA has been shown to inhibit cell proliferation and induce *myoD,* thus promoting differentiation of myoblasts (Alric et al., 1998). Given the shortened axis observed in double knockdown conditions, the possibility arises that tailbud progenitors destined to form somites are exposed to an encroaching domain of RA thus causing premature differentiation and inhibiting proliferation in presomitic mesoderm. The expression pattern of the RA synthesis gene *aldh1a2* may provide insight into this question.

Antagonizing the activity of RA is the cytochrome *cyp26a1.* This gene is expressed in the tailbud during somitogenesis in an area slightly separated from the more anterior RA domain. The gene product of *cyp26a1* metabolizes RA and prevents it from acting in the taibud (Martin and Kimelman, 2008).

aldh1a2 is expressed in the posterior somites anterior to presomitic mesoderm at 5-6 somite stage, and *cyp26* is expressed in the tailbud (Figure 10, A, E). In *chd* knockdown embryos, the *aldh1a1* domain is shifted slightly anteriorly but is otherwise unchanged (Figure 10, C). However, the *cyp26a1* domain is significantly expanded both anteriorly along the axis of the embyo, and in a less intense pattern that covers much of the posterior half of the embryo (Figure 10,

G). This expansion may explain the larger somites observed in these embryos at 24 hpf. When *wnt8* is knocked down, domains of both *cyp26a* and *aldh1a2* are substantially reduced as would be expected in these embryos, but the general A/P orientation of expression appears to be maintained (Figure 10, B, F). Lastly, in *wnt8;chd* loss of function the *aldh1a2* domain is again essentially unchanged in size compared to wild type, but is moved posteriorly somewhat, likely due to the shortened A/P axis in these embryos (Figure 10, D). *cyp26a1* posterior expression is robust in these embryos and extends anteriorly along the A/P axis, abutting and nearly overlapping the *aldh1a2* domain (Figure 10, H).

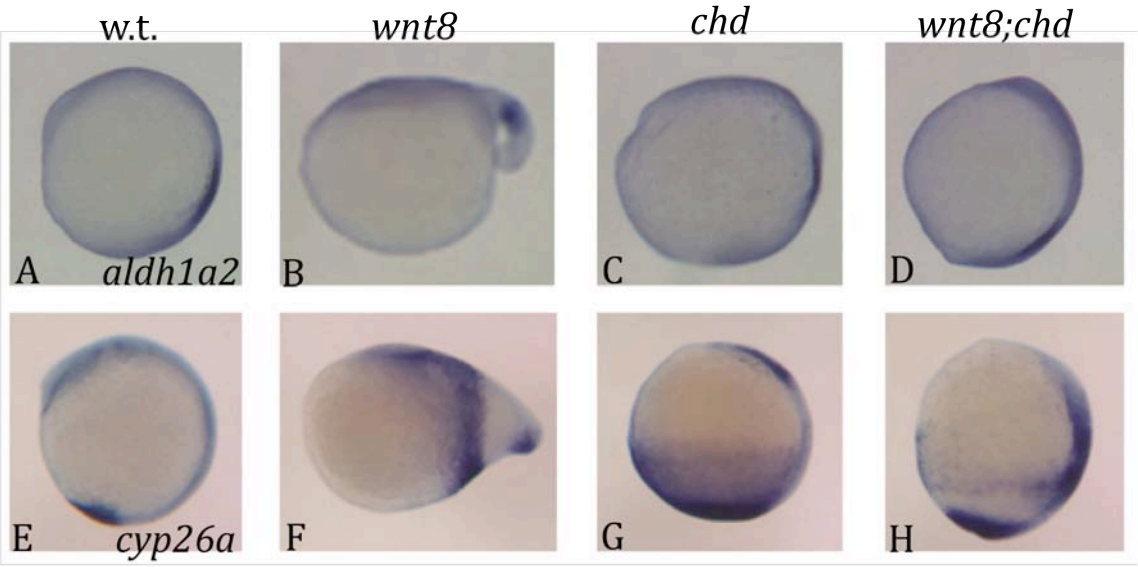


Figure 10. Regulation of retinoic acid by *wnt8.* In situ hybridization at 5-6 somite stage to detect the expression of *aldh1a2* (A-D) and *cyp26a* (E-H). Genotypes are listed at the top of each column. Lateral view with doral oriented to the right. The expression domain of *aldh1a2* remains in the same position relative to *cyp26a* in all conditions. Note however the expanded domain of *cyp26a* when BMP signaling is elevated (G,H).

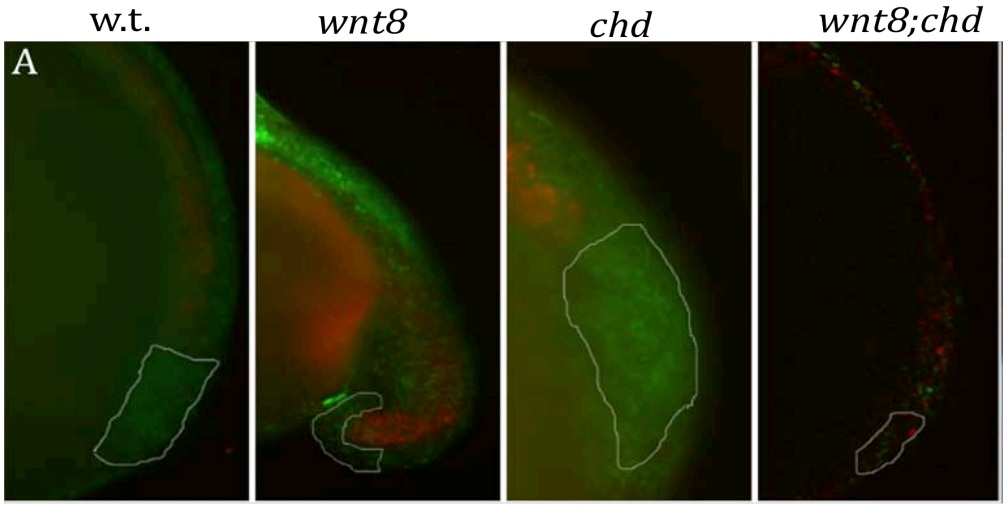
These results suggest that RA does not play a role in preventing posterior development and elongation in double knockdown embryos, as its expression is not altered relative to established somitic and pre-somitic mesoderm. However, elevated BMP appears to be capable of inducing the expression of *cyp26a* which may provide insight into the mechanism responsible for the large field of tailbud progenitors we observe in these embryos.

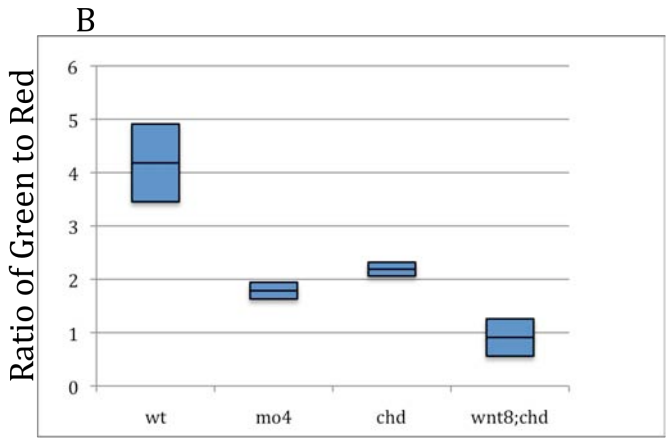
Our previous findings had suggested that *wnt8*, after its early role in regulating the size of the organizer, has a role in promoting cell proliferation. In order to assess this potential effect we utilized the transgenic Fucci (Fluorescent Ubiquinitin dependent Cell Cycle Imaging) system (Sugiyama et al., 2009). This cell cycle visualization tool works by illuminating cells according to their progression through mitosis. Cells in S, G2, or M phase fluoresce green and those in G1 or G0 fluoresce red.

Using Image J software to process z stack images from the red and green channels, we selected identical patches of cells in the tailbud domain from each channel with the Sync Measure 3D plug in (Figure 11, A). These selections were then measured for fluorescent intensity, and an average intensity was calculated for each channel in the stack. The overall ratio of green to red channels in each embryo was then normalized for area. We then took an average of these values from wild type, *wnt8* MO, *chd* MO, and *wnt8/chd* double loss-of-function.

In the wild type, the calculated average of green to red was 3.46 indicating that the intensity of the green was over three times that of red and as previously reported, the tailbud is a region of robust mitotic activity (Sugiyama et al., 2009). The *wnt8* loss of function embryos showed a significantly reduced level of mitotic activity with an average ratio of 1.63 (Figure 11 B, C). This result is the first evidence that explains the *wnt8* phenotype as an outcome of reduced cell proliferation as well as a patterning defect. Of critical importance then, is the mitotic ratio in the *wnt8/chd* double loss of function, where proper dorsal ventral patterning has been restored (Baker et al. 2010).

The average ratio of mitotic to non-mitotic cells in *wnt8/chd* embryos is .57, indicating an even greater number of cells in G1/G0 relative to cells actively dividing (Figure 11, B, C). Our previous findings indicated that the tailbud and other posterior tissues were being properly specified but were composed of fewer cells, this latest data supports that conclusion and suggests a possible mechanism in the form of *wnt8* activation of the cell cycle.





C

Knockdown Condition	Average Ratio of Green to Red	Standard Deviation	Number of Samples
w.t.	3.42	.73	3
wnt8	1.63	.16	5
chd	2.06	.13	3
wnt8;chd	.57	.35	6

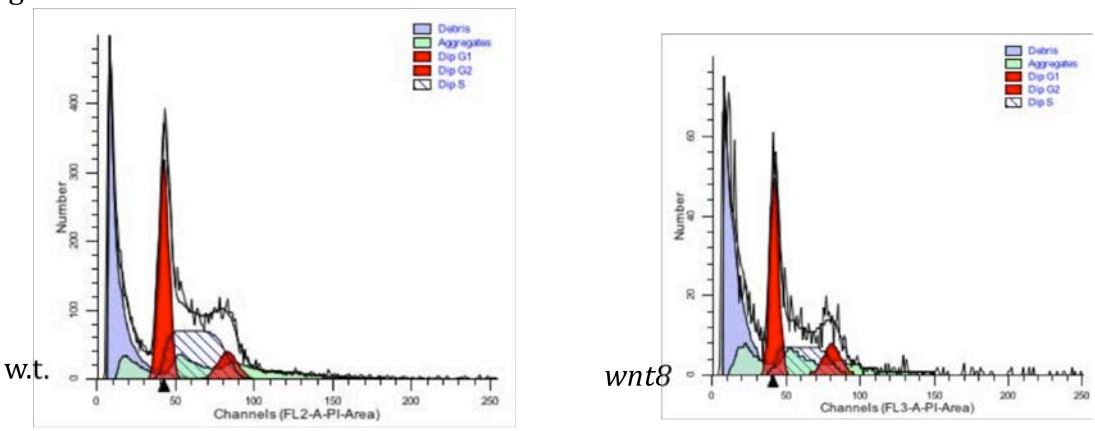
Figure 11. Cell cycle visualization in Fucci transgenic embryos. Z stack images of the tailbud from a representative embryo of each genotype at 2-3 somite stage. (A). Red and green intensities were taken from within the outlined area. Green cells are in $S/G_2/M$ phase and red cells are in G_1. (B) Box graph showing the ratio of green to red intensity. The midline through each box represents the mean from all samples of each genotype, and the area above and below is one standard deviation from the mean. Note that the intensity ratio of dividing cells in wild type is nearly four times that observed in *wnt8;chd* morphants. (C) Table showing actual values of green to red intensity, standard deviation, and number of samples.

In order to corroborate the data we obtained using the Fucci transgenic fish, we used flow cytometry to attempt to acquire cell cycle information from individual cells. This technique measures the amount of DNA in each cell from a given embryo and from it we can discern the percentage of cells that are in each phase of the cell cycle.

Our preliminary data indicate that there are cells actively dividing when *wnt8* is knocked down. In wild type embryos at the 5-6 somite stage, we found that 66.1% of cells were in S, G2, or M phase while only 34% were in G1/G0 (Figure 12, B). When *wnt8* function is lost, 54% of cells were found to be in S/G2/M and 45% were determined to be in G1/G0 (Figure 12, B). Although preliminary, these figures recapitulate the data we have acquired by measuring cell cycle in the tailbud using the Fucci line.

Figure 12

A



B

Cell Cycle Phase

	G0/G1	S/G2/M
w.t.	34%	66%
wnt8	45%	55%

% of cells in each phase

Figure 12. Direct measurement of cell cycle regulation using flow cytometry. (A) Histograms representing a single sample of dissociated cells from wild type and *wnt8* morphant embryos. The Y axis represents the number of cells counted and each peak on the X axis represents a different phase of the cell cycle. (B) Table showing the percentage of cells in each phase of the cell cycle from each genotype.

Our previous findings indicated that *wnt8;chd* loss-of-function embryos properly specify posterior fates, but these domains are smaller and are populated with fewer cells. This data may be explained by one of two potential hypotheses: that cell proliferation is decreased in these tissues or that the fate map at shield stage is altered such that fewer mesodermal progenitors are established at mid-gastrulation. It has been previously established that a cell's fate can be predicted

by its position at shield stage (Kimmel et al. 1990), so we tested the latter of the two hypotheses by performing a fate map study.

We have previously shown that although the tailbud in *wnt8/chd* morphants is nearly wild type, progenitors for mesoderm derived tissues such as pronephros, pre-somitic mesoderm, and heart are reduced (Baker et al., 2010). We used the photo-convertible Eos protein (Wiedenmann et al., 2004) to discern if these differences are the result of changes at shield stage. We photoconverted Eos in marginal blastomeres at 50% epiboly and then tracked their position until 2-3 somite stage. Cells that divided generally stayed in close association and were treated as one unless otherwise noted.

In wild type embryos, our data matches previous findings (Figure 13, Kimmel et al. 1990). All cells labeled in an arc between 150° and 180° (position I) from the dorsal midline occupied the tailbud (n=3). Half of the cells labeled in position II (120°-150°) occupied the tailbud while the other two moved away from the marginal leading edge and converged towards the midline at 80% epiboly (n=4). Of the cells labeled in position III (between 90°-120°), two moved anteriorly while the rest converged towards the midline by 80% epiboly (n=7). Six cells were labeled in position IV (a large arc 0°-90°). Four of them were on or nearly on the dorsal midline and they remained on that axis as expected while the other two rapidly converged to the dorsal side by 60% epiboly.

In *chd* morphant embryos, 100% of cells in position I occupied the enlarged tailbud (Figure 13). Of the three cells labeled in position II, one of the cells inhabited the tailbud while the other two moved towards the vegetal pole and divided to produce two daughter cells that did not converge towards the midline (n=3). At bud stage, all four daughter cells paused at an indeterminate position very near the tailbud and three remained at that position until 2-3 somite stage. Interestingly, the remaining cell moved away from the midline toward the ventral side and ultimately occupied the tailbud. Two of three cells labeled in position III occupied the tailbud (n=3) while the remaining cell and all cells in position IV (n=9) converged on the midline during gastrulation.

24 cells in individual embryos that had *wnt8* knocked down were labeled in positions I, II, and III (Figure 13). Of these, none occupied the tailbud. Interestingly, although all of these cells generally seemed to undergo gastrulation normally none of them converged towards the midline. Further, six cells in position IV had also failed to converge at 2-3 somite stage suggesting the intriguing possibility that *wnt8* embryos have a convergence defect in addition to a patterning defect.

Finally, in double loss of function conditions we find that the fate map is largely restored, but the convergence defect seen in *wnt8* embryos may persist. Five out of the eight cells labeled in positions I and II inhabited the tailbud, but the

remaining three moved away from the margin and did not converge on the midline (Figure 13). Cells labeled in position III did not occupy the tailbud and while two moved vegetally, none converged on the midline (n=4). Only two cells in position IV were more than 20° away from the midline and both of these moved anteriorly (n=11). In sum, these results argue that wnt8 is not required specifically to specify tailbud progenitor cells, but our results are consistent with a role for wnt8 signaling in the regulation of cell proliferation of tailbud progenitor cells. The flow cytometry results suggest that this may be through the regulation of progenitor cell cycle progression, although another possibility is that progenitors differentiate more rapidly and therefore exit the cell cycle earlier than in the presence of wnt8. This is an interesting observation that will require further analysis, but Wnt signaling is known to be an important factor in promoting cell proliferation in cancers. These results would indicate that this is a role normally required of wnt8 during mesoderm development.

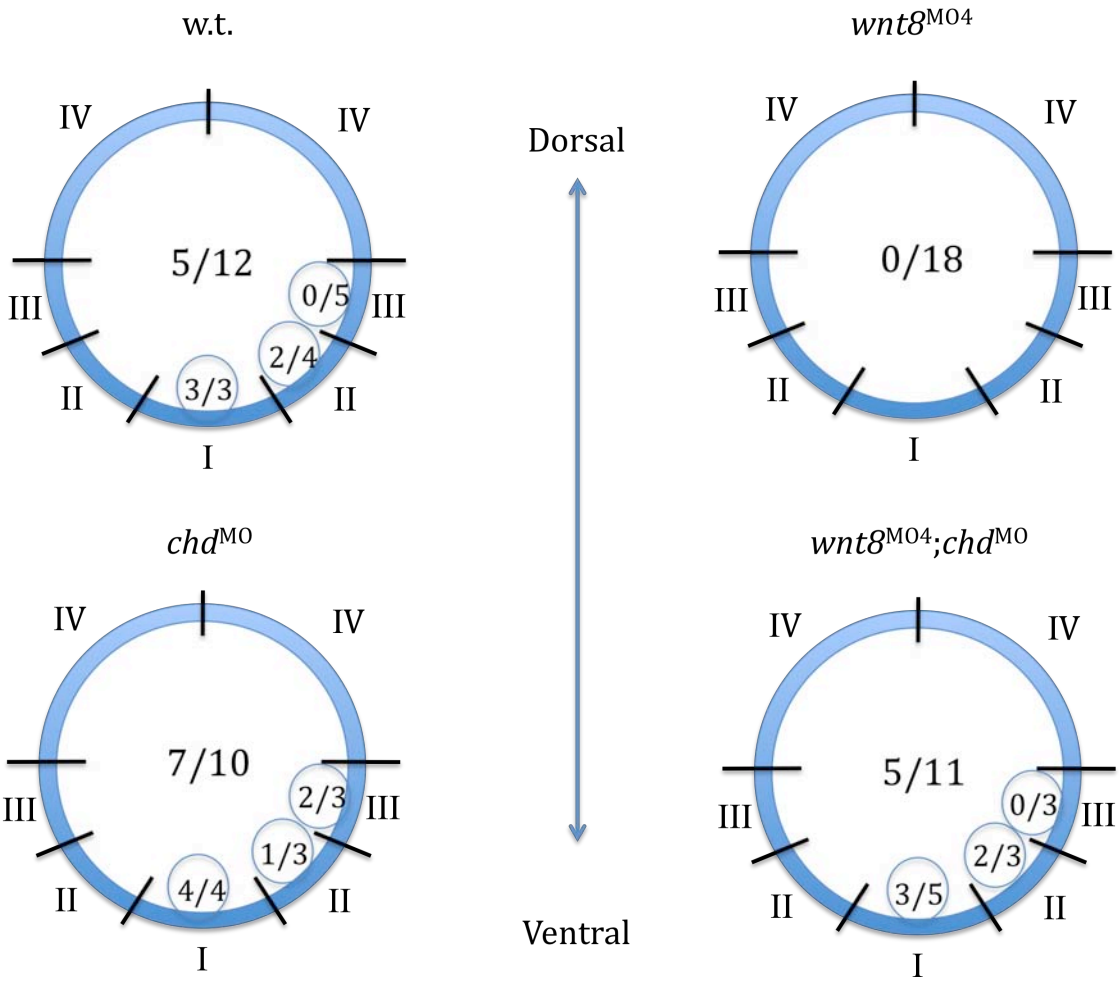


Figure 13. Fate mapping to determine if *wnt8;chd* morphants have fewer tailbud progenitors at shield stage. Each circle represents an animal pole view of a shield stage embryo. Cells were labeled at shield stage and monitored throughout gastrulation. The final position of each cell at 2-3 somite stage was documented. The numbers in bubbles reflect the number of embryos with labeled cells in the tailbud, over the number over the total number of embryos with cells labeled in that position at shield stage. Note the large number of cells in *wnt8* morphants (n=18) that failed to converge on the dorsal midline or occupy the tailbud.

Experimental Procedures

Fish Maintenance and Strains

Fish were maintained as described (Westerfield, 2000). Embryo staging was performed according to Kimmel et al (Kimmel et al., 1995). Injections of morpholinos were performed into wild type embryos derived from an intercross of AB and TL wild type lines.

MO Injection

2ng/nL of each splice blocking *wnt8* MO and 6 ng/nL translation blocking *chd* MO were used as previously described (Ramel et al., 2005). Coinjection experiments were performed as previously described. MOs were diluted in Danieau's buffer as described (Genetools, LLC).

In Situ Hybridization and Immunostaining

In situ hybridization of whole mount embryos was performed as previously described (Jowett, 2001). Embryos were mounted in glycerol and photographed using a Spot RT color camera (Diagnostic Instruments).

Fate Mapping

Fate mapping was performed using the photo-convertible protein Eos (Wiedenmann et al., 2004). Plasmid DNA containing the Eos coding sequence driven by a 2.6 kilobase fragment of the *wnt8* promoter was injected into wild type embryos at a concentration of 80 ng/μl. At shield stage a single cell was converted using a pinhole apparatus attached to an Olympus compound microscope. The position of the converted cell at both shield stage and 24 hpf was recorded.

Flow Cytometry

Embryos were staged and dechorionated in Pronase (2 mg/ml). Embryonic cells were washed twice in Holtfretters Buffer and then covered in collagenase (1 mg/ml in Holtfretters Buffer). Brief trituration with a small bore pipette tip was used to disrupt yolk mass followed by an incubation of 20 minutes at 37 C. The suspension was then centrifuged (300 g, 4 minutes, 4° C) after which the supernatant was discarded and the cells were resuspended in Ginzburg Fish Ringers. The solution was then vortexed at low speed and centrifuged (300g, 4 minutes, 4° C) after which the supernatant was discarded and the cells were resuspended in Ginzburg Fish Ringers and then fixed in 70% ethanol. Following fixation, embryos were incubated for 20 minutes in propidium iodide staining solution (P.I. 50 μg/ml, sodium citrate 4 mM, RNase 200 μg/ml) before being

injected into a Becton-Dickinson FACSCalibur 488 nm argon laser, flow cytometry instrument.

Fucci Visualization

Wild type and treated embryos were mounted in 3% methyl celluose and images were obtained using a 10X objective on a fluorescence microscope (ImagerZ1; Carl Zeiss) fitted with a fluorescence microscope slider module (ApoTome; Carl Zeiss).

CHAPTER IV

SUMMARY OF EXPERIMENTS AND DISCUSSION

The purpose of this study was to uncover the function of *wnt8* in patterning ventrolateral mesoderm, independent of its role in regulating the organizer. It has been established that *wnt8* indirectly maintains ventral fates by its role in limiting the size of the organizer, but what role this ligand plays in direct regulation of mesoderm is not clear. In order to more fully understand this role we have used zebrafish as a model and taken advantage of morpholinos to create *wnt8;chd* double loss of function embryos.

wnt8 Signaling is Necessary for BMP Activity

Our findings indicate that when *wnt8* function is inhibited, BMP function is reduced as well. *chd,* which has expanded expression in *wnt8* morphants, prevents BMPs from binding to cellular receptors. This implies that the function of BMP should be reduced in this condition and we find that by looking at the output gene *sizzled*, BMP signaling is effectively lost at bud stage when *wnt8* is knocked down. This result is supported by expression data from the three BMP ligands, *bmp2b, bmp7,* and *bmp4*, which show acute loss of mesodermal expression in *wnt8* mutant embryos. Also, BMP ligands are under positive autoregulatory control, making their expression an additional indicator for BMP signaling (Kishimoto et al., 1997; Nguyen et al., 1998) Finally, we have found

that BMP activity itself is degraded in *wnt8* knockdown embryos. Phosphorylated smad immunochemistry reveals that BMP signal transduction pathway activity is diminished when *wnt8* signaling is compromised. The data from the expression profiles of each of the BMP ligands indicate that the relative contribution of each may not be equal.

While *bmp7* and *bmp4* show nearly full recovery in the margin at shield stage in double morphants, the recovery of *bmpb2* expression is much less robust. In fact, *bmp2b* expression in the margin is not observed to recover in *wnt8;chd* double morphants, suggesting that this expression domain of *bmp2b* is downstream of Wnt signaling. Nonetheless, phospho-Smad staining is observed to recover in *wnt8;chd* morphants, indicating that *bmp2b* may be dispensable for gastrula-stage BMP signaling. One possible hypothesis to explain the smaller ventral and posterior domains we observe in *wnt8;chd* double morphants is that although the distribution of cells expressing activated Smad levels are recovered, the stability of phospho-Smad is reduced. This is supported by the observation of Fuentealba et al. that Smad1/5/8 proteins are phosphorylated by GSK3-β. This phosphorylation results in the inhibition of Smad activity and targeting for destruction in the proteasome. In the presence of Wnt signaling, GSK3- β is inhibited, and thus activated Smads are not degraded as quickly (Fuentealba et al., 2007). As applied to our findings, this would be consistent with the recovery of BMP signaling toward the dorsal organizer in wnt8;chd

morphants because of the reduction in Chordin-dependent BMP antagonism (i.e. phospho-Smad staining expands towards dorsal), but the absence of Wnt8 signaling results in a shorter half-life of activated Smad in the mesoderm (i.e. phospho-Smad staining in the mesoderm of wnt8;chd morphants is less intense).

This hypothesis does raise the possibility that reduced potency of Smad signaling in the mesoderm of *wnt8;chd* morphants has an effect on mesodermal development. Clearly, the integration of Wnt and BMP signaling is a complex issue, and interactions such as these must be taken into account when analyzing the functions of signaling pathways on differentiation. One way to address this is to test whether expression activated Smad is sufficient to rescue mesoderm development in wnt8;chd morphants, though such an approach would require the ability to spatially control the induction of a stabilized Smad variant.

wnt8, BMP and Patterning

Our approach to navigating the obstacle of BMP loss-of-function in *wnt8* knockdown embryos was to simultaneously knock down *chd* with the expectation that the capacity of BMP to regulate *ved, vox,* and especially *vent* would compensate and restore proper patterning of ventral mesoderm (Ramel and Lekven, 2004). Our data bore out this expectation as established by the

restoration of expression of *bmp7* and *bmp4* and the partial restoration of *bmp2b* in double loss of function conditions. Importantly, BMP activity was rescued and initially restored to above wild type levels at shield stage before returning to near wild type levels at 80% epiboly. Further, patterning was largely restored based on our assessment of mesodermal and organizer genes at shield stage.

wnt8;chd Morphants Display Normal Patterning at Shield Stage

Our data indicate that ventrolateral mesoderm is restored in embryos where both *wnt8* and *chd* have been knocked down. Although prechordal plate mesoderm was not rescued at shield stage, axial mesoderm fated to become notochord does return to wild type levels when BMP levels are elevated. Critically, ventral mesoderm as assayed by *vent* and *ved* is rescued and extends dorsally in an even larger domain than seen in wild type. Taken together, our data suggest that elevated BMP is sufficient to maintain a pool of mesodermal progenitors independent of *wnt8* signaling. However, a key aspect of this finding is whether non-axial mesodermal derivatives are properly specified.

In order to assay if the pool of mesodermal progenitors maintained by BMP signaling is able to undergo proper specification, we tested whether cephalic mesoderm and tailbud populations were maintained at the end of gastrulation. We found that head mesoderm is essentially unaffected by loss of *wnt8* but elevated BMP is able to suppress specification of this population. Currently, it is

believed that BMPs are necessary to maintain a population of undifferentiated mesodermal cells to occupy posterior tissue (Tucker et al., 2008). In keeping with this, our findings suggest that presumptive cephalic mesoderm is lost at the expense of an enlarged tailbud, as expression of *eve1* in this population is enlarged in *wnt8* morphants and well maintained in double loss of function embryos. Indeed, a population of *eve1* expressing cells can be found at the posterior end of truncated double loss of function embryos, providing more evidence of the potency of BMPs in maintaining posterior cell fates. However, it is possible that either the observed levels of BMP suppress not only anterior mesoderm, but also prevent specification of progenitors that arise from lateral mesoderm as has been previously reported for blood precursors (Pyati et al., 2006), or that these progenitors fail to differentiate in the absence of *wnt8.* In order to test these two hypotheses, we looked at a variety of posterior markers that arise from ventrolateral mesoderm.

wnt8 is Not Required for Posterior Specification

Observing the data from the *pax2a* in situ, it becomes clear that neither of the above hypotheses is true. *pax2a,* which it marks lateral mesoderm derived pronephros, is nearly lost when *wnt8* is knocked down, but recovers in the double loss of function. Nevertheless, this domain is truncated and somewhat thinner than that observed in wild type and we see similar results when looking at *cmlc2* (heart), *gata1* (blood), and *myoD* (muscle). Our data indicate that

observed levels of BMP maintain a progenitor population of posterior cells but do not repress lateral mesoderm derived precursors. Of particular importance, we find that *wnt8* is not necessary for the specification of posterior tissues *per se*, but rather may be required to ensure that posterior tissues are adequately populated. Further, the fact that *chd* embryos produce robust posterior tissue but *wnt8;chd* embryos do not suggests that these genes play differing roles in the way they act on posterior progenitors.

We attempted to parse out the differing contributions of *wnt8* and BMPs by looking at the expression of *myf5* and *mesogenin.* Both of these genes are expressed in the tailbud, but while *myf5* extends into the posterior somites, *mesogenin* is limited to presomitic mesoderm. Interestingly, *myf5* is excluded from a small region in the posterior part of the tailbud in which previous mapping work has identified as a proliferative zone. However, although this zone is greatly expanded in both the *chd* and *wnt8;chd* embryos, only in the presence of *wnt8* does posterior extension occur. One possible hypothesis that may explain the large tailbud but lack of posterior extension and the small number of poorly formed somites in these embryos as visualized by *myf5,* can be explained by the reduced domain of presomitic mesoderm as seen in *mesogenin* expression. *mesogenin* has been shown to necessary for maturation of presomitic mesoderm in mice (Wittler et al., 2007). While the role of mesogenin has not been established in zebrafish, it is possible that loss of this gene in these cells

prevents somite formation. Although we see the tailbud recover in double knockdown embryos, the relative recovery of presomitic mesoderm is considerably less robust and trunk somites are small and malformed. Reduced presomitic mesoderm raises the possibility of a *wnt8* mediated mechanism wherein cells in the tailbud are not only non-proliferative, but also prevented from prematurely exiting presomitic mesoderm. According to this model, when *wnt8* is lost, cells are allowed to enter the somites inappropriately and without concomitant replenishment of the tailbud by cell division.

The Role of *wnt8* in Posterior Growth

The problem of reduced BMP signaling in *wnt8* morphants has made it difficult to parse the relative contributions of these inputs in posterior development. Another strategy utilized by a different team of researchers has been to use partial knockdown of both *wnt8* and *wnt3a* (Shimizu et al., 2005). The rationale behind this strategy was to avoid patterning defects at shield stage and thus avoid BMP loss-of-function, while making the assumption that *wnt8* and *wnt3a* function redundantly in posterior development. However, although they only found a partial defect of patterning at shield stage, the expression of *chd* was considerably expanded at this stage suggesting the possibility of a reduction of BMP signaling (see fig. 2 g, Shimizu et al., 2005). Because our strategy allows for a much stronger knockdown of *wnt8* without concomitant loss of BMP, we

attempted to explicate the specific role this gene plays in the regulation of posterior development genes.

Our data confirm the reduction of *cdx4* expression, and in fact we found a more severe phenotype than previously seen (see fig. 3m, Shimizu et al., 2005) as expression in the *wnt8;chd* double loss of function does not extend into the somites. Shimizu et al. found that expression of *fgf3* was completely lost in the tailbud and *fgf8* was significantly reduced. We attempted to confirm this by looking at the expression of two genes regulated by FGF signaling, *erm* and *spry4.* Our data confirms the reduction of FGF signaling, however, this may be due to loss of tailbud progenitors and pre-somitic mesoderm rather than reflecting a loss of FGF activity *per se*. Further, similar to what we have observed in *cdx4* expression, we found that neither *erm* nor *spry* expression extends into the somites.

Of significant interest, we found that *ntl* expression in the tailbud was reduced but notochord expression remains normal, albeit truncated in proportion to the embryo proper. This data is recapitulated by similar experiments using an inducible *dkk* transgene performed by Martin and Kimmelman in 2008 (Martin and Kimmelman 2008). However, they also found that *ntl* expression was completely lost in both the tailbud and notochord when they induced a dominant negative form of the transcription factor TCF, concluding that *wnt3a* is directly

regulated by Wnt signaling. This apparent contradiction between their data and ours can be reconciled by assuming a shared role by *wnt8* and *wnt3a* in regulation of *ntl* in the tailbud, but exclusive regulation by *wnt3a* in the notochord. Additional support for this may be seen via *myoD* expression in *wnt8;wnt3a* double knockdown embryos, which show signs of significant perturbation of the axial domain (Shimizu et al., 2005).

The individual requirements of *wnt8* and wnt3a on posterior development

In mice, *wnt3a* is required for posterior development (Dunty et al., 2008). Although *wnt3a* has been shown to be involved in posterior development in zebrafish (Shimizu et al., 2005; Thorpe et al., 2005), it is not clear if it acts redundantly with *wnt8* or whether its expression is dependent on *wnt8*. We attempted to dissect out the similar function of these two genes by first determining if *wnt3a* expression is dependent on *wnt8.* Our data indicate that *wnt3a* is not dependent on *wnt8* and is rescued to near wild type levels in *wnt8;chd* double loss of function embryos, suggesting that these genes act in parallel in posterior development. Further, these data imply that although *wnt3a* is required for normal posterior development (Shimizu et al., 2005), the truncated phenotype witnessed in double loss of function embryos suggest that *wnt3a* requires *wnt8* for posterior elongation. Furthermore, *wnt3a* morphants exhibit only mild defects in posterior growth indicating that these genes are only partially redundant (Shimizu et al., 2005). We dissected out the ability of *wnt3a*

to compensate for loss of *wnt8* in tailbud formation by knocking down all Wnt signaling at 90% epiboly using a *hs:dkk* transgene. Using *mesogenin* as a read out for tailbud formation, our data show that as expected the remnant of tailbud seen in *wnt8* morphants is nearly lost. When we knocked down Wnt signaling in *wnt8;chd* embryos, we found that *wnt3a* has a substantial contribution to tailbud formation. It appears that the pool of tailbud progenitors when all Wnt signaling is inhibited is approximately half that seen in double morphants. Based on this, it might be expected that the tail in *wnt3a* embryos would also be 50% shorter. However, as previously reported, *wnt3a* morphants have only a mild defect and a normal number of somites (Shimizu et al., 2005, Thorpe et al., 2005). Taken together with our previously reported data that shows that *wnt8* is not required for specification, this raises the intriguing possibility that *wnt8* is required as a proliferative agent in posterior growth.

Wnt8 Signaling Promotes Cell Proliferation

In order to address whether lack of cell proliferation is the mechanism responsible for inhibited posterior growth in *wnt8;chd* embryos, we first asked if the causative factor was premature differentiation into somitic mesoderm by exposure to retinoic acid. Soaking embryos in RA causes truncation, and it has been shown that RA is responsible for restricting cardiac progenitors (Keegan et al., 2005). Our assay shows that the relative positions of *aldh1a2* and the RA inhibitor *cyp26a* expression are relatively normal. Indeed, *cyp26a* expression is

somewhat expanded compared to wild type which would seem to preclude RA exposure as the responsible mechanism for the double morphant phenotype.

wnt8 mediated cell division has been demonstrated in other systems. For example, in the regenerating tail *wnt8* overexpression can increase the rate of proliferation (Stoick-Cooper et al., 2007). If *wnt8* mediated mitosis is indeed the means by which posterior growth occurs, a more direct approach may answer that question. Accordingly, we found that Fucci transgenic fish that have been injected with *wnt8* or *wnt8;chd* morpholino show a marked decrease in cells that are in S/M/G2 phases compared G1/G0. Preliminary data from flow cytometry experiments further support the possibility that cell proliferation is reduced, as our comparison of cells from wild type and *wnt8* morphant embryos shows. This experiment will need to be completed in *chd* and *wnt8;chd* conditions, but reduced mitotic activity remains a parsimonious explanation for the observed truncated phenotype.

Another explanation for the smaller posterior domains observed in *wnt8;chd* morphants is that, although we have shown that patterning is generally restored, the fate map has been altered such that there are fewer progenitors destined for posterior tissues at shield stage. It has been known for some time that it is possible to predict the determination of a cell by its position at shield stage (Kimmel et al., 1990), so we undertook a cell tracking experiment to ascertain if

changes at shield stage partially explain the *wnt8* and *wnt8;chd* phenotype. As expected, we found that in *wnt8* morphants cells in the ventral-most region do not occupy the tailbud. Interestingly, we also observed what appears to be a defect in convergence. It has been reported that the cells on the ventral-most margin occupy a “no convergence no extension” zone (Myers et al., 2002), but our observations suggest that cells more lateral to this region may also fail to converge. Coordination of mesodermal genes and genes involved in cell movement are coordinated, and it has been shown in *Xenopus* that *wnt8* and *wnt11* (*siberblick*; required for convergence and extension) are co-regulated by Gli protein (Myers et al., 2002).

Our fate mapping data further suggest that in the *wnt8;chd* double knockdown, normal patterning as it pertains to tailbud progenitors is largely restored in accordance with what we observed previously. It appears that the convergence defect seen in *wnt8* morphants may also be rescued. Technical issues have prevented us from determining whether more lateral mesodermal progenitors are also rescued at shield stage, but the near normal ventral domain supports this hypothesis. Another possibility is that the relatively normal tailbud has increased in size at the expense of more lateral tissues. This hypothesis is supported by the fact that we see elevated BMP signaling at shield stage combined with the generally accepted belief that high BMP dosage is necessary to maintain a pool of tailbud progenitors during gastrulation (Tucker et al., 2008).

Further, loss of BMP signaling can increase the progenitor pool for blood and pronephros (Pyati et al., 2006) Thus, it remains possible that reduced domains of pronephros and heart precursors are a result of higher than normal BMP activity during gastrulation.

We have observed that *wnt8;chd* morphants are generally patterned correctly at shield stage, and that a relatively normal pool of tailbud progenitors is maintained until the end of gastrulation. Furthermore, we have determined that several genes necessary for posterior growth exhibit tailbud expression in *wnt8;chd* embryos. Finally, we have observed that *wnt8* morphants have reduced cell proliferation. These data suggest a biphasic model for Wnt8 activity, wherein *wnt8* is initially required during gastrulation to limit the size of the organizer and is later required to promote posterior elongation by activating cell proliferation.

The Correlation of Our Work with Cancer Research

Our work here has important implications for cancer research. In addition to the role it plays in embryo development, abnormal Wnt signaling has been observed in tumors from patients with colorectal cancer, gastric cancer, hepatocellular carcinoma, hepatoblastoma, and myeloma. (reviewed in Takebe et al., 2011).

Mutations in APC, a member of the Wnt signal transduction pathway have been implicated in familial adenomatous polyposis (FAP), a form of colon cancer. Patients with FAP develop large numbers of polyps, which then rapidly accumulate mutations in other oncogenes such as *p53* and *K-Ras* (reviewed in Reya and Clevers, 2005). APC mutations prevent the formation of the β-catenin destruction complex, allowing for the inappropriate stabilization of β-catenin (Rubinfeld et al., 1996). β-catenin is involved not only in cell proliferation but also adhesion, which is indispensable for cancer invasion and metastasis. Thus, improper stabilization of β-catenin plays a key role in cancer progression.

Our data suggest a developmental role for *wnt8* in cell proliferation. This activity has also been observed in abnormal Wnt pathway regulation during tumor growth. It has been reported that β-catenin can activate the cell cycle regulator *cyclin D1.* The *cyclin D1* promoter sequence contains consensus TCF/LEF binding sites, and a dominant negative form of TCF can arrest the cell cycle in cancer cells (Tetsu and McCormick, 1999).

Persistent expression of the oncongene *c-Myc* has also been implicated in tumorigenesis. This gene acts a regulator for downstream genes that are involved in metastasis and cell proliferation, and it has been observed that the Wnt pathway is involved in its regulation. The *c-Myc* promoter has consensus TCF binding sites, and APC has been shown to repress its expression (He et al.,

1998). Further, β-catenin acts as an activator of *c-Myc*, once again demonstrating the involvement of Wnt pathway components in cancer development.

Taken together, these findings indicate a common function for the Wnt pathway in both development and cancer biology. Continuing to decipher the critical role this pathway plays in development will provide a direct benefit in human health as it pertains to birth defects as well as cancer prevention.

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APPENDIX

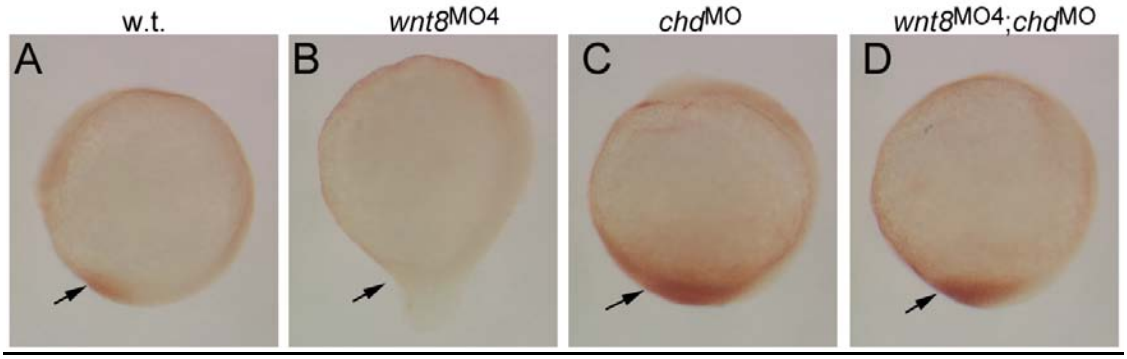

Figure S1. Wnt8 is not required for BMP signaling activity after gastrulation. Images of ant phosphorylated Smad1/5 staining at the 5-6 somite stage. Genotypes are indicated abov i- row. Arrows indicate tail domain where nuclear P-Smad1/5 indicates active BMP signali e each Lateral views, dorsal right, anterior up. Note absence of staining in tailbud of *wnt8* morp ng. elevated staining in *chordin* morphant (C) and strong staining in *wnt8;chordin* double mo hant (B), (D). rphant

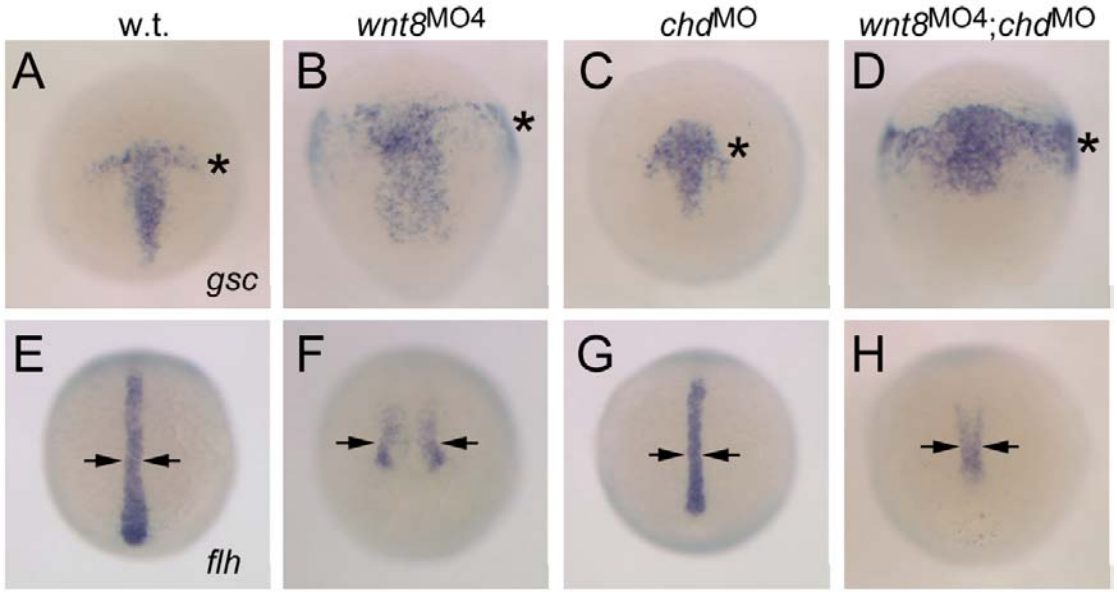

Figure S2. Differential dorsal mesoderm regulation by Wnt8 and BMP signaling. In situ hybridizati ons on bud stage embryos to detect *gsc* (A-D) and *flh* (E-H). Genotypes are indicated above each colum n. Asterisks in (A-D) indicate prechordal plate mesoderm domain. Note expansion in *wnt8* and *wnt8;* morphants (B,D), but not *chordin* morphant (C). Arrows in (E-H) indicate width of notochord domai *chordin* expansion in *wnt8* morphant (F) but not *chordin* or *wnt8;chordin* morphants (G,H). These results s n. Note the observations made at shield stage (see Fig. 4) continue to hold true through gastrulation. how that

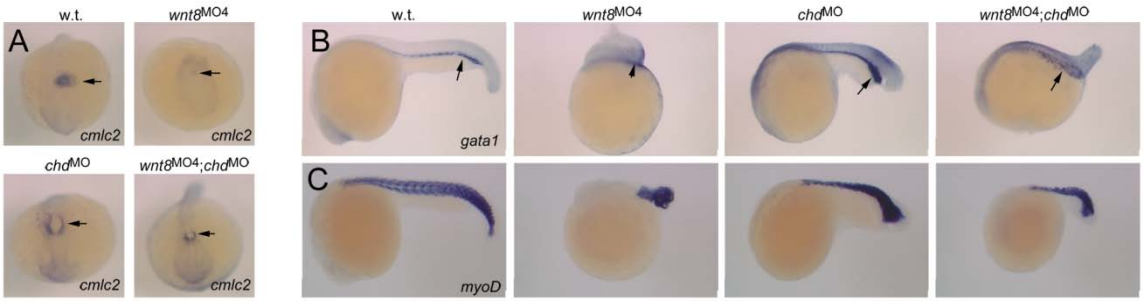

Figure S3. Differential response of additional ventrolateral mesoderm domains to Wnt8 and BMP regulation. In situ hybridizations at 24 hpf to detect *cmlc2* in the heart (A), *gata1* in blood progenitors (B) and *myoD* in body musculature (C). (A) Dorsal views of the anterior embryo, anterior pointing down. Arrows indicate *cmlc2*-expressing cardiac cells. Note near-absence of staining in *wnt8* morphant, but presence in *chordin* and *wnt8;chordin* morphants. (B) Lateral views, anterior left. Arrows indicate *gata1*-expressing blood progenitors. Note severe reduction in *wnt8* morphant, expansion in *chordin* morphant and mild reduction in *wnt8;chordin* morphant. (C) Lateral views, anterior left. *myoD* expression marks somitic muscles. Note reduction of somites in *wnt8* morphant, increased myoD expression in the most posterior region of *chordin* morphant, and additive phenotype in *wnt8;chordin* morphant. These results mirror our observations of *pax2a* expression domains as shown in Fig. 5, i.e. defects of mesodermal patterning observed in *wnt8* morphants is rescued by elevated BMP signaling, but posterior growth of tissues is not.

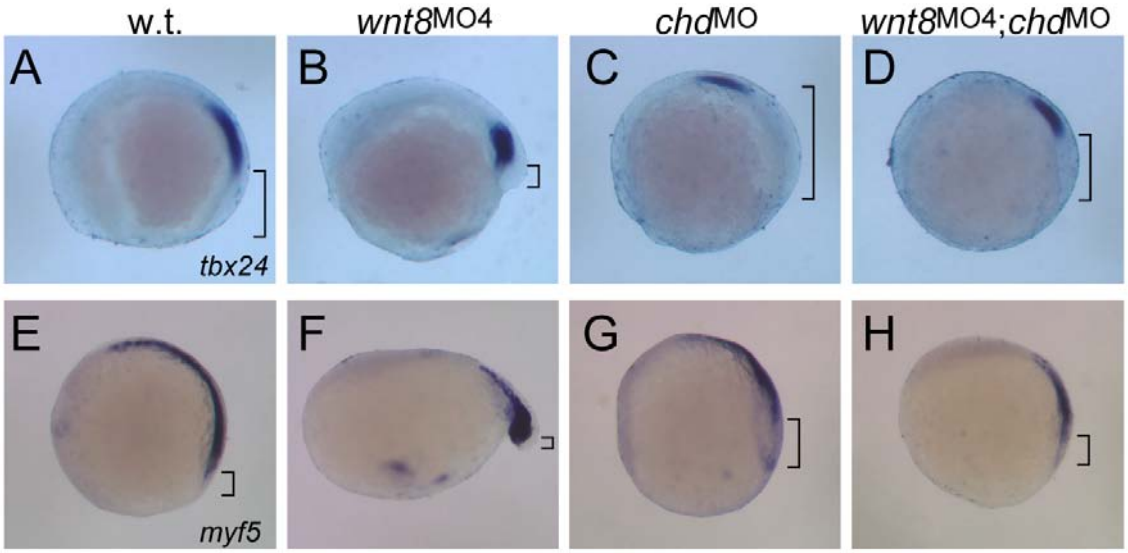

Figure S4. Alternate view of the tailbud and presomitic mesoderm in *wnt8, chordin* and *wnt8;chordin* morphants. All images are lateral views, 5-6 somite stage embryos, anterior to left, dorsal up. Genotypes are indicated above each column. Brackets indicate the tailbud, defined as tissue posterior to presomitic mesoderm. (A-D) *tbx24* is expressed in presomitic mesoderm. Note the strong reduction in tailbud tissue observed in *wnt8* morphants (B), significant expansion in *chordin* morphants (C), and approximately wild-type size observed in *wnt8;chordin* morphant (D). (E-H) Lateral views of embryos stained for myf5 expression reveal a similar result. Note that myf5 expression is also in adaxial cells that flank the notochord into the tailbud, thus the apparent size of the tailbud in this view is smaller than that observed in *tbx24* stained embryos. This is thus an optical effect, yet the result is not different between the two probes used.