BMP AND NONCANONICAL WNT SIGNALING CO-REGULATE THE TAIL DEVELOPMENT IN ZEBRAFISH

by

YI YANG

B.S., Sichuan University, 2003
M.S., Sichuan University, 2006

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010
Abstract

Multiple signaling pathways regulate development of the posterior zebrafish body, which is derived from a population of progenitor cells called the tailbud, a structure formed at the end of gastrulation. Fate specification and differentiation are closely linked with cell migration to ensure that, as some cells exit the tailbud and differentiate, other cells are retained in the tailbud as undifferentiated precursors to support later growth. The role of BMP signaling in specifying cell fate in the tailbud has been well-characterized. Among the lost ventral tissues like ventral tailfin and cloaca, embryos with compromised BMP signaling produce a curious phenotype—a ventrally located secondary tail containing both somitic muscle and notochord. This phenotype is proposed to be a fate-patterning defect when the BMP gradient lowered to a precise level. However, this morphogen mode is insufficient to explain secondary tail formation without considering BMP also regulates morphogenetic movements during gastrulation, promoting the convergence of lateral mesodermal cells towards the dorsal axis. In this study, we provide evidence that BMP signaling continues to mediate cell movements during tail development. Our data indicate that BMP signaling is activated in the ventroposterior tailbud to promote cell migration during tailbud protrusion, and that it is the defective migration of these cells which ultimately leads to bifurcation of the CNH domain, a presumptive stem cell pool in the tailbud, and formation of a secondary tail in BMP mutants. In parallel, the morphogenesis of tailbud cells is known to be under the control of noncanonical Wnt signaling, although the exact nature of the defect remains unclear. We find that inhibition of noncanonical Wnt signaling also leads to secondary tail formation. Additionally, we show that noncanonical Wnt signaling interacts with BMP signaling to maintain CNH integrity by affecting cadherin localization in CNH cells, possibly disrupting cell cohesion. We propose a model that BMP and a noncanonical Wnt pathway regulate tail morphogenesis by controlling cell migration and cell adhesion within the tailbud.
BMP AND NONCANONICAL WNT SIGNALING CO-REGULATE THE TAIL DEVELOPMENT IN ZEBRAFISH

by

YI YANG

B.S., Sichuan University, 2003
M.S., Sichuan University, 2006

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Approved by:

Major Professor
Alexander Beeser
Copyright

YI YANG

2010
Abstract

Multiple signaling pathways regulate development of the posterior zebrafish body, which is derived from a population of progenitor cells called the tailbud, a structure formed at the end of gastrulation. Fate specification and differentiation are closely linked with cell migration to ensure that, as some cells exit the tailbud and differentiate, other cells are retained in the tailbud as undifferentiated precursors to support later growth. The role of BMP signaling in specifying cell fate in the tailbud has been well-characterized. Among the lost ventral tissues like ventral tailfin and cloaca, embryos with compromised BMP signaling produce a curious phenotype—a ventrally located secondary tail containing both somitic muscle and notochord. This phenotype is proposed to be a fate-patterning defect when the BMP gradient lowered to a precise level. However, this morphogen mode is insufficient to explain secondary tail formation without considering BMP also regulates morphogenetic movements during gastrulation, promoting the convergence of lateral mesodermal cells towards the dorsal axis. In this study, we provide evidence that BMP signaling continues to mediate cell movements during tail development. Our data indicate that BMP signaling is activated in the ventroposterior tailbud to promote cell migration during tailbud protrusion, and that it is the defective migration of these cells which ultimately leads to bifurcation of the CNH domain, a presumptive stem cell pool in the tailbud, and formation of a secondary tail in BMP mutants. In parallel, the morphogenesis of tailbud cells is known to be under the control of noncanonical Wnt signaling, although the exact nature of the defect remains unclear. We find that inhibition of noncanonical Wnt signaling also leads to secondary tail formation. Additionally, we show that noncanonical Wnt signaling interacts with BMP signaling to maintain CNH integrity by affecting cadherin localization in CNH cells, possibly disrupting cell cohesion. We propose a model that BMP and a noncanonical Wnt pathway regulate tail morphogenesis by controlling cell migration and cell adhesion within the tailbud.
# Table of Contents

List of Figures .................................................................................................................. ix  
List of Tables ..................................................................................................................... x  
Acknowledgements .......................................................................................................... xi  
Dedication .......................................................................................................................... xii  
CHAPTER 1 - Introduction ................................................................................................. 1  
  Tail development in zebrafish ......................................................................................... 2  
  Formation of the tailbud ................................................................................................. 3  
  Tail development .......................................................................................................... 4  
The BMP signaling pathway in zebrafish ........................................................................... 4  
  BMP signaling in fate specification ............................................................................... 5  
  Secondary tail formation in BMP-deficient embryos ..................................................... 7  
  BMP signaling in cell movements ................................................................................. 8  
The noncanonical Wnt signaling pathway ......................................................................... 9  
  The PCP pathway in *Drosophila* ............................................................................... 9  
  The noncanonical Wnt pathway in zebrafish ................................................................. 10  
Cadherin in zebrafish development ................................................................................. 13  
Summary .......................................................................................................................... 14  
Figures and Tables ........................................................................................................... 17  
Copyrights ......................................................................................................................... 22  
References ......................................................................................................................... 23  
CHAPTER 2 - BMP and Noncanonical Wnt Signaling are Required to Inhibit Secondary Tail  
  Formation in Zebrafish .................................................................................................. 31  
Abstract ......................................................................................................................... 31  
Introduction ....................................................................................................................... 31  
Materials and methods .................................................................................................... 35  
  Zebrafish strains ........................................................................................................... 35  
  *In situ* hybridization and antibody staining ............................................................... 35  
  Morpholino and RNA injection ..................................................................................... 36
Time lapse confocal microscopy ................................................................. 36
Chemical treatment .................................................................................. 36
Results ........................................................................................................ 38
Mesoderm tissues produced in the secondary tail of mini fin ...................... 38
Secondary tail formation is not accompanied by changed mesoderm fates ... 38
BMP signaling is required during early somitogenesis to inhibit secondary tail formation. 39
Noncanonical Wnt signaling functions with BMP in inhibiting secondary tail formation... 40
BMP and noncanonical Wnt signaling are required to prevent bifurcation of the
chordoneural hinge during tail outgrowth .................................................. 42
N-Cadherin is required to maintain tailbud integrity ..................................... 43
CNH bifurcation occurs during tailbud protrusion ....................................... 44
BMP regulates migration of posterior mesoderm cells in the tailbud .............. 46
Discussion................................................................................................... 47
BMP signaling inhibits secondary tail formation ........................................ 47
Noncanonical Wnt signaling and Cadherin localization .............................. 50
Noncanonical Wnt signaling promotes plasma membrane localization of cadherin .... 51
A model for the roles of BMP and noncanonical Wnt signaling in tail morphogenesis ..... 51
Figures and Tables ..................................................................................... 53
References ................................................................................................ 68
Supplement ............................................................................................... 73
Materials and methods (supplementary) .................................................... 73
   In situ hybridization and antibody staining .............................................. 73
Morpholino injection ............................................................................... 73
Figures and tables (supplementary) .......................................................... 74
References (supplementary) .................................................................... 84
CHAPTER 3 - Smad6 Influences Dorsoventral Patterning by Inhibiting BMP Activity in
Zebrasish .................................................................................................. 85
Abstract .................................................................................................. 85
Introduction ............................................................................................. 85
Results ..................................................................................................... 87
Clone and sequence analysis of zebrafish Smad6.1 and Smad6.2 ............... 87
Comparison of spatial expression patterns of smad6.1 and smad6.2 in early development. 88
Expression of smad6.1 and smad6.2 is regulated by BMP signaling during gastrulation.... 88
Overexpression of smad6.1 and smad6.2 inhibits BMP signaling ............................... 90
Inhibition of smad6.1 and smad6.2 impacts dorsoventral patterning ...................... 90
Discussion.................................................................................................................. 92
Materials and methods .......................................................................................... 93
   Cloning of smad6.1 and smad6.2 ....................................................................... 93
   Phylogenetic analysis ......................................................................................... 94
   In situ hybridization and antibody staining .................................................... 94
   Morpholino and RNA injection ........................................................................ 95
Figures and Tables ................................................................................................. 96
References ............................................................................................................... 111
Appendix A - strabismus and prickle interact with Noncanonical Wnt and BMP Signaling to
   Affect the Secondary Tail Formation in Zebrafish............................................. 114
Abstract .................................................................................................................. 114
Introduction ............................................................................................................ 114
Material and Methods ......................................................................................... 116
Results and Discussion ....................................................................................... 116
Figures and Tables ................................................................................................. 118
References ............................................................................................................... 122
List of Figures

Figure 1.1 Tail development in zebrafish ................................................................. 17
Figure 1.2 BMP signaling in zebrafish ................................................................. 18
Figure 1.3 Models of Planar Cell Polarity (PCP) and noncanonical Wnt signaling pathways ... 20
Figure 2.1 Mesoderm tissues are mis-specified in the secondary tails of mfn ...................... 53
Figure 2.2 Secondary tail formation is independent of the role of BMP signaling in fate patterning ................................................................. 54
Figure 2.3 Noncanonical Wnt signaling functions with BMP in inhibiting secondary tail formation .................................................................................. 56
Figure 2.4 Bifurcation of the chordoneural hinge (CNH) in BMP and noncanonical Wnt mutants during tail outgrowth ..................................................................... 58
Figure 2.5 N-cadherin synergizes with BMP and noncanonical Wnt signaling to prevent secondary tail formation ............................................................. 60
Figure 2.6 Defects of CNH in mfn and kny initiate during tailbud protrusion ......................... 62
Figure 2.7 BMP regulates posterior mesoderm cells of tailbud to inhibit secondary tail formation .................................................................................. 64
Figure 2.8 A model for the roles of BMP and noncanonical Wnt signaling in regulating tail development .................................................................................. 65
Figure 3.1 Clone and sequence analysis of Smad6.1 and Smad6.2 ..................................... 96
Figure 3.2 smad6.1 and smad6.2 show redundant and unique domains of expression during development .................................................................................. 103
Figure 3.3 Decreased and increased expression of smad6.1 in response to down and up regulated BMP signaling at early gastrula stage .................................................. 105
Figure 3.4 Overexpressing smad6.1 and smad6.2 dorsalizes embryo by inhibiting BMP signaling .................................................................................. 107
Figure 3.5 Inhibiting smad6.1 and smad6.2 ventralizes embryo as increasing BMP signaling. 108
Figure A.1 ............................................................................................................ 118
Figure A.2 ............................................................................................................ 120
List of Tables

Table 2.1 Ectopic tail tissues produced in mini fin mutants .................................................. 66
Table 2.2 Ectopic tail tissues produced in knypek mutants.................................................. 67
Table 3.1 Percentage of embryos showing changes of designated markers after injection of RNAs.............................................................................................................. 109
Table 3.2 Percentage of embryos showing changes of designated markers after injection of morpholinos. ............................................................................................................ 110
Acknowledgements

I give my most sincere thanks and gratefulness to Dr. Chris Thorpe for leading me into the wonderland of zebrafish research. His hand-to-hand training and word-by-word education prepared me for the future academic career. My achievements cannot be fulfilled without other professors’ efforts. My committee members: Dr. Michael Herman, Dr. Alexander Beeser and Dr. Anna Zolkiewska kept giving me advices and providing very insightful suggestions on my research projects. Also I feel very honored to have Dr. Susan Brown and Dr. Richard Todd in my defense panel.

As an international student, I would like to give my hugs to people I worked with, especially Susan Hettenbach and Katelyn O’Neill, who taught me a lot in languages and cultures and gave me the happiest moments at work.

Thanks to Division of Biology for taking good care of my study life and MCDB program for creating harmonious academic atmosphere.

Last but not least, I thank my parents and friends who are very supportive all these years.
Dedication

I dedicate this work to my wife Yang, Chun-hui. She is one of the major reasons that make me want to pursue a Ph.D. and her blesses from across the Pacific Ocean helped me through all those hard times.
CHAPTER 1 - Introduction

Developmental biology is the study of the process by which organisms grow and develop, and it is originated from classical experimental embryology. Since the beginning of the 20th century, through extensive microsurgical experiments on embryos of frogs and sea urchins, people started to believe that chemical signals induce embryonic development, but it was not until the mid-1890s the first signals (fibroblast growth factors and activins) were actually identified (Slack, 2001). With the advent of molecular biology and genetics, it is now possible for scientists to identify these previously mysterious inducing factors and the genes regulated by them.

Inducing factors are mainly proteins secreted by signaling cells that exert effects on target cells via binding to specific receptors and activating signal transduction pathways within the cell, which eventually cause a series of cellular responses such as proliferation, migration and differentiation. By comparing development of diverse species, scientists have identified many important inducing factors that are widely used by most animals during embryogenesis.

The conserved inducing factors include Bone Morphogenic Protein (BMP), Fibroblast Growth Factor (FGF), Hedgehog (a polypeptide ligand), Nodal (a subset of proteins in the transforming growth factor beta superfamily) and Wnt (a glycoprotein named by combining of Wg/wingless in fly and Int-1 in mouse). These signaling-molecule-activated pathways control the formation of body plans as well as cellular organization from the zygote to a complete organism. Cell fate specification and morphological changes (by cell movements) are two concurrent processes in body plan formation. Recently, growing evidence indicates that many signaling pathways, such as BMP, Nodal and FGF instruct cell fate and also regulate cell movements (Heisenberg and Solnica-Krezel, 2008), which supports the idea that cell fate and cell movement are intrinsically connected (Ip and Gridley, 2002; Myers et al., 2002a; Myers et al., 2002b). However, it is still unclear to what extent these two seemingly distinct processes are mechanistically linked. How does a cell behave in the context of multiple signaling inputs, is cell fate pre-determined before its migratory behavior, or is migration required for cell fate acquisition?
Over the last three decades, *Danio rerio*, commonly called the zebrafish, has become an ideal model system for the study of vertebrate development. The zebrafish embryo is fertilized and develops in ex utero in optically transparent embryos, which makes the manipulation and visualization of embryos very easy and cost-efficient compared to the efforts to study mouse embryos. The three-month generation time and high fecundity (hundreds of embryos from one pair can be obtained weekly) make zebrafish optimal to perform genetic tests and produce statistically significant results. Large-scale random mutant screening of zebrafish in the 1990s identified sets of mutations responsible for a series of patterning defect phenotypes during embryonic development (Driever et al., 1996; Haffter et al., 1996). With morpholino MO anti-sense oligonucleotide induced gene silencing and the completed genome sequence, reverse genetics can be performed in zebrafish by analyzing the loss-of-function phenotypes and biological functions of any genes involved in early development (Nasevicius and Ekker, 2000).

GFP (green fluorescent protein) transgenic reporter is a very useful modern molecular tool widely used in zebrafish. Briefly, the GFP gene is fused downstream of a tissue-specific promoter and this construct is then inserted into the genome of zebrafish to generate a transgenic line (Long et al., 1997). The in vivo tissue-specific expression of GFP in optically transparent zebrafish embryos can highlight the cells within a particular region, which allows for the real time imaging of cells undergoing morphogenetic movement.

**Tail development in zebrafish**

In vertebrates, posterior body (posterior trunk and tail) growth is a central event of body plan formation, which requires properly coordinated cell fate specification and movements to ensure normal development. Generally, tail development involves the addition of segmented muscle tissue (somites) and growth of the spinal cord that extends between somites along the midline of the body axis. In chordates, the post-anal tail is one of the definitive features, which is different from the tail-like appendages in invertebrates. The internal composition of a tail--with neural tube, notochord and muscle--is the same as that of the main body axis, so tail formation is expected to share similar molecular mechanisms underlying pattern formation, morphogenesis, cell determination and differentiation with that of the vertebrate body plan as a whole. Humans also have a tail during embryogenesis but it is a vestigial structure that transiently exists during the first two months of development (Fuhrmann, 1965).
Formation of the tailbud

After fertilization, the zebrafish zygote multiplies by mitotic cell division during the first 3 hours, which results in a cell mass in the animal pole region (developmental stages are thoroughly described in Kimmel et al., 1995). Then, this clump of cells becomes motile and thins in a sheet of cells called the blastoderm that moves towards the vegetal pole to envelope the yolk in a process is called epiboly. Stages of zebrafish development are identified as percentage of epiboly, depending on how much of the yolk has been covered. At 50% epiboly (5.5 hpf), cells at the leading edge of the migrating blastoderm, also called the margin, begin to involute. This process, called gastrulation, generates three distinct germ layers, endoderm, mesoderm and ectoderm (Fig. 1.1A). Beginning during early stages of gastrulation, a series of morphogenic movements known as ‘convergence and extension’ (CE) start to elongate the body axis from the original oval shape and consequently push the tail away from the head (Keller, 2002). Laterally positioned cells migrate toward the dorsal midline (dorsal convergence). When they reach the dorsal midline at late gastrulation, cells insert into the presumptive notochord region and get rearranged by changing their neighbors (mediolateral intercalation), which consequently narrows the body axis and contributes to anteriorposterior extension (Wallingford et al., 2002). (Fig. 1.1A). Epiboly finishes as blastoderm marginal cells come together at the vegetal pole to form a bud-like structure at 11-12 hpf (Fig. 1.1B).

Fate mapping evidence demonstrates this bud-like cell aggregate is capable of giving rise to the entire posterior body including tail, so it is called the tailbud (Kanki and Ho, 1997; Agathon et al., 2003) (Fig. 1.1C). Formation of the tailbud is generally considered the end of gastrulation and the starting point of tail development. This group of cells gradually differentiates into the complete posterior body (posterior trunk and tail) over the next 12 hours (Kanki and Ho, 1997) (Fig. 1.1A-C). Transplantation experiments in zebrafish embryos show that the tailbud is not homogenous. The anterior tailbud, derived from dorsal mesoderm, is primarily patterned into axial structures like the notochord, while the posterior tailbud, derived from ventral mesoderm, gets patterned into paraxial tissues like tail somites (Agathon et al., 2003).
Tail development

During post-gastrula stages, tail development is divided into several steps: extension (12-16 hpf, when the tailbud extends along the surface of yolk), protrusion (16-18 hpf, when the tailbud begins to leave yolk) and eversion (>18 hpf, when the tailbud protrudes out away from the yolk and the tail starts to emerge) (Kanki and Ho, 1997). During these steps, the gastrula-like convergence and extension movements continue to shape the tail. As the tailbud extends, paraxial tissues converge towards the midline to facilitate the posteriorward extension of the body (Fig. 1.1D). Meanwhile, there are unique tailbud movements: the posterior tailbud cells diverge away from the midline by moving laterally and anteriorly and subduct underneath the posteriorly migrating anterior tailbud cells (Fig. 1.1D).

As in many teleosts, a transiently ciliated organ called Kupffer’s vesicle is found in the zebrafish tailbud (Essner et al., 2005). It originates from the dorsal forerunner cells during gastrulation and is involved in the establishment of left and right symmetry (Essner et al., 2005). The Kupffer’s vesicle is located between the yolk and tailbud during the extension stage, and gradually incorporates into the tailbud and eventually disappears during tail eversion. Since physical ablation of Kupffer’s vesicle or perturbation of its formation by morpholinos result in a normal tail (Essner et al., 2005), whether Kupffer’s vesicle plays a role in tail development is still unknown, but it is normally treated as a morphological landmark that marks the interface between the anterior and posterior tailbud (Fig. 1.1B, inset).

Within the developing tailbud, not all cells are determined. A minority of dorsal derived cells lying posterior to the notochord, termed the chordoneural hinge (CNH), are suggested to be a pluripotent stem cell-like population (Cambray and Wilson, 2002; Cambray and Wilson, 2007). In *Xenopus*, CNH cells contribute to notochord, neurons and somites and are capable of producing ectopic tails when grafted to host embryos (Charrier et al., 1999). In mice, in addition to giving rise to equivalent tissue types as in *Xenopus*, CNH cells contribute to the tailbud mesoderm and retain the self-renewable ability(Cambray and Wilson, 2002). In zebrafish, CNH can differentiate into notochord, hypochord and ventral neural tube (Agathon et al., 2003).

The BMP signaling pathway in zebrafish

The BMP signaling pathway in vertebrates has been demonstrated to have a crucial role in trunk and tail formation (reviewed in von Bubnoff and Cho, 2001) (see Fig. 1.2E for
schematic BMP pathway). BMP proteins are members of the TGFβ superfamily, and dimerized BMP ligands (BMP2/4/7) bind to type I and type II transmembrane receptors of activin receptor-like kinase (Alk) family, which result in the phosphorylation of type I receptors (von Bubnoff and Cho, 2001). Activated type I receptors phosphorylate downstream effectors r-Smads (Smad1/5/8) to enable their association with co-Smad (Smad4). The complex of r-Smads and Smad4 shuttles into the nucleus to initiate transcription of targets. BMP signaling activity is regulated by a myriad of inhibitory proteins such as extracellular antagonists: Chordin, Noggin and Follistatin as well as intracellular inhibitory Smads (I-Smads): Smad6 and Smad7. Excessive Chordin is degraded by metalloproteinase, Tolloid (Little and Mullins, 2006).

**BMP signaling in fate specification**

In zebrafish, during the early blastula stage, *bmp2b* and *bmp7* expression is initially ubiquitous (Kishimoto et al., 1997; Dick et al., 2000) (Fig. 1.2A), but then becomes extinguished from the dorsal side (Fig. 1.2B). During this stage, BMP starts to define the presumptive ventral side by inducing the ventrolateral expression of transcription factor genes *vox*, *vent* and *ved* (Imai et al., 2001; Shimizu et al., 2002). BMP is suppressed dorsally by *bozozok*, a transcriptional repressor that directly inhibits expression of *bmps* (Fekany et al., 1999) (Fig. 1.2B). The initial ventral and dorsal domains are defined through the establishment of BMP-positive and BMP-negative regions during the blastula stage.

During gastrulation, BMP act as a morphogen to pattern DV fates (reviewed in Little and Mullins, 2006). According to this model, a gradient of BMP activity is established along the DV axis due to interaction of BMP agonists and antagonists. Ventral and ventrolateral mesoderm that receives high and intermediate BMP signaling adopts the fates of blood, vasculature, pronephros and tail somites, while low BMP activity specifies dorsal fates such as notochord and trunk somites (Fig. 1.2D). Loss of BMP signaling causes dorsalized phenotypes with expansion of dorsal fates at the expense of ventral fates (Fig. 1.2F).

BMP signaling also patterns ectoderm in a gradient. A high level of BMPs is required for the specification of epidermis; an intermediate level of BMPs is required for the specification of neural crest and BMPs need to be inhibited to promote the fates of neurectoderm (Fig. 1.2D). Inhibiting BMPs by inducing overexpression of *hsp70:chd* (*chordin/chd* under control of heatshock inducible promoter *hsp70*) at 30 minutes progression from early to late gastrulation (4
hpf-6.5 hpf) reveals BMP signaling progressively patterns ventrolateral ectodermal tissues along the anterior-posterior axis with more anterior cells being patterned prior to posterior ones (Tucker et al., 2008).

This BMP morphogen model in the gastrula is supported by a series of mutants that are defective in BMP signaling. swirl/bmp2b mutants represent the most severe loss of BMP signaling, in which derived trunk somites (specified on the dorsal side of embryo during gastrulation) are expanded, ventrally derived blood, vasculature and pronephros are reduced and the tail is literally absent (Kishimoto et al., 1997) (Fig. 1.2F C5). Other mutants that are defective in components of the BMP pathway, including snailhouse/bmp7 (Dick et al., 2000) (Fig. 1.2F C4), piggytail/smad5 (Kramer et al., 2002) (Fig. 1.2F C3), lost-a-fin/alk8 (Mintzer et al., 2001) (Fig. 1.2F C2) and minifin/tolloid (Connors et al., 1999) (Fig. 1.2F C1), exhibit progressively less severe phenotypes than swirl. In minifin embryos, which are defective in the BMP agonist Tolloid, which promotes BMP signaling by degrading Chordin (an inhibitor that directly binds to BMP ligands), tail structures are largely complete, except for reduced ventral tail fin and vascular structures (Connors et al., 1999). In contrast, chordino/chordin mutants with excess BMP activity, show reduced notochord with fused tail somites and extra blood, kidney, vasculature and tail fins (Schulte-Merker et al., 1997) (Fig. 1.2F V1).

In zebrafish, the tail organizer is a group of cell from the ventral margin that, when transplanted to a different region of the embryo, can direct the formation of an extensive tail, lacking only notochord (Agathon et al., 2003). Ectopic expression of BMP, together with Wnt and Nodal, in blastula cells is capable of specifying a new tail organizer (Agathon et al., 2003). Transplantation experiments suggest ventral marginal cells from the blastula and early gastrula have the more potential to induce tail structure than donor cells from later stages (Agathon et al., 2003). These results suggest BMP signaling starts to exert its effect in tail development from the blastula stage, almost ten hours before the emergence of the tailbud itself.

Inhibition of BMP signaling by using the BMP-specific small-molecule inhibitor Dorsomorphin (Yu et al., 2008) or transgenic zebrafish lines carrying a heat-shock inducible dominant-negative truncated BMP receptor (dnBMPR) (Pyati et al., 2005) or Chordin (hsp70:chd) (Tucker et al., 2008), reveals the temporal roles of BMP signaling in specifying ventral mesoderm tissues. Inhibiting BMP signaling in embryos at an early gastrula stage phenocopies swirl and snailhouse mutants, confirming an early role for BMP signaling in
specifying primary tail tissue. From late gastrula to early somitogenesis stage, inhibiting BMP phenocopies those weakly dorsalized mutants such as piggytail, lost-a-fin, minifin and recently characterized bmp4 mutants (Stickney et al., 2007) that commonly have reduced tail fin and cloaca (opening of gut and kidney). Taken together, the role of BMP signaling during early development is to pattern ventral derivatives and specify a primary tail, while the late phase BMP signaling patterns ventroposterior fates like the ventral tail fin and cloaca.

**Secondary tail formation in BMP-deficient embryos**

Interestingly, inhibiting BMP signaling at post-gastrula stages in dnBMPR (transgenic zebrafish lines carrying a heat-shock inducible dominant-negative truncated BMP receptor) embryos sometimes results in the formation of a short secondary tail, a phenotype also observed in some minifin zygotes (Pyati et al., 2005). The secondary tail always emerges from the ventral side of the primary tail and is smaller in size, which makes the end of the tail looks bifurcated (Fig. 1.2F arrow). It has been shown that secondary tails can have many of the same tissues as primary tails, including tailbud cells, somatic tissue, notochord, hypochord, epidermis and pigment (Pyati et al., 2005). So far, there are two distinct models that have been proposed to explain these phenotypes. One model proposes that BMP signaling functions to prevent secondary tail formation independently from its fate-patterning role in the tailbud (Pyati et al., 2005). The other model proposes that BMP continues to act as morphogen after gastrulation and that a secondary tail is formed as a result of expanded mesoderm fates (Stickney et al., 2007). More specifically, the morphogen model predicts that a high level of BMP signaling is required for the ventral tail fin and cloaca fates and that an intermediate level of BMP signaling is required for the tail mesoderm and blood fates. When BMP signaling is slightly reduced, ventral tail fin and cloaca-fated cells fall into the intermediate level range of BMP signaling and acquire the fates of tail mesoderm and blood cells. This expansion of tail mesoderm accounts for the emergence of a secondary tail. When BMP signaling is reduced below the intermediate level, tail mesoderm and blood fates are reduced, which results in a shortening of the primary tail, thus there are no mesodermal cells that could be patterned to form a secondary tail. However, the later model is not fully supported by the experimental results. Several experiments suggest that the extreme ventral mesoderm is mis-fated into blood and vascular progenitors from ventral tail fin and cloaca only when BMP signaling is inhibited within a short time frame at mid-gastrula stage.
This does not explain the secondary tail formed when BMP is inhibited at later stages. The model is weakened by the observation that bmp4 mutants that show the expected expansion of somites and blood do not develop secondary tails (Stickney et al., 2007). So far, why and how BMP-deficient embryos produce secondary tails is still unclear.

**BMP signaling in cell movements**

In other systems, BMP has been suggested to be involved in cell movement regulation. For example, in the mouse embryo, bmp4 negatively regulates ectoderm migration during lung bud morphogenesis (Weaver et al., 2000). In zebrafish, BMP signaling has also been demonstrated to play a role in the regulation of cell movements. A BMP gradient appears to regulate cell movements during gastrulation. Cell-tracking experiments indicate that gastrula cells along the DV axis can be divided into three domains based on their convergence (mediolateral narrowing) and extension (anterioposterior elongation) (CE) movements (Myers et al., 2002a). On the ventral side, high levels of BMP signaling create a no-convergence-no-extension domain where cells move directly to the vegetal pole to participate in tailbud formation. Lower BMP activity promotes the CE movements of lateral gastrula cells by polarizing them along the mediolateral axis, where they actively translocate to the dorsal side with increasing speed. In the dorsal domain, where BMP activity is lowest, cells exhibit strong extension but only modest convergence movements as a result of intercalation of dorsolateral cells. BMP has been suggested to affect cell movements in parallel to cell fate specification by suppressing the expression of wnt11 and wnt5a, two genes involved in a noncanonical Wnt pathway that specifically regulates CE movements without impacting cell fate (see below for more detailed description of the noncanonical Wnt pathway) (Myers et al., 2002a). However, a recent study has shown that the migratory behavior of gastrula cells is able to respond to BMP independently of wnt5a or wnt11 (von der Hardt et al., 2007). Moreover, during gastrulation, BMP negatively regulates Cadherin-mediated cell-cell adhesion (for a more detailed description, see ‘Cadherin’ section, below) (von der Hardt et al., 2007). Along the DV axis of gastrula-stage embryos, cell adhesion is lowest on the ventral side and highest on the dorsal side, which opposes the BMP gradient. This adhesion between cells is important for lamellipodia-driven directional migration of mesodermal progenitors (Bakkers et al., 2004). As lateral cells converge to the dorsal midline with increasing speed, they require higher adhesion with neighboring cells.
in order to exchange places and move. The ventral cells do not converge, as there is little adhesion between these cells. This BMP-mediated adhesion gradient provides an explanation to the three CE domains described earlier. More importantly, this mechanism does not alter cell fates, which indicates that BMP signaling has separable roles in cell fate specification and morphogenesis regulation (von der Hardt et al., 2007). However, the current studies only address the role of BMP signaling during gastrulation. Whether BMP signaling continues to regulate cell movements after gastrulation is largely unknown.

**The noncanonical Wnt signaling pathway**

Over the past two decades, an abundance of evidence from multiple experimental systems has demonstrated a conserved role for the noncanonical Wnt signaling pathway (also known as β-catenin independent Wnt signaling) in the regulation of cell polarity and cell movements (reviewed in Veeman et al., 2003a). Unlike so-called canonical Wnts, noncanonical Wnt ligands such as Wnt5 and Wnt11 transduce signals without stabilization of β-catenin (Moon et al., 1993; Du et al., 1995). In vertebrates, a major function of noncanonical Wnt signaling is to regulate CE movements without affecting cell fates. The noncanonical Wnt pathway is also referred to as the Wnt/PCP pathway, because it governs CE movements in vertebrates in a mechanism similar to planar cell polarity (PCP) pathway in the fruit fly, *Drosophila melanogaster* (Veeman et al., 2003a). In vertebrates, the mesenchymal cells undergoing CE movements need to be polarized to move collectively, just as the epithelial cells in the wing and eye of *Drosophila* need polarity to be arrayed consistently (Keller, 2002). Many of the components involved in the PCP pathway in *Drosophila* have been shown to function in noncanonical Wnt pathways in vertebrates.

**The PCP pathway in Drosophila**

In *Drosophila*, the PCP pathway has a clear requirement in the polarity of wing hair and ommatidia (reviewed in Adler, 2002). Upon disruption of PCP signaling, distally-pointing wing hairs become mis-oriented and ommatidia become disorganized. The establishment of cellular polarity is thought to require the asymmetric subcellular localization of the core PCP factors, including the transmembrane proteins Frizzled (Fz) and Strabismus (Stbm), the cytoplasmic protein Dishevelled (abbreviated as Dsh in *Drosophila* and *Xenopus*; Dvl in mouse and
zebrafish) and Prickle (Pk). Pk and Dsh are recruited to the cell membrane in response to active PCP signaling. As modeled in the wing epithelia cell (Fig. 1.3A), upon activation of the PCP pathway, Fz-Dsh complexes become anchored at the distal surface, while Stbm-Pk complexes become localized at the opposite side of the cell, at the proximal surface. It has been suggested that this asymmetry leads to polarity establishment via the rearrangement of cytoskeletal components (Harris and Peifer, 2007; Kim et al., 2010). Genetic studies so far find these opposing components of the PCP pathway genetically antagonize each other. For instance, in the Drosophila wing, the overexpression phenotype of stbm resembles the loss-of-function phenotype of fz, and the converse is also true (Adler et al., 1997; Bastock et al., 2003). In addition, a loss of stbm is able to suppress the fz mutant phenotype (Wu and Mlodzik, 2008). A generally accepted model suggests that Fz promotes the Dsh-dependent activation of a downstream cascade, while the Stbm-Pk complex functions to inhibit Dsh activation (Tree et al., 2002; Jenny et al., 2005).

**The noncanonical Wnt pathway in zebrafish**

In zebrafish, CE movements involve complex polarized behaviors including directional migration (i.e., dorsal convergence), orientation of cell division, cell-shape changes and cell rearrangement (intercalation of cells at the midline, for example) (reviewed in Wallingford et al., 2002). Zebrafish silberblick/slb/wnt11 (Ulrich et al., 2005) and pipetail/ppt/wnt5 (Kilian et al., 2003) are two mutants that affect noncanonical Wnt genes and show morphogenic defects in CE movements. Phenotypically, these mutant embryos have a shortened body axis, thinner and wider somites and occasional cyclopia. This is quite different from the phenotype of canonical Wnt mutants, wnt3 and wnt8, which affect cell fate specification, particularly in the posterior (Thorpe et al., 2005).

The first evidence that correlates noncanonical Wnt signaling to PCP signaling came from the studies of the Dsh protein (reviewed in Wallingford and Habas, 2005). In Drosophila, Dsh is a central protein involved both in canonical Wnt and PCP signaling (Axelrod et al., 1998; Boutros et al., 1998). Deletion constructs of Dsh show that three separate functional domains (DIX, PDZ and DEP domains) are involved in different signaling pathways: for canonical Wnt signaling the DIX and PDZ domains are used and for PCP signaling the PDZ and DEP domains are used (Axelrod et al., 1998; Boutros et al., 1998; Moriguchi et al., 1999). Overexpressing Dsh
variant (Dsh-DEP+: DEP domain only) that blocks the PCP pathway in the fly could also disrupt CE movements in frog and zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000). Correspondingly, overexpressing a variant Dsh (DshΔDIX: DIX domain truncated) that blocks the canonical Wnt pathway in the fly could rescue wnt11 mutants in frog and zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000). With the identification of more vertebrate homologs of PCP pathway components, the conserved Wnt/PCP pathway becomes recognized, including the core components of the PCP pathway such as Pk, Fz, Stbm and Dsh/Dvl. It is worth noting that the noncanonical Wnt pathway in vertebrates involves PCP-exclusive Wnt ligands that have yet to be identified in the Drosophila PCP pathway. In zebrafish, the trilobite(tri)/stbm (Park and Moon, 2002), knypek(kny)/glypican4 (a gene encoding a putative co-receptor for noncanonical Wnt proteins) mutants (Topczewski et al., 2001), together with pk (Carreira-Barbosa et al., 2003; Veeman et al., 2003b) and dvl2/3 morphants (Angers et al., 2006) all exhibit CE defects without obviously affected cell fates. Morpholino-induced loss-of-function studies in zebrafish embryos suggest that dvl2 and dvl3 are exclusively involved in the noncanonical Wnt pathway (Angers et al., 2006). In the epithelial wing cells of the fly, the asymmetric distribution of core PCP factors is well characterized, but whether this is consistent in the highly motile vertebrate cells is just beginning to be investigated.

Several experiments provide evidence suggesting that the mechanism underlying the Wnt/PCP pathway might be conserved between fly and fish. In zebrafish embryo, GFP-tagged Pk and Dsh do become asymmetrically localized in migrating cells (Yin et al., 2008). Pk-GFP is preferentially localized anteriorly on the membrane of axial and presomitic mesoderm cells that are undergoing a cell rearrangement called mediolateral intercalation (Yin et al., 2008). Further, this localization is dependent on the functions of kny, stbm and dvl (Yin et al., 2008). In kny;tri double mutants, and in embryos overexpressing dominant-negative Xenopus Dsh, the anterior localization of Pk is abolished and become more cytoplasmic, accompanied by defective cell intercalation (Yin et al., 2008). Moreover, in axial mesoderm, Dsh-GFP is enriched along the posterior membrane, which is mediated by kny and tri (Yin et al., 2008). In kny and tri mutants, Dsh becomes more cytoplasmic and in the kny;tri double mutant the posterior localized Dsh is nearly absent (Yin et al., 2008). This suggests Dsh and Pk have opposite localization in migrating gastrula cells of the zebrafish embryo. The results are consistent with the protein interaction model in the fly wing that suggests that Dsh and Pk become localized to opposite
sides of the cell. The results also suggest kny functions mutually with stbm to regulate CE movements. In zebrafish, serial double-mutant, double-morpholino, and morpholino/mutant experiments with following genes: slb;ppt (Kilian et al., 2003), pk1 and stbm (Carreira-Barbosa et al., 2003; Veeman et al., 2003b), pk1 and slb (Carreira-Barbosa et al., 2003) and pk1 and ppt (Carreira-Barbosa et al., 2003) all show a more severe CE phenotype.

In searching for the mechanism underlying noncanonical Wnt signaling, three downstream cascades have been proposed. One pathway downstream of Dsh leads to the activation of small GTPases of the Rho family, including Rho, Rac and Cdc42 (Habas et al., 2001; Choi and Han, 2002; Habas et al., 2003; Penzo-Mendez et al., 2003). Overexpression of these GTPases in Xenopus causes a CE phenotype. In zebrafish, Rho-bridging protein Daam1 (Kida et al., 2007) and Rho kinase (ROK2) (Marlow et al., 2002) have been show to affect CE movements in downstream of noncanonical Wnt signaling. Another potential target of noncanonical Wnt signaling is the c-Jun N-terminal kinases, JNK pathway. In the fly, blocking the JNK pathway suppresses the Dsh overexpression phenotype (Boutros et al., 1998). In vertebrate cells, Dvl is also shown to activate JNK signaling (Boutros et al., 1998; Li et al., 1999; Moriguchi et al., 1999). The third noncanonical Wnt pathway involves calcium signaling. As shown in zebrafish embryos, overexpression of Wnt5a or Pk1 at the blastula stage stimulates calcium flux (rapid, aperiodic transient increase of intracellular free Ca\(^{2+}\)) (Slusarski et al., 1997). In Xenopus, overexpression of Wnt5 or Wnt11 activates two calcium-dependent kinases, PKC and CamKII (Sheldahl et al., 1999; Kuhl et al., 2000). These are potential targets downstream of noncanonical Wnt signaling that could affect CE movements. However, these branches of noncanonical Wnt signaling still require further characterization and validation.

Most of the current studies mainly concern the roles of noncanonical Wnt signaling in the regulation of CE movements during gastrulation. Less is understood about the role of noncanonical Wnt signaling after gastrulation. From the study of ppt and kny, noncanonical Wnt signaling appears to regulate tail morphogenesis. The ppt and kny single mutants have shortened tails without loss of tail tissues (Marlow et al., 2004). A T-box transcription factor gene, ntl cooperates with ppt and kny to regulate CE-like movements within the tailbud, and to promote the tailbud-specific subduction movements (Marlow et al., 2004). Double mutants kny;ntl and ppt;ntl exhibit a synergistic tail shortening phenotype (Marlow et al., 2004). By cell labeling experiments, it was found that posterior tailbud cells in ppt and kny have a reduced rate of
extension and lateral divergent movements (Marlow et al., 2004). In kmy;ntl and ppt;ntl, the labeled cells are retained in the posterior tailbud, suggesting a total loss of movements (Marlow et al., 2004). It was further demonstrated that the shortened tail is due to cell movement defects but not fate mis-specification, reduced proliferation or increased apoptosis (Marlow et al., 2004). The function of other components of noncanonical Wnt signaling after gastrulation is still unclear. How this signaling is used in distinct contexts and developmental stages is one of the key questions that needs to be addressed in the future.

**Cadherin in zebrafish development**

Tissue morphogenesis during development requires cadherin-mediated cell-cell adhesion. Migrating cells rapidly de-adhere and re-adhere to neighboring cells during translocation, and this is achieved through dynamic disassembly and reassembly of adhesion complexes (Ulrich et al., 2005). The cadherin family is composed of classical cadherins, protocadherins and atypical cadherins (reviewed in Halbleib and Nelson, 2006). Generally, classical cadherins are characterized by an extracellular domain that forms Ca\(^{2+}\)-dependent homophilic interactions, a transmembrane domain and an intracellular domain that directly binds to β-catenin and p120. Protocadherins and atypical cadherins are distinguished from classical cadherins by their additional extracellular motifs and different cytoplasmic tails that interact with selective binding partners (Halbleib and Nelson, 2006).

In zebrafish, *flamingo*, a gene encoding an atypical cadherin, functions with *stbm* and *wnt11* to regulate CE movements during gastrulation (Formstone and Mason, 2005). Inhibiting *flamingo* suppresses the extension of the anterior-posterior body axis (Formstone and Mason, 2005; Carreira-Barbosa et al., 2009). A hanging drop aggregation assay (measuring adhesive properties of cells) shows that embryonic cells with compromised *flamingo* have different cohesive properties from wild type cells (Carreira-Barbosa et al., 2009). Zebrafish *paraxial protocadherin (papc)* encodes a protocadherin that is expressed in trunk mesoderm during gastrulation (Yamamoto et al., 1998). Blocking *papc* activity by expressing a dominant-negative secreted construct blocks proper dorsal convergence movements during gastrulation, indicating that *papc* is required for early morphogenetic movements in the embryo (Yamamoto et al., 1998).
Classical cadherins, including E-cadherin (epithelial) and N-cadherin (neuronal) are also required for mesodermal morphogenesis (Halbleib and Nelson, 2006). Zebrafish embryos carrying different mutated alleles of *e-cadherin* exhibit a series of movement defects during gastrulation, including arrested epiboly, defective intercalation and impaired overall CE movements (Kane et al., 2005). N-cadherin in zebrafish has a broad expression pattern during early development and N-cadherin deficient mutants have abnormal brain and tail morphogenesis (Lele et al., 2002). Moreover, a semi-dominant *N-cadherin* allele genetically interacts with *stbm* to mediate elongation of the posterior body (Warga and Kane, 2007).

Growing evidence has shown that distinct signaling pathways regulate cell movements by impinging on cadherin through a variety of mechanisms. During gastrulation, Wnt11 has been proposed to regulate the endocytosis of E-cadherin in migrating prechordal plate cells via the GTPase Rab5 (Ulrich et al., 2005). Wnt11-deficient cells were also shown to adhere more weakly to an E-cadherin coated substrate, suggesting that noncanonical Wnt signaling may regulate morphogenesis by promoting intercellular adhesion (Ulrich et al., 2005). As mentioned previously, BMP signaling guides gastrula cell movements by negatively regulating cadherin-dependent cell adhesion. Thus, a gradient of intercellular adhesion is established across the DV axis in response to graded BMP activity. Adhesion strength is highest dorsally, where BMP activity is lowest, and declines in more lateral and ventral regions, where BMP activity is higher. When mesodermal cells converge to the dorsal midline, they project lamellipodia in all directions. Lamellipodia that contact more dorsally positioned neighboring cells are more likely to form a stable contact with that cell (due to the higher adhesive properties) and pull the cell extending the lamellipodia in a dorsal direction. It will be interesting to see whether and how cadherin interacts with these signaling pathways to regulate tail morphogenesis after gastrulation.

**Summary**

Morphogenesis refers to the cell movements that shape the organism in three dimensions. This controls the organized spatial distribution of cells during embryonic development. How the cells move in response to the signals induced by multiple morphogenetic cues is not well understood. This dissertation focuses on the roles of intercellular signaling pathways underlying tail development and dorsoventral patterning in the zebrafish embryo. Chapter 2 describes our investigations into how BMP and noncanonical Wnt signaling function together to regulate tail
morphogenesis. The studies described in this chapter form the basis of a manuscript currently under consideration by the journal *Development*. The formation of secondary tails in BMP-compromised embryos has been observed for a long time, going back to the isolation of the first BMP pathway mutants, but no convincing explanation for their development has been put forward. We discovered that this phenotype in BMP-deficient embryos is primarily due to the mis-migration of a group of posterior mesoderm cells, not due to the production of excess tail mesoderm, as had been previously proposed (Stickney et al., 2007). BMP signaling has to be activated at the right time and right place to ensure normal tail morphogenesis. Additionally, we found that noncanonical Wnt signaling functions redundantly but independently with BMP signaling to prevent secondary tail formation. As in BMP-compromised embryos, the secondary tails are primarily composed of mesoderm tissues. Defective cell migration early in tail development results in a bifurcation of a population of pluripotent cells in the tailbud called the chordoneural hinge (CNH). Ectopic CNH cells subsequently give rise to secondary tails.

We also assessed the potential role of cadherin in tail morphogenesis. We find that noncanonical Wnt signaling regulates the localization of cadherin within the CNH, likely affecting the cohesion of this group of cells. We propose a model that during tail morphogenesis BMP signaling is activated in the posterior mesoderm of tailbud to ensure their proper migration and noncanonical Wnt signaling is required in the CNH region of tailbud to promote cadherin-dependent cell cohesiveness. This study demonstrates that BMP and noncanonical Wnt pathways are also required after gastrulation to control morphogenetic movements in the embryo.

In Chapter 3, we describe the characterization of two homologs of *smad6* (*smad6.1* and *smad6.2*), and *smad6.2* was identified in a yeast two hybrid screen for candidate proteins that bind to Spork (the ortholog of human ARHGAP21 in zebrafish). The rationale of this screening is that knocking down *spork* leads to secondary tail formation and we are looking for other Spork-associated proteins and study their roles in tail development. However, loss-of-function studies of the two *smad6* homologs do not show any obvious tail phenotype. From initial studies, we found that *smad6* functions as BMP signaling inhibitor to impact dorsoventral patterning during gastrulation, and that *smad6* expression is responsive to down- and up-regulated BMP activity. These results suggest that in zebrafish *smad6* functions as a feedback inhibitor of BMP signaling in order to fine tune BMP activity.
Lastly, we describe further genetic studies concerning secondary tail formation in the Appendix. We found that \textit{pk1} and \textit{stbm} genetically antagonize \textit{dvl} and \textit{kny} in secondary tail formation, similar to the inhibitory interactions between homologs of these genes observed in studies of \textit{Drosophila} PCP signaling. Unexpectedly, we observed that inhibition of \textit{pk1} suppressed secondary tail formation in \textit{mfn}, while inhibition of \textit{stbm} promoted secondary tail development in \textit{mfn}. The mechanisms behind these genetic interactions await further investigation. In addition to their well described roles in development, BMP and noncanonical Wnt signaling play a critical role in cancer progression and metastasis (reviewed in Jessen, 2009; Ye et al., 2009). As many tumors initiate with aberrant cell movements, secondary tail formation may also serve us a tumorigenesis model, so understanding the genetic regulation and identifying enhancers and suppressors involved in this process could give us insight into areas potentially impacting human health.
Figures and Tables

Figure 1.1 Tail development in zebrafish.

(A) Position and movement of ventral (red) and dorsal (blue) marginal cells at the shield stage. Green arrows indicate the involution at the margin. Yellow arrow indicates the convergence of lateral margin. Purple arrow indicates the epibolic movement.

(B) At the end of gastrulation, ventral (red) and dorsal (blue) marginal cells form tailbud. Tailbud is formed as shown in the inset. kv, Kupffer’s vesicle.

(C) At 24 h after fertilization, the dorsal margin contributes to axial structures (blue); ventral margin contributes to non-axial tissues (red).

(D) Dorsal view of schematic cell movements in tail development.

(A-C) is adapted from (Agathon et al., 2003). (D) is adapted from (Kanki and Ho, 1997).
**Figure 1.2 BMP signaling in zebrafish.**

(A-C) Schematic of BMP signaling in the zebrafish embryo. (A) At early blastula, genes encoding BMP ligands are expressed throughout the blastoderm. (B) BMP expression is subsequently eliminated in the dorsal domain by *bozozok*. (C) By the onset of gastrulation, an established BMP signaling gradient establishes three CE domains. A high level of BMP on the ventral side creates a no-convergence-no-extension domain where cells move directly to the vegetal pole. Lower BMP activity promotes the CE movements of lateral gastrula cells, which eventually converge on the dorsal side. In the dorsal domain, where BMP activity is lowest, cells exhibit strong extension.

(D) Low resolution fate map of the zebrafish embryo at the start of gastrulation.

(E) Model of the BMP signal transduction pathway. Extracellular BMP dimers bind to the type I and type II BMP receptor complexes at the cell membrane. The BMP type II receptor phosphorylates the type I receptor, which transduces the signal by phosphorylating an R-Smad protein. The phosphorylated R-Smad binds with a Co-Smad, and the complex is translocated into the nucleus, where it activates transcription of target genes. Regulators of BMP pathway include Tolloid, Chordin and I-Smads. P, phosphorylations.

(F) Morphologies of normal (N), dorsalized (C1–C5) and ventralized (V1) embryos. V1 and C1 embryos are weakly ventralized or dorsalized, respectively. Arrowhead indicates the secondary tail formed in part of the C1 embryos.

(A-D) is adapted from (Little and Mullins, 2006). (E-F) is adapted from (Kondo, 2007).
Figure 1.3 Models of Planar Cell Polarity (PCP) and noncanonical Wnt signaling pathways

(Adapted from Veeman et al., 2003a).

(A) The *Drosophila* PCP pathway includes Frizzled, Dishevelled, Rho, Rho Kinase, Flamingo, Strabismus, and Prickle. It is not a simple, linear pathway and is best appreciated in a spatial context. Frizzled, Dishevelled, and Rho, become localized specifically to the distal side, whereas Prickle and Strabismus become localized to the proximal side. The function of all of these proteins is required to ensure both their correct segregation into proximal and distal domains and the subsequent development of correct planar polarity.

(B) Vertebrate noncanonical Wnt signaling requires Frizzled receptors and the proteoglycan coreceptor Knypek. This pathway involves the cytoplasmic signal transduction protein Dishevelled. A main branch downstream of Dishevelled involves the small GTPases of the Rho family. Dishevelled activation of Rho requires the bridging molecule Daam1. The precise roles of Rho, Rac and Cdc42 remain unclear, as is the potential role of the JNK pathway. Dsh can also stimulate calcium flux and the activation of the calcium-sensitive kinases PKC and CamKII, suggesting a Wnt/calcium pathway. As in the *Drosophila* PCP pathway, Prickle and Strabismus appear to have some role in this process, but not in a linear or well-understood way.
Copyrights

Permissions of reusing figures of published journals in this dissertation were granted.

Figure 1 A-C was adapted from Agathon, A., Thisse, C. and Thisse, B. (2003). The molecular nature of the zebrafish tail organizer. *Nature* 424, 448-452. Permission from Nature Publishing Group was provided by Copyright Clearance Center.

Figure 1 D was reproduced/adapted with permission. Kanki, J. P. and Ho, R. K. (1997). The development of the posterior body in zebrafish. *Development* 124, 881-893.

Figure 2 A-D was adapted from Little, S. C. and Mullins, M. C. (2006). Extracellular modulation of BMP activity in patterning the dorsoventral axis. *Birth Defects Res C Embryo Today* 78, 224-242. This material was reproduced with permission of John Wiley & Sons, Inc.

Figure 2 E,F was adapted from Kondo, M. (2007). Bone morphogenetic proteins in the early development of zebrafish. *FEBS J* 274, 2960-2967. Permission from John Wiley and Sons was provided by Copyright Clearance Center.

Figure 3 was adapted from Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003a). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* 5, 367-377. Permission from Elsevier was provided by Copyright Clearance Center.
References


Adler, P. N., Krasnow, R. E. and Liu, J. (1997). Tissue polarity points from cells that have higher Frizzled levels towards cells that have lower Frizzled levels. Curr Biol 7, 940-949.


CHAPTER 2 - BMP and Noncanonical Wnt Signaling are Required to Inhibit Secondary Tail Formation in Zebrafish

Abstract

The role of Bone Morphogenetic Protein (BMP) signaling in specifying cell fate in the tailbud has been well-established. Among the lost ventral tissues like ventral tailfin and cloaca, embryos with compromised BMP signaling produce a curious phenotype—a ventrally located secondary tail containing both somitic muscle and notochord. This phenotype is proposed to be a fate-patterning defect when the BMP gradient is lowered to a precise level. However, this morphogen model is insufficient to explain secondary tail formation. BMP also regulates morphogenetic movements during gastrulation, promoting the convergence of lateral mesodermal cells towards the dorsal midline. Here, we provide evidence that BMP signaling continues to mediate cell movements during tail development. Our data indicate that BMP signaling is activated in the ventroposterior tailbud to promote cell migration during tailbud protrusion, and that it is the defective migration of these cells which ultimately leads to bifurcation of the chordoneural hinge (CNH) domain, a presumptive stem cell pool in the tailbud that results in formation of a secondary tail in BMP mutants. In parallel, the morphogenesis of tailbud cells is known to be under the control of noncanonical Wnt signaling, although the exact nature of the defect remains unclear. Additionally, we show that noncanonical Wnt signaling interacts with BMP signaling to maintain CNH integrity by affecting cadherin localization in CNH cells, possibly disrupting cell cohesion. We propose a model that BMP and a noncanonical Wnt pathway regulate tail morphogenesis by controlling cell migration and cell adhesion within the tailbud.

Introduction

The vertebrate tailbud is a mass of undifferentiated cells that gives rise to posterior tissues of the body during tail development. In zebrafish, the tailbud is derived from cells originating at both the dorsal and ventral margins, which come together at the vegetal pole of the embryo upon completion of epiboly (Kimmel et al., 1995). Ventrally-derived cells comprise the posterior portion of the tailbud, and are fated to develop into somitic muscle during tail
formation, while the dorsally-derived cells form the anterior tailbud and form primarily axial tissues such as notochord and floor plate (Kanki and Ho, 1997; Myers et al., 2002; Agathon et al., 2003).

Extensive genetic analyses have demonstrated roles for several conserved signaling pathways including the BMP, FGF, Nodal, and both the Wnt/β-catenin and noncanonical Wnt pathways in the specification, patterning, and morphogenesis of the tailbud and its derivatives (Schier and Talbot, 2005). Some of these pathways are required at distinct stages both for proper initial specification of the tailbud and for correct fate patterning of cells derived from it. For example, the BMP pathway is required (along with Wnt/β-catenin and Nodal signaling) during gastrulation for the formation of a tail organizer, located at the ventral margin (Agathon et al., 2003). In embryos completely lacking BMP activity during gastrulation, such as swirl(swr)/bmp2b mutants, tail development is essentially nonexistent (Mullins et al., 1996; Kishimoto et al., 1997). During gastrulation, BMP is thought to function as a morphogen, patterning cell fates along the dorsal-ventral (DV) axis, with higher BMP activity inducing the ventral-most fates, including tail mesoderm, intermediate levels specifying lateral fates such as trunk mesoderm, and with the absence of BMP activity allowing for the development of dorsal fates, such as the notochord (Little and Mullins, 2006). Later, during somitogenesis stages, BMP also acts to pattern cell fates derived from the tailbud (Pyati et al., 2005). Post-gastrula inhibition of BMP signaling using Dorsomorphin, a small molecule BMP inhibitor, or inducible overexpression of either a dominant-negative, truncated BMP receptor (dnBMPR) or the BMP antagonist Chordin, results in embryos showing reduced ventral tailfin and cloacal tissue (Pyati et al., 2005; Pyati et al., 2006; Tucker et al., 2008; Yu et al., 2008). Such embryos show a phenotype similar to that observed in embryos carrying mutations in several conserved components of the BMP pathway-bmp4, minifin(mfn)/tolloid, lost-a-fin(laf)/Alk8, as well as hypomorphic smad5 alleles (Connors et al., 1999; Mintzer et al., 2001; Kramer et al., 2002; Stickney et al., 2007). Based on phenotypic analysis of embryos carrying certain combinations of bmp4, bmp2, and bmp7 alleles, it has been suggested that a gradient of BMP signaling specifies distinct cell types in posterior tissues, with the highest levels of BMP activity being required for production of ventral tailfin and cloaca and a lower amount being sufficient for presomitic mesoderm and blood (Stickney et al., 2007). Further, bmp4 has recently been shown to regulate tail development by promoting proliferation of cells that reside in the chordoneural
hinge (CNH) (Esterberg et al., 2008), a population of cells at the caudal end of the notochord which has been shown in other vertebrates to act as a pool of progenitor cells that can give rise to multiple tail tissues, including notochord, floor plate, and somitic muscle (Charrier et al., 1999; Davis and Kirschner, 2000; Cambray and Wilson, 2002; Cambray and Wilson, 2007). Recent studies have shown that, independent of its role in fate patterning, BMP also regulates morphogenetic movements during gastrulation, promoting the convergence of lateral mesodermal cells towards the dorsal midline (Myers et al., 2002; von der Hardt et al., 2007). The BMP activity gradient is proposed to negatively regulate calcium-dependent cell adhesion, establishing distinct patterns of migratory behavior among mesodermal cells along the DV axis (von der Hardt et al., 2007). The ventral-most cells, exposed to the highest levels of BMP, exhibit very low levels of intercellular adhesion, and do not converge dorsally at all, but move vegetally to occupy the tailbud. In contrast, laterally positioned cells move preferentially towards regions of lower BMP/higher adhesion (dorsally). Presumably, simultaneous control of both cell fate patterning and of cell movements by BMP ensures the necessary tight coupling of these two essential processes. BMP’s role in promoting dorsalward migration of mesodermal cells is independent of the role of noncanonical Wnt signaling, with BMP being required predominantly for migration of ventrolateral mesoderm and noncanonical Wnts functioning in more dorsolateral regions of the gastrula.

Cell tracing experiments have shown that the anterior and posterior portions of the tailbud undergo distinct morphogenetic movements during tail outgrowth (Kanki and Ho, 1997). Cells in the anterior (dorsally derived) tailbud continue the convergence and extension (CE) movements observed during gastrulation, with presomitic and somitic mesoderm converging to the midline, driving the posteriorward extension of the embryo. In contrast, posterior (ventrally derived) tailbud cells move laterally away from the midline and subduct beneath the posteriorly migrating anterior tailbud cells.

Relatively little is known concerning the signaling pathways that regulate morphogenetic movements during tail development. However, it is known that some genes that regulate cell movements during gastrulation have also been shown to contribute to proper tail morphogenesis. For example, when noncanonical Wnt signaling is disrupted, as in pipetail(ppt)/wnt5 or knypek(kny)/glypican mutant embryos, posterior tailbud cells correctly undergo subduction and move laterally away from the midline, but tail extension is reduced, suggesting a requirement for
noncanonical Wnt signaling in promoting continued CE movements during tail development (Marlow et al., 2004). Further, in embryos deficient for both noncanonical Wnt signaling and the T-box transcription factor no tail (ntl), posterior tailbud cells fail to undergo subduction movements, and tail extension is completely absent (Marlow et al., 2004). Clearly, noncanonical Wnt signaling is essential for proper morphogenesis during tail development, although the exact nature of the defect remains unclear.

As described above, recent studies have shown that post-gastrula BMP signaling is required for patterning of ventral mesoderm in the developing tail. A subset of embryos with compromised BMP signaling also produce a ventrally located secondary tail containing both somitic muscle and notochord (Connors et al., 1999; Pyati et al., 2005; Stickney et al., 2007; Yu et al., 2008). It has been proposed that these secondary tails form as a result of a mis-specification of cell fates due to a change in the slope of the postulated gradient of BMP activity in the tail (Stickney et al., 2007). According to this hypothesis, a mild reduction in BMP activity could lead to a shallower slope of the gradient such that it would be insufficient to specify ventral tailfin, which requires the highest level of BMP signaling, but would expose more cells to the intermediate level of activity sufficient for formation of tail mesoderm, leading to an expansion of this tissue.

However, this model does not explain why the excess tail mesoderm would form such a morphologically distinct structure or why the secondary tails observed in BMP-compromised embryos include notochord, which is not hypothesized to be responsive to the BMP gradient. Also, while bmp4 mutant embryos show the loss of ventral tailfin and expansion of blood progenitors predicted by the gradient model, these embryos do not form secondary tails (Stickney et al., 2007). Further, in dnBMPR (transgenic line carrying dominant negative BMP receptor) embryos, when expression is induced at bud stage, the population of blood progenitors is not expanded, although approximately 15% of these embryos form secondary tails (Pyati et al., 2005). Taken together, these observations are inconsistent with the hypothesis that secondary tails form as a consequence of defects in fate patterning and raise questions about the BMP gradient model as it relates to secondary tail formation.

Earlier analysis of secondary tail formation in dnBMPR embryos had indicated that initial tailbud specification occurs normally, and that the secondary tail might be composed of cells that are inappropriately left behind by the tailbud during tail extension (Pyati et al., 2005). This
suggests that a defect in morphogenesis may be underlying secondary tail formation in BMP compromised embryos. To test this hypothesis, we undertook a detailed analysis of tail development in several BMP-defective backgrounds. We found that, contrary to the gradient hypothesis, secondary tails are formed in several BMP pathway mutants, irrespective of the severity of dorsalization observed. We further found that formation of secondary tails is presaged during early tail extension by bifurcation of the CNH, with the ectopic CNH cells contributing to the secondary tail. Time-lapse confocal videomicroscopy shows that BMP is required for anterior migration of ventral-posterior presomitic mesoderm that serves to lift the CNH away from the yolk. In the absence of this migration, the CNH remains closely associated with the yolk and gradually becomes bifurcated during tail elongation. We further demonstrate that noncanonical Wnt signaling functions in a parallel pathway to prevent secondary tail formation. We show that in kny embryos, the adhesion protein cadherin is mislocalized in CNH cells, suggesting that noncanonical Wnts may promote cohesion of CNH cells, in addition to their previously described role in CE movements.

**Materials and methods**

**Zebrafish strains**

Wild-type line AB was used. Mutant lines used were *mfn*tc263a (a point mutation, undescribed) (Connors et al., 1999), *kny*fr6 (a nonsense mutation results in a truncated protein) (Topczewski et al., 2001), *smad5*em169 (an insertional mutation) (Kramer et al., 2002), *smad5*hy40 (a missense mutation) (Kramer et al., 2002) and *snh*268a (a missense mutation leads to exchange from Val to Gly at amino acid position 130 in the proregion of Bmp7 protein) (Dick et al., 2000). *mfn/+;Tg(flh:EGFP)* and *kny/+;Tg(flh:EGFP)* strains were created by crossing *mfn* and *kny* heterozygotes to the *Tg(flh:EGFP)* transgenic line (Gamse et al., 2003).

**In situ hybridization and antibody staining**

Whole amount *in situ* hybridization was carried out using standard methods (Oxtoby and Jowett, 1993). The following probes were used: *col2a* (Yan et al., 1995), *myoD* (Weinberg et al., 1996), *neurog1* (Blader et al., 1997), *ntl* (Schulte-Merker et al., 1992), *gata1* (Detrich et al., 1995) and *flh* (Talbot et al., 1995). Fluorescent *in situ* hybridization (FISH) for *flh* expression was performed using a Fast Red color reaction (Hauptmann and Gerster, 1994), followed by an
incubation of 1:100 diluted P-Smad1/5/8 antibody (Cell Signaling Technology). β-catenin antibody (BD Transduction Laboratories) was applied at 1:400; pan-cadherin antibody (Sigma) was applied at 1:100; Tbx6 antibody (ZIRC) was applied at 1:1000. Appropriate Alexa Fluor conjugated (Molecular Probes) secondary antibodies were used.

**Morpholino and RNA injection**

Morpholino antisense oligonucleotides: (dvl1 MO: 5’-ATATGATTTTAGTCTCCGCCATGAG-3’) were purchased from Gene Tools. mfn, dvl2, dvl3 and cdh2 morpholinos have been previously described (Lele et al., 2002; Angers et al., 2006; Jasuja et al., 2006). The standard control morpholino by Gene Tools was used in some experiments. Morpholinos were diluted in Danieau’s buffer before injection. mfn MO was injected at a concentration of 1 mg/ml; concentrations of 0.5 mg/ml and 1.5 mg/ml were injected as low and high dose. dvl1, dvl2 and dvl3 MOs were injected at a sub-optimal concentration of 1 mg/ml each; a concentration of 1.5 mg/ml was injected as high dose; to fully inhibit dvl2 and dvl3, the concentration was 3 mg/ml. cdh2 MO was injected at a sub-optimal concentration of 0.1 mg/ml and a high concentration of 0.25 mg/ml was injected to achieve strong synergistic effects. Standard deviation of three replicated experiments was calculated by Excel. Synthetic mRNA of membrane-bound RFP (mRFP) was made from pCS2+ constructs with mMessage mMACHINE kit (Ambion) and injected at a concentration of 25 mg/ml. In all experiments, a volume of 3-5 nl was injected in the yolk of one-cell stage embryos.

**Time lapse confocal microscopy**

Tg(flh:EGFP), mfn;Tg(flh:EGFP) and kny;Tg(flh:EGFP) embryos were injected with mRFP RNA. To analyze tail morphogenesis, embryos were mounted at 10 somites in 0.7% low-melt agarose in Ringer’s solution. Agarose surrounding the posterior body was mechanically removed to ensure the free extension of the tailbud. Images were acquired using a Zeiss LSM 5 Pascal confocal microscope and a 20× lens every 1.5 min. Videos were re-aligned by ImageJ.

**Chemical treatment**

To inhibit BMP signaling, embryos were treated with Dorsomorphin (AMPK Inhibitor, Compound C, Calbiochem) as described with slight modifications (Yu et al., 2008).
Dorsomorphin resuspended in DMSO was dissolved in fish water at 60 µM. Embryos were manually dechorionated and reared in the solution for the duration of the experiment.
Results

*Mesoderm tissues produced in the secondary tail of mini fin*

To develop a clearer understanding of the origin of secondary tails in BMP-compromised embryos, we carefully analyzed the process of secondary tail formation in the *mfn/tolloid* mutant, which, at the time we began this study, was the only reported mutant to consistently produce secondary tails (Connors et al., 1999). To determine which tissues are located in the secondary tail domain, we examined the expression of the notochord marker *col2a*, muscle marker *myoD*, neural marker *neurog1* and the notochord/tailbud marker *ntl* by in situ hybridization. In *mfn* embryos at 26 hpf, ectopic *col2a* expression (Fig. 2.1A, B) was observed in 53% of the embryos and ectopic *myoD* expression (Fig. 2.1C, D) was observed in 30% of the embryos (Table 2.1). Consistent with previous studies, no embryo showed ectopic *neurog1* expression (Fig. 2.1E, F), indicating that secondary tails do not contain neural tissue (Pyati et al., 2005). Ectopic *ntl* expression (Fig. 2.1G, H) was detected in 64% of the embryos. These data suggest that different mesodermal tissues are not equally represented in the secondary tail. The majority of the secondary tails contain ectopic notochord tissue, while only a subset of them have ectopic somitic muscle.

*Secondary tail formation is not accompanied by changed mesoderm fates.*

The BMP morphogen model in tail development suggests that secondary tail formation only occurs at a modestly reduced level of BMP signaling in the tailbud, while stronger reduction of BMP activity results in reduced ventral mesoderm, with no secondary tails formed (Stickney et al., 2007). Secondary tail formation is proposed to occur only in embryos with a phenotype intermediate between C1 (losing partial ventral fin) and C2 (slightly twisted tail) (Mullins et al., 1996), which is included in the range of phenotypes typically seen in *mfn* embryos (Stickney et al., 2007). To test this model, we examined *ntl* expression in mutants that more significantly reduce BMP signaling- *piggytail (smad5)* and *snailhouse (bmp7)*. These mutants display a range of dorsalized phenotypes from weak (C1) to severe (C4) (Mullins et al., 1996) ( Also see Chapter 1, Fig1.2F). In the weak *piggytail (smad5<sup>m169</sup>*) allele, we observed that 49% of the C1 homozygotes had a secondary tail (Table 2.S1). In clutches of embryos from an incross of *piggytail (smad5<sup>dvy40</sup>*) carriers, we observed a range of phenotypes spanning the C1, C2 and C3
classes. The overall penetrance of secondary tail formation in all the mutant embryos was 36% (Table 2.S1). Notably, we observed secondary tail formation in each phenotypic class (Fig. 2.2A-D). Further, in snailhouse (bmp7<sup>70,68</sup>) embryos, which are severely dorsalized (C4), 38% formed secondary tails (Fig. 2.2E, Table 2.S1). Taken together, we observed secondary tails in multiple BMP mutant lines independent of the severity of dorsalization. These results suggest that secondary tail formation does not require that BMP signaling is reduced to a specific level, and it is unlikely to result from expanded mesoderm since secondary tails can even be specified in embryos that have limited tail mesoderm. Further, our earlier observation that only a subset of secondary tails contain somitic mesoderm is also inconsistent with secondary tails forming as a consequence of an overproduction of presomitic mesoderm.

**BMP signaling is required during early somitogenesis to inhibit secondary tail formation**

A previous study using transgenic zebrafish carrying a heat shock inducible, dominant-negative BMP receptor construct (Tg:dnBMPR) had shown that inhibition of BMP signaling post-gastrulation can induce secondary tails in a subset of embryos (Pyati et al., 2005). To confirm this result, we used a small molecule BMP antagonist, Dorsomorphin (DM) (Yu et al., 2008). We treated embryos at progressively later stages, beginning at the 1 somite stage, then scored for the presence of a secondary tail at 24 hpf by fixing the embryos and staining with ntl probe. Nearly all embryos treated with DM beginning at 6 somites lacked ventral tailfin at 24 hpf, with about 30% also forming a secondary tail (Fig. 2.2F). Embryos treated with DM after 7 somites showed normal or slightly reduced ventral tail fin with only a few embryos forming a secondary tail (Fig. 2.2F). In rare cases, embryos treated with DM at 7 somites formed a secondary tail even in the presence of a fully formed ventral tail fin (Fig. 2.2F, G). Our data indicate that the critical periods for specifying ventral tailfin and for prevention of secondary tail formation overlap significantly. However, the two processes appear to be separable, indicating that secondary tails might be formed in the absence of overt fate patterning defects.

Blood and muscle fates are expanded in the tails of bmp4 mutant embryos (Stickney et al., 2007). Although bmp4 mutants do not form secondary tails, it has been suggested that these expanded tissues could contribute to their formation in other BMP-compromised embryos. To investigate whether expansion of blood and tail mesoderm is associated with secondary tail
formation, we examined *gata1* (a blood progenitor marker) and *myoD* expression in BMP-compromised embryos. First, we treated 60 embryos with DM beginning at 5-somite stage. We fixed half of these at the 13-somite stage and performed whole amount *insitu* hybridization with *gata1* probe. 27 embryos were indistinguishable from wild-type (Fig. 2.2H, I), while 3 showed weak expansion of *gata1* expression to the posterior end (data not shown). To get an estimate of the penetrance of secondary tails in this clutch of embryos, we stained the other 30 embryos with *ntl* at 24 hpf. 11/30 embryos showed a secondary tail in this assay, suggesting that secondary tails can form in the absence of *gata1* expansion.

To determine whether somitic muscle is expanded in embryos producing secondary tails, we stained *mfn* embryos at 22 hpf with *myoD*. Whereas *myoD* expression has been reported to be expanded across the midline of the caudal tail in *bmp4* embryos (Stickney et al., 2007), we observed no such expansion in *mfn* embryos (Fig. 2.2J, K). These observations suggest a lack of association between secondary tail formation and an expansion of somitic muscle and blood precursors. Our data suggest that BMP is required to regulate the integrity of the tailbud during early somitogenesis, and this function of BMP signaling is independent of its early role of establishing dorsoventral axis and late role of specifying ventral tail fin, somitic mesoderm and blood.

**Noncanonical Wnt signaling functions with BMP in inhibiting secondary tail formation**

Noncanonical Wnt signaling plays an essential role in governing morphogenesis of the posterior body (Veeman et al., 2003; Seifert and Mlodzik, 2007). *ppt/wnt5* and *kny/glypican4* mutants are defective in components of the noncanonical Wnt signaling pathway and have short tails due to impaired cell movements within the tailbud (Marlow et al., 2004). Strikingly, *kny* mutants have also been reported to exhibit ectopic *eve1* (a gene expressed in the tailbud) and *shh* (*sonic hedgehog*, a gene expressed in notochord and floorplate) expression domains in the tail (Solnica-Krezel et al., 1996; Marlow et al., 1998), resembling the expression pattern of these markers in secondary tails of dnBMPR embryos (Pyati et al., 2005). This suggested that *kny* embryos might also produce secondary tails, with the dramatic shortening of the axis perhaps precluding identification of the ectopic tissue in living embryos. In looking at later stage (72 hpf) *kny* embryos, we could observe clear bifurcations of the posterior notochord (Fig. 2.3C),
suggesting that a secondary tail may form in these embryos. We confirmed this by examining ntl expression at 24 hpf (Fig. 2.3D, E). Further, we observed ectopic expression of col2a (Fig. 2.3F, G) as well as myoD (Fig. 2.3H, I; Table 2.2). These results suggest that inhibition of non-canonical Wnt signaling results in secondary tail formation. We next tested whether this phenotype is unique to kny mutants, or might be common to other embryos in which noncanonical Wnt signaling is impaired. We screened the tail phenotype of disheveled, strabismus and prickle morphants by examining ntl expression at 22 hpf. Three disheveled homologs, dvl1, dvl2 and dvl3 were knocked down systematically by morpholino (MO).

Knockdown of each dvl gene individually did not result in a secondary tail phenotype (data not shown). However, we found that inhibition of dvl2 and dvl3 simultaneously did give rise to a secondary tail in a subset of embryos (Fig. 2.3A, B) in addition to the previously reported mild CE defect.

As described above, approximately 50% of kny embryos form secondary tails. To test whether this partial penetrance was due to residual activity of the noncanonical Wnt pathway or to genetic redundancy with another pathway, we injected dvl2/dvl3 MOs into kny embryos. We did not observe any increased penetrance of secondary tail formation (data not shown) in these embryos, suggesting that even in embryos lacking most, if not all, noncanonical Wnt activity, other pathways such as the BMP pathway might partially compensate (see below). Lastly, we tested several known noncanonical Wnt ligands-wnt5, wnt11, and wnt11r for a role in secondary tail formation-knocking them down using morpholinos, singly and in combination, and observed no evidence for a role in this process for any of these genes (data not shown).

In contrast to our observations in kny or dvl2/dvl3 MO embryos, we never observed secondary tail formation in embryos in which strabismus, prickle 1a and prickle 2b had been inhibited, separately or in combination (Table 2.S2). These data suggest that prickle and strabismus do not regulate secondary tail formation, and that secondary tail formation is not likely to be merely a secondary consequence of earlier defects in CE. Our data support the involvement of at least some elements of a non-canonical Wnt pathway in preventing secondary tail formation.

To investigate whether non-canonical Wnt signaling interacts with BMP signaling in inhibiting secondary tail formation, we tested for synergy between mfn and dvl by injecting suboptimal amounts of morpholinos for each gene and scoring embryos for secondary tail
formation by examining *ntl* expression (Fig. 2.3J). Partial knockdown of *mfn* showed a very low penetrance of secondary tail formation, while partial knockdown of either *mfn* and *dvl1* or *mfn* and *dvl2* did not affect the penetrance of secondary tail formation. Moderate enhancement of secondary tail penetrance was observed with partial knockdown of *mfn* and *dvl3* simultaneously, while partial knockdown of *mfn*, *dvl2* and *dvl3* greatly enhanced the secondary tail penetrance to over 50%.

Next, we confirmed this interaction by using *mfn* mutants. Injection of suboptimal amounts of *dvl2*/*dvl3* MO into *mfn* embryos also caused a robust enhancement (Fig. 2.3J). Lastly, to test whether *mfn* interacts with *kny*, we scored the penetrance of secondary tails in *mfn; kny* double mutant embryos. In *mfn; kny*, strong enhancement of secondary tail formation was observed (88% of double mutant embryos formed a secondary tail, Fig. 2.3K), approximately what one would expect for a simple additive effect on penetrance of this phenotype. Together, the genetic interactions are consistent with a model in which noncanonical Wnt signaling and BMP signaling function together to inhibit secondary tail formation.

**BMP and noncanonical Wnt signaling are required to prevent bifurcation of the chordoneural hinge during tail outgrowth**

The chordoneural hinge (CNH) is a stem cell pool lying posterior to the developing notochord in the tailbud. In *Xenopus*, progenitors of neural tube, notochord and somites reside in the CNH, and when the CNH is grafted to host embryos, it is able to produce ectopic tails (Gont et al., 1993). In mice, the CNH hosts both differentiating axial progenitors and self-renewing stem cells that contribute to differentiated tissues along the anterior/posterior axis (Cambray and Wilson, 2002). Our observation that secondary tails contain both notochord and somitic muscle suggested the possibility that a defect in CNH specification or morphogenesis could underlie the defect.

We first tested whether the secondary tails contained ectopic CNH cells by performing *in situ* hybridization with *flh*, which is expressed in the CNH during tail development. In both *mfn* and *kny* embryos at 24 hpf, *flh* was ectopically expressed in the region of the secondary tail, suggesting the existence of an extra CNH domain (Fig. 2.4A-C). We confirmed this result by observing ectopic expression of a second CNH marker *fgf4* (Fig. 2.S1).
The above experiments did not distinguish whether two distinct CNH domains were specified in these embryos or a single domain was initially specified that later split into two separate domains. In order to understand the emergence of the ectopic CNH domain, we performed a time-course experiment by fixing mutant embryos hourly during tail outgrowth and using flh expression to determine the onset of the secondary CNH domain. At 17 hpf, all mfn embryos showed one intact CNH domain in the tailbud (Fig. 2.4D). Over the next few hours, an increasing percentage of mfn embryos showed an elongated CNH stretched along the ventral side of the tailbud, with embryos showing two distinct CNH domains beginning to appear at 20 hpf (Fig. 2.4D). By 24 hpf, the penetrance of two CNH domains reached its peak and no stretched CNH were observed at this stage. In snh embryos, we observed similar progress of the onset of two CNH domains (Fig. 2.S2). In the case of kny embryos, the kinetics of secondary tail formation were roughly similar, with the onset of the appearance of two CNH domains beginning slightly later (23 hpf) (Fig. 2.4E). By 25 hpf, the formation of two CNH domains in kny embryos was close to completion (Fig. 2.4E). These results suggest that the CNH domain is specified normally in both mfn and kny embryos, but becomes split during tail extension, suggesting that a defect in morphogenetic movements could underlie the secondary tail phenotype.

**N-Cadherin is required to maintain tailbud integrity.**
Proper regulation of intercellular adhesion is important for proper cell movements (Halbleib and Nelson, 2006). Both the BMP pathway and noncanonical Wnt signaling have been demonstrated to regulate cell movements during gastrulation at least in part by regulation of the localization or activity of cadherins (Ulrich et al., 2005; von der Hardt et al., 2007). As a recent study has shown that a semidominant allele of N-cadherin (cdh2) perturbs cell cohesion in the tailbud (Harrington et al., 2007), we asked whether disruption of cell adhesion in the tailbud affects CNH morphogenesis. We performed genetic interaction assays between dvl2/3 and cdh2. Simultaneous injection of suboptimal amounts of dvl2/3 MO and cdh2 MO, which did not cause any tail defects when injected separately, induced secondary tail formation (Fig. 2.5A, C-E). At higher doses of dvl2/3 MO and cdh2 MO, we observed dramatically scattered tailbud cells rather than simple bifurcation (Fig. 2.5F). Interestingly, in kny embryos, we occasionally observed
embryos with three CNH domains, triple-branch notochords and peeled off hyphochord (Fig. 2.S3), which resemble these severe dvl/cdh2 MO morphants (Fig. 2.5F).

To test for genetic interactions between BMP signaling and N-cadherin in secondary tail formation, we combined partial loss of BMP with partial loss of N-cadherin function. cdh2 MO significantly enhanced the secondary tail penetrance in mfn morphants (Fig. 2.5B). Together, these results indicate that proper regulation of cell adhesion is essential for maintaining the coherence of the CNH during tail development, and suggests that BMP and the noncanonical Wnt pathway may act to prevent tail bifurcations by regulating intercellular adhesion in the tailbud.

During gastrulation, wnt11 promotes coherence of the anteriorly migrating prechordal plate population of cells (Ulrich et al., 2005). In wild-type migrating prechordal plate cells, cadherin is believed to cycle between the plasma membrane and endosomes, but is found predominantly in endosomes. In embryos with impaired wnt11 function, E-cadherin was localized more exclusively to the plasma membrane, suggesting that proper cycling of cadherin localization, regulated by noncanonical Wnts, is important for maintaining coherence of migrating prechordal plate cells (Ulrich et al., 2005). To determine whether cadherin localization is regulated by non-canonical Wnt signaling, we performed confocal imaging of embryos stained with a pan-cadherin antibody. In both mfn and wild-type embryos, cadherin was localized to the plasma membrane of CNH cells (Fig. 2.5H). In contrast, we observed an apparent shift to a more intracellular localization of cadherin within the CNH of kny embryos (Fig. 2.SI). We also analyzed the localization of β-catenin in mfn and kny embryos, and observed no defects in the normal membrane localization of β-catenin in tailbud cells (Fig. 2.S4). These results indicate that noncanonical Wnt signaling specifically affects the localization of cadherin without affecting the β-catenin.

**CNH bifurcation occurs during tailbud protrusion.**

Although BMP signaling clearly acts to promote the coherence of the CNH, it was unclear whether BMP acts directly on the CNH, or indirectly. The fact that known BMP ligands in the tailbud-bmp2, bmp4, and bmp7, are expressed most intensely in the posterior tailbud, away from the CNH, suggested that BMP’s action could be indirect (Dick et al., 2000; Thissé et al., 2004). To determine more precisely where in the tailbud BMP is active, we performed
immunostaining with phosphorylated Smad1/5/8 (P-smad5) antibody in embryos at the 6-somite stage, and combined this assay with fluorescent *in situ* hybridization (FISH) using *flh* probe to visualize the CNH. Consistent with a recent study (Esterberg et al., 2008), we observed activation of BMP signaling in the posterior mesoderm of the tailbud, separate from the CNH (Fig. 2.6A). This localization of BMP activity to the posterior tailbud persisted at least until the 14-somite stage (Fig. 2.6B). In contrast, we observed no activation of BMP signaling in the tailbud of *mfn* embryos (Fig. 2.6C) although these embryos showed normal phosphorylation of Smad1/5/8 in the notochord. These results suggested that BMP regulates CNH morphogenesis indirectly.

To better understand how BMP signaling regulates CNH morphogenesis by its activation in the posterior mesoderm of the tailbud, we performed confocal time-lapse imaging of the developing tail in Tg(*flh*:EGFP) (Video 2.S1.) and *mfn*;Tg(*flh*:EGFP) embryos (Video 2.S2). In wild-type embryos, during tailbud extension stage, the CNH moved along the surface of the yolk cell in close association with Kupffer’s vesicle (KV) (Fig. 2.6D). As the tailbud entered the protrusion phase at about the 12-somite stage, the most ventral posterior tailbud cells, coming from a region of high BMP activity (see Fig. 2.6B), moved anteriorly and started to undercut the KV (Fig. 2.6D’). Progressively, more cells from the ventral side moved in between the KV and yolk, and this was accompanied by notochord extension and yolk constriction (Fig. 2.6D’’). As a result, the CNH together with the KV was lifted away from the surface of yolk and the tail began extending off of the yolk (Fig. 2.6D’’’). In the case of *mfn* embryos, after a comparatively normal extension stage (Fig. 2.6E), the tailbud entered a protrusion phase also at the 12-somite stage. In contrast to wild type, the most ventral cells failed to undercut the KV. Instead, they moved inward, protruding into the yolk, often forming a very notable indentation (Fig. 2.6E’). This had the effect of drawing the KV and the CNH ventrally towards, or even into, the yolk. As tail extension continued, the lengthening notochord eventually caused the CNH to adopt the elongated “stretched” state during yolk constriction (Fig. 2.6E’’). At the end of tailbud protrusion, the stretching of the CNH became more severe as the most ventral cells continued their irregular movements. This finally led to part of the CNH embedded into the yolk with the KV (Fig. 2.6E’’’). Depending on the embryo, these ectopic CNH domains could either be completely ‘left behind’ during tail elongation, or remain connected to the main axis by a branching of the notochord (data not shown).
We also performed confocal time-lapse in vivo imaging with *kny*;*Tg(flh:EGFP)* transgenic line (Video 2.S3). We did not notice any change of CNH morphology until about the 14-somite stage, when the tailbud began to protrude off the yolk (Fig. 2.6F’). As the most ventral cells in *kny* undercut beneath the KV, we observed cells gradually detaching away from the CNH (Fig. 2.6F’’'). Over time, the ectopic CNH cells formed an elongated cluster nearly the size of the main CNH (Fig. 2.6F’’’). Our observations suggest that stereotypical cell movements occur roughly normally in *kny* embryos, albeit at a reduced rate, and that the CNH, perhaps due to the mislocalization of cadherin, is unable to maintain coherence during tail extension and protrusion. Our confocal microscopy videos plus results of immunostaining experiments with pan-cadherin antibody suggest that non-canonical Wnt and BMP regulate CNH cohesion by direct and indirect mechanisms, respectively.

**BMP regulates migration of posterior mesoderm cells in the tailbud**

To determine the identity of the cells that undercut the KV and the CNH, we performed immunostaining with a Tbx6 antibody to label presomitic mesoderm cells (Szeto and Kimelman, 2004) in *Tg(flh:EGFP)* and *mfn;Tg(flh:EGFP)* embryos at several stages of tail development. In wild type, we observed a band of Tbx6-positive cells located beneath the CNH at the 16-somite stage (Fig. 2.7A), suggesting that those cells migrating along the ventral side of the CNH during tail protrusion are from the posterior mesoderm. Notably, cells in this region also show high levels of phospho-Smad staining (Fig. 2.S5). In *mfn* embryos, a cluster of Tbx6-positive cells is retained posteriorly in the tailbud, reflecting the defective movement of these undercutting cells (Fig. 2.7B). Consistent with our time-lapse video of *mfn* embryos, at least some of these posterior mesoderm cells eventually moved anteriorly and gradually surrounded the ectopic CNH domain, eventually ending up in the secondary tail domain (Fig. 2.7D). Taken together, our data suggest that split of the CNH upon loss of BMP signaling is likely a secondary consequence caused by the aberrant migration of a group of ventral-posterior presomitic mesoderm cells.

To determine whether blocking non-canonical Wnt signaling also affects the movement of posterior mesoderm cells, we performed a similar time course experiment in *kny;Tg(flh:EGFP)* embryos. Migration of the Tbx6-positive cells is delayed in *kny* embryos—these cells are retained posteriorly at 18 hpf (Fig. 2.7F). Only later, at the 21-somite stage do we observe some Tbx6-positive cells located ventrally beneath the CNH (Fig. 2.7H). At 23 hpf, we
observed Tbx6 positive cells colocalized with ectopic CNH cells (Fig. 2.7J), suggesting these Tbx6 cells were pushed along as the ectopic CNH cells drifted out from the main axis. Consistent with our time-lapse video (Fig. 2.6F”, also see Video 2.S3), the undercutting movement of the posterior mesoderm cells also occurred in kny embryos, though with delayed timing.

Discussion

The role of BMP signaling in specifying cell fate in the tailbud has been well-established. Here, we provide evidence that BMP signaling also mediates cell movements during tail development. Our data indicate that BMP signaling is activated in the ventroposterior tailbud to promote cell migration during tailbud protrusion, and that it is the defective migration of these cells which ultimately leads to bifurcation of the CNH domain and formation of a secondary tail in BMP mutants.

We also show that noncanonical Wnt signaling interacts with BMP signaling to maintain CNH integrity by affecting cadherin localization in CNH cells, possibly disrupting cell cohesion. Our data suggest a model in which BMP and noncanonical Wnt signaling regulate tail morphogenesis by controlling cell migration and cell adhesion within the tailbud.

**BMP signaling inhibits secondary tail formation**

It has been observed in previous investigations that reducing BMP signaling after gastrulation results in secondary tail formation (Connors et al., 1999; Pyati et al., 2005), and multiple explanations have been suggested to account for this unusual phenotype. Pyati and Kimelman proposed that after gastrulation, the role of BMP switches from that of promotion of tail development to one of inhibition, although a mechanism was not proposed (Pyati et al., 2005). In contrast, a recently proposed model suggests that post-gastrula BMP’s continue to act as morphogens, patterning the fates of tail mesoderm, and that when BMP is inhibited mildly, ectopic presomitic mesoderm is produced that leads to formation of a secondary tail (Stickney et al., 2007). Two main lines of evidence indicate that the gradient model does not adequately explain the formation of secondary tails. First, our observation that secondary tails are produced by several BMP pathway mutants of widely varying strengths, spanning the strongly dorsalized snh mutants to the weakly dorsalized mfn mutant. The severity of the phenotype of mutants represents the level of loss of BMP signaling. The observation that secondary tail formation is
not restricted in a specific phenotypic category indicates that secondary tails are not a consequence of a precise adjustment of a BMP activity gradient within a narrow range (also see Fig 1.2F). Secondly, secondary tails are formed in the absence of the fate patterning defects predicted by the BMP gradient model. Thus, our findings do not support the BMP morphogen model as an explanation of secondary tail formation. Rather, we propose that BMP signaling independently regulates morphogenesis and cell fate patterning in the tailbud during early somitogenesis stages.

This model is similar to the proposed separable roles for BMP signaling during gastrulation in DV fate patterning and regulation of CE movements (von der Hardt et al., 2007). During gastrulation, BMP is thought to create a gradient of intercellular adhesion along the DV axis, from low levels on the ventral side, where BMP activity is highest, to high levels dorsally, where BMP activity is low. Migrating mesodermal cells therefore move away from regions of high BMP signaling/low adhesion towards regions of low BMP activity/higher adhesion (Myers et al., 2002; von der Hardt et al., 2007). While we observe a similar directionality (movement of mesodermal cells away from a region of high BMP activity), we do not know if an analogous gradient of adhesion is established in the tailbud by BMP. A key phenotypic distinction between the role of BMP in regulation of cell migration during gastrulation and the defects we observe here is that during gastrulation, dorsalward movement of lateral mesoderm is completely blocked in BMP-compromised embryos, while in the tailbud, posterior mesodermal cells still move anteriorly in mfn mutants, but move aberrantly into the yolk instead of undercutting Kupffer’s vesicle. Precisely why these cells take this path remains unclear, though one possible explanation is that Kupffer’s vesicle becomes more tightly associated with the yolk cell in BMP-compromised embryos, thereby blocking the movement of the presomitic mesoderm cells. However, in embryos where the dorsal organizer region was surgically removed near the end of gastrulation, and also in embryos where formation of Kupffer’s vesicle was blocked by blastula-stage injection into the yolk of ntl and spt MOs, we still observed ectopic tbx6-positive cells associated with yolk extension (data not shown), indicating that these cells migrate aberrantly even in the absence of Kupffer’s vesicle or the CNH. Therefore, we favor the hypothesis that BMP signaling is required for ventroposterior mesodermal cells to undertake a migratory path between the surface of the yolk cell and Kupffer’s vesicle. When these cells move inappropriately into the yolk when BMP signaling is blocked, Kupffer’s vesicle and the CNH
can be drawn at least partially into the yolk as well. As tail elongation proceeds, the CNH can become bifurcated, resulting in a branched notochord (see Fig. 2.1B, 1G). In some cases the ectopic CNH cells remain associated with the yolk extension, sometimes generating a short stretch of notochord that does not connect with the main notochord at all (data not shown). In general, such embryos do not form any morphologically obvious secondary tail and can only be detected by staining with the appropriate marker.

Our results are consistent with observations of dnBMPR embryos by Pyati and Kimelman, which led them to suggest that some population of mesodermal progenitors were being left behind during tail extension, and subsequently formed the secondary tail (Pyati et al., 2005). Our observations confirm and extend their hypothesis by identifying the population of mesodermal progenitors whose migration is regulated by BMP, by showing that defective migration of these cells leads to a bifurcation of the CNH, and by demonstrating that BMP signaling genetically interacts with N-cadherin and noncanonical Wnt signaling to regulate tail morphogenesis.

During gastrulation, BMP is proposed to regulate cell adhesion in a cadherin-dependent manner. For example, when BMP signaling is abrogated, as in alk8 MO embryos, mesodermal cells exhibit behaviors (high stability of intercellular contacts) consistent with an increase in intercellular adhesion (von der Hardt et al., 2007). When cadherin function is also blocked via coinjection of a dominant negative Cadherin, cells show decreased stability of intercellular contacts, indicating that cell-cell adhesion is reduced (von der Hardt et al., 2007). These and other data led to a model in which BMP signaling negatively regulates an as yet unknown aspect of cadherin function.

We observed a strong enhancement of the secondary tail phenotype when cdh2/N-cadherin was knocked down in conjunction with partial knockdown of BMP. While this result indicates that regulation of cell adhesion is important for proper morphogenesis of the tailbud, whether BMP functions in this process via regulation of cadherin function, as it does during gastrulation, is unclear. For example, cdh2 is expressed in the notochord as well as the tailbud (Lele et al., 2002; Harrington et al., 2007), raising the possibility that the synergy we observe is due to separate effects of BMP on the posterior tailbud and N-cadherin on the notochord/CNH. Alternatively, cdh2 and BMP might both regulate migration of posterior mesoderm without necessarily functioning in the same pathway. More work, including a detailed examination of
morphogenetic movements occurring in the tailbuds of mfn;cdh2 MO embryos will be required to address these possibilities more definitively.

**Noncanonical Wnt signaling and Cadherin localization**

Our results are the first to demonstrate that secondary tails are formed in embryos deficient for noncanonical Wnt signaling, although we note that the first published report of the kny mutant presciently described the presence of ectopic eve1-expressing cells near the yolk (Solnica-Krezel et al., 1996). As tail extension in kny embryos is so dramatically reduced, the presence of ectopic tail structures is generally not nearly as obvious as in mfn embryos. Indeed, we first observed secondary tails due to defective noncanonical Wnt signaling in the course of earlier experiments on dvl2/dvl3 MO embryos (Angers et al., 2006), which have a much milder tail extension defect, allowing for easier identification of secondary tails.

We observed secondary tails in kny and dvl2/dvl3 MO embryos, although the ligand for this putative noncanonical Wnt pathway remains unidentified at this time. Our observation that other genes that are required for CE movements during gastrulation, such as stbm, pk1, and pk2, do not appear to play a role in secondary tail formation, suggests that secondary tails are not formed merely as a by-product of earlier defects in morphogenesis. This, along with the defects in cadherin localization in CNH cells of kny embryos (see below), suggests a role for noncanonical Wnt signaling specifically during tail development to govern morphogenetic movements.

As the CNH moves posteriorly during tail extension, it encounters a continuous stream of anteriorly-migrating mesodermal progenitors from the tailbud, which move around the CNH via subduction and by moving laterally away from the midline (Kanki and Ho, 1997). We hypothesize that the CNH experiences some shear stress as the result of moving through this field of tailbud cells, and that, in the absence of noncanonical Wnt signaling, is unable to maintain sufficient cohesion to support CNH integrity during tail extension. Our time-lapse imaging of kny embryos shows that the ectopic CNH cells slough off the ventral portion of the CNH during tail extension, consistent with this group of cells being less cohesive. Our observation that partial inhibition of cdh2, which has previously been shown to be important in maintaining cohesion of the notochord (Harrington et al., 2007), synergizes strongly with
inhibition of noncanonical Wnt signaling, is consistent with cell-cell adhesion playing a key role in maintaining the integrity of the CNH.

**Noncanonical Wnt signaling promotes plasma membrane localization of cadherin**

During gastrulation, Wnt11 has been proposed to promote the cycling of E-cadherin between the plasma membrane and endosomes in migrating prechordal plate cells (Ulrich et al., 2005). In the absence of Wnt11, E-cadherin is predominantly localized at the plasma membrane, while in Wnt11-overexpressing cells, E-cadherin is observed most often in endosomes. Wnt11-deficient cells were also shown to adhere more weakly to an E-cadherin coated substrate, indicating that the noncanonical Wnt signaling promotes intercellular adhesion among prechordal plate cells as they migrate past other cells, such as the overlying epiblast (Ulrich et al., 2005). Curiously, in the CNH, we observe the opposite effect on cadherin localization—cadherin becomes more intracellularly localized in the absence of Wnt signaling, rather than in its presence. The significance of this difference remains unclear. One possibility is that prechordal plate cells, being actively migrating across a substrate of other cells and extracellular matrix, need to constantly remodel adhesion complexes in order to both move over their substrate and remain associated with other prechordal plate cells. It is not clear if CNH cells behave in the same way—posteriorward extension of the CNH appears more passive, driven predominantly by CE movements occurring more anteriorly. Perhaps in this situation, more static localization of cadherin to the plasma membrane is sufficient to maintain coherence of CNH cells as they move posteriorly through a stream of anteriorly migrating posterior tailbud cells.

**A model for the roles of BMP and noncanonical Wnt signaling in tail morphogenesis**

Our genetic and phenotypic data suggest that BMP and noncanonical Wnt signaling function independently of each other to ensure integrity of the CNH and proper morphogenesis of the tail (Fig. 2.8). First, BMP signaling, acting on ventroposterior mesodermal presursors, promotes the anteriorward migration of these cells, undercutting Kupffer’s vesicle and disrupting the association of the CNH with the yolk prior to tail protrusion. When BMP signaling is blocked, aberrant migration of the mesodermal progenitors into the yolk can capture the CNH, leading to its bifurcation as tail development proceeds. Meanwhile, noncanonical Wnt signaling promotes the localization of cadherin to the plasma membrane in CNH cells, increasing intercellular adhesion among these cells and allowing them to remain associated as CE
movements push them posteriorly into the tailbud. When noncanonical Wnt signaling is impaired, cohesion of the CNH is reduced, leading to a sloughing off of CNH cells and formation of a secondary tail. When both pathways are blocked, nearly all embryos form a secondary tail, possibly because a less cohesive CNH will nearly always split when ‘caught’ in the yolk. Thus, we propose that these two pathways, which function independently to regulate convergence-extension during gastrulation, work in separate pathways later in development in distinct regions of the tailbud to govern proper morphogenetic movements.
Figures and Tables

Figure 2.1 Mesoderm tissues are mis-specified in the secondary tails of *mfn*.

(A-G) Lateral view of expression of *col2α* (A,B), *myoD* (C,D), *neurog1* (E,F) and *ntl* (F,G) in the posterior tail of wild-type and *mfn* embryos fixed at 26 hpf. *col2α* (B), *myoD* (D) and *ntl* (G) are ectopically expressed in the secondary tail, but not *neurog1* (F). Embryos in all images are mounted with anterior to the left.
Figure 2.2 Secondary tail formation is independent of the role of BMP signaling in fate patterning.

(A-E) Lateral view of tails in wild-type and dorsalized embryos of indicated genotypes at 24 hpf, after whole amount in situ with ntl. Primary and secondary tails are marked with arrowheads.

(F) Distribution of secondary tail phenotype in embryos following DM treatment at indicated time points. Secondary tails were scored by the presence of ectopic ntl expression.

(G) Lateral view of the tail of a live embryo at 24 hpf, after DM treatment at 7 somites. Note the secondary tail with fully developed ventral fin.

(H,I) Dorsal view of gata1 expression in embryos at 12-somite stage, after treatment with DMSO (H) and DM (I) at 5-somite stage.

(J,K) Dorsal view of myoD expression in the tails of wt (J) and mfn (K) embryos at 22 hpf. Inset shows the lateral view.

Expression of gata1 (I) and myoD (K) is unaffected.
Figure 2.3 Noncanonical Wnt signaling functions with BMP in inhibiting secondary tail formation.

(A-C) Lateral view of wild-type (A, 24 hpf), dv12/3 morphant (B, 24 hpf) and kny mutant (C, 72 hpf). Insets of (A) and (B) show ntl expression in the posterior tail. Inset of (C) shows a close-up of posterior tail. Primary and secondary tails are marked with arrowheads.

(D-I) Lateral view of expression of ntl (D,E), col2α (F,G), and myoD (H,I) in the tail of wild-type and kny embryos fixed at 24 hpf. ntl (E), col2α (G), and myoD (I) exhibit ectopic expression within the tails of kny.

(J) Percentages of secondary tail formation in embryos injected with indicated combination of morpholinos (1 mg/ml each, except for ‘dv12/dv13 MO’ condition, where a concentration of 3 mg/ml for each MO was injected). For each column embryos from at least three separate experiments were scored.

(K) Percentages of secondary tail formation in embryos from incross of mfn/+;kny/+ double mutants carriers. Embryos are categorized by phenotypes.

Secondary tails in (J) and (K) were scored by ntl expression. Percentage of secondary tail of each separate experiment is calculated. Then the arithmetic mean of percentage of all replicates is calculated by dividing the sum of percentages with numbers of replicates. Then the variation from the mean is represented by calculated standard deviation. Error bars represent standard deviations of at least 3 replicates. Number of embryos scored for each column is shown in figures.
Figure 2.4 Bifurcation of the chordoneural hinge (CNH) in BMP and noncanonical Wnt mutants during tail outgrowth.

(A-C) Lateral view of expression of CNH marker, flh in the tail of wild-type, mfn and kny embryos fixed at 24 hpf. flh is expressed in the secondary tail of mfn (B) and kny (C).

(D,E) Distribution of morphological phenotypes of flh-expressing CNH in mfn (D) and kny (E) embryos fixed at indicated time points. Right panel shows the lateral view of representative embryo in each phenotypic class; inset, close-up of posterior tail of kny. For each time point around 40 embryos were scored.
Figure D: Percent of 2\(^{nd}\) tail in mfn

- 17hpf: 0%
- 19hpf: 0%
- 20hpf: 0%
- 21hpf: 0%
- 22hpf: 0%
- 23hpf: 0%
- 24hpf: 0%
- 25hpf: 0%

Legend:
- 1 tail
- stretched
- 2\(^{nd}\) tail

Figure E: Percent of 2\(^{nd}\) tail in kny

- 18hpf: 0%
- 19hpf: 0%
- 20hpf: 0%
- 21hpf: 0%
- 22hpf: 0%
- 23hpf: 0%
- 24hpf: 0%
- 25hpf: 0%

Legend:
- 1 tail
- stretched
- 2\(^{nd}\) tail
**Figure 2.5 N-cadherin synergizes with BMP and noncanonical Wnt signaling to prevent secondary tail formation.**

(A, B) Percentages of secondary tail formation in embryos injected with indicated combination of morpholinos. For low concentration, *dvl2/3* (1 mg/ml each), *mfn* (0.5 mg/ml) and *chd2* (0.1 mg/ml) MOs were injected. Embryos from three separate experiments were scored. Percentage of secondary tail of each separate experiment is calculated. Then the arithmetic mean of percentage of all replicates is calculated by dividing the sum of percentages with numbers of replicates. Then the variation from the mean is represented by calculated standard deviation. Error bars represent standard deviations of at least 3 replicates. Number of embryos scored for each column is shown in figures.

(C-G) Lateral view of tails of embryos at 24 hpf injected with indicated combination of morpholinos, after *in situ* hybridization with *ntl*. *ntl* expression in *cdh2* and *dvl2/3* MO morphants (C,D) is similar to wide-type. Note that co-injection of high concentration of *chd2* and *dvl2/3* MOs further enhanced the *ntl* expression from a single fork shaped pattern in (E) to a multi-fork pattern in (F), and co-injection of high concentration of *chd2* and *mfn* MOs in embryo (G) did not result in similar phenotype. This difference is consistent with our following antibody staining results (see below). For high concentration, *dvl2/3* (1.5 mg/ml each), *mfn* (1.5 mg/ml) and *chd2* (0.25 mg/ml) MOs were injected.

(H-J) Representative confocal microscopy images of tailbud in wild-type, *kny* and *mfn* embryos stained with pan-cadherin antibody at 21 somites; inset, close-up of CNH region. Membrane staining pattern in CNH is shown in wild-type (H) and *mfn* (J), while diffused staining pattern is shown in *kny* (I).
**Figure 2.6 Defects of CNH in mfn and kny initiate during tailbud protrusion.**

(A) Dorsal view of flat-mounted tailbud of 6-somite wild-type embryo, after FISH with flh (red) and antibody staining with P-Samd5 (green), and no colocalization (merge) is seen.

(B,C) Lateral view of tails of 14-somite wild-type and mfn embryos exposed to P-Smad5 (green) and β-catenin (red) antibodies. P-Smad5 staining is absent in the posterior tailbud of mfn (C, merge). Arrowheads indicate the ventroposterior cells in the tailbud that are normally positive for P-Smad5 (B, merge).

(D-F’’) Confocal time-lapse recording of tailbuds in embryos expressing Tg(flh:EGFP) transgene (green) and mRFP (red) from 10-somite stage. Shown are wild-type embryo (D-D’’), mfn (E-E’’) and kny mutants (F-F’’). Note that besides CNH transgenic EGFP is retained in notochord, floorplate and periphery of Kupffer’s vesicle (KV), marked by white line. Ends of stretched CNH are indicated by blue line in (E’’) and (F’’). Arrowheads mark the ventroposterior cells in the tailbud. Embryos were all mounted laterally.
Figure 2.7 BMP regulates posterior mesoderm cells of tailbud to inhibit secondary tail formation.

(A-J) all panels show the midline section of tails of Tg(flh:EGFP) expressing embryos (green) at indicated time points after anti-Tbx6 (red) immunostaining. Tbx6 positive mesoderm cells in mfn embryo (B) displays a detention in the posterior tailbud, when compared to wild-type embryo (A) at the same stage. Ectopic A more wild-type like movement of posterior mesoderm cells in kny embryo is shown in (F) and (H). Note eventually ectopic Tbx6 positive mesoderm cells are shown in the secondary tail domain of mfn (D) and kny embryos (J). Arrowheads mark the ventroposterior mesoderm cells in the tailbud. All tails are flat mounted laterally.
Figure 2.8 A model for the roles of BMP and noncanonical Wnt signaling in regulating tail development.

Depiction of the tailbud of a 10-somite wild-type embryo. During tailbud protrusion, BMP signaling in the posterior tailbud plays a crucial role in guiding the ventroposterior mesoderm cells to move beneath the Kupffer’s vesicle properly. Noncanonical Wnt signaling promotes intercellular adhesion in CNH cells, allowing them to maintain cohesion during tail elongation. nt: notochord; PM: posterior mesoderm; KV: Kupffer’s vesicle.
Table 2.1 Ectopic tail tissues produced in *mini fin* mutants

<table>
<thead>
<tr>
<th></th>
<th><em>col2α</em></th>
<th><em>myoD</em></th>
<th><em>neurog1</em></th>
<th><em>ntl</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mfn</em> embryos scored (n)</td>
<td>77</td>
<td>83</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>ectopic tissue (%)</td>
<td>53.2</td>
<td>30.1</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>
**Table 2.2** Ectopic tail tissues produced in *knypek* mutants

<table>
<thead>
<tr>
<th></th>
<th><em>col2α</em></th>
<th><em>myoD</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>knyp</em> embryos scored (n)</td>
<td>112</td>
<td>107</td>
</tr>
<tr>
<td>2° tail (%)</td>
<td>56.3</td>
<td>51.4</td>
</tr>
</tbody>
</table>
References


Supplement

Materials and methods (supplementary)

In situ hybridization and antibody staining

Whole amount in situ hybridization with fgf4 (Thisse et al., 2004), and other mentioned probes and antibody staining were performed as described previously in the Chapter 2.

Morpholino injection

pk1a, pk2b and stbm morpholinos have been previously described (Park and Moon, 2002; Carreira-Barbosa et al., 2003) and are injected at a concentration of 1 mg/ml each in embryo at one cell stage.
Figures and tables (supplementary)

Figure 2.S1

Ectopic expression of CNH marker, \textit{fgf4} in the secondary tail of \textit{mfn} embryo (B) and \textit{dvl2/3} morphant (C) fixed at 24 hpf. Lateral view with anterior to the left.
Figure 2.S2

Distribution of morphological phenotypes of flh-expressing CNHs in snh embryos fixed at indicated time points. Right panel shows the lateral view of representative embryo in each phenotypic class. For each time point around 40 embryos were scored.
Figure 2.S3

(A-E) Lateral view of expression of \textit{ntl} (A,B) and \textit{col2α} (C-E) in the tail of wild-type and \textit{kny} embryos fixed at 24 hpf. Note the three separate \textit{flh}-expressing CNH domains in (B), triple-branched notochord (nt) in (D) and birfurcated notochord with detached hypochord (hc) (the tissue that is localized immediately ventral to the notochord) in (E). Each region is indicated by arrowheads.
Figure 2.S4

(A-C) Confocal microscopy images of tailbud in wild-type, kny and mfn embryos labeled with β-catenin antibody at 21 somites; inset, close-up of CNH region. Lateral view with anterior to the left.
Figure 2.S5

(A,B) Confocal microscopy images of tailbud in wild-type and mfn embryos labeled with β-catenin (red) and P-Smad5 (green) antibody at 21 somites. Note in wild-type embryo (A) P-Smad5 staining is expressed in the posterior tailbud and extends under the notochord, while absent in mfn (B).
Video 2.S1

Time-lapse video of normal tailbud morphogenesis in mRFP-labeled alive Tg(flh:EGFP) embryo starting at 10-somite stage. Lateral view, anterior to the left.
Video 2.S2

Time-lapse video of defective tailbud morphogenesis in mRFP-labeled alive
mfn;Tg(flh:EGFP) embryo starting at 10-somite stage. Lateral view, anterior to the left.
**Video 2.S3**

Time-lapse video of defective CNH morphogenesis in mRFP-labeled alive *kny:tg(flh:EGFP)* embryo starting at 10-somite stage. Lateral view, anterior to the left.
Table 2.S1 Penetrance of secondary tail in BMP mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>$smad5^{m169}$</th>
<th>$smad5^{dty40/+},$</th>
<th>$bmp7a^{ly68a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2° tail</td>
<td>49% (n=84)</td>
<td>36% (n=186)</td>
<td>38% (n=95)</td>
</tr>
</tbody>
</table>

Table 2.S2 Penetration of secondary tail in embryos injected with prickle1a, prickle 2b and strabismus morpholinos

<table>
<thead>
<tr>
<th>MOs</th>
<th>pk1a</th>
<th>pk2b</th>
<th>stbm</th>
<th>pk1a+ pk2b</th>
<th>pk1a+ stbm</th>
<th>pk2b+ stbm</th>
<th>pk1a+ pk2b+stbm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2° tail</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(n=70)</td>
<td>(n=82)</td>
<td>(n=79)</td>
<td>(n=75)</td>
<td>(n=69)</td>
<td>(n=84)</td>
<td>(n=77)</td>
<td></td>
</tr>
</tbody>
</table>
References (supplementary)


CHAPTER 3 - Smad6 Influences Dorsoventral Patterning by Inhibiting BMP Activity in Zebrafish

Abstract

Bone morphogenetic protein (BMP) signaling is crucial for the patterning of embryonic dorsoventral axis in vertebrates. BMPs transduce signals by serine/threonine kinase receptor mediated phosphorylation of designated Smad proteins (Smad1/5/8). This process is negatively regulated by a subclass of Smad proteins, inhibitory Smads including Smad6 and Smad7. In this study, we have characterized two zebrafish homologs of smad6 (smad6.1 and smad6.2) that share high structural similarity. We show that smad6.1 and smad6.2 are broadly expressed throughout early development and their expression patterns are largely overlapped by 24 hpf. Also, we show that smad6.1 and smad6.2 expression is mediated by BMP signaling during gastrulation. By examining the gain-of-function phenotypes, we determine that both genes function as BMP inhibitors. In addition, knockdown of smad6.1 and smad6.2 by morpholinos (MO) results in mild dorsoventral patterning defects at the mid-gastrula stage, suggesting that smad6.1 and smad6.2 fine tune BMP signaling to ensure proper dorsoventral patterning by negatively regulating the BMP activity in the zebrafish embryo during gastrulation.

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and play a central role in specifying cell fate during embryogenesis (Little and Mullins, 2006). As demonstrated in Xenopus and zebrafish, BMP signals act as morphogens and form an activity gradient along the dorsal-ventral (DV) axis of the embryo during gastrulation (Schier and Talbot, 2005; Kimelman, 2006; Little and Mullins, 2006). On the ventral side, the highest level of BMP activity is found to induce ventral cell fates, and on the dorsal side, the BMP activity is eliminated by a series of secreted inhibitory proteins such as Chordin, Noggin and Follistatin to promote the dorsally expressed genes (Schier and Talbot, 2005; Kimelman, 2006; Little and Mullins, 2006). Activation of BMP signaling requires BMP ligands binding to type I and type II serine/threonine kinase receptors, which leads to the phosphorylation of receptor-regulated transcription factors Smad1/5/8 (R-Smads) (Feng and Derynck, 2005;
Massague et al., 2005). This phosphorylation allows R-Smads to translocate nucleus with cofactor Smad4 to propagate the signal (Feng and Derynck, 2005; Massague et al., 2005).

In contrast to the R-Smads, another class of Smad proteins known as inhibitory Smads (I-Smads), including Smad6 and Smad7 in vertebrates, function as intracellular antagonists to inhibit BMP signaling (Feng and Derynck, 2005; Massague et al., 2005). The I-Smads share a conserved C-terminal MH2 (Mad-homology 2) domain present in all Smad proteins but lack the N-terminal MH1 (Mad-homology 1) domain that is highly conserved in all R-Smads and Smad4 (Massague et al., 2005). I-Smads. Although Smad6 and Smad7 both function to negatively regulate BMP, Smad7 displays additional inhibition of TGF-β/activin signaling (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Hata et al., 1998). Smad6 has been proposed to inhibit BMP signaling by physically binding to type I receptors to prevent the phosphorylation of R-Smads (Imamura et al., 1997) and by binding to Smad1 in competition with Smad4 (Hata et al., 1998), which consequently interferes signal transduction. Additionally, Smad6 has also been suggested to act as a transcriptional corepressor to inhibit BMP signaling by recruiting other components such as corepressor C-terminal binding protein (CtBP) (Lin et al., 2003), class I histone deacetylase (HDAC) (Bai et al., 2000) and homeobox (Hox) c-8 proteins (Bai and Cao, 2002).

*In vitro* studies using mouse and human cell lines have shown that Smad6 expression can be induced by BMP signaling (Afrakhte et al., 1998; Ishida et al., 2000). In chick embryos, it has been shown that Smad6 is strongly expressed in the cardiogenic region and developing limb where high amount of BMPs are expressed (Yamada et al., 1999; Vargesson and Laufer, 2009). BMP2 protein can induce the ectopic expression of Smad6 in explant culture and Noggin protein treatment reduces the expression of Smad6 in cardiogenic cells (Yamada et al., 1999). Activating BMP signaling by retrovirus-mediated expression of a constitutively-active form of type I BMP receptor in the limb tissues induces the expression of Smad6 while expressing of dominant negative BMP receptor or Noggin reduces Smad6 expression in limb tissues (Vargesson and Laufer, 2009). These results suggest Smad6 functions in a negative feedback loop to limit the BMP signal (Park, 2005). In *Xenopus* Smad6 is expressed through the gastrulation, and overexpression of Smad6 in ventral cells forms a partial secondary axis which phenocopies the embryos with suppressed BMP signaling, while overexpression of Smad6 in dorsal cells result in no patterning defect (Nakayama et al., 1998). This suggests in *Xenopus* Smad6 has a potential
role in DV patterning during gastrulation. However, since there are limited loss-of-function studies of Smad6, the exact role of Smad6 in fish development is still unclear. Insertion of a LacZ reporter to the MH2 domain of Smad6 in mice demonstrates Smad6 expression is largely restricted to the heart and blood vessels (Galvin et al., 2000). These Smad6 mutant mice exhibit multiple cardiovascular defects suggesting a specific role of Smad6 in regulating endocardial cushion transformation and homeostasis of adult cardiovascular system (Galvin et al., 2000), but do not show any obvious gastrulation defects (Zhao, 2003). In zebrafish, the role of Smad6 has yet to be addressed. Here we provide evidence to show that two homologs of smad6 in zebrafish, smad6.1 and smad6.2 are broadly expressed throughout early development and their expression patterns are similar to each other. Also, we show that smad6.1 and smad6.2 expression is mediated by BMP signaling during gastrulation. By examining the phenotypes after overexpression of smad6.1 and smad6.2, we determine that both genes are able to inhibit BMP signaling. Lastly, inhibition of smad6.1 and smad6.2 results in mild dorsoventral patterning defects at the mid-gastrula stage, suggesting that smad6.1 and smad6.2 fine tune BMP signaling to ensure proper DV patterning by negatively regulate the BMP activity in the zebrafish embryo during gastrulation.

Results

Clone and sequence analysis of zebrafish Smad6.1 and Smad6.2

One of our hits from a yeast two hybrid screen for proteins interacting Spork (homolog of human ARHGAP21 in zebrafish) was characterized as zebrafish Smad6.2 (also named Smad6b) on the linkage group 18 (Fig. 3.1A red letters). The paralog of Smad6.2, Smad6.1 (also named Smad6a) on linkage group 7 was found in the BLAST results with mRNA sequence of Smad6.2. Multi-alignments with amino acid sequences of Smad6.1, Smad6.2 and zebrafish Smad7, together with Smad6 and Smad7 in Xenopus, mouse and human showed the position of MH2 domain (predicted by Prosite) is conserved (Fig. 3.1C). The conserved domain analysis showed this domain is highly conserved. Smad6.1 and Smad6.2 showed higher similarity and identity scores when comparing amino acids sequences with Xenopus, mouse and human Smad6 than those scores when comparing with listed Smad7 sequences (Fig. 3.1 D). Phylogenetic tree of amino acid sequences of Smad6.1, Smad6.2 and zebrafish Smad7, together with Smad6 and Smad7 in frog, mouse and human clearly show Smad6.1 and Smad6.2 are more closely related to
other Smad6 homologs than Smad7 (Fig. 3.1E) and this result is supported by those representative amino acids that differentiate Smad6 and Smad7 in conserved MH2 domain (Fig. 3.1C, red box). To get a more precise phylogenetic tree we built a tree by only aligning the MH2 domains, which showed Smad6.2 is closely related to Smad6 in mammals while Smad6.1 is more distantly related. Considering the possible functional redundancy, we began study of these two Smad6 homologs by isolating full-length cDNAs of Smad6.1 and Smad6.2 from a 24hpf zebrafish cDNA library (Fig. 3.1A, B). For Smad6.2 an alternative ORF (in frame of annotated ORF, based on the sequences from our yeast two hybrid screening data) was cloned (Fig. 3.1B).

**Comparison of spatial expression patterns of smad6.1 and smad6.2 in early development**

To determine the expression patterns of *smad6.1* and *smad6.2*, we performed in situ hybridization analysis in embryos at multiple stages during early development. At the shield stage, in contrast to other BMP antagonists such as *chordin*, which is exclusively expressed on the dorsal margin, *smad6.1* and *smad6.2* were both ubiquitously expressed throughout the embryo (Fig. 3.2A,B,A’,B’). At bud stage, both *smad6* homologs were expressed broadly, with more intense expression in the midline axis and tailbud (Fig. 3.2C,D,C’,D’). At the 10-somite stage, *smad6.1* and *smad6.2* transcripts were evenly distributed all over the embryo (Fig. 3.2E,F,E’,F’). At 24 hpf, *smad6.2* was more intensly expressed in the posterior tail compared to *smad6.1*, while both genes were strongly expressed in the anterior region (Fig. 3.2G,G’). In the head, both *smad6* homologs showed strong expression in the eye and throughout the brain (Fig. 3.2H,H’). In addition, *smad6.2* was more clearly expressed on the periphery of the otic vesicle (Fig. 3.2H’). At 48 hpf, we observed expression of *smad6.1* and *smad6.2* in the head, heart, pectoral fin and somites (Fig. 3.2I,J,I’,J’). We also observed unique expression of *smad6.1* near the hindbrain (Fig. 3.2I), and high expression levels of *smad6.2* in the lens (Fig. 3.2J’). Our data shows that both *smad6s* have a comparatively broad and largely overlapping expression pattern during early development, suggesting that these two homologs could be functionally redundant.

**Expression of smad6.1 and smad6.2 is regulated by BMP signaling during gastrulation**

The BMP expression gradient is established from shield stage: high ventrally and low dorsally. This expression pattern resembles that of the concurrently expressed *smad6.1* and *smad6.2*, which led us to determine whether *smad6* expression is controlled by BMP signaling in
zebrafish. At early gastrula stage (60% epiobly), we analyzed the smad6.1 and smad6.2 expression patterns by in situ hybridization when down- and up-regulating BMP signaling. Consistent with the expression profile of smad6 in Xenopus during gastrulation (Nakayama et al., 1998) (also see Fig. 3.2A,A'), smad6.1 showed ubiquitous expression in the embryo with slightly more intensive signal on the dorsal versus the ventral side at 60% epiboly (Fig 3.3A,B).

Knocking down admp (encoding anti-dorsalizing morphogenetic protein, ADMP) and mfn (encoding BMP agonist Tolloid) was previously shown to result in weakly dorsalized embryos due to mild loss of BMP signaling. Here, we found inhibiting mfn or admp causes absence of smad6.1 expression from the ventral side (Fig. 3.3C-F). However, this effect is weak and penetrance is very low: around 8% (10/122) in mfn morphants and 15% (16/105) in admp morphants. However, we did not find any apparent change in smad6 expression in snh/bmp7 and Dorsomorphin (a chemical inhibitor of BMP pathway)-treated embryos (data not shown).

Enhancing BMP signaling by inhibiting the BMP antagonist chordin by morpholino resulted in a moderate enhancement of smad6.1 expression level in 52% (46/88) of the embryos (Fig. 3.3G,H). Overexpression of a constitutively active form of the BMP receptor (caBMPR) potently enhanced BMP signaling and resulted in severely ventralized embryo at 24 hpf (data not shown).

We observed strong induction of smad6.1 transcripts in 80% (90/112) of embryos injected with caBMPR mRNA (Fig. 3.3I,J). Enhancing BMP signaling by injecting Smad5 mRNA, also induced expression of smad6.1 in 65% (70/108) of embryos (Fig. 3.3K,L). So far, our data suggests the expression of smad6.1 is regulated, at least in part, by BMP signaling. To test the possibility that expression of smad6.1 is partially regulated by a second family member in TGFβ signals, Nodal signaling, we knocked down oep (encoding Nodal co-receptor) and then checked smad6.1 expression. We did not observe a significant change in smad6.1 expression, suggesting that smad6.1 expression is independent of Nodal signaling (Fig. 3.3M,N). The concentrated signal along the margin is likely due to accumulation of un-internalized marginal cells resulting from the loss of Nodal signaling (Fig. 3.3M). Our parallel experiments with smad6.2 display the same changes in expression pattern as smad6.1 (data not shown) and the penetrance is similar to that of smad6.1 (Fig. 3.3O). Together, we show that enhancing BMP signaling induces the expression of smad6.1 and smad6.2 and suppressing BMP signaling slightly reduces their expression. The result that smad6.1 and smad6.2 are responsive to both up- and down-regulation of BMP signaling suggests that they may act cooperatively as feedback inhibitors.
Overexpression of smad6.1 and smad6.2 inhibits BMP signaling

Overexpression of *smad6* in *Xenopus* embryos has previously been shown to result in secondary axis formation, a patterning defect due to loss of BMP signaling (Nakayama et al., 1998). In order to test whether *smad6* can function as a BMP inhibitor in zebrafish, we examined the effect of overexpression of *smad6.1* and *smad6.2* on DV patterning during early gastrulation (60% epiboly). Embryos injected with either *smad6.1* and *smad6.2* mRNAs showed reduced expression domain of the ventral marker *eve1* (Fig. 3.4A-C), and expanded expression of two dorsal markers: *flh*, expressed in the chordamesoderm (Fig. 3.4E-G), and *chd*, expressed in axial mesoderm (Fig. 3.4I-K). Combined injection of *smad6.1* and *smad6.2* mRNA showed slightly stronger effect in restricting the expression of *eve1* (Fig. 3.4D). However, no further expansion of *flh* and *chd* is detected (Fig. 3.4H,L). At shield stage, *chd* is expressed in a comparatively smaller domain and we observed significant expansion of *chd* in embryos injected with both *smad6.1* and *smad6.2* mRNAs (Fig. 3.4M,N). The ventrally shifted expression patterns of *eve1*, *flh* and *chd* resulting from overexpression of *smad6.1* and *smad6.2* reflect disrupted DV patterning during gastrulation, a typical early defect expected in BMP-compromised embryos (Schier and Talbot, 2005). Consistent with this result, embryos injected with *smad6.1* and *smad6.2* mRNA at one-cell stage exhibited a noted dorsalization phenotype at 24 hpf evidenced by reduced ventral tail tissues, which phenocopies the BMP mutants *piggytail* and *minifin* (data not shown). To confirm that overexpression of both *smad6.1* and *smad6.2* perturbs transduction of BMP signaling, we performed antibody staining with P-Smad5 as readout of BMP activity. Overexpressing *smad6.1* and *smad6.2* together resulted in a moderately reduced domain of active BMP signaling (Fig. 3.4O,P). In addition, the overall signal intensity in these injected embryos is comparatively weaker than in wild type (Fig. 3.4O,P). The numbers of embryos injected are given in Table 3.1. Overall, our results suggest that *smad6.1* and *smad6.2* both act as antagonists of BMP signaling in zebrafish embryos.

Inhibition of smad6.1 and smad6.2 impacts dorsoventral patterning

To further investigate the function of *smad6.1* and *smad6.2* in early gastrulation, we used antisense morpholino oligonucleotides (MO) to knock down both *smad6* homologs. In embryos injected with *smad6.1* MO and *smad6.2* MO respectively, expression of *eve1* exhibited little, if any, lateral expansion (Fig. 3.5A-C), and expression domains of *flh* (Fig. 3.5E-G) and *chd* were
reduced (Fig. 3.5I-K). Comparing to single knockdown, double knockdown of *smad6.1* and *smad6.2* resulted in a further reduction of *chd* expression (Fig. 3.5L), but no stronger change in *eve1* and *flh* expression (Fig. 3.5D,H). The more dorsally shifted expression of *flh* and *chd* indicates slight ventralization (Schier and Talbot, 2005). This result is consistent with an increase in BMP signaling in *smad6.1* and *smad2* knockdown embryos. However, we did not observe apparently ventralized embryos at 24 hpf (data not shown). To confirm that knockdown of *smad6.1* and *smad6.2* could increase the activity of BMP signaling during gastrulation, we performed immunostaining assay using P-Smad5 antibody in embryos injected with both *smad6.1* MO and *smad6.2* MO. We observed a mild expansion of the BMP-active domain in *smad6.1/2* MO morphants (Fig. 3.5M,N). This result further supports the idea that *smad6.1* and *smad6.2* antagonize BMP signaling. The ventralization phenotype observed early during gastrulation in our loss-of-function assay suggests one function of *smad6.1* and *smad6.2* is to help establish proper DV patterning by inhibiting BMP signaling. The numbers of embryos injected are given in Table 3.2.
Discussion

In this study, we have characterized two homologs of Smad6 in zebrafish and provide evidence that they share high sequence similarity with Smad6 in other species (human, mouse and *Xenopus*) and both function as inhibitory Smads that antagonize BMP signaling during gastrulation. This is consistent with the role of Smad6 proteins as BMP inhibitors previously described in other systems (Afrakhte et al., 1998; Nakayama et al., 1998; Ishida et al., 2000). The spatial expression patterns of *smad6.1* and *smad6.2* are largely overlapping during early development. Besides a broad early expression pattern, both *smad6* homologs exhibit high expression in the brain, eye and heart, which is consistent with the expression pattern of *smad6* in chick embryos (Yamada et al., 1999; Vargesson and Laufer, 2009). This expression pattern also overlaps with the expression of *bmp* genes (Dick et al., 1999; Stickney et al., 2007; Shawi and Serluca, 2008) suggesting that Smad6 may function in those BMP-responsive cells to adjust the strength or duration of the BMP signaling. Unlike Chordin, which is expressed locally to abolish BMP activity (Schulte-Merker et al., 1997), Smad6 has a much broader expression pattern suggesting it may be an inhibitor used to fine-tune overall BMP signaling.

In line with the ubiquitous expression of *Xenopus smad6* during gastrulation as previously reported (Nakayama et al., 1998), we observe that both *smad6* homologs are expressed broadly in zebrafish embryos at early gastrula stage with a perceivably increasing intensity from ventral to dorsal pole. This expressing pattern suggests Smad6 may help major inhibitors like Chordin to establish the DV axis by suppressing BMP signaling along the margin of embryo, and this idea is supported by our results from gain- and loss-of-function assays. Interestingly, we find *smad6* expression is more responsive to up-regulated BMP signaling while showing less response to down-regulated BMP signaling, suggesting a partial dependence of BMP signaling in *smad6* expression. In contrast, suppressing BMP signaling by viral expression of Noggin in chick limb tissue abolishes *smad6* expression and directly applying Noggin protein to chick cardiogenic tissues results in significant reduction of *smad6* expression. This discrepancy might suggest *smad6* is differentially regulated by BMP signaling depending on the system. It has been shown that expression of mouse *smad6* is regulated by a Smad binding element (SBE) in the promoter region, and this SBE is preferentially recognized by BMP-activated Smad1/5 (Ishida et al., 2000). In the putative promoter region of *smad6.1* (1k bp
upstream of the coding sequence), we found one DNA motif was identical to the Smad5 SBE (TGTCTAGAC) identified in the mouse smad7 promoter (Li et al., 2001), however we did not find such motif in the similar region upstream of smad6.2 (data not shown). Possible cross-talk of Smad6 to TGF-β signaling may interfere our results as well. It has been shown that TGF-β and activin can transiently induce the expression of smad6 while BMP results in comparatively stable expression of smad6 (Afrakhte et al., 1998).

Inhibiting smad6.1 and smad6.2 simultaneously results in slightly further expansion of eve1 expression, which suggests the two genes work somewhat redundantly. Our P-Smad5 antibody staining assays show the active BMP region is reduced/increased in size as well as in amplitude when expressing/suppressing smad6. This suggests smad6 is utilized to finely adjust and strength of BMP gradient along the DV axis during gastrulation. smad6 may also regulate BMP signaling by adjust its duration. Smad6 knockout mice are reported to have phenotypes in cardiovascular system but no gastrulation defect (Galvin et al., 2000; Zhao, 2003). Here we observe a slight shifting in the DV markers in smad6.1/2-MO morphants during gastrulation, though no overtly ventralized phenotypes at 24 hpf. This difference may be due to varied biological activity of Smad6 among organisms, considering Xenopus Smad6 could inhibit activin but human Smad6 could not (Hata et al., 1998; Nakayama et al., 1998). The weak effect of smad6 on DV patterning in zebrafish indicates it may function transiently to suppress BMP activity during gastrulation. Another possible explanation is the loss of Smad6 function could be compensated by closely related Smad7, since in chick limb tissue Smad7 exhibits similar response to down- and up-regulated BMP signaling as Smad6 (Vargesson and Laufer, 2009). It will be necessary to perform comparative studies between Smad6 and Smad7 in future to see how these I-Smads work together to regulate the gastrulation process in zebrafish.

**Materials and methods**

*Cloning of smad6.1 and smad6.2*

Smad6.2 was originally identified from a yeast two hybrid screen for Spork (homolog of human ARHGAP21) interactive protein (screening service was provided by Hybrigenics). This sequence was used to BLAST the zebrafish Ensembl genome database. These approaches identified two smad6 paralogs, smad6.2 on linkage group 18 and smad6.1 on linkage group 7. PCR primers designed to clone smad6.2 were 5’-
GCGGATCCAAATTCCCAAGACGGAGTTTC-3’ (forward) and 5’-
CCCTCGAGATTGTGGAGAATCCTCTCG-3’ (reverse). Primers designed to clone smad6.1
were 5’-GCGGATCCGGTATGGTTCAGGACGAGAC-3’ (forward) and 5’-
CCCTCGAGAAGGTGGATTGGTTATCTGT-3’ (reverse). The BamHI and XhoI enzymatic
sites were introduced in the forward and reverse primers respectively for subsequent clone to
pCS2+ vector. We used these primers to amplify full-length cDNAs of smad6.1 and smad6.2
from a 24 hpf cDNA library made using the SMART cDNA kit (Clontech) (see Fig. 3.1A,B for
cloned sequences). The PCR product was inserted the PGEM vector (Promega) for sequencing as
well as into the vector pCS2+ for mRNA synthesis (Turner and Weintraub, 1994).

**Phylogenetic analysis**

Amino acid sequences were aligned using ClustalW algorithm with the default
parameters performed by MacVector® software. The phylogenetic trees were built using the
method UPGMA (Unweighted Pair Group Method with Arithmetic Mean, assuming the rate of
divergence is constant in different evolutionary lineages). Distance is estimated by total number
of differences between sequences and confidence of particular nodes in a tree is estimated by
bootstrap resampling approach. Other parameters were set at default values.

**In situ hybridization and antibody staining**

*In situ* hybridization with digoxigenin-labeled mRNA probes was performed as described
previously (Oxtoby and Jowett, 1993). The smad6.1 probe recognizes a 675 bp sequence
beginning at the 298bp nucleotides from the 5’ ends of smad6.1 cDNA. The smad6.2 probe
recognizes a 612 bp sequence beginning from the start codon of smad6.2 cDNA. The exact
sequences used to design *in situ* probes are indicated in Figure 3.1A,B. The hybridization
temperature was set to 65 °C to minimize non-specific binding. Other probes used have been
previously described: chd (Miller-Bertoglio et al., 1997), eve1 (Joly et al., 1993), and flh (Talbot
et al., 1995). For antibody staining, embryos were fixed, then dechorionated and incubated with
1:100 diluted P-Smad1/5/8 antibody (Cell Signaling Technology), and then followed by 488
Alexa Fluor conjugated to secondary antibody (Molecular Probes). Embryos were mounted in
3% methylcellulose and scanned by a Zeiss LSM 5 Pascal confocal microscope.
Morpholino and RNA injection

Morpholino antisense oligonucleotides: (smad6.1 MO: 5’-TAACTTAAACTACTTACTCAGTGCC-3’; smad6.2 MO: 5’-TCGACAGGACCTTCTACCTACGTGGC-3’) were purchased from Gene Tools. mfn, admp, chd and oep morpholinos have been previously described (Miller-Bertoglio et al., 1997; Feldman and Stemple, 2001; Willot et al., 2002; Jasuja et al., 2006). Morpholinos were diluted in Danieau’s buffer before injection. Morpholinos were injected at a concentration of 3 mg/ml. Synthetic mRNAs of caBMPR (1 mg/ml), smad5 (120 mg/ml), smad6.1 (50 mg/ml) and smad6.2 (50 mg/ml) made from pCS2+ constructs with mMessage mMACHINE kit (Ambion) were injected except for when Smad6.1 and Smad6.2 mRNA were co-injected, a concentration of 25 mg/ml each were used. In all experiments, a volume of 3-5 nl was injected in the yolk of one-cell stage embryos.
Figures and Tables

Figure 3.1 Clone and sequence analysis of Smad6.1 and Smad6.2.

(A,B) Complete mRNA sequences of Smad6.2 (on linkage group 18) and Smad6.1 (on linkage group 7). The sequence characterized in yeast two hybrid screen for proteins interacting Spork is marked red. The open reading frame (ORF) is indicated by bold letters. Note, for smad6.2 cDNA an alternative ORF is cloned (in frame with the larger ORF). The nucleotides where PCR primers bind to amplify cDNA are indicated by underline. The start codon of cloned cDNA is indicated by green capitalized letter. The sequences used to make in situ hybridization RNA probe is indicated by gray shade. lg, linkage group.

(C) Clustal alignment of amino acid sequences of zebrafish Smad6.1, Smad6.2 and Smad7 together with Smad6/Smad7 in mouse, Xenopus and humans. Outlined gray shading indicates identity. The conserved MH2 domains is indicated by underline. The representative amino acids in MH2 domains that distinguish Smad6 and Smad7 are indicated by red box.

(D) Similarity and indentity matrix of alignments of amino acid sequences of zebrafish Smad6.1, Smad6.2 and Smad7 together with Smad6/Smad7 in mouse, Xenopus and humans.

(E) Phylogenetic tree software built using UPGMA upon the amino acid sequences of zebrafish Smad6.1, Smad6.2 and Smad7 together with Smad6/Smad7 in mouse, Xenopus and humans by MacVector®.

(F) Phylogenetic tree built using UPGMA upon the amino acid sequences of the MH2 domains of zebrafish Smad6.1, Smad6.2 and Smad7 together with Smad6/Smad7 in mouse, Xenopus and humans by MacVector® software.

In (E) and (F), the method UPGMA (assuming the rate of divergence is constant in different evolutionary lineages) is used to build phylogenetic tree. Distance is estimated by total number of differences between sequences and confidence of particular nodes in a tree is estimated by bootstrap resampling approach.
A Smad6.2 (lg18) complete mRNA

1 aaaaaaa aaaaagqqa gaaacgqaqa aagaqagaga qagctgqgtt gcaagtttgac
61 caggtctgaa cagcggacacct gacgctacatt aagatgtcag aacattttcc ttctcctgcc
121 cccggcgcgcg cacaaggata ttttggattc gaaaaaagag gacgctagctg qaatgcttga
181 aaaaaacgcgg gaaatgtcag aaccaagcg aatgttcttgat gatttttaatt gacgccgag
241 cagcttttctt cagatgctcct cagccgcggctt gtaaagtggtt gtgtaagctt gttcctaatgt
301 cgggagacac ttaaacgcag gctggctacgt taaaatattag tctgggtgcg ttcgctagatca
361 catttttttt atgttgattta aacccggttgt gcatttttaaa tctggaaaaa aatatacatcg
421 gacccctcggt ctacattttct aacagttggtc aacatggttcc atcgttcaac gatctttgattgtga
481 aaagatcagct cgtgctgtgct agccagttgtgc ggttttcatat taattatatg ccatttttaatcg
541 ttcgctgcttc ccaactactag ttcgctgtttt ggcaccgctt cagctggtgtc gcacgtgtgccac
601 ggcctacgg acgcacagcgc cacaatttaag ttcgcacgca tccagaaataq ttccaaatactg
661 ctgcagacagt cacgatataa aactcatctag agttccaaagt agtgcgtatgt tcaacgagaca
721 acgcctccggt ctctgtgctgc gacactgtgct aacgccaggt attctgagata aacagggggg
781 cggagaggttt tggagctttgt gttcgagagc cgggtgca gttcgaggtc gccagaggtt gttcgagagc
841 gaaaatctcc aacagcaggct tcaagcggtatt cccccaga aacgacggtt gttcgagagc
901 tgggggaggtg tgtacagagga atcccccgtga gacgcagcgc ggttcagcct cacgggggttct
961 tttgacccaa gatgtctgagt gcgtttagag gtttgagagc cttcttgggtg tggagagc
1021 gcggtgattac gcacatcacat gttgtttttat caagagatcgc ggacacactt ctgtccagtcg
1081 aagtcggcgg ccagcgttctg gttcagctgt gttccccagctt aacaggggct cccggcgcagc
1141 cgggtctcttg gagacgcaggc agttcagaccc tccgctgagtt atggagcaggg gctcaagacg
1201 cgcaccctct tccactcattaa aagaacctaa ggagaaacc ttggataactt ttgtgagagc
1261 tgtgccacgt gcggggtgtgc gcagcgccagc tggagactggt tgtgtgactg tcggagccagc
1321 aggggcccac acgggcttctc cacaccctgtg tatcctgtag ctacttgggtt gttcaagcct
1381 gcacacacag gccgccgtaaa aacgtcctgtg tgtgacgcaag atcctctgggt ctcagagcgg
1441 ttcctgtagtt tgtgctatgct ctaactctac cactcatctata cccctgttgcg gacgaggggt
1501 tccctccacac tcacagctaat cccccagcgc gaaagacacag cccacgtgactgc tttggttggct
1561 cacactgtct ttcagactaa cggggggtttc cgcgttcatgac gtcacacttta cggcctgccg
1621 ctctagatcgg aagtttgttag tggagctgttc cagcagttggt caagcgattg aagtgcaagc
1681 tccggagctct ccggcagcgc gcgggggtttc cggcctgctc agcagtgggta cgtgggtgga
1741 tttcttatgac ctacccctagc gtagggctttg gaggagccgc gaaagggctt cgggggttgg
1801 cacagagcagg cggcagacgag aagtcggtcag ggtgcgtcat cgggagtctac gcaaggggagt
1861 gcggagaggt tgttgggcttt aacagggcag gagcgcggccc atctttgctca aaaactctcgac
1921 tgtggagttc ccagggggagc gagcttcggtt ggtgctggag cgtgatggctg gatctccgcct
1981 taagttgttt gacattcagc gcgtgccttac gctagctgattt ggaagggctt cggagtttgcg
2041 agacgagccgcc tattgacgct aagtcggttc gcgcagtgctcg caagctttgttg gcgagcccttg
2101 ttactccggt ccggttcgctt cttggtgcgatt gttggctgagc ttaaactctat ccacactgaa
2161 cagattaccess aagggccagt aagggcttgctg gaggagctta aacagcggttc aacaacccag
2221 ttcattactt cctaggttagt ctaatattaatt cttttttataa tttatatata tatataaatat
2281 acttgttaaat acctgcctgc tttttcagat gttattattt atgttggtct taatgttgac
2341 atggagacag gaaaaaagag cgggagagct cttggtgctgg ttatatataa tttatatataa
2401 tcattataag tctaaactac aattggaaaa tttataagcga aaattagagga
2461 gcgggttttg gattgtgattt gttccccatgt actatattac cccagtgaga aagctacacac
2521 cattcacaaca tttataataaa agtatcggca taatataaaa aaaaaaaa aaaa
E

Method: UPGMA; Bootstrap (100 reps); tie breaking = Systematic
Distance: Absolute (# differences)
Gaps distributed proportionally

F

Method: UPGMA; Bootstrap (100 reps); tie breaking = Systematic
Distance: Absolute (# differences)
Gaps distributed proportionally
Figure 3.2 *smad6.1* and *smad6.2* show redundant and unique domains of expression during development.

(A,B,A’,B’) Shield stage, (A,A’) lateral view, (B,B’) animal pole view, dorsal towards the right. (C,D,C’,D’) Bud stage, (C,C’) lateral view, anterior towards the top and dorsal towards the right; (D,D’) caudal view, dorsal towards the top. (E,F,E’,F’) 10-somite stage, (E,E’) lateral view and (F,F’) dorsal view with anterior towards the top. (G,H,G’,H’) 24 hpf, (G,G’) lateral view and (H,H’) dorsal anterior view. (I,J,I’,J’) 48 hpf, (I, I’) lateral view and (J,J’) dorsal anterior view. tb, tailbud. ml, midline. ey, eye. br, brain. pt, posterior tail. ht, heart. so, somites. pf, pectoral fin. ov, otic vesicle. le, lens. Open arrow indicates the unique expression of *smad6.1* near the hindbrain.
104
Figure 3.3 Decreased and increased expression of *smad6.1* in response to down and up regulated BMP signaling at early gastrula stage.

(A-N) Common spatial expression of *smad6.1* in representative embryos at 60% epiboly, (A,C,E,G,I,K,M) lateral view and (B,D,F,H,J,L,N) animal pole view with dorsal towards the right. Embryos were injected with *mfn* MO (C,D), *admp* MO (E,F), *chd* MO (G,H), *caBMPR* RNA (I,J), *smad5* RNA (K,L) and *oop* MO (M,N). Decreased expression of *smad6.1* is indicated by arrowheads while increased expression is indicated by open arrow heads. Belt shaped expression pattern along the margin is indicated by arrow. (O) Penetrance of altered *smad6.1* and *smad6.2* expression in response to changed BMP signaling. Wild type like expression is denoted as value zero. Positive percentage represents the penetrance of increased expression and negative percentage represents the penetrance of decreased expression.
Percentage of embryos showing changes in *smad6* expression
Figure 3.4 Overexpressing *smad6.1* and *smad6.2* dosalizes embryo by inhibiting BMP signaling.

(A-L) Embryos after whole mount *in situ* hybridization at 60% epiboly; animal pole views, dorsal towards the right. Reduced ventral domain of *eve1* expression (A-D) and expanded *flh* (E-H) and *chd* expression in dorsal regions (I-L) in injected compared with uninjected embryos was observed. (M,N) Dorsal view of expansion of *chd* expression in embryos at shield stage. (O,P) Animal pole view of embryos at 60% epiboly after P-Smad5 antibody staining, dorsal towards the right. Expression domain is delineated by arrowheads.
Figure 3.5 Inhibiting *smad6.1* and *smad6.2* ventralizes embryo as increasing BMP signaling.

(A-L) Embryos after whole mount *in situ* hybridization at 60% epiboly; animal pole views, dorsal towards the right. Expanded ventral domain of *eve1* expression (A-D) and reduced *flh* (E-H) and *chd* expression in dorsal regions (I-L) in injected compared with uninjected embryos was observed. (M,N) Animal pole view of embryos at 60% epiboly after P-Smad5 antibody staining, dorsal towards the right. Arrowheads indicates the expression region.
Table 3.1 Percentage of embryos showing changes of designated markers after injection of RNAs

<table>
<thead>
<tr>
<th></th>
<th>smad6.1 RNA</th>
<th>smad6.2 RNA</th>
<th>smad6.1/2 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>eve l</td>
<td>50% (n=102)</td>
<td>49% (n=87)</td>
<td>60% (n=91)</td>
</tr>
<tr>
<td>filh</td>
<td>43% (n=85)</td>
<td>44% (n=90)</td>
<td>55% (n=82)</td>
</tr>
<tr>
<td>chd</td>
<td>40% (n=95)</td>
<td>45% (n=107)</td>
<td>50% (n=98)</td>
</tr>
<tr>
<td>P-Smad5</td>
<td>--</td>
<td>--</td>
<td>49% (n=35)</td>
</tr>
</tbody>
</table>
Table 3.2 Percentage of embryos showing changes of designated markers after injection of morpholinos.

<table>
<thead>
<tr>
<th></th>
<th>smad6.1 MO</th>
<th>smad6.2 MO</th>
<th>smad6.1/2 MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>eve1</td>
<td>0% (n=110)</td>
<td>0% (n=112)</td>
<td>0% (n=118)</td>
</tr>
<tr>
<td>flh</td>
<td>38% (n=115)</td>
<td>35% (n=118)</td>
<td>42% (n=120)</td>
</tr>
<tr>
<td>chd</td>
<td>32% (n=115)</td>
<td>28% (n=104)</td>
<td>34% (n=122)</td>
</tr>
<tr>
<td>P-Smad5</td>
<td>--</td>
<td>--</td>
<td>35% (n=32)</td>
</tr>
</tbody>
</table>
References


Appendix A - *strabismus* and *prickle* interact with Noncanonical Wnt and BMP Signaling to Affect the Secondary Tail Formation in Zebrafish

**Abstract**

Based on our results that inhibition of noncanonical Wnt and BMP signaling leads to secondary tail formation in zebrafish, we studied the genetic interactions between *pk* and *stbm*, two genes involved in noncanonical Wnt signaling, and other players in secondary tail formation. By morpholino-induced gene knockdown, we found that inhibiting *stbm* and *pk1* suppresses the secondary tails induced by losing *kny* or *dvl2/dvl3*. This genetic interaction is consistent with the Planar Cell Polarity (PCP) signaling pathway in *Drosophila* where *pk* and *stbm* genetically antagonize *dsh*. Although we do not know whether there is corresponding asymmetrical localization of these proteins in tailbud cells, our data suggest that in tail development, noncanonical Wnt signaling functions similar to PCP signaling in *Drosophila*. We tested the genetic interactions between *stbm/pk* and BMP signaling in secondary tail formation. Surprisingly, knocking down *stbm* enhances the secondary tail phenotype when losing BMP signaling, whereas knocking down *pk1* suppresses the secondary tail phenotype in BMP mutants.

**Introduction**

In zebrafish, tail development is shaped by two forces: convergence and extension (CE) movements of the anterior (dorsally derived) tailbud, which converge the presomatic and somitic mesoderm to the midline and drive the posteriorward extension of the embryo, and the lateral subductive movements of the posterior (ventrally derived) tailbud cells, which avoids the posteriorly migrating anterior tailbud cells by diverging from the midline and moving anteriorly beneath them (Kanki and Ho, 1997).

Noncanonical Wnt signaling, the equivalent of *Drosophila* planar cell polarity (PCP) cascades that establish polarity in planar epithelial cells, is required in zebrafish tail morphogenesis. Two noncanonical Wnt signaling components, the heparan sulfate proteoglycan encoded by *glypican4/knypek* (*kny*) and the secreted glycoprotein of Wnt family encoded by...
wnt5/pipetail (ppt), control cell movements within the tailbud (Marlow et al., 2004). Zebrafish mutants of kny and wnt5 have shortened tails and thinner and wider somites due to reduced CE and impaired laterad divergence movements (Topczewski et al., 2001; Kilian et al., 2003). We previously reported that inhibiting two dvl homologs, dvl2 and dvl3, which are involved in noncanonical Wnt signaling, results in secondary tail phenotype (described in Chapter 2). We further confirmed this phenotype in the kny mutant, suggesting that one role of noncanonical Wnt signaling is regulating the integrity of the tailbud. We found that kny is required in the CNH domain of the tailbud to positively regulate the membrane localization of N-cadherin. Upon loss of noncanonical Wnt signaling, the CNH cells become less tightly associated with each other, which results in aberrant cell movement. This serves as the initiation of subsequent secondary tail formation. In parallel to the noncanonical Wnt signaling pathway, BMP signaling is required in the posterior mesoderm that surrounds the CNH to prevent secondary tail formation.

Whether other components of noncanonical Wnt signaling involved in maintaining the integrity of the tailbud is largely unknown. Most of the work in vertebrates concentrates on the role of noncanonical Wnt signaling in regulating CE movements during gastrulation, suggesting this process shares much similarity in the mechanism of PCP signaling in Drosophila (Veeman et al., 2003). In the Drosophila wing epithelium, asymmetrical localization of the core PCP components provides the cue for the establishment of cell polarity. When PCP signaling is activated, transmembrane receptor Frizzled brings the cytoplasmic docking protein Dishevelled (Dsh/Dvl) to the distal membrane and activated Dsh can transduce the signal to its downstream effectors. Strabismus (Stbm) recruits the intracellular protein Prickle (Pk) to the proximal cell membrane to suppress Frizzled and Dsh. Since the tailbud is a different context from the epithelium and gastrula cells, how genes involved in noncanonical Wnt signaling interact with each other needs to be clarified.

Here, we studied the genetic interactions between pk and stbm and the other players (mfn, kny, dvl2 and dvl3) in secondary tail formation. By morpholino-induced gene knockdown, we found that inhibiting stbm and pk1 suppresses the secondary tails induced by losing kny or dvl2/dvl3. This genetic interaction fits the PCP pathway in Drosophila that pk and stbm genetically antagonize dsh. Although we do not know whether there is corresponding asymmetrical localization of these proteins in tailbud cells, our data suggest that in tail development, noncanonical Wnt signaling functions similar to Drosophila PCP. We tested the
genetic interactions between stbm/pk and BMP signaling in secondary tail formation. Surprisingly, knocking down stbm enhances the secondary tail phenotype when losing BMP signaling, whereas knocking down pk1 suppresses the secondary tail phenotype in BMP mutants.

### Material and Methods

Morpholino Injections experiments were carried out as mentioned in the supplementary section of Chapter 2.

### Results and Discussion

In *Drosophila*, dsh is genetically inhibited by stbm and pk. Interfering with the function of Dsh leads to disoriented wing hairs (Axelrod et al., 1998). Knocking down stbm or pk rescues the dsh loss of function phenotype (Jenny et al., 2005). As for the secondary tail phenotype in zebrafish, the kny and dvl linear pathway of noncanonical Wnt signaling has a demonstrated role (see Chapter 2). Our previous experiments show that inhibiting stbm and pk separately or cooperatively does not result in secondary tail formation. To test whether stbm and pk suppress dvl as in the PCP pathway in the fly, we knocked down stbm or pk by morpholino to see whether this inhibits the secondary tail phenotype in dvl2/3 morphants or kny mutants. Knocking down stbm consistently reduces the penetrance of secondary tail formation in dvl2/3 morphants (Fig. A.1A) and kny embryos (Fig. A.1B). In the two homologues of pk we tested, knockdown of pk1a exhibited great efficacy in suppressing secondary tails in kny (Fig. A.1D) while pk2b has no obvious effect on secondary tail formation (Fig. A.1C,E). This is in line with the differential expression patterns of these two pk genes, suggesting a local requirement of these genes in the regulation of tail development. Our results fits the genetic interactions as deduced from the *Drosophila* PCP pathway, suggesting the noncanonical Wnt and PCP signaling are conserved in fish and fly. There seems to be no direct linkage between the severity of CE and occurrence of secondary tail, since knocking down stbm in kny show strong defect of CE movements causing extremely short embryos (data not shown) as knocking down dvl2/3 in kny, but with much lower penetrance of secondary tail. Since we observed losing membrane localization of cadherin in the CNH is the key to cause secondary tail in kny (Fig. 2.5), it will be important to determine whether inhibiting stbm or pk1a is able to reestablish the membrane anchoring of cadherin in kny embryos. So far, we have not found any role for known noncanonical Wnt ligands in secondary
tail regulation, so identifying the roles of additional components in noncanonical Wnt signaling will be important to understand how noncanonical Wnt signaling functions to regulate tail morphogenesis. One good candidate is Diego/Diversion, an ankyrin repeat protein that functions to promote PCP signaling in *Drosophila* (*Jenny et al., 2005*) and acts downstream of *wnt5/11* to regulate CE in zebrafish, and is capable of binding to Dsh (*Moeller et al., 2006*).

We have previously shown that inhibiting *dvl2/3* together with *mfn* strongly enhances the secondary tail phenotype. To investigate whether other genes (*pk* and *stbm*) in the noncanonical Wnt pathway interact with BMP signaling in secondary tail formation, we performed serial knockdown experiments. Our results show that knocking down *stbm* greatly enhances the penetrance of secondary tails in *mfn* (Fig. A.2A,C). In contrast, *pk1a* knockdown suppresses the secondary tail in *mfn* (Fig. A.2B). *pk2b* knockdown does not result in any change in secondary tail formation in BMP-compromised embryos (Fig. A.2D). My future studies plan to better define these genetic interactions in secondary tail development in zebrafish.
Figures and Tables

Figure A.1

Percentage of secondary tail formation in embryos injected with indicated combination of morpholinos (1 mg/ml each, except for ‘dvl2/dvl3 MO’ condition, where a concentration of 3 mg/ml for each MO was injected). Embryos from at least three separate experiments were scored. Percentage of secondary tail of each separate experiment is calculated. Then the arithmetic mean of percentage of all replicates is calculated by dividing the sum of percentages with numbers of replicates. Then the variation from the mean is represented by calculated standard deviation. Error bars represent standard deviations of at least 3 replicates. Number of embryos scored for each column is shown in figures. Error bars represent the standard deviation of at least three replicates.
Figure A.2

Percentage of secondary tail formation in embryos injected with indicated combination of morpholinos (1 mg/ml each, except for mfn MO, where a concentration of 2 mg/ml was injected). Embryos from at least three separate experiments were scored. Note the penetrance of secondary tails in mfn embryo is varied due to different wild-type background. Percentage of secondary tail of each separate experiment is calculated. Then the arithmetic mean of percentage of all replicates is calculated by dividing the sum of percentages with numbers of replicates. Then the variation from the mean is represented by calculated standard deviation. Error bars represent standard deviations of at least 3 replicates. Number of embryos scored for each column is shown in figures. Error bars represent the standard deviation of at least three replicates.
References


