MOLECULAR MECHANISMS OF *wnt8a* REGULATION: INSIGHTS INTO VERTEBRATE MESODERM PATTERNING AND DEVELOPMENT

A Dissertation

by

ANAND NARAYANAN

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

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Biology

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Approved by:

Chair of Committee,	Arne C. Lekven
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Head of Department,	U.J.McMahan

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ABSTRACT

Molecular Mechanisms of *wnt8a* Regulation: Insights Into

Vertebrate Mesoderm Patterning and Development. (May 2012)

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Chair of Advisory Committee: Dr. Arne C. Lekven

Vertebrate *wnt8a* occupies a position at a crossroads linking anteroposterior and dorsoventral axis patterning. While functional aspects of *wnt8a* are beginning to be understood, the regulation of *wnt8a* expression and its relationship to mesoderm induction and maintenance pathways are unclear. Three inputs that control *wnt8a* expression in the zebrafish embryonic margin have been identified: the Brachyury-related T-box transcription factors No tail a (Ntla) and No tail b (Ntlb, previously called Bra) and the maternal zinc-finger transcription factor Zbtb4 (previously called Kzp) are known direct regulators, and Nodal signaling is genetically upstream of *wnt8a* expression. The transcriptional mechanisms by which the *wnt8a* locus integrates these diverse temporal inputs are not yet known. We have generated zebrafish transgenic for a modified genomic PAC clone that expresses EGFP from the *wnt8a* locus. The EGFP reporter transgene is expressed in a pattern nearly identical to *wnt8a*, including maternal deposition, expression in the ventrolateral mesoderm and in the yolk syncytial layer. Using this transgenic line, we identified two phases of *wnt8a* transcriptional regulation in zebrafish: phase I comprises Nodal-dependent activation during early gastrulation and

phase II comprises No tail (Ntl)-dependent regulation from mid to late gastrula stages onwards. These phases mirror the transition from Nodal-dependent mesoderm induction to Ntl-dependent mesoderm maintenance.

To further understand how the *wnt8a* locus integrates these signals to achieve its transcriptional output, we analyzed upstream cis-regulatory regions through transgenic reporter assays. We identified three promoters in the bicistronic *wnt8a* locus, two of which drive expression of the upstream coding region (*wnt8a.1*). We identified two regulatory regions, proximal and distal: the proximal regulatory region contains a mesodermal enhancer with potential binding sites for FoxH1 and Ntl that is required for both the Nodal and Ntl responses. Phase I expression requires Nodal signaling through the mesoderm enhancer in combination with the distal regulatory region, which bears a Zbtb4 consensus binding site. Phase II expression requires Ntl regulation of the mesoderm enhancer in the context of the proximal regulatory region. The distal regulatory region negatively impacts phase II expression driven by the proximal regulatory region, indicating a complex relationship of regulatory elements.

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CHAPTER I

INTRODUCTION

The development of nearly all multicellular organisms begins from a single cell- the fertilized egg, or zygote, which undergoes mitotic division to form a blastula, gastrula and then an adult (Gilbert, 2010). During early development of the embryo, the setting up of the primary axes: dorsoventral (D/V), anteroposterior (A/P), and left-right, are crucial steps that establish the body plan. During this process, the three germ layers; ectoderm, mesoderm and endoderm, are formed, from which tissues and organs of the body will develop. Formation of germ layers is one of the earliest subdivisions that occur in embryonic development. The ectoderm (outer layer) gives rise to several different structures such as epidermis, spinal cord and brain. The mesoderm (middle layer) gives rise to notochord, bone tissue, kidneys, blood cells, heart and muscles. The endoderm (inner layer) gives rise to the digestive tube, pharynx and respiratory tube. Fate map studies have revealed that the precursor cells of these tissues are arranged in a specific order along the D/V axis of the embryo as shown in Fig.1. The formation of the germ layers and its specification into distinct subpopulation of precursors are critical steps in early development (Gilbert, 2010).

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

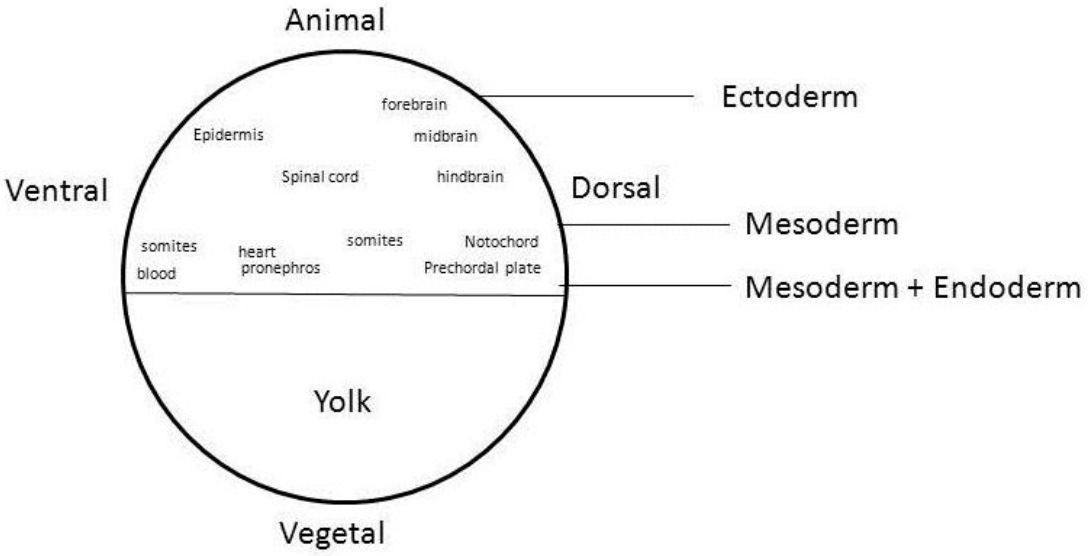

Fig. 1. Fate map of a zebrafish embryo at gastrula stage. The three germ layers are shown above. Ectoderm gives rise to different parts of the brain and epidermis. Mesoderm gives rise to notochord, prechordal plate, somites, pronephros and blood. They are arranged along the dorsal-ventral axis. Endodermal cells are mixed with mesoderm. (adapted Schier and Talbot, 2005).

MESODERM INDUCTION

In vertebrates, mesoderm has received special attention because it is thought to arise as a result of the earliest induction known in the embryo (Schulte-Merker et al., 1992). Induction refers to a process in which extracellular signals bring about a change from one cell fate to another in a particular group of cells (Kimelman, 2006). Mesoderm induction is the process in which undifferentiated cells receive extracellular signals and differentiate into mesoderm. The mechanisms of mesoderm induction are more or less conserved across species.

In amphibian embryos, Nieuwkoop demonstrated that explanted tissue from the bottom of the blastula stage embryo (the vegetal cap) could convert prospective ectodermal cells from the top of the embryo (the animal cap) into mesodermal tissues that normally reside in between the two germ layers. Thus, he showed that mesoderm is induced by a signal that is released by the most vegetal cells, the prospective endoderm, which converts the overlying prospective ectoderm toward a mesodermal fate, creating the three germ layers of the embryo (reviewed in Harland and Gerhart, 1997). In *Xenopus*, a maternally expressed, vegetally localized mRNA encoding a member of the TGF-β family, Vg1 (Weeks and Melton, 1987) along with the maternally deposited T Box transcription factor, VegT (Zhang et al., 1998) have been shown to be involved in mesoderm induction. VegT has been shown to activate Xenopus nodal related (Xnr) genes, which are important in mesoderm induction (Kimelman, 2006). In zebrafish, it has been shown that the signals that induce the mesoderm originate from the YSL (Yolk Syncytial Layer), an extra-embryonic tissue in teleost. Mizuno et al. (1996) showed the inductive capacity of this tissue through transplantation assays. In order to demonstrate the importance of the YSL, the blastoderm was separated from the yolk cell. This yolk cell with the YSL was biotin-labelled and transplanted to the animal pole region of unlabeled host embryos. The transplantation procedure ensured that the yolk cells from the donor embryos came in contact with the animal pole region (prospective ectoderm) of the host embryos. These recombinant embryos were incubated and assayed for mesodermal markers. In addition to the endogenous mesoderm formed in host embryos, they also observed that the host embryo developed mesoderm at the region where it came in

contact with the donor cells. This ectopic mesoderm developed as a result of the signals derived from the donor yolk cells. Chen and Kimelman, (2000) further confirmed that RNA in the YSL is required for ventrolateral mesoderm induction. They also demonstrated that the RNA from the YSL is important for expression of the Nodal genes in zebrafish, *cyc* and *sqt*, in the ventro-lateral blastomeres right above the YSL. Nodal genes have been shown to be critical to mesoderm development.

NODAL SIGNALING IN MESODERM

Nodal is a member of the transforming growth factor β (TGF β) super family. Nodal was shown to be required for mesoderm formation by the isolation and cloning of a retroviral insertion mutation in the mouse Nodal gene (Zhou et al., 1993; Conlon et al., 1994). Mouse Nodal mutants lack a primitive streak-a thickening caused by ingression of mesoderm and endoderm progenitors- and most mesoderm, displaying only sporadic formation of posterior mesoderm (Reviewed in Schier and Shen, 2000.). The requirement for Nodal genes in mesoderm development is conserved across vertebrate species. Feldman et al., (1998) characterized mutations in the zebrafish Nodal genes, *cyc* and *sqt*, and demonstrated that these genes are important for dorsal mesoderm formation, similar to mouse Nodal. Ventro-lateral mesoderm development was unaffected in these zebrafish mutants. They also showed that the *sqt* gene encodes a Nodal related protein and the protein shares structural similarities with mouse and Xenopus Nodal proteins. *sqt* acts as a secreted morphogen which induces downstream genes in a concentration and

distance-dependent manner. Sqt can act at a distance without any relay mechanism while Cyc acts locally (Chen and Shier, 2001).

The importance of Nodal genes in dorsal mesoderm induction was confirmed by studies on zebrafish *MZoep* (Maternal zygotic one eyed pinhead) mutants (Gritsman et al., 1999). The zebrafish ortholog of Epidermal Growth Factor-Cripto-FRL1-Cryptic (EGF-CFC) gene, *oep* is an extracellular co-factor indispensible for Nodal signaling, similar to Cripto in mouse.

NODAL SIGNALING PATHWAY

Nodal ligands initiate signal transduction through type I and type II serine/threonine kinase receptors, in the presence of the coreceptor EGF-CFC. Upon formation of the ligand-receptor complex, the type II receptor kinase activates type I receptors by phosphorylating their intracellular kinase domain. Activated type I receptors phosphorylate and activate the downstream effectors Smad 2&3. These activated effectors form complexes with the co-smad Smad 4 that are translocated into the nucleus, where they interact with other transcription factors to regulate expression of specific genes as shown in Fig. 2. (Reviewed in Tian and Meng, 2006; Schier, 2003).

Nodal and Nodal-related genes have been identified in several vertebrate species. One Nodal gene has been identified in Humans, mice and chicks. At least three zebrafish Nodal genes have been identified: *squint* (*sqt*), *Cyclops* (*cyc*) and *southpaw* (*spaw*). In

the model organism, *Xenopus laevis* (African clawed frog); at least six Nodal related genes (Xnr 1-6) have been identified. However, Nodals have not been identified in Drosophila or *Caenorhabditis elegans* (Reviewed in Tian and Meng, 2006; Schier, 2003).

Besides mesoderm induction and endoderm development, Nodals have also been shown to play critical roles in neural patterning along the anterior-posterior axis and in specification of the left-right axis (Reviewed in Tian and Meng, 2006; Schier, 2003). In zebrafish, out of the three Nodal ligands, *squint* and *cyclops* are required for mesoderm and endoderm formation (Feldman et al., 1998). Absence of Nodal signaling in zebrafish embryos leads to the loss of all endoderm, head and trunk mesoderm, including heart, kidney, blood, liver, pancreas and gut (Feldman et al., 1998; Gritsman et al., 1999). The third gene, *southpaw*, is only expressed after gastrulation and is required to establish left-right asymmetry (Long et al., 2003). One transcription factor, FoxH1, belonging to the winged helix transcription factor family, has been identified as a mediator of Nodal signaling (Pogoda et al., 2000; Sirotkin et al., 2000; Pei et al., 2007; Fan et al., 2007). Forkhead transcription factors have also been shown to mediate Nodal signaling in mouse and frogs (Chen et al., 1996; Watanabe and Whitman, 1999; Osada et al., 2000).

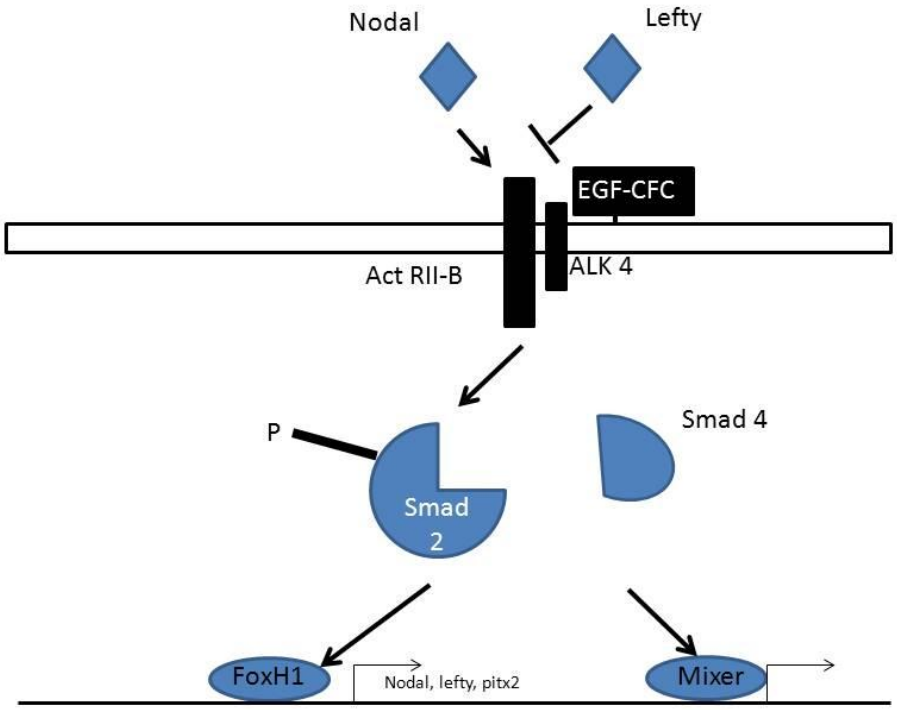

Fig. 2. **Simplified representation of the Nodal signaling pathway.** Nodal ligands bind to type I and II serine-threonine kinase receptors. Upon binding, the type II receptor phosphorylates the kinase domain of the type I receptor. This leads to the activation of receptor Smads, which later associate with Smad4 and translocate in the nucleus to modify gene transcription. (Adapted from Schier, 2003).

ORGANIZER FORMATION AND AXIS FORMATION OF THE VERTEBRATE EMBRYO

The process of mesoderm induction and axis patterning of the vertebrate embryo are tightly linked. The earliest known studies conducted in this field were by Hans Spemann on amphibian embryos. In the first set of experiments, he divided a newly fertilized salamander egg along different planes. He then isolated the cells and allowed them to

grow separately. He observed that the plane of cleavage determines whether the cell develops into a normal embryo or gives rise to an abnormal embryo which lacked dorsal structures. This was the first evidence that molecules responsible for determining the embryonic axis may be localized asymmetrically between the cells. These experiments led to transplantation experiments that finally helped to determine the reasons behind the normal and abnormal embryo development. Spemann and his graduate student Hilde Mangold transplanted the dorsal lip of the blastopore into the future ventral region of another embryo. They observed that the transplanted blastopore was able to induce neural differentiation (a dorsal fate) in the host embryo where it was transplanted. Later transplantation studies led to the development of an embryo which had duplicated head and other dorsally derived structures. The dorsal blastopore lip was renamed the Spemann Organizer in honor of this important discovery.

Research in subsequent years confirmed the importance of the Organizer as a signaling center essential for specifying the D/V and A/P axis. However, the mechanisms of Organizer formation and the molecules that give it inductive abilities were still not known. Experiments in frog embryos have shown that exposure to UV light leads to loss of dorso-anterior structures (Scharf and Gerhart, 1980; Elinson and Pasceri, 1989). UV treatment of newly fertilized zebrafish eggs at the vegetal pole and prolonged exposure to cold temperatures (18 deg C) resulted in ventralized embryos (Jesuthan and Stähle, 1997). This suggested that the UV treatment and cold temperature may be inhibiting a molecular pathway or mechanism important for Organizer formation. On the other

hand, embryos treated with lithium chloride (LiCl) results in dorsalized embryos (Kao and Elinson, 1989; Stachel et al., 1993). This suggested that there is an increase in the dorsalizing signal, and also that the treatments that disrupted Organizer formation affected normal patterning of the embryo.

The current model of Organizer formation in *Xenopus* suggests that dorsal determinants are located in membrane vesicles in the vegetal pole of the embryo. Biochemical changes associated with fertilization cause the transport of these vesicles to the dorsal side by cortical microtubules. This accumulation correlates with the activation of canonical Wnt signaling pathway on the dorsal side, causing the stabilization and nuclear localization of the β-catenin protein. This nuclear localization leads to the activation of Nodal genes. High levels of Nodal genes turn on other genes that are important for the Organizer (Reviewed in De Robertis et al., 2000; Kimelman, 2006).

In zebrafish, the role of microtubule mediated transport and nuclear localization of β-catenin protein in the dorsal margin as essential steps for Organizer formation is well documented (Jesuthan and Stähle, 1997; Kelly et al., 2000). β-catenin accumulation in the dorsal YSL is essential for activating Nodal, *sqt* and *bozozok* (Kelly et al., 2000; Fekany-Lee et al., 2000). The Bozozok and Nodal genes function cooperatively in Organizer formation (Solnica-Krezel and Driever, 2001). Sqt, in turn, activates Cyclops and Squint in a tier of blastoderm cells above YSL (Kelly et al., 2000; Fan et al., 2007; Kimelman, 2006; Kimelman and Griffin, 2000). High levels of Nodal signaling in the

dorsal mesoderm lead to the activation of *goosecoid* (Feldman et al., 1998; Gritsman et al., 2000). Lower levels of Nodal are required to turn on *notail*, *floating head* and *bhikari* (Gritsman et al., 2000; Chen and Schier, 2001). The set of genes that gets activated in the Organizer gives it its inductive capacity. These genes are also responsible for patterning the embryo.

INDUCTIVE CAPABILITIES OF THE ORGANIZER

The establishment of the Organizer in the dorsal mesoderm is a very critical step in the embryonic patterning and survival of the embryo. The Organizer is the source of secreted proteins that antagonize genes that are active in the ventral embryo. These antagonists include Noggin, Chordin, Cerebrus, Frzb-1, Crescent and Dickkopf (DKK) (De Robertis, 2000). As mentioned earlier, the Organizer is also a site of *bozozok* and *goosecoid*, which are transcriptional repressors (Kessler, 1997; Fekany-Lee et al., 2000).

MECHANISMS OF INHIBITION OF VENTRAL GENES BY ORGANIZER

Chordin, Noggin and Follistatin were identified separately as BMP antagonists (Smith and Harland, 1992; De Robertis, 2000). BMPs are members of the TGFβ superfamily of secreted molecules. BMP ligands bind to extracellular domains of type I and type II receptors, which are transmembrane proteins with intracellular serine/threonine kinase domains. Upon binding of the BMP ligand, transcription factors Smad 1/5/8 are phosphorylated. The phosphorylated Smads translocate to the nucleus, bind to the DNA with Smad 4 and activate downstream target genes. The BMP pathway is involved in

inducing ventral fates, differentiation, proliferation and apoptosis (Reviewed in Schier and Talbot, 2005). One of the main functions of Organizer genes is to limit BMP activity to the ventral half of the embryo. Chordin and Noggin inhibit BMP signaling by binding to BMP ligand directly in extracellular space, thereby preventing the ligand from binding to the receptor (Piccolo et al., 1996; Zimmerman et al., 1996). Follistatin binds BMP ligands and this complex can bind to the receptor, but this complex is unable to affect any downstream signaling.

In the zebrafish embryo, BMP is expressed in the ventral half of the embryo. BMP activity is important for formation of ventral mesoderm and ventral half of the ectoderm. The ventral half of the ectoderm has a non-neural fate, i.e. epidermis. The limiting of BMP activity to the ventral half of the ectoderm by antagonists secreted by the Organiser is critical for inducing Neural (dorsal ectoderm) fates and non-neural fates (ventral ectoderm) (Reviewed in De Robertis et al., 2000).

In zebrafish, loss of function mutations in *chordino v*entralizes the mesoderm and ectoderm (Hammerschmidt et al., 1996). In *chordino* mutants, the epidermis and ventral mesoderm are expanded with a concordant reduction of the neural plate and dorsal mesoderm (Schulte-Merker et al., 1997; Fisher and Halpern, 1999). In zebrafish there are four zygotic BMP signaling mutants: type I BMP receptor *alk8* mutant (*lost-a fin*), smad 5 mutant (*somitabun and piggy tail)*, two BMP ligand mutants- *bmp2b* mutant (*swirl*) and *bmp7* mutant (*snailhouse*). All these mutants have a dorsalized phenotype,

confirming the requirement of BMP in promoting ventral fates in the embryo (Hammerschmidt and Mullins, 2002; De Robertis and Kuroda, 2004). In zebrafish, it has been established that Chordino specifically functions to inhibit Bmp as shown in Fig. 3. The homozygous *swirl* mutant embryo is dorsalized, while *chordino* mutants are ventralized. The *swirl* dorsalized phenotype is epistatic to *chordino* in *chordino:swirl* double mutants (Hammerschmidt et al., 1996). This suggests that the primary role of Chordin is to function as a specific inhibitor of BMP.

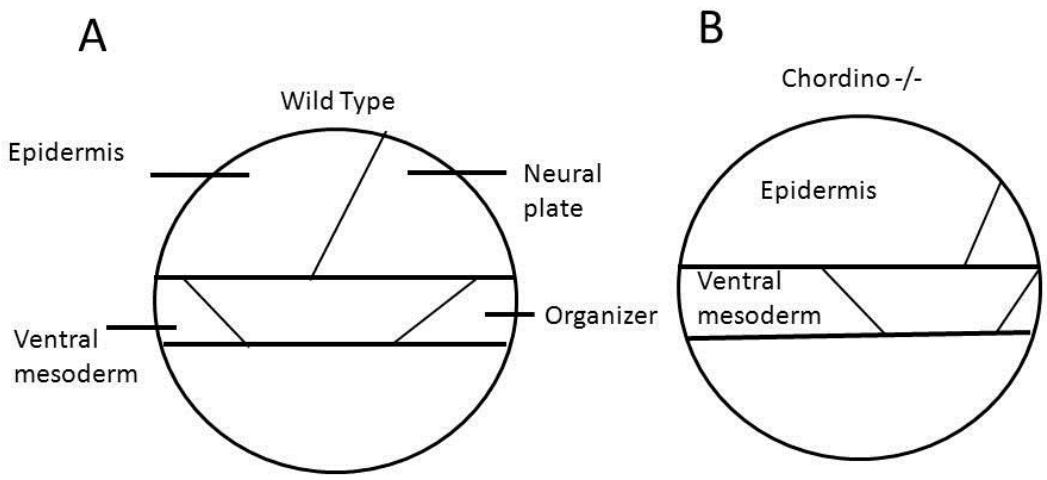

Fig. 3. Role of BMP in early zebrafish embryo. A: normal zebrafish embryo, BMP is expressed in the ventral half of the embryo. The domain of BMP expression defines epidermis and ventral mesoderm. B: chordino mutant lacking the BMP inhibitor chordin, the BMP expression domain is expanded leading to an expansion of epidermis and ventral mesoderm at the loss of neural plate and Organizer. (adapted from De Robertis et al., 2000).

When the BMPs are inactivated by mutation, Chordin is not necessary for dorsal development (Reviewed in Schier and Talbot, 2005; Robertis and Kuroda, 2004; De Robertis et al., 2000).

The BMP signaling pathway is conserved in other model systems such as Drosophila, *Xenopus* and mouse. In Drosophila, *Decapentaplegic* (*dpp*) and *screw* (*scw*) are BMP growth factors which function as dorsal-ventral morphogens of the fly embryo. The functional homologue of vertebrate Chordin in Drosophila is encoded by the *short gastrulation* (sog) gene and it antagonizes *dpp* and *scw*. Loss of function mutations in *sog* result in an expansion of dorsal ectoderm with a loss of neurogenic ectoderm. Dpp acts as a dorsalizing gene in Drosophila (Reviwed in Gilbert, 2010; Schier and Talbot, 2005; Robertis and Kuroda, 2004; De Robertis et al., 2000). The Sog protein functions specifically to inhibit DPP. *dpp* mutants are epistatic to *sog* in *sog:dpp* double mutants. These results are similar to the mutant studies in zebrafish. In Xenopus, knockdown of Chordin expression using morpholino oligonucleotide shows a phenotype similar to that of *chordino* mutants in zebrafish (Schulte-Merker et al., 1997). They display a reduction in neural plate and other neural structures derived from dorsal mesoderm and a concomitant expansion of ventral mesoderm. In mouse, *chordin:noggin* double mutants lack forebrain and anterior notochord. They display expansion of ventral mesoderm and randomized left-right symmetry (Bachiller et al., 2000). Results in these model organisms suggest the importance of BMP pathway in the patterning of germ layers.

Besides BMP, Wnt genes are also repressed by the Organizer. In zebrafish, two *wnt* genes are important for ventral fates. They are *wnt8a* and *wnt3a* (Thorpe et al., 2005; Lekven et al., 2001). In zebrafish, Wnt8a is a secreted molecule expressed in the non axial mesoderm during gastrulation (Christian et al., 1991). Proper regulation of Wnt signals is essential for the patterning and survival of the embryo. Wnt inhibitors secreted by the Organizer are of two types, Frizzled related proteins (Frzbs) and Dkks. Frzb-1 is a secreted protein that has a cysteine rich domain (CRD) similar to the Wnt-binding region of the Frizzled Wnt receptors as shown in Fig. 4. Frizzled receptors are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (CRD) (Bhanot et al., 1996). Frzb-1 functions by binding to Wnt proteins and antagonizing their activity (Moon et al., 1997; Leyns et al., 1997). During Wnt signal transduction, upon binding to a Wnt ligand, Frizzled cooperates with a single-pass transmembrane molecule of the LRP family, LRP5 or LRP6. Dkk-1 is a secreted Wnt inhibitor that binds directly to the LRP5/6 receptor and inhibits its function as a Wnt coreceptor (Fedi et al., 1999; Glinka et al., 1998). Dkk-1 and Frzb-1 cooperate in the formation of head structures (Glinka et al., 1997). Crescent is another protein that is expressed in the Spemann Organizer and functions as a Wnt antagonist (Pera and De Robertis, 2000). Cereberus protein can function as an antagonist to Nodal and Wnt genes in *Xenopus* and also to BMP4 in the extracellular space (Piccolo et al., 1999).

Loss of *wnt8a* leads to expansion of the Organizer and loss of mesodermally derived tissues. The repression of Organizer is mediated by transcriptional repressors Vent and

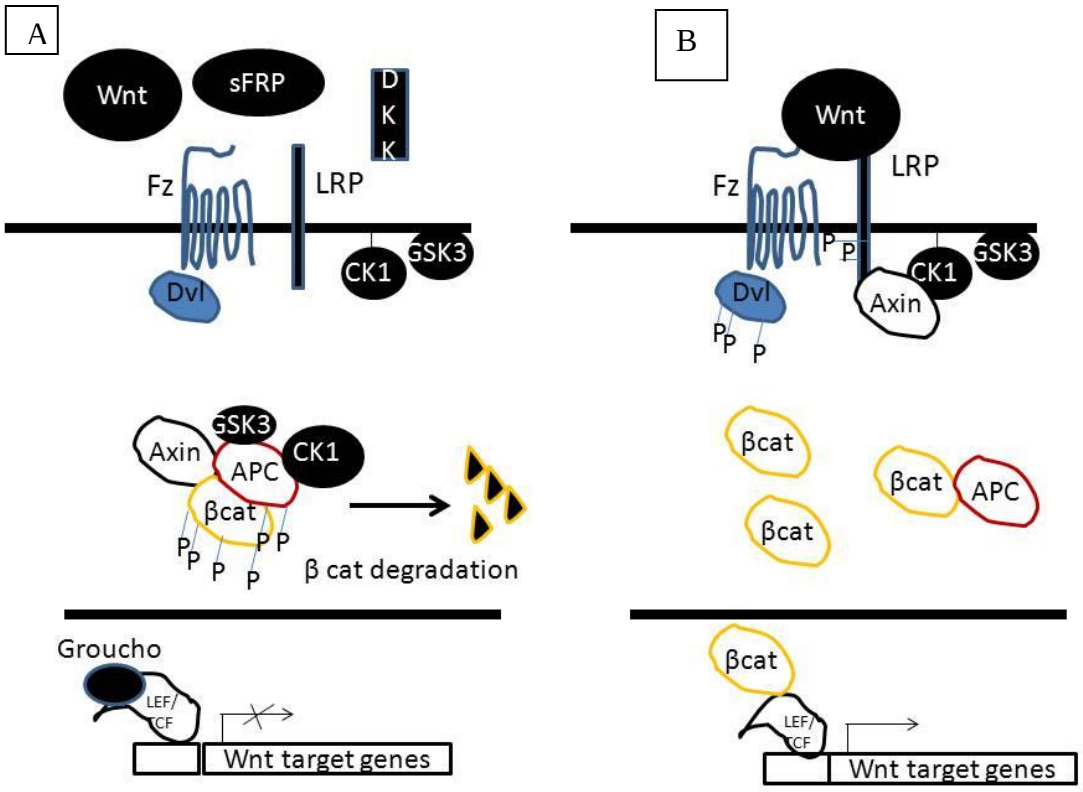

Fig. 4. Schematic representation of canonical Wnt signaling. A: in the absence of Wnt ligand, β- catenin is phosphorylated by GSK3 in the Axin-scaffolded complex. Phosphorylated β- catenin is ubiquitinated and degraded. B: Upon binding by the Wnt ligand, the Axin complex is inhibited leading to its cytoplasmic accumulation and nuclear translocation. It activates the transcription of Wnt target genes by binding to Lef/Tcf transcription factors and recruiting transcription machinery (Adapted from Reya and Clevers, 2005).

Vox (Ramel and Lekven, 2004). Wnt8a functions cooperatively with BMP to pattern the mesoderm during gastrulation (Ramel et al., 2005). Wnt8a also plays a direct role in promoting ventrolateral mesoderm which is independent of its role in repressing the Organizer (Ramel and Lekven, 2004; Baker et al., 2010).

ROLE OF *wnt8a* IN NEURAL PATTERNING

In zebrafish, gain of function of Wnts, as seen in headless/Tcf3 mutants, blocks head formation (Kim et al., 2000). In *headless* mutants, there is a reduction in expression of anterior neural specific genes and an expansion of posterior neural specific markers in the midbrain-hindbrain boundary as shown by *pax2* and *eng2*. In *wnt8a* mutants, the reverse is true. There is an expansion of the anterior neural marker *otx2* and a reduction of the posterior neural marker *pax2* (Lekven et al., 2001). These results are in agreement with the expanded anterior neural plate markers and decreased posterior neural fates seen in enhanced Nodal mutants which show a loss of *wnt8a* expression (Erter et al., 2001). The homeoprotein Bozozok that is expressed in the Organizer has been shown to negatively regulate the *wnt8a* and BMP pathways. Bozozok mutants have reduced anterior neurectoderm and enhanced posterior neurectoderm. The negative regulation of *wnt8a* by Bozozok promotes anterior neuroectoderm formation (Fekany-Lee et al., 2000). A morpholino knockdown of *wnt8a* in *boz* mutants rescues the neurectoderm domains, further confirming the significance of *wnt8a* repression by *bozozok* and the role of *wnt8a* in neurectoderm specification (Erter et al., 2001). *wnt8a* expression in the ventrolateral mesoderm is not only sufficient but necessary for normal mesodermal development and plays a crucial role in posteriorization of the early neurectoderm.

REGULATORS OF *wnt8a* EXPRESSION

In zebrafish, Nodal mutants have reduced *wnt8a* expression (Erter et al., 2001). Misexpression of Nodal results in ectopic induction of *wnt8a* expression (Agathon et al.,

2003). The reduction of *wnt8a* in Nodal mutants does not conclusively confirm whether the reduction of *wnt8a* expression is a result of loss of mesoderm or loss of *wnt8a* transcription. The T-Box transcription factors, No tail and Brachyury, have been shown to be direct transcriptional activators of *wnt8a* (Martin and Kimelman, 2008). *wnt8a* is reduced in *ntl* mutants at later gastrulation stages (Goering et al., 2003). Ntl binding sites have been identified at the *wnt8a* locus (Morley et al., 2009). The maternal zinc-finger protein Zbtb4 also has been found to be essential for the early activation of *wnt8a* (Yao et al., 2010).

In zebrafish embryos, it has been shown that *bozozok* negatively regulates *wnt8a* expression (Fekany-Lee et al., 2000). Tcf3, encoded by the *headless* locus, negatively regulates *wnt8a* transcription in the Organizer (Kim et al., 2000). Hhex is expressed in the dorsal YSL and can repress *wnt8a* (Ho et al., 1999). In *Xenopus*, *Xwnt8* is directly repressed in the dorsal mesoderm by Goosecoid (Yao and Kessler, 2001).

Wnt8a occupies a very critical position in embryonic patterning. Even though these regulators have been shown to affect the transcription of *wnt8a*, it is not known how these multiple inputs function cooperatively to regulate *wnt8a* expression in a spatial and temporal manner in a developing embryo. Understanding the transcriptional regulation of *wnt8a* is very important in understanding the different facets of vertebrate axis patterning.

WNT SIGNALING PATHWAYS

The first Wnt gene identified was the mouse *wnt1* gene, originally named *Int-1* in 1982 by Nusse and Varmus. *Wnt1* was identified as the cause of breast tumors in the majority of mice that were infected with Mouse Mammary Tumor Virus in virally induced mammary tumors (Nusse and Varmus, 1982). Sequencing of the Wnt1 gene revealed that it encoded a secreted protein that is cysteine rich. Later, the *Drosophila* segment polarity gene, *wingless (wg)*, was identified as the orthologue of Wnt-1 (Cabrera et al., 1987; Rijsewijk et al., 1987). Wnt genes have been identified from humans to *C.elegans*. Further proof of the conserved nature of the Wnt signaling pathways between vertebrates and invertebrates was revealed by McMahon and Moon in 1989. They observed a duplication of the body axis in Xenopus upon injection of mouse *Wnt1* mRNA into the ventral blastomeres of embryos at the 4 cell stage (McMahon and Moon, 1989). All the Wnt genes encode proteins with an N-terminal signal peptide and 23 cysteine residues (Wodarz and Nusse, 1998). Wnt proteins are relatively insoluble and this has been attributed to a protein modification, cysteine palmitoylation, which is essential for Wnt function (Willert et al., 2003). All Wnt proteins are secreted from cells and act through surface receptors either on the producing cells or on adjacent cells to affect cell fate determination, cell proliferation or cell differentiation (Reviewed in Clevers, 2006; Wodarz and Nusse, 1998). In addition to regulating early development, misregulation of Wnt signaling is implied in human birth defects including spina bifida (Logan and Nusse, 2004; Wallingford and Habas, 2005).

Currently, three different pathways are believed to be activated by the binding of Wnt ligand to the receptor. They are the canonical Wnt/β catenin pathway, the non-canonical planar cell polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway. All these Wnt pathways are activated by the binding of the Wnt ligand to a receptor complex comprizing Frizzled (Fz) and Low-density Lipoprotein related protein LRP5/6 (He et al., 2004). The binding of the ligand to the receptor recruits the cytoplasmic protein, Dishevelled (Dvl) to the membrane and phosphorylates it. At the level of Dvl, three independent pathways of Wnt signaling become apparent.

In the canonical signaling pathway, the phosphorylation and recruitment of Dvl to the membrane is also accompanied by the phosphorylation of LRP5/6 by GSK3β and casein kinase1. The phosphorylation of LRP regulates docking of Axin. The central player in the canonical Wnt cascade is a cytoplasmic protein, β-catenin, whose stability is regulated by a destruction complex. Axin acts as a scaffold for a protein complex that holds together β-catenin and APC and two kinase families (CK1and GSK 3) (Lee et al., 2003). When the WNT receptor complex is not formed, CK1 and GSK3 phosphorylate β-catenin. Phosphorylated β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome (Aberle et al., 1997). When WNT ligand binds to the receptor, Axin is docked and is recruited away from the protein complex. This leads to stabilization of the non phosphorylated β-catenin into the nucleus, where it binds to TCF and replaces Groucho, a repressor, and induces transcription of Wnt-target genes (Reviewed in Clevers, 2005; MacDonald and Silver, 2009).

The Wnt/PCP pathway acts through small GTPases such as RhoA and Rac and activates c-Jun amino terminal kinase (JNK). This pathway regulates planar cell polarity and morphogenetic movements (Reviewed in Peifer and McEwen, 2002; van Es et al., 2003). The Wnt/Ca^{2+} pathway involves the activation of phospholipase C and leads to elevated Ca^{2+} levels and the activation of protein kinase C and Ca^{2+} - Calmodulin dependent protein kinase II (Kuhl et al., 2000). The elevated levels of Ca^{2+} can activate the phosphatase Calcineurin, which leads to the dephosphorylation of the transcription factor NF-AT. This leads to the accumulation of NF-AT in the nucleus which activates target genes. This pathway regulates cell adhesion and motility (Saneyoshi et al., 2002; van Es et al., 2003).

ROLE OF WNTS IN DIFFERENT MODEL ORGANISMS

Wnt proteins have characteristic cysteine residues and are defined by sequence rather than by functional properties (Nusse, 2005). 19 Wnt genes have been identified in humans, 16 in *Xenopus*, 17 in Mouse, 7 in Chicks and 12 in zebrafish, 7 in *Drosophila* and 5 in *C.elegans* (Nusse, 2005).

In Drosophila, *wg* is one of the best characterized Wnt family members. It is a segment polarity gene in that it is required for the formation of parasegment boundaries for maintenance of *engrailed* expression in adjacent cells (Nusse, 2005). Wnt genes have been identified in *C. elegans* and implicated in embryogenesis. Wnt (*mom*) is required

for mesoderm and gut specification. In the loss of function mutation, it leads to the absence of gut formation.

In humans, mutation in the *wnt3* gene has been implied in tetramelia. It is a developmental defect in humans characterized by the loss of limbs and is embryonic lethal (Falcon et al., 2005). In mouse, hyperactivity of Wnt signaling causes anencephaly. Mouse knockouts of the Wnt inhibitor *dickkopf* (*dkk*) are anencephalic (Mukhopadhyay et al., 2001). The condition is characterized by defects in neural tube closure and loss of forebrain. Zebrafish embryos carrying a mutation in Tcf3, a downstream component of the Wnt signal transduction cascade, also are characterized by the loss of forebrain fates (Kim et al., 2000). This phenotype in zebrafish is functionally similar to mammalian anencephaly. These results suggest that proper regulation of Wnt activity during embryogenesis is a critical factor in preventing anencephaly and other brain patterning birth defects. Thus, understanding regulation of Wnt activity during early embryogenesis may shed light on this common birth defect. In this study, I am using zebrafish to understand the regulation of *wnt8a*.

wnt8a EXPRESSION IS CONSERVED IN VERTEBRATES

wnt8a expression is generally conserved in vertebrate model organisms and is seen in the region of mesoderm induction. For example, in mouse, *mWnt-8* is expressed in the early primitive streak (Bouillet et al., 1996). In chick, *Cwnt-8C* is expressed in the primitive streak and is involved in regulation of axis formation (Hume and Dodd, 1993). In

Xenopus, *X-wnt8* expression is primarily restricted to ventral mesodermal cells (Christian et al., 1991). As mentioned, zebrafish *wnt8a* is expressed in the ventro-lateral mesoderm and plays an important role in axis specification (Kelly et al., 1995; Lekven et al., 2001). The functional conservation between vertebrate species has been demonstrated by the ability of *Cwnt-8C* to generate a secondary axis when injected into *Xenopus* embryos. A duplication of the body axis was also observed upon injection of mouse *wnt-1* mRNA into the ventral blastomeres of *Xenopus* (Sokol et al., 1991; Wodarz and Nusse, 1998; McMahon and Moon, 1989). Despite the conserved nature of expression pattern and similar functions of *wnt8a*, very little is known about the regulators of *wnt8a* across species. As only a few regulators of *wnt8* have been identified in *Xenopus* and *Zebrafish*, in this study, I have used the zebrafish to understand the possible transcriptional regulators of *wnt8a*.

ROLE OF *wnt8a* IN PROMOTING VENTROLATERAL FATES AND POSTERIOR BODY IN ZEBRAFISH

In teleosts, the *wnt8a* locus comprises two *wnt8a* coding regions, *wnt8a.1* and *wnt8a.2*. Cloning and sequencing of the zebrafish *wnt8a* locus have revealed that both the coding regions are cotranscribed as a single bicistronic transcript encoding both *wnt8a.1* and *wnt8a.2* as well as a *wnt8.2a* specific transcript (Lekven et al., 2001). In addition, there is an alternate splice product that comprises coding regions of *wnt8a.1* and *wnt8a.2*. The transcripts are generally coexpressed in the ventrolateral margin at shield stage. However, during gastrulation, in addition to the expression in the margin, *wnt8a.2*

transcripts are also seen in the axial mesoderm and neurectoderm (Lekven et al., 2001). The bicistronic mRNA is capable of encoding two Wnt8 proteins (Lekven et al., 2001). The two proteins expressed from the locus are ~70% identical and either one can rescue the molecular markers in a *wnt8* mutant. Further proof of the functional redundancy of *wnt8a.1* and *wnt8a.2* is that a combined knockdown of both transcripts is required to recapitulate the phenotype of *wnt8a* mutants. The duplication of the *wnt8a* locus is also seen in pufferfish, *Takifugu rubripes*, suggesting that this duplication event may have occurred before the divergence of the teleosts, approximately 150 million years ago (Ramel et al., 2004). In teleosts, the activity of both *wnt8a.1* and *wnt8a.2* are required to carry out all the functions of the *wnt8* gene in comparison to non teleosts that have a single *wnt8a* coding region (Ramel et al., 2004). This could be due to the genome duplication that occurred in the teleosts that resulted in the division of gene into two and functional specification for each gene.

wnt8a EXPRESSION IN ZEBRAFISH

The focus of my research is transcription regulation of *wnt8a*. In zebrafish, *wnt8a* transcripts are deposited maternally. At the 128-256 cell stage, just before mid-blastula transition, there is a reduction in *wnt8a* transcripts. This corresponds to approximately 2.5 hours after fertilization. The transcripts then increase in levels at the midblastula transition (Kelly et al., 1995). This increase corresponds to the onset of zygotic transcription. The transcripts continue to increase and are detectable by in-situ hybridization at 30% epiboly at the ventrolateral margin (Kelly et al., 1995) and also in

the Yolk Syncitial Layer (Ho et al., 1999). At shield stage (6 hours after fertilization) and during gastrulation, *wnt8a* transcripts are seen in the ventrolateral margin and are excluded from the dorsal organizer. After gastrulation, *wnt8a* transcripts are expressed in the tailbud, presomitic mesoderm and prospective pronephros (Kelly et al., 1995).

INTERACTIONS OF *wnt8a* WITH OTHER GENES IN VENTROLATERAL MESODERM DEVELOPMENT OF THE POSTERIOR BODY IN ZEBRAFISH EMBRYO

wnt8a functions with different genes at different stages of development. Before gastrulation, interactions of *wnt8a* with other genes are very crucial for ventrolateral mesoderm development and patterning (Ramel and Lekven, 2004; Ramel et al., 2005; Shimuzu et al., 2005). After gastrulation, *wnt8a* has been shown to function with another set of genes to maintain posterior mesodermal progenitors as well as to regulate the size and patterning of presomitic mesoderm (Baker et al., 2010, Martin and Kimelman, 2008).

Upon induction of mesoderm, *wnt8a* functions with *bmp2b* to repress the Organizer and to maintain and pattern the ventrolateral mesoderm. At the 40% epiboly stage, Wnt8a is required to maintain high levels of *vent* and *vox* expression. Vent and Vox are transcriptional repressors that repress Organizer genes. Later, during gastrulation, at 70% epiboly, Wnt8 along with zygotic BMP is required to maintain *vent* expression. BMP is also required for ectodermal *vox* expression (Ramel and Lekven, 2004). *wnt8a* and

bmp2b also coregulate the maintenance of ventrolateral mesoderm domain and pattern it into subdivisions.(Ramel et al., 2005).

INTERACTIONS WITH T BOX GENES

The interaction of *wnt8a* with T box genes is very important for posterior mesoderm formation and tail development. Wnt8a has been shown to regulate or interact with at least four T-Box genes during posterior mesoderm development. They are *tbx6* (Szeto and Kimelman, 2004), *spt* (Lekven et al., 2001), *ntl* and *bra* (Martin and Kimelman, 2008). *tbx6* is expressed in the ventrolateral mesoderm and is important for posterior body formation (Chapman et al., 1996; Hug et al., 1997). Characterization of the promoter of the *tbx6* gene reveals that *wnt8a* directly regulates its expression along with BMP (Szeto and Kimelman, 2004). Ntl and Bra are two zebrafish Brachyury homologues which function together to positively regulate *wnt8a* transcription after gastrulation (Martin and Kimelman, 2008). These T box genes also regulate *wnt3a* expression. Wnt8 and wnt3a have been shown to regulate the T box genes, thereby forming an autoregulatory loop. This loop promotes the maintenance of the paraxial mesodermal precursors throughout somitogenesis and regulates patterning of presomitic mesoderm (Martin and Kimelman, 2008). *wnt8a* has been shown to activate *ntl* expression along with BMP (Harvey et al., 2010). The T-box gene *spt* is coexpressed with *ntl* and is required for posterior mesoderm development (Amacher et al., 2002). *wnt8a* has been shown to function synergistically with *ntl* and *spt* in the formation of trunk and tail mesoderm (Lekven et al., 2001).

ROLE OF *wnt8a* IN TAIL FORMATION

Wnt8a, Bmp and Nodal are required in the ventral margin which functions as a tail organizer. Transplantation of this region leads to formation of ectopic tails. These three pathways function together for the induction of the tail (Agathon et al., 2003). Injections of *bmp4* and *wnt8a* have shown to induce ectopic tails in wild type embryos suggesting that these two pathways are sufficient for tail induction (Harvey et al., 2010). *wnt8a* and BMP function together to regulate *tbx6* expression. *wnt8a* and *wnt3a* are expressed in the tail bud and inhibition of both these genes simultaneously can block tail formation. Both these Wnt genes activate *sp5l* (sp5-like), a transcription factor belonging to the Sp1 family of zinc finger transcription factors. *sp5l* acts downstream of these Wnt genes and controls tail development (Thorpe et al., 2005). *sp5l* redundantly functions with its paralog, *sp5* to mediate the functions of *wnt8a* in dorsoventral patterning. *sp5l* also acts downstream of *wnt8a* and specifically functions in neuroectoderm posteriorization (Weidinger et al., 2005). *sp5l* has been shown to act downstream of FGF signaling in mesoderm induction (Zhao et al., 2003). FGF signaling and *wnt8* seem to be regulating *sp5l* expression independently in mesoderm induction and neuroectoderm patterning (Weidinger et al., 2005). *wnt8a* and *wnt3a* also regulate another set of genes that are important in the formation of posterior body, Cdx1a and Cdx4. These, in turn activate *hox* genes (*hoxa9a* and *hoxb7a*) that are important in formation of posterior body. FGF signaling is also required for Cdx-mediated *hox9a* expression (Shimuzu et al., 2005; Martin and Kimelman, 2009). As mentioned above, the autoregulatory loop consisting of canonical Wnts, *wnt8a* and *wnt3a* with the T box genes, *ntl* and *bra* is important for

maintaining a population of cells as precursors at the tip of the tail (Martin and Kimelman, 2008). Thus, *wnt8a* holds a key position in the gene regulatory network that is critical for mesoderm patterning, establishing A/P and D/V axis and tail formation. Understanding the transcription regulation of *wnt8* will give us a better understanding of the complex gene regulatory networks that are important in dorsoventral patterning and formation of the posterior body.

USE OF TRANSGENICS TO STUDY CIS-REGULATORY ELEMENTS

Cis-regulatory elements refer to DNA sequences that regulate a gene on the same stretch of DNA (i.e. promoters and enhancers). Enhancers can regulate temporal and tissue specific expression upon binding of transcription factors. Enhancer sequences can be identified by cloning DNA sequences flanking the gene and fusing them to reporter genes. These reporter genes are thus driven by the gene-specific DNA sequences and provides an easily recognizable product if they get activated in certain tissues at certain times of development. To visualize tissue specific expression, these transgenes are introduced into the early embryo. Integration of the transgene into the genome and subsequent analysis of the expression of the transgene helps to identify the importance of the cis regulatory region that is driving the reporter gene (Gilbert, 2010). Trangenesis is an important means to study the function of genes in vivo (Kawakami, 2007). The common reporter genes used are the lac Z gene, an *E. coli* gene for β-galactosidase (the *lacZ* gene) and green fluorescent protein (GFP) from jellyfish. Transgenic analysis

makes it possible to define genomic intervals containing regulatory elements sufficient for recapitulating aspects of endogenous expression.

Transgenic analysis has helped to identify important regulatory regions within genomic intervals in different model organisms. In mouse, transgenic analysis of the *pax2* locus has identified two separate enhancers for activation and maintenance of Pax2 expression at the mid-hindbrain boundary. In this study, they identified the enhancers by cloning regions of the *pax2* gene to drive expression of *lacZ* gene (Pfeffer et al., 2002). In the vertebrate model organism, *Xenopus*, a nodal related gene, *Xnr1* plays central roles in induction of mesendoderm and left-right axis specification. Transgenic analysis of the *Xnr1* locus has revealed the functional significance of nodal response elements in the intron1 of the gene (Osada et al., 2000). In *Drosophila*, the transcription factor Brinker plays an essential role in regulating Dpp, the fly homologue of vertebrate BMP. Cis-regulatory properties of the *brk* promoter were studied by generating transgenic β-gal reporter lines containing regions upstream of the coding sequence. This study revealed multiple enhancer modules that integrate multiple inputs that drive *brinker* expression (Yao et al., 2008). In chick, Sox2 is expressed in neural and sensory primordia at various stages of development. Transgenic analyses have helped to identify enhancers responsible for Sox2 expression in various domains at different stages of development. This study also helped to identify highly conserved regions in the *Sox2* locus between chicken and mammals (Uchikawa et al., 2003). In zebrafish, the microscopy-friendly optical clarity of the developing embryos makes it ideal for transgenic analysis of cis

regulatory regions. Transgenic analyses of several genes that play key roles in axis patterning and mesoderm development have been studied. These studies have revealed temporal and spatial interactions of genes involved in axis patterning and mesoderm development. They have helped to unravel the complex gene regulatory networks involved in patterning the embryo. For example, transgenic analysis of the transcription factor Tbx6, a posterior mesodermal gene, has revealed that BMP and Wnt regulate its expression in a combinatorial manner. The study also revealed that a genomic fragment that includes 1.7kb upstream of the translation start site through the second exon is sufficient to recapitulate the endogenous expression of the gene. This region was also able to drive expression in *Xenopus* embryos suggesting that the regulatory regions are conserved across species. Mutational analysis of conserved enhancer elements also revealed that this promoter responds to both Wnt and Bmp signaling pathways (Szeto and Kimelman, 2004). Ntl is a pan mesodermal marker which is expressed in both dorsal and ventrolateral mesoderm at early stages of development. Transgenic analysis of the *ntl* promoter have revealed an upstream region that has two different regulatory elements: one responding to Nodal signaling and the other responding to BMP and Wnt. Regulation by Nodal is essential for activating Ntl throughout the margin, while regulation by BMP and Wnt is required for expression in the ventral margin (Harvey et al., 2010). The Nodal ligand *sqt* has been shown to function as a morphogen and is required to activate mesoderm specific genes (Yu Chen and Schier, 2001). Transgenic analysis of the *sqt* locus has identified a 1.9KB region that is sufficient for recapitulating

the spatio-temporal expression of endogenous *sqt* gene. This study also revealed that Nodal regulates *sqt* expression in a feedback loop (Fan et al., 2007).

DISSERTATION OBJECTIVES

As mentioned in the above paragraphs, transgenic analysis of genes involved in axis patterning and mesoderm formation has revealed important interactions and combinatorial regulations involved in early development of zebrafish embryo. Wnt8a plays a critical role in these events in zebrafish. However, no studies have been undertaken in a vertebrate model organism to dissect the cis-regulatory regions necessary for the function of *wnt8a*. In this study, I have undertaken a thorough transgenic analysis of the *wnt8a* genomic locus. Chapters II and III address the expression patterns of transgenic lines that express *EGFP* under the control of different cis- regulatory regions of *wnt8a* locus.

Chapter II describes a new transgenic zebrafish line in which EGFP expression faithfully recapitulates *wnt8a* expression. This transgenic line was created through homologous recombination in a *wnt8a* PAC clone comprising ~80 kb of flanking sequence. Using this transgenic line, I show that *wnt8a.1* is regulated in a biphasic manner. This biphasic expression is regulated by Nodal at the early stage (Phase I; 6 hpf or before) and by Ntl/Bra in the late stages (Phase II; later than 6 hpf).

In Chapter III, I have further dissected the cis-regulatory elements upstream of the zebrafish *wnt8a* locus. I generated a series of reporter constructs that recapitulate different aspects of the *wnt8a* expression pattern in transient and stable transgenic assays. The constructs identified two functional cis-regulatory units, referred to as the distal regulatory region (DRR) and the proximal regulatory region (PRR). This chapter also discusses the identification of an evolutionarily conserved mesoderm enhancer that helps to achieve biphasic *wnt8a* expression.

Chapter IV gives the summary of experiments and discusses the impact of the findings. This chapter also talks about the future directions.

CHAPTER II

A TRANSGENIC *wnt8a:PAC* REPORTER REVEALS BIPHASIC REGULATION OF VERTEBRATE MESODERM DEVELOPMENT *

INTRODUCTION

Vertebrate mesoderm is the source of progenitors for multiple tissues and organs, including heart, blood, kidney and body muscles. In the zebrafish, mesoderm initially forms in two morphologically and genetically distinct domains: dorsal and ventrolateral, that are both induced by Nodal signaling (Schier and Talbot, 2005). Subsequent to induction, an interplay between Wnt and Bmp signals emanating from the ventrolateral domain and their antagonists secreted by dorsal mesoderm (the Organizer) patterns mesoderm into distinct fate domains in the dorsoventral axis (Itasaki and Hoppler, 2010). Posterior growth of the body then requires an additional suite of interactions downstream of Wnt and FGF signaling (Kimelman, 2006). Despite a wealth of knowledge regarding the signals and effectors that mediate early mesoderm development, far less is understood about the molecular basis of vertebrate mesodermal gene regulatory networks.

One factor central to multiple phases of mesoderm development is the Wnt/β-catenin ligand, Wnt8a. In zebrafish, Wnt8a signaling in the ventrolateral mesoderm of the early

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gastrula is required to maintain high expression levels of *vent*, *vox* and *ved*, which encode transcriptional repressors of dorsal mesoderm genes (Ramel and Lekven, 2004; Ramel et al., 2005). In *wnt8a* mutants, *vent*, *vox* and *ved* expression is reduced, leading to an expansion of dorsal mesoderm genes into ventrolateral domains (Lekven et al., 2001; Ramel et al., 2005). The expanded organizer secretes Bmp antagonists that reduce Bmp signaling and cause a morphologically dorsalized phenotype at 24 hours post fertilization (Baker et al., 2010). Expansion of dorsal mesoderm is accompanied by a reduction in ventrolateral mesoderm progenitors, as indicated by reduced expression of ventral markers such as *eve1*, *cdx4* and *tbx6* (Ramel et al., 2005). Analysis of embryos simultaneously knocked down for *wnt8a* and *wnt3a* expression showed that Cdx and Sp5 transcription factors function downstream of Wnt and Fgf signaling to promote posterior development (Shimizu et al., 2005; Thorpe et al., 2005), and *wnt8a* is known in multiple vertebrates to function in a feedback interaction with Brachyury orthologs that are essential for posterior development (Yamaguchi et al., 1999; Vonica and Gumbiner, 2002; Martin and Kimelman, 2008). Zebrafish mutants that lead to *wnt8a* gain-of-function, such as *bozozok* or *headless*, display defects in neural anteroposterior patterning and notochord formation (Fekany-Lee et al., 2000; Kim et al., 2000). These phenotypes are associated with ectopic *wnt8a* transcription in the dorsal mesoderm at early gastrula stages and are likely related to the role for Wnt8a in posteriorizing the early neurectoderm (Erter et al., 2001; Lekven et al., 2001; Rhinn et al., 2005). Therefore, Wnt8a occupies a key developmental position by integrating and coordinating dorsoventral and anteroposterior mesoderm patterning mechanisms. Understanding the

transcriptional regulation of *wnt8a* is thus key to understanding multiple facets of vertebrate axis patterning.

The *wnt8a* expression pattern is largely conserved in vertebrates and marks the region of mesoderm production, for example the primitive streak in mouse and chick and the marginal zone of *Xenopus* (Christian et al., 1991; Hume and Dodd, 1993; Bouillet et al., 1996). In the zebrafish, *wnt8a* is deposited maternally, declines prior to the midblastula transition but increases again at MBT (3 hours post fertilization; Kelly et al., 1995). Transcripts cannot be detected by in situ hybridization until 30% epiboly (4.66 hpf; Kelly et al., 1995), when *wnt8a* expression is detected as an arc in the ventral embryonic margin and also in the yolk syncytial layer (Ho et al., 1999; Ramel and Lekven, 2004). Expression expands up to the dorsal organizer shortly thereafter, and is maintained in the margin during gastrulation (Kelly et al., 1995). Post-gastrulation expression is observed in the tailbud. Two aspects of *wnt8a* expression are key to its zygotic function: exclusion from dorsal mesoderm in the early gastrula and maintenance in the ventral embryonic margin through gastrulation.

Despite its critical position in the mesoderm gene regulatory network hierarchy and its role across taxa in posterior development (McGregor et al., 2008; Martin and Kimelman, 2009; McGregor et al., 2009), very little is known about the mechanism of *wnt8a* transcriptional regulation. In invertebrates, *wnt8a* regulation is best understood in the sea urchin, where Runt1, Blimp1 and Tcf regulate a dynamic pattern of *wnt8a* transcription

through a defined cis-regulatory element (Minokawa et al., 2005; Smith et al., 2007; Robertson et al., 2008; Smith and Davidson, 2008). In *Xenopus*, Yao and Kessler, (2001) showed that *Xwnt8a* is directly repressed in the dorsal mesoderm (Spemann's organizer) by Goosecoid (Yao and Kessler, 2001), although the temporal dynamics of this interaction are still unknown. In the zebrafish, Dharma (encoded by the *bozozok* locus) and Tcf7l1a (previously referred to as Tcf3a, encoded by the *headless* locus) prevent *wnt8a* transcription in the organizer but it is unknown whether this is a direct effect (Fekany-Lee et al., 2000; Kim et al., 2000). Similarly, *hhex* is expressed in the dorsal YSL and can repress *wnt8a* (Ho et al., 1999), but an in vivo role for *hhex* repression of *wnt8a* is contradicted by a deletion mutant lacking *hhex* that does not show expanded *wnt8a* expression (Bischof and Driever, 2004). Thus, the functions of several candidate *wnt8a* regulators on *wnt8a* repression in the organizer are not known.

Several data suggest Nodal signaling is upstream of *wnt8a* activation in ventrolateral mesoderm. For instance, *wnt8a* transcription is reduced in *cyc;sqt* double mutants (Erter et al., 2001), and ectopic Nodal signaling can activate *wnt8a* (Agathon et al., 2003). While these data argue that Nodal signaling activates *wnt8a*, it is unclear whether this is a direct effect or an indirect consequence of mesoderm induction. The only direct transcriptional activators of *wnt8a* that have been identified are the T-box transcription factors No Tail and Brachyury: *wnt8a* is reduced in *no tail* mutants at late gastrulation (Goering et al., 2003; Martin and Kimelman, 2008), and Ntl and Bra are both able to directly activate *wnt8a* (Martin and Kimelman, 2008) through Ntl binding sites at the

wnt8a locus (Morley et al., 2009). While these studies reveal Ntl and Bra as crucial regulators of *wnt8a*, this does not answer how *wnt8a* expression is initiated, whether other factors impact *wnt8a* transcription and how multiple inputs, such as Nodal and Ntl/Bra, are integrated to achieve the wild-type *wnt8a* transcription pattern.

To enable studies of *wnt8a* transcriptional regulation, we have generated a zebrafish line that is transgenic for a modified PAC genomic clone that expresses EGFP under *wnt8a* regulatory element control. We show that the PAC interval contains regulatory elements sufficient to recapitulate endogenous *wnt8a* expression and we use loss-of-function experiments to reveal Nodal and Ntl/Bra regulation of our transgenic reporter. We further use EGFP fluorescence of the reporter to examine mesodermal lineages in Nodal and Ntl/Bra loss-of-function backgrounds. Our results reveal a biphasic regulatory program for *wnt8a* that correlates with the transition from Nodal-dependent mesoderm induction to Ntl/Bra-dependent posterior mesoderm growth, and in each case *wnt8a* expression functions as a lineage marker for mesodermal tissue.

MATERIALS AND METHODS

PAC Recombineering and transgenesis

A zebrafish PAC clone spanning from -13kb to +60 kb flanking the bicistronic *wnt8a* locus was isolated from a library (Amemiya et al., 1999). PAC 42J21 was engineered through a modification of the protocol of Tischer et al. (Tischer et al., 2006). Briefly, we designed a targeting cassette, pLC10, which contains the EGFP coding sequence

followed by an I-SceI restriction site and a chloramphenicol resistance gene upstream of the SV40 poly adenylation sequence in pCS2+. Targeting DNA was amplified from pLC10 with primers that contain 50 bp of homology to the first coding exon of *wnt8aa*. Homologous recombination was performed via two-step Red-recombination as described (Tischer et al., 2006). Correct targeting was verified by sequencing and resulted in an EGFP-SV40 polyA cassette placed at the ATG initiation codon of Wnt8aa. Primer sequences will be provided upon request. PAC DNA was isolated with a Qiagen Plasmid Maxi kit. To generate transgenics, PAC DNA was dissolved at 25 ng/µl in water + 1% green food coloring, then injected into 1-cell stage wild-type embryos. Injected embryos were raised to adulthood, then outcross embryos were screened for fluorescence. One founder that produced 4/176 GFP+ embryos was identified from 112 fish screened. Transgenic embryos were raised and outcrossed to establish a line, with the allele designation *Tg(wnt8aPAC:EGFP)*^{x26}.

Fish care, morpholinos and in situ hybridization

Zebrafish were maintained as described (Westerfield, 2000). Embryo staging was according to (Kimmel et al., 1995). An AB-TL hybrid line serves as our wild-type stock. To knock down Ntl and Bra levels, we used morpholinos described in Martin and Kimelman (2008). MOs were dissolved in 1X Danieau's buffer and 1-3 nl injected at 10 mg/ml (*bra* MO) and 5 mg/ml (*ntl* MO). Groups of injected embryos were assayed for morphology at 24 hpf to confirm that our morpholino protocol recapitulated the effects described by Martin and Kimelman (2008). In situ hybridizations were performed as

described (Ramel et al., 2004). To assay knockdown effects on the *Tg(wnt8aPAC:EGFP)* transgene, embryos from a cross of a *Tg(wnt8aPAC:EGFP)*/+ male to a wild-type female were injected with morpholinos, then embryos were fixed en masse and processed for hybridization. Because embryos were not prescreened to identify transgene carriers, each group is expected to comprise 50% heterozygous, 50% wild-type.

Nodal drug treatments

5mg of SB-431542 (Sigma) was dissolved in 1300µl of DMSO to make a 10mM stock. 30 embryos were incubated in one well of a 24 well plate with 500 µl of 150 µmolar SB-431542 at 29^{o} C. Dilutions were made in fish water.

As a control, embryos were treated with the equivalent volumes of DMSO and fish water. Embryos were incubated in either the drug or DMSO control beginning at the one cell stage. The embryos were washed in fish water and fixed at shield stage for in situ hybridization. Some embryos were left in the well and the incubation was continued until 24 hours post fertilization to confirm the morphological phenotype indicative of Nodal loss-of-function. In three separate trials to test this protocol, 25-27 of 30 treated embryos phenocopied MZoep, with the remaining 3-5 embryos of each batch showing a more severe phenotype. Each drug incubation experiment included one group of embryos that were treated and observed for the MZoep phenotype at 24 hpf to confirm the drug efficacy. Only embryos from trials showing equivalent severity were used for in

situ hybridization. To assay SB-431542 effects on the *Tg(wnt8aPAC:EGFP)* transgene, embryos from a cross of a *Tg(wnt8aPAC:EGFP)*/+ male to a wild-type female were treated with the drug, then embryos were fixed en masse and processed for hybridization. Because embryos were not prescreened to identify transgene carriers, each group is expected to comprise 50% heterozygous, 50% wild-type.

RESULTS

Tg(wnt8aPAC:EGFP) reports *wnt8a*

To understand how *wnt8a* transcription in the embryonic margin is controlled, we sought to define a genomic interval that spans necessary regulatory elements to drive normal *wnt8a* expression. We identified a genomic PAC clone, 42J21, that spanned an 80 kilobase (kb) interval comprising approximately 13 kb upstream of the bicistronic *wnt8a* locus (Lekven et al., 2001; Ramel et al., 2004) to 60 kb downstream (Fig. 5A). We used a modification of the protocol of Tischer et al. (Tischer et al., 2006) to insert EGFP at the translation start site of Wnt8a.1 (Fig. 5B; see Experimental Procedures). Our targeting procedure produced a modified PAC with no portions of the *wnt8a* locus deleted and that should express EGFP in place of Wnt8a.1.

To determine whether the modified PAC contained essential *wnt8a* regulatory elements, we assayed EGFP reporter expression in transient assays. PAC DNA was injected into wild type embryos at the one-cell stage, then fluorescence was monitored at mid-somitogenesis. We found EGFP to be expressed in the expected position of posterior

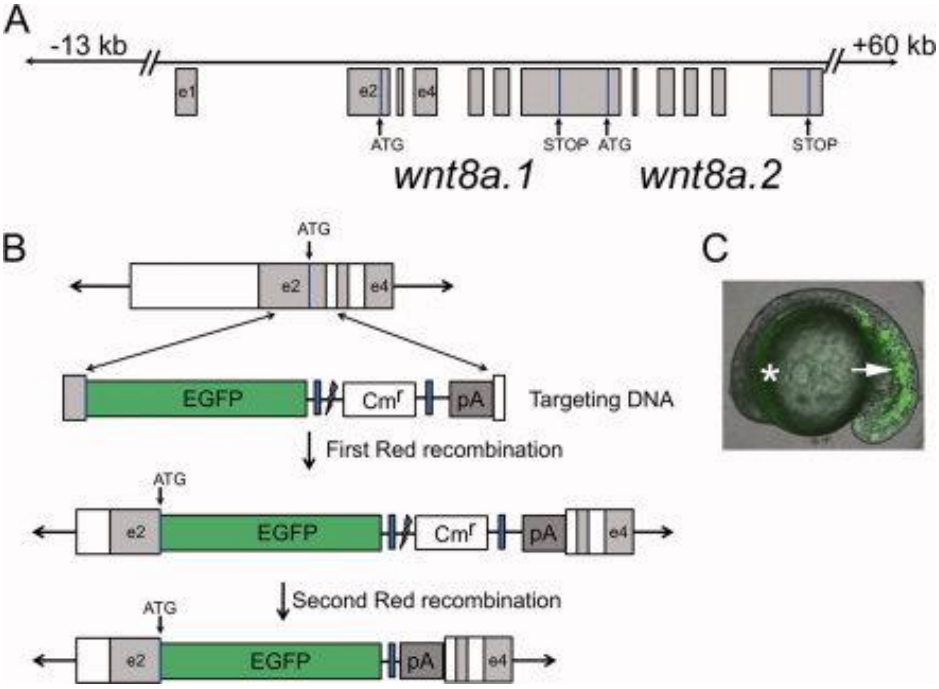

Fig.5. *wnt8a* gene structure and generation of an EGFP reporter PAC. A: Schematic diagram (not to scale) of the *wnt8a* locus within PAC clone 42J21. Line represents the PAC insert. Boxes indicate exons (e.g., e1). Start and stop codons are shown for both wnt8a coding sequences, although we targeted our insertion only into *wnt8a.1*. B: An outline of the homologous recombination procedure. Targeting DNA contains 50-bp ends homologous to exon 2 upstream of the translation start site and to the second intron (double arrows). Correct recombination results in an insertion of EGFP at the Wnt8a.1 translation start site. Blue boxes represent 50-bp repeated vector sequences that recombine during the second Red-recombination step that removes the chloramphenicol resistance gene (Cm^r). This second Red recombination is stimulated by I-SceI cleavage at its target sequence, indicated by the jagged line. The EGFP coding sequence is followed by the SV40 poly adenylation signal (pA), then the rest of the *wnt8a.1* locus. C: Transient EGFP expression from the modified PAC. Lateral view, anterior to the left. Note fluorescence in posterior mesoderm (arrow) and the YSL surrounding the yolk (asterisk).

mesoderm (Fig. 5C), thus we generated a stable transgenic line with the modified PAC. Injected embryos were raised to adulthood, then test-crossed to wild-type. We identified one founder fish that transmitted the PAC transgene through its germline. EGFP positive

embryos from this founder were raised to adulthood and outcrossed to a wild-type stock to generate a stable line.

We first characterized the pattern of EGFP fluorescence in transgenic embryos at several developmental stages. EGFP fluorescence is first observable at approximately shield stage (6 hpf) as a narrow band of expressing cells at the embryonic margin and as a bright fluorescence around the yolk (not shown). By bud stage, strong fluorescence is observed in the mesoderm of the posterior half of the embryo and also around the yolk cell (Fig. 6A, B). At mid somitogenesis stages, expression continues in the posterior embryo, where expression is clearly observable throughout the somites, with an apparent gradient of fluorescence becoming brighter toward the posterior (Fig. 6C, D). By 24 hpf, expression is observed in mesoderm posterior to the head (Fig. 6E, F). We noted expression in several mesodermally derived organs including the heart (Fig. 6G) and pronephros (Fig 6H). Fluorescence around the yolk and yolk extension is diminished by 24 hpf, but is still visibly brighter than yolk auto-fluorescence (Fig. 6E, F; data not shown). We also observed that embryos derived from heterozygous females crossed to wild-type males were uniformly and ubiquitously fluorescent (Fig. 6J), while progeny from heterozygous males crossed to wild-type females exhibited the expected 1:1 ratio of fluorescent to non-fluorescent individuals (Fig. 6I). This is consistent with maternal deposition of EGFP, reflecting the maternal expression of *wnt8a* (Kelly et al., 1995; data not shown). Thus, the transgene appears to be expressed in all mesodermal derivatives of

the ventrolateral embryonic margin, is maternally deposited and is expressed in the YSL, consistent with the *wnt8a* expression pattern.

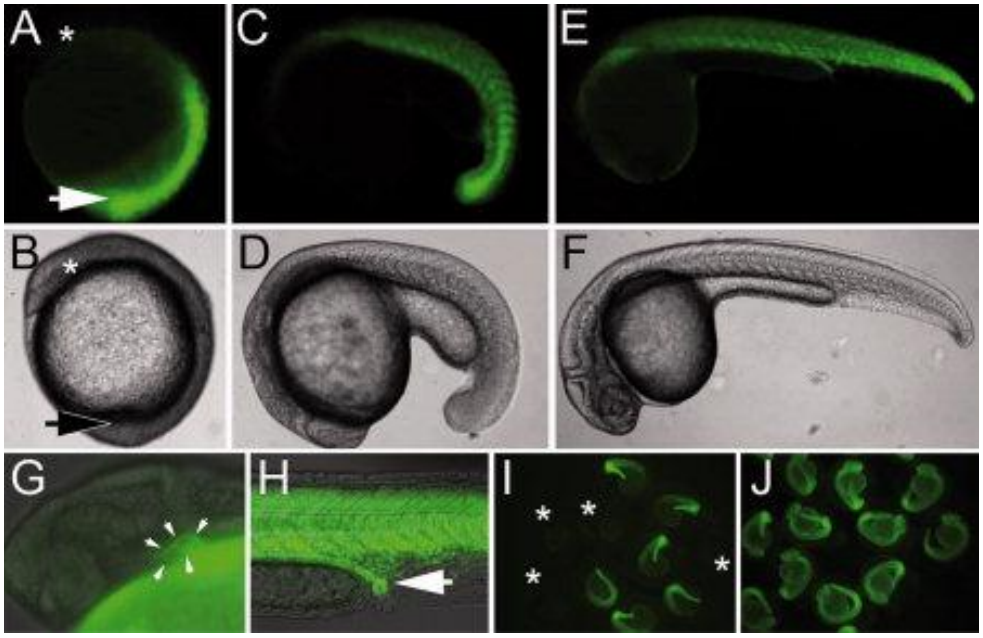

Fig.6. EGFP fluorescence profile of *Tg(wnt8aPAC:EGFP)* heterozygotes. A, B: Expression at bud stage, lateral view, anterior to upper left. Arrow indicates tailbud, asterisk indicates anterior neural plate. Note strong fluorescence limited to the posterior embryo. C, D: Expression at ~18-somite stage, lateral view, anterior to left. Fluorescence is observed in somites with an increasing gradient toward the tailbud. E, F: Expression at 24 hpf, lateral view. Strong fluorescence continues in somitic mesoderm, with continuing fluorescence detectable around the yolk. Expression is also observed in the heart (G, outlined by arrows) and pronephros (H, arrow). I, J: Comparison of transgene expression in embryos derived from males or females. I: Progeny from a heterozygous male crossed to wild-type female. Fifty percent of embryos fluoresce, 50% do not (non-fluorescing embryos indicated with asterisks). Note easily scored expression in somites of trunk and tail and around yolk. J: In contrast, 100% of progeny from a heterozygous female crossed to a wild-type male fluoresce throughout all tissues.

To determine whether EGFP fluorescence reflects a similar pattern of transcription, we performed in situ hybridizations for the reporter at multiple developmental time points and compared this to endogenous *wnt8a* transcription. We first detect patterned EGFP transcription at 30% epiboly. At this stage, EGFP transcripts are detected in the YSL and some blastomeres in the ventral margin (Fig. 7A, inset). Expression begins in an arc at the ventral-most region of the margin, while transcripts are not detected on the prospective dorsal side. Endogenous *wnt8a* is very difficult to detect by in situ hybridization at this stage, and, in particular, expression in the YSL is difficult to visualize but has been reported (Ho et al., 1999). This pattern continues at 40% epiboly, where the *Tg(wnt8aPAC:EGFP)* and *wnt8a* patterns are clearly congruous (Fig. 7A,B). By shield stage, EGFP reporter transcripts are detected in the entire ventrolateral embryonic margin and the YSL but not the shield or the dorsal YSL (Fig. 7C, D), matching endogenous *wnt8a*. After the onset of gastrulation, EGFP transcripts persist in the ingressing hypoblast layer so that reporter transcripts are detected in a broad band at the margin in the mid to late gastrula (Fig. 7E). While *wnt8a* is expressed in a similar pattern, transcripts are not as broadly distributed in involuted mesoderm due to destabilizing effects of the 3' UTR (Butler and Lekven, manuscript in preparation) thus producing a narrower staining region at the margin (Fig. 7F). Also at this stage, reporter transcripts are found in the dorsal margin, although at a much reduced intensity compared to the ventrolateral margin (data not shown). This may reflect low level *wnt8a* that is detected in the dorsal margin at late gastrula stages (see for example Martin and Kimelman, 2008, Fig. 6E or G). At bud stage, *wnt8a* transcripts are detected in the

ventral tailbud and in an arc of cells around the edge of the posterior mesoderm that corresponds to prospective pronephros (Fig. 7H). EGFP expression in *Tg(wnt8aPAC:EGFP)* embryos is remarkably similar at this stage with the exception that strong expression is seen in adaxial cells adjacent to the notochord and in paraxial mesoderm (Fig. 7G, asterisk). The presence of reporter transcripts in the somitic mesoderm is likely attributable to the absence of 3' UTR elements on the reporter transcripts (Butler and Lekven, manuscript in preparation). During somitogenesis stages, *wnt8a* is expressed in the tailbud exclusively (Fig. 7J), while EGFP reporter expression is observed in tailbud and somitic mesoderm, also likely reflecting the absence of destabilizing 3' UTR elements (Fig. 7I) (Butler and Lekven, in prep; Tian et al., 1999). By 24 hpf, EGFP transcription is observed only at the tip of the tail, while endogenous *wnt8a* is very difficult to detect at this stage, but an extended staining period reveals *wnt8a* transcripts in a similar distribution to that of the reporter (Fig. 7K,L). Interestingly, EGFP fluorescence is high in all ventrolateral mesoderm derivatives at 24 hpf despite the lack of transcription in these tissues (compare Fig. 6E, G, H, Fig. 7K), indicating that EGFP protein persists in cells that previously transcribed the reporter. Therefore, our results show that *Tg(wnt8aPAC:EGFP)* encompasses sufficient regulatory elements to recapitulate all expression domains of endogenous *wnt8a*. Further, the fluorescence of the reporter serves as a lineage label for all ventrolateral mesoderm derivatives.

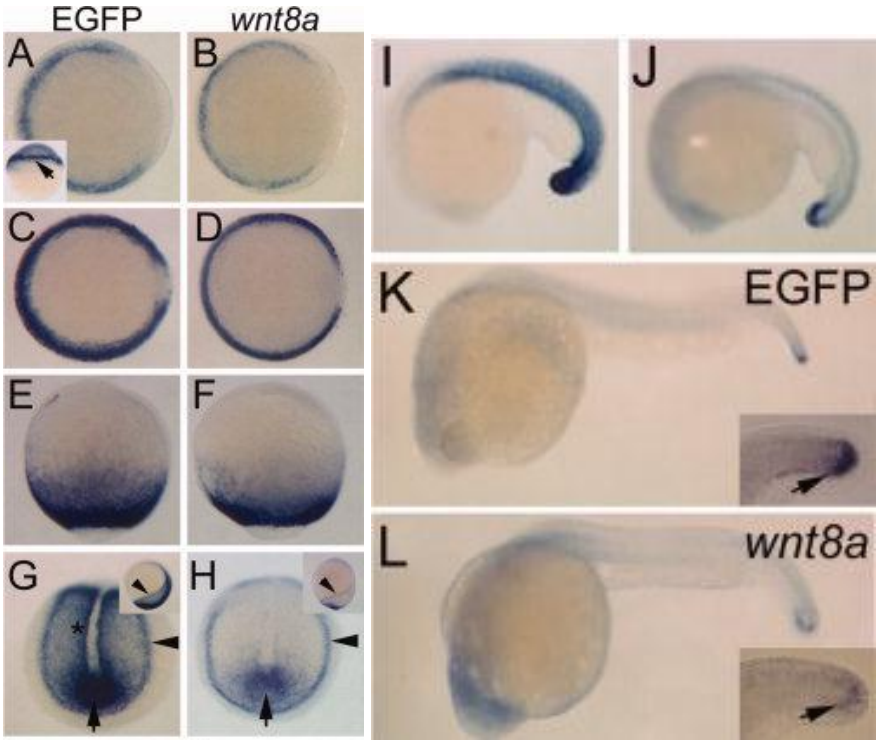

Fig. 7. Transcription profile of *Tg(wnt8aPAC:EGFP)* compared to *wnt8a*. (A,C,E,G,I,K) In situ hybridization for EGFP transcripts. (B,D,F,H,J,L) In situ hybridizations for *wnt8a* transcripts. (A, B) 40% epiboly, animal pole view, dorsal right. Both the transgene and wnt8a are expressed in the ventrolateral margin and are excluded from the dorsal margin. Inset in A: lateral view of EGFP expression at dome-30% epiboly to illustrate YSL expression (arrow). (C, D) Shield stage, animal pole view, dorsal right. Note continued expression in the ventrolateral margin and exclusion from the organizer. (E, F) 90% epiboly, lateral view, dorsal right. Note expression of EGFP and *wnt8a* in the embryonic margin. EGFP transcripts are detected in a broader band at the margin, likely reflecting a longer transcript half-life. (G, H) Late bud stage, posterior view. Note strong expression of EGFP and *wnt8a* in the tailbud (arrows) and prospective pronephros (arrowheads, also in inset). EGFP transcripts are also detected in the paraxial mesoderm and adaxial cells (asterisk). Insets: lateral views, anterior up. (I, J) 18 somite stage, lateral view, anterior left. EGFP transcripts are detected strongly in the tailbud, similar to *wnt8a*. EGFP transcripts continue to be detected in paraxial mesoderm. (K, L) 24 hpf, lateral view, anterior left. EGFP transcripts are detected only at the tip of the tail (K) similar to *wnt8a* (L). Insets: higher magnification of the tail. *wnt8a* is expressed at barely detectable levels at the tip of the tail (arrows).

Nodal regulates early phase *wnt8a* and *Tg(wnt8aPAC:EGFP)* expression

Nodal signaling has been shown to be upstream of *wnt8a* expression in zebrafish, thus we tested whether the *Tg(wnt8aPAC:EGFP)* reporter responds to Nodal signaling similarly to the endogenous locus. To test this, we relied on the small molecule TGF-β receptor antagonist SB-431542, which has been shown to induce a Nodal loss-of-function phenotype in the zebrafish (Fan et al., 2007; Hagos et al., 2007). At a dose of SB-431542 that induces a strong Nodal loss-of-function phenotype (see Experimental Procedures), both *Tg(wnt8aPAC:EGFP)* and *wnt8a* transcription were considerably reduced at shield stage (Fig. 8A-D; 27/30 embryos with reduced *wnt8a* as shown, remaining embryos with little to no staining; 14/30 *Tg(wnt8aPAC:EGFP)* outcross embryos with reduced expression as shown, expected 15/30 for Mendelian transmission. Similar results in three separate trials.). We confirmed Nodal antagonism under our experimental conditions by assaying *ntl* expression, a pan mesodermal marker which is absent from dorsal mesoderm but retained in the ventrolateral margin of Nodal loss-of-function embryos (Fig. 8E,F; 30/30 with expression pattern as shown, similar results in three separate trials)(Feldman et al., 1998; Gritsman et al., 1999). We observed that the dorsal clearing of *wnt8a* and EGFP was enlarged in drug-treated embryos, and that expression in the ventrolateral margin was significantly reduced but not absent (Fig. 8B,D). The dorsal clearing corresponds to the absence of dorsal mesoderm caused by Nodal loss, while the reduction in the ventrolateral margin suggests that Nodal signaling is required for the induction of normal levels of *wnt8a*. However, persistence of *wnt8a* and EGFP expression indicates that an alternate pathway to Nodal is sufficient to

stimulate *wnt8a* expression in ventrolateral mesoderm progenitors, albeit at a reduced level.

We further examined *wnt8a* and EGFP reporter expression in SB-431542-treated embryos at late bud stage. Consistent with Nodal-independent initiation of *wnt8a*, expression of both *wnt8a* and EGFP persists in the tailbud of treated embryos (Fig. 8H,J; 29/30 embryos with *wnt8a* pattern as shown, repeated once; 14/15 identified *Tg(wnt8aPAC:EGFP)/+* embryos with EGFP pattern as shown, repeated once), while EGFP reporter transcripts remain detectable in the tail presomitic mesoderm (Fig. 8I, J, asterisks).

In contrast, both *wnt8a* and EGFP expression is absent from the prospective pronephros domain in Nodal deficient embryos (Fig. 8H,J, arrows). Thus, *Tg(wnt8aPAC:EGFP)* expression is a sensitive reporter of mesoderm that develops in Nodal-deficient embryos. Because EGFP perdurance makes the reporter transgene a tracer for mesodermal lineages, we examined the distribution of $EGFP^{+}$ cells in SB-431542 treated embryos at 24 hpf to determine whether *wnt8a* expression correlates with tail mesoderm that forms in Nodal deficient embryos. Indeed, we found that EGFP fluorescence perfectly labels all tail mesoderm that forms in SB-431542-treated embryos (Fig. 8K-N), even those treated with high doses of the drug that induce more severe phenotypes. At this stage, reporter transcription is observed only in the tailbud of treated embryos (Fig. 8O,P; 30/30 embryos as shown). Importantly, EGFP fluorescence around the yolk, reflecting

reporter expression in the YSL, is relatively unaffected by SB-431542 treatment, consistent with the hypothesis that YSL expression of *wnt8a* is not regulated by Nodal. Further, we did not observe EGFP fluorescence in non-mesodermal tissue, indicating that the *wnt8a*$^{+}$ ventrolateral mesoderm progenitors in Nodal loss of function embryos do not contribute to other germ layers, in contrast to prospective dorsal mesoderm that changes fate to ectoderm (Feldman et al., 2000). Thus, Nodal is a major input into early *wnt8a* expression, but in its absence *wnt8a* expression is activated by an alternate pathway, perhaps the tail mesoderm induction pathway.

Ntl/Bra regulates late phase *wnt8a* and *Tg(wnt8aPAC:EGFP)* expression

The T-box proteins Ntl and Bra are known direct regulators of *wnt8a* (Martin and Kimelman, 2008), thus we tested whether the *Tg(wnt8aPAC:EGFP)* transgene responded to Ntl/Bra loss of function as predicted. By in situ hybridization, we found that transgene expression was only modestly reduced at shield stage in embryos co-injected with morpholinos against *ntl* and *bra* (Fig. 9A,D; 23/23 *Tg(wnt8aPAC:EGFP)/+* embryos with modest reduction as shown).

In contrast, transgene expression was severely reduced in *ntl;bra* morphants at 90% epiboly (Fig. 9B,E; 18/20 *Tg(wnt8aPAC:EGFP)/+* embryos with severe reduction as shown), similar to the reduction observed for *wnt8a* (Fig. 9B,E, insets; 45/50 embryos as shown). Reporter expression continued to be reduced after gastrulation, but the decrease in expression did not appear uniform throughout the embryo (Fig. 9C,F; 20/25

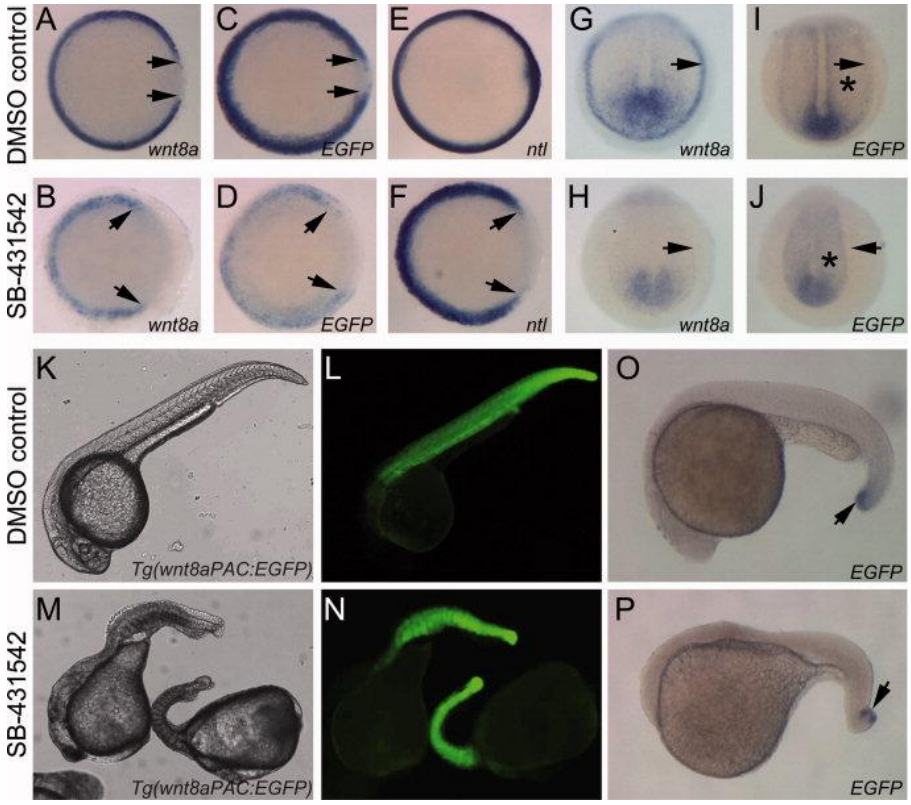

Fig.8. ***Tg(wnt8aPAC:EGFP)*** **expression depends on Nodal**. A–J: In situ hybridizations in control (A,C,E,G,I) or SB-431542 treated embryos (B,D,F,H,J). A–F: Shield stage, animal pole views, dorsal right. A,B: *wnt8a* levels in the ventrolateral margin are reduced when Nodal signaling is inhibited, and the dorsal clearing is expanded (arrows). C,D: *Tg(wnt8aPAC:EGFP)* expression responds in an identical way to wnt8a. E,F: In situ hybridization for ntl to confirm effectiveness of SB-431542 treatment. Note dorsal clearing of ntl (arrows), corresponding to the loss of dorsal mesoderm; this phenotype resembles the *cyc;sqt* double mutant (compare to fig. 6U in Dougan et al., 2003). G–J: Late bud stage, posterior views. G,H: wnt8a expression persists in the tailbud of SB-431542-treated embryos but is not detected in the prospective pronephros. Arrows indicate prospective pronephros expression domain. I,J: *Tg(wnt8aPAC:EGFP)* expression responds identically to wnt8a. Persistent transcripts are observed in the tail paraxial mesoderm (asterisks). Note that no EGFP transcripts are detected in the position of the prospective pronephros, lateral to the presomitic mesoderm domain (arrow). K–P: The $EGFP^{+}$ lineage forms tail mesoderm in SB-431542-treated embryos. K,L: Control *Tg(wnt8aPAC:EGFP)* 24-hpf embryo. Note strong expression in trunk and tail mesoderm. **M,N**: 24-hpf SB-431542-treated *Tg(wnt8aPAC:EGFP)* embryos. Note that developing tail mesoderm expresses EGFP. **O**: EGFP transcripts in a control *Tg(wnt8aPAC:EGFP*) embryo. Note expression in tailbud (arrow) but not in trunk or tail somitic mesoderm. **P**: EGFP transcripts are also detected in the tailbud of SB-431542-treated *Tg(wnt8aPAC:EGFP)* embryos (arrow).

Tg(wnt8aPAC:EGFP)/+ embryos with severe reduction as shown): that is, while expression in paraxial mesoderm was not detectable (Fig. 9C,F, asterisks), expression in the tailbud and prospective pronephros was still evident (Fig. 9F; arrows indicate pronephros domain). Thus, *Tg(wnt8aPAC:EGFP)* expression is strongly regulated in the embryonic margin by Ntl/Bra function after shield stage, suggesting that the PAC interval includes the Ntl/Bra response cis-regulatory element as expected (Morley et al., 2009). However, Ntl/Bra function does not appear to be essential for *Tg(wnt8aPAC:EGFP)* transcription prior to shield stage or in domains outside of the paraxial mesoderm.

We next examined the distribution of $EGFP^{+}$ mesodermal cells in 24 hpf *ntl;bra* morphants. In contrast to the strong EGFP expression observed in the tail somites that form in Nodal loss-of-function embryos, only weak EGFP fluorescence was observed in somites of *ntl;bra* morphants (Fig. 9H,J, arrows). This may reflect the dramatic loss of EGFP transcription in somitic mesoderm progenitors observed at bud stage (Fig. 9F, asterisk). In contrast to reduced fluorescence in the somitic mesoderm, we observe strong reporter fluorescence in the pronephros of *ntl;bra* morphants (Fig. 9J, arrowhead), consistent with transcription in this domain at bud stage (Fig. 9F, arrow). Reporter fluorescence reflects earlier transcription, as no reporter transcripts are detected in *ntl;bra* morphants at 24 hpf (Fig. 9J, inset; 0/53 embryos from *Tg(wnt8aPAC:EGFP)/+* outcross stained, expected 27 stained for Mendelian ratio), similar to what is observed for *wnt8a* (data not shown; 55/60 embryos with no *wnt8a* expression). As in SB-431542

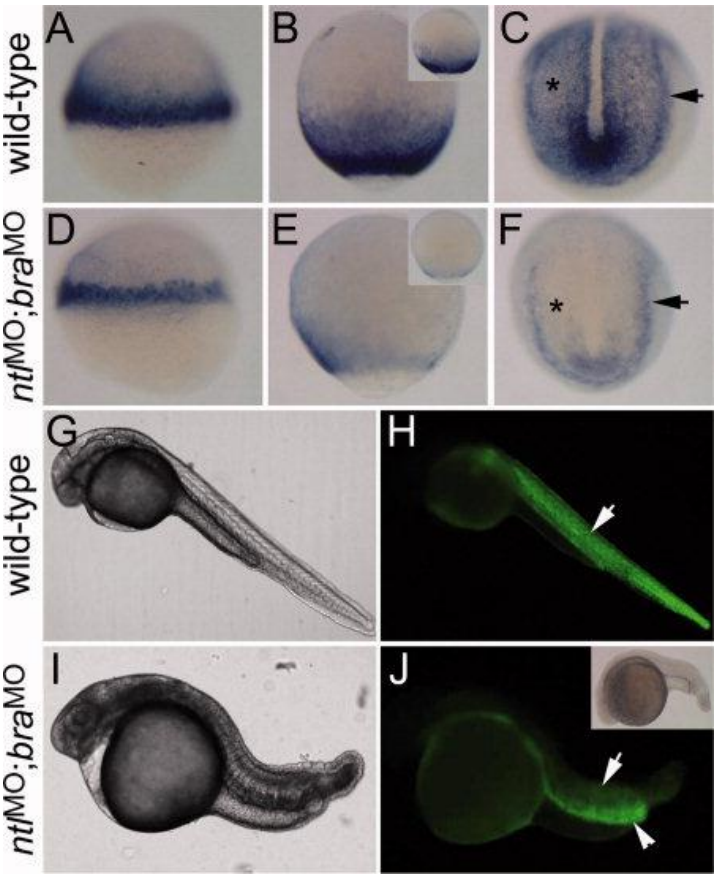

Fig.9. *Tg(wnt8aPAC:EGFP)* expression depends on Ntl/Bra. A–F: In situ hybridizations to detect EGFP transcripts in wild-type (A–C) or *ntl;bra* morphants (D–F). A,D: Expression at shield stage, lateral view, dorsal right. Note slightly reduced EGFP staining in *ntl;bra* morphant. B,E: Expression at 90% epiboly, lateral view, dorsal right, anterior up. Note significantly reduced expression in the morphant. Insets: In situ hybridization for *wnt8a* expression to confirm knockdown. C,F: Bud stage, posterior view, dorsal up. Note significant reduction in EGFP transcripts in the morphant, with slight expression remaining in the tailbud and prospective pronephros (arrows). Asterisks indicate presomitic mesoderm. G–J: EGFP expression at 24 hpf. Note reduced EGFP fluorescence in somitic mesoderm that forms in *Tg(wnt8aPAC:EGFP*) embryos injected with *ntl* and *bra* morpholinos (arrows in H,J), but pronephros expression is not significantly diminished (arrowhead in J). J, Inset: In situ hybridization for EGFP in 24-hpf *ntl;bra* morphant. Expression is not detected, similar to the behavior of *wnt8a*.

treated embryos, YSL expression appeared relatively unchanged in *ntl;bra* morphants, which is consistent with this expression domain being independent of Ntl/Bra function. Thus, Ntl/Bra is not required for early *wnt8a* expression but is required for *wnt8a* expression in prospective paraxial mesoderm, after shield stage.

DISCUSSION

To understand the molecular basis of vertebrate ventrolateral mesoderm induction and patterning, we have initiated an analysis of transcriptional regulation of zebrafish *wnt8a*, a crucial patterning gene that links dorsoventral and anteroposterior patterning networks. To enable our analysis and identify a genomic interval spanning critical *wnt8a* regulatory elements, we inserted an EGFP reporter gene into the *wnt8a* locus of a genomic PAC clone, then generated a stable transgenic line with the modified PAC. *Tg(wnt8aPAC:EGFP)* expression matches the pattern of *wnt8a* in both maternal deposition and zygotic expression. Differences between expression of the reporter transgene and *wnt8a* are likely due to EGFP mRNA stability compared to *wnt8a* mRNAs, which are destabilized by 3‘ UTR elements (Tian et al., 1999; Butler and Lekven, in prep). EGFP fluorescence labels all ventrolateral mesoderm derivatives, and perduring fluorescence serves as a lineage tracer for ventrolateral mesoderm. We showed that EGFP reporter expression is regulated by both Nodal signaling and Ntl/Bra proteins in a biphasic manner: Nodal is essential for early *wnt8a* transcription while Ntl/Bra function is required for late phase *wnt8a* expression. *wnt8a* expression marks tail mesoderm that forms via a Nodal-independent mechanism, and Ntl/Bra appear to be

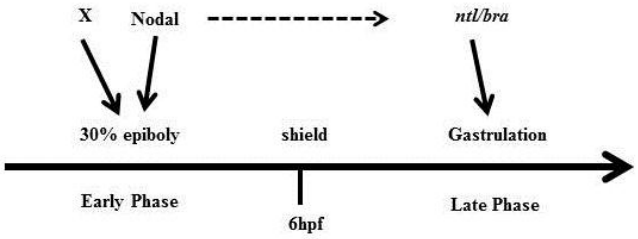

Fig.10. A model for biphasic control of *wnt8a* transcription. At 30% epiboly, Nodal signaling is the primary inducer of *wnt8a*. However, another input exists, indicated by "X." This mechanism controls early phase *wnt8a* expression. After shield stage, Nodal regulation of *wnt8a* is superseded by a Ntl/Bra/*wnt8a* network that maintains *wnt8a* expression in paraxial mesoderm progenitors. However, additional inputs likely exist that are responsible for *wnt8a* expression in pronephros progenitors and perhaps other mesodermal domains.

essential for *wnt8a* expression in paraxial mesoderm but may play a less important role in *wnt8a* expression in the pronephros. Thus, *wnt8a* expression marks multiple mesodermal lineages that are under separate regulatory control, and our reporter transgene is an effective reagent to understand mesodermal patterning.

Our results suggest a model for the temporal control of *wnt8a* transcription (Fig. 10). Early *wnt8a* expression, i.e. expression at or before shield stage, is controlled by Nodal, but another non-Nodal factor must also stimulate early *wnt8a* expression, as *wnt8a* and *Tg(wnt8aPAC:EGFP)* transcripts persist at a reduced level in the absence of Nodal. Late phase *wnt8a* expression, i.e. expression after shield stage, is controlled primarily by Ntl/Bra function, although spatially this mode of regulation appears to be greatest in paraxial mesoderm and not in pronephric progenitors.

The ventrolateral mesoderm gene regulatory network: mesoderm induction and posterior development

Several reports have begun to illuminate the genetic interactions behind ventrolateral mesoderm specification, and they point to a complex interaction of Wnt, Nodal and Bmp signaling with Ntl/Bra-dependent transcriptional regulation. For instance, Agathon et al. (2003) showed that the ventral embryonic margin constitutes the fish tail organizer, which is dependent upon Wnt, Bmp and Nodal signaling for the formation of tail structures. Nodal is known to be crucial for mesoderm induction, but tail somites form in zebrafish embryos devoid of Nodal signaling (Gritsman et al., 1999), demonstrating the existence of a Nodal-independent pathway for tail mesoderm formation. In this context, tail somite formation correlates with *ntl* and *wnt8a* expression. A study of *ntl* regulation suggests that Bmp and Wnt8a signaling induce *ntl* expression through a separate enhancer that integrates these signals independently of Nodal (Harvey et al., 2010). Further, tail induction by ectopic sources of Bmp and Wnt8a requires Ntl function, suggesting that Bmp and Wnt8a signaling act upstream of *ntl* during tail mesoderm induction. Intriguing recent results indicate that Ntl regulates *wnt8a* and *wnt3a* transcription directly, and that these Wnts appear to function downstream of Ntl in posterior development (Martin and Kimelman, 2008; Morley et al., 2009). Thus, *ntl* integrates Nodal and combinatorial Bmp and Wnt8a signaling during mesoderm induction, and Ntl then functions within a feedback network with *wnt8a* during posterior mesoderm development.

This model suggests two potential factors as candidates for the unidentified factor "x" that acts in parallel to Nodal to induce *wnt8a* (Fig. 10). One candidate is Bmp, although evidence against this includes the finding that *wnt8a* expression is unaffected in zebrafish *Bmp2b* mutants (Ramel and Lekven, 2004), ectopic Bmp expression was reported to be unable to induce *wnt8a* (Agathon et al., 2003), and we have found that overexpression of *Bmp2b* via mRNA injection is unable to induce *Tg(wnt8aPAC:EGFP)* expression (Baker and Lekven, unpublished results). However, this does not rule out a permissive role for Bmp in regulating competence of prospective ventral mesoderm to another input. A second potential candidate is Ntl, since it is known to directly bind *wnt8a* regulatory elements and *Tg(wnt8aPAC:EGFP)* expression is slightly reduced in *ntl/bra* morphant embryos. This raises an interesting potential feedback interaction: Wnt8a and Bmp induce *ntl* through a specific cis-regulatory module, and Ntl feeds back to positively regulate *wnt8a* transcription in parallel to Nodal. Clearly, the identification of the critical *wnt8a* cis-regulatory modules is essential for understanding the molecular dynamics of this mesoderm gene regulatory network. Experiments are currently underway to identify the important cis-regulatory modules within the PAC-defined genomic interval.

wnt8a in the YSL: an input into mesoderm induction?

One potentially important but unexplored *wnt8a* expression domain is the YSL, an extra-embryonic tissue that plays essential roles in mesoderm induction and gastrulation (Carvalho and Heisenberg, 2010). Several studies established that the YSL secretes mesoderm inducing factors (Mizuno et al., 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999). Nodal ligands are the predominant mesoderm inducing factors (Kimelman and Schier, 2002), and the zebrafish Nodal ligands *cyclops* and *squint* are expressed in the YSL (Fan et al., 2007). Chen and Kimelman showed that mesoderm induction requires a signal from the YSL between 3 and 4 hpf, and that this signal is encoded by at least one mRNA (Chen and Kimelman, 2000). However, *sqt* and *cyc* are likely not the YSL factors identified by Chen and Kimelman, as the destruction of mRNAs in the YSL produces a far more severe phenotype (e.g. absence of *ntl* expression) than is observed in Nodal-null embryos (Gritsman et al., 1999; Chen and Kimelman, 2000). The expression of *wnt8a* in the YSL raises the intriguing possibility that it, together with *Bmp2b* which is also expressed in the YSL (Kishimoto et al., 1997), may at least contribute to the YSL mesoderm inducing activity identified by Chen and Kimelman. Identification of YSL cis-regulatory modules from the *wnt8a* locus may help unravel the molecular basis of YSL-dependent *ntl* induction. In sum, the generation of our *Tg(wnt8aPAC:EGFP)* line reveals several novel aspects of *wnt8a* regulation and promises to assist in unraveling the complex genetic interactions behind vertebrate mesoderm development and axis patterning.

CHAPTER III

AN EVOLUTIONARY CONSERVED MESODERM ENHANCER INTEGRATES NODAL AND NO TAIL/BRACHYURY ACTIVITY TO ACHIEVE BIPHASIC *wnt8a* EXPRESSION IN ZEBRAFISH

INTRODUCTION

The vertebrate mesoderm is critical in establishing positional information in the early embryo upon which axis patterning depends. Mesoderm is induced by Nodal signaling, and then forms two morphologically distinct domains, dorsal and ventrolateral, that function antagonistically to balance dorsoventral patterning systems in the early embryo (Kimelman, 2006; Schier and Talbot, 2005). Although induced in common by Nodal signaling, dorsal and ventrolateral mesoderm development depends on distinct but overlapping molecular mechanisms. The mechanisms of mesoderm induction and subsequent maintenance are also separable, as Nodal is responsible for induction, but maintenance depends on a gene network centered around Brachyury-related transcription factors (Ntla and Ntlb in zebrafish, we will use Ntl to refer to both; (Conlon et al., 1994; Jones et al., 1995; Martin and Kimelman, 2008, 2010; Morley et al., 2009; Toyama et al., 1995). However, these systems are not independent, as *ntla* expression is dependent upon Nodal signaling (Chen and Schier, 2001; Harvey et al., 2010). To understand the molecular basis of mesoderm development and the integration of induction and maintenance mechanisms, it is crucial to unravel the transcriptional basis of the gene regulatory networks behind the separable events of mesoderm induction, dorsoventral identity specification and progenitor maintenance.

One factor critical to mesoderm development is the secreted ligand, Wnt8a. In the early gastrula, Wnt8a signaling prevents organizer expansion through the maintenance of *vent*, *vox* and *ved* expression in the ventrolateral mesoderm (Ramel and Lekven, 2004). During and after gastrulation, Wnt8a functions downstream of Brachyury-related T-box transcription factors and regulates posterior mesoderm maintenance and proliferation (Baker et al., 2010; Martin and Kimelman, 2008). Wnt8a signaling is also crucial to nervous system anteroposterior patterning (Erter et al., 2001; Lekven et al., 2001; Rhinn et al., 2005). Wnt8a mutants are marked by severe dorsoventral and anteroposterior patterning defects (Baker et al., 2010; Lekven et al., 2001; Ramel and Lekven, 2004). Conversely, ectopic Wnt8a expression, for instance in the zebrafish mutants *headless* and *bozozok*, leads to defects in neural anteroposterior patterning and notochord formation (Fekany-Lee et al., 2000; Kim et al., 2000). Thus, *wnt8a* expression is a critical component of the mesoderm gene regulatory network with ramifications for global embryonic axis patterning. Consequently, understanding the transcriptional regulation of *wnt8a* is a critical step in unraveling multiple aspects of early vertebrate development.

The expression pattern of *wnt8a* is largely conserved in vertebrates (Bouillet et al., 1996; Christian et al., 1991; Hume and Dodd, 1993; Kelly et al., 1995). Notably, teleost *wnt8a* comprises a tandem duplication encoding two Wnt8a proteins, Wnt8a.1 and Wnt8a.2 (Lekven et al., 2001; Ramel et al., 2004). Zebrafish *wnt8a* produces a bicistronic transcript, encoding both Wnt8a.1 and Wnt8a.2 proteins, as well as a *wnt8a.2*-specific

transcript; together, their expression recapitulates the tetrapod *wnt8a* expression pattern (Lekven et al., 2001). Zebrafish *wnt8a* transcripts are deposited maternally, decline by ~2.5 hours post fertilization (hpf, 128-256 cell stage) and again increase at the midblastula transition (Kelly et al., 1995). In situ hybridization reveals *wnt8a* transcripts at the ventrolateral embryonic margin and in the yolk syncytial layer (YSL) starting at 30% epiboly (Ho et al., 1999; Kelly et al., 1995; Narayanan et al., 2011). Expression of *wnt8a* expands up to the dorsal organizer and is maintained in the ventrolateral margin during gastrulation. After gastrulation, *wnt8a* is expressed in the tailbud, presomitic mesoderm and prospective pronephros (Kelly et al., 1995; Narayanan et al., 2011).

Three positive regulators of *wnt8a* have so far been identified. Consistent with Nodal being an upstream regulator, zebrafish *wnt8a* expression is reduced in *cyc;sqt* double mutants, while ectopic Nodal expression can induce *wnt8a* (Agathon et al., 2003; Erter et al., 2001; Narayanan et al., 2011). Whether this is a direct or indirect interaction is unknown. Two direct positive regulators of *wnt8a* are the T-box paralogs Ntla and Ntlb (Goering et al., 2003; Martin and Kimelman, 2008), and the maternal zinc-finger protein Zbtb4 (previously called Kzp; (Yao et al., 2010). Zbtb4 is essential for *wnt8a* activation, while Ntla and Ntlb are required for expression during gastrulation (Goering et al., 2003; Martin and Kimelman, 2008; Yao et al., 2010). Thus, three upstream factors are known to be required for *wnt8a* transcription in the zebrafish embryonic margin, and they are essential for different phases of *wnt8a* transcription.

While three factors that activate *wnt8a* transcription have been identified, it is not understood how *wnt8a* locus cis-elements integrate these inputs to achieve normal expression. We recently demonstrated that zebrafish *wnt8a.1* is regulated in a biphasic manner via cis-elements within an 80 KB genomic interval (Narayanan et al., 2011). This biphasic regulation correlates with the transition from Nodal-dependent mesoderm induction to Ntl dependent posterior growth. Here we have dissected the *wnt8a* upstream region to identify crucial cis-regulatory elements. Our results show that *wnt8a* transcription is regulated by two independent enhancers that drive expression in the YSL and in mesoderm progenitors in the embryonic margin. The YSL and mesoderm enhancers lie in the first intron, situated between two alternate promoters. We identified two functional cis-regulatory modules, which we call the Proximal and Distal Regulatory Regions (PRR and DRR), each containing one of the alternative promoters. The PRR and DRR integrate the three known inputs to achieve the *wnt8a* expression pattern. Our cis-regulatory analysis allowed us to define the phases of *wnt8a* expression: phase I comprises activation at 30% epiboly through 60% epiboly (4.7-~6.5 hours post fertilization) while phase II comprises 70% epiboly and beyond (after ~7 hpf). Phase I expression requires Nodal activation of the mesoderm enhancer which is Zbtb4-dependent. Phase II expression requires Ntl activation of the mesoderm enhancer, which is independent of both Nodal and Zbtb4. Phase I expression requires both the PRR and DRR, but phase II expression requires only the DRR. Curiously, the DRR interferes with the ability of the PRR to generate normal phase II expression. We also present evidence that this mechanism may be conserved in teleosts, although biphasic regulation of *wnt8a*

in other species may be mediated through separate enhancer elements. Thus, *wnt8a.1* expression results from a complex interaction of multiple enhancers with alternative promoters.

MATERIALS AND METHODS

Fish care

Zebrafish were maintained as described (Westerfield, 2000). An AB-TL hybrid line serves as our wild-type stock. Vertebrate animal procedures were approved by the Texas A&M Institutional Animal Care and Use Committee.

Molecular biology and transgenesis

To map *wnt8a* transcription start sites, 5' RACE was performed on 80% epiboly RNA with the GeneRacer kit (Invitrogen). Full-length *wnt8a* cDNAs were ligated into pGemTeasy and sequenced. Multiple clones showing the furthest 5' base identified transcription start sites. Transgene construction initiated with an EcoRI genomic fragment clone extending 4.8 kb upstream of the *wnt8a.1* translation start site. We created an NcoI site at the *wnt8a.1* start codon, then ligated in the EGFP and SV40 polyA sequences to create pE8G. p-2.8*wnt8a.1:EGFP* was created by ligating a 2.8 kb NarI-NcoI fragment from pE8G into pT2AW1, our own derivative of pT2AL200R150G (kind gift of Dr. Koichi Kawakami). Constructs 2-5 were generated as subclones of p-2.8*wnt8a.1:EGFP* through inverse PCR using a common reverse primer: 5' CTTCCATATGCGACAAGATATCCG 3' with forward primers: -1.8: 5'

TAAACGCTGTGGTGATGGTCTTC 3‘; -1.3: 5’ TATAATCACCCTCATGATTATCC 3’; -.8: 5’ CGAATGATTTTATGGCTTTTTGTATA 3’; -.3: 5’ TCCCCAGGAAATATATAGTTTAATG 3’. Construct 6 was generated by inverse PCR with primers: 5’ CTCACCTTGCTGCACAGATG 3’ and 5’ TTTACACTTCTCAGCTGTTGATCC 3’. Construct 7 was made with 5’ AAGCTTGGGAGATCCCTGTCACTC 3’ and 5’ ACACCATGTGTCATACAAGAC 3’. Construct 8 used 5’ CTGCCCATTCATGAATACACTACAC 3’ and 5’ TGAATCAATACGATTTGTATGTCGA 3’. For construct 9, the region spanning FoxH1 and Ntl binding sites was amplified with 5’ TTTACACTTCTCAGCTGTTGATCC 3’ and 5’ TGGTGTAGTGTATTCATGAATGGG 3’ and ligated into construct 5. For construct 10, a 570 bp portion of the mesoderm enhancer was amplified with 5’ CACTAATCCACACAACCGATC 3’ and 5’ CGCTAACGTTTGTGGAGACAG 3’ and ligated into pT2AW2, a derivative of pT2AL200R150G with the cfos minimal promoter. Stickleback genomic DNA sequence was analyzed via the UCSC Genome Browser. Positions of exons were inferred manually and through Blast comparisons with zebrafish and *Fugu wnt8a* cDNA sequences. Stickleback genomic DNA was isolated through standard procedures from a wild-caught male (gift of Clay Small, Texas A&M University). Fragments were amplified with primers: control: 5’ GATACACAGCAAATCCAGGAGC 3’ and 5’ AGTCAGCGTTCATGTAATACCG 3’; 5’ fragment: 5’ GAGCATGTATATTATGGCTTGCC 3’ and 5’ AGCCGTATTCCAAACGGATCC 3’; 3’ fragment: 5’

CTCCATGATGCGGCTGAATAAC 3' and 5' GTAACCTCTCTGGGACGTTG 3'. The control and 3' fragments were ligated into pT2AW2. The 5' fragment was ligated into pT2AW3, a promoter-less derivative of pT2AL200R150G. In situ hybridizations and probes were as described previously (Ramel et al., 2004; Narayanan et al., 2011). For transgenesis, Tol2 plasmids were coinjected with transposase mRNA (both at 25 ng/µl) into 1-cell stage embryos, which were raised and outcrossed to wild-type. Progeny were screened for EGFP fluorescence and positives were raised to generate lines. At least two independent lines were recovered to verify expression patterns. All stable lines transmit reporters in standard Mendelian ratios.

Overexpression and inhibition methods

For the analysis of reporter expression under treated or control conditions, embryos were collected from an outcross of a heterozygous transgenic male (Tg/+) to a wild-type female. Embryos were collected, fixed and processed for in situ hybridizations. 50% of each resulting clutch is expected to be heterozygous for the transgene. TARAM-D cDNA was a generous gift of Dr. Frederic Rosa (INSERM, France). The cDNA was digested using XbaI and sense transcripts were synthesized using the Sp6 Message Machine kit (Ambion, Inc., Austin TX). 1-3 nl of 100ng/µl solution was injected. In all experiments, groups of injected embryos were assayed for morphology at 24hpf to confirm injection efficacy. For the HS-*ntl* plasmid, the *ntl* coding sequence was ligated downstream of the hsp70/4 promoter. Embryos were injected with 1-3 nl of 100 ng/µl solution. SB-431542 was prepared and applied as previously described (Narayanan et

al, 2011). *kzp* MO1 was used as described (Yao et al., 2010). Knockdown efficiency was assayed with *eve1* and *wnt8a* in situs at shield stage and by 24hpf morphology. *ntl* and *bra* morpholinos were as previously described (Martin and Kimelman, 2008). In situ hybridizations were as described (Ramel et al., 2004).

RESULTS

The zebrafish *wnt8a* locus contains three promoters

We sought to identify cis-regulatory elements capable of driving reporter expression that recapitulates *wnt8a*, and began by identifying the sites of *wnt8a* transcription initiation. The teleost *wnt8a* locus comprises tandem *wnt8a* coding sequences, referred to as *wnt8a.1* and *wnt8a.2* (Ramel et al., 2004). In zebrafish, these are transcribed as a bicistronic message and a *wnt8a.2* specific message (Lekven et al., 2001). Initial reports identified cDNAs with 5' regions immediately upstream of the *wnt8a.1* translation initiation codon (Kelly et al., 1995), one cDNA (Genbank accession no. BC044143) suggested that transcription may initiate from an upstream promoter. To resolve this discrepancy, we performed 5' RACE and primer walking to amplify the 5' ends of full length transcripts from 80% epiboly stage mRNA samples. We identified three transcription start sites, TSS-A, TSS-B and TSS-C (Fig. 11), suggesting that all three promoters are active during embryogenesis. TSS-A and TSS-B are upstream of *wnt8a.1*, while TSS-C will generate *wnt8a.2*-only transcripts. Importantly, exon 1 of TSS-A transcripts splice to an acceptor site 35 bases downstream of TSS-B (Fig. 11). We were not able to identify the exact position of the TSS-A start site, but primer walking

experiments suggested it may lie ~400-500 bp upstream of the intron 1 splice donor site (data not shown). These findings suggest that *wnt8a* transcriptional regulation is complex and involves multiple promoters.

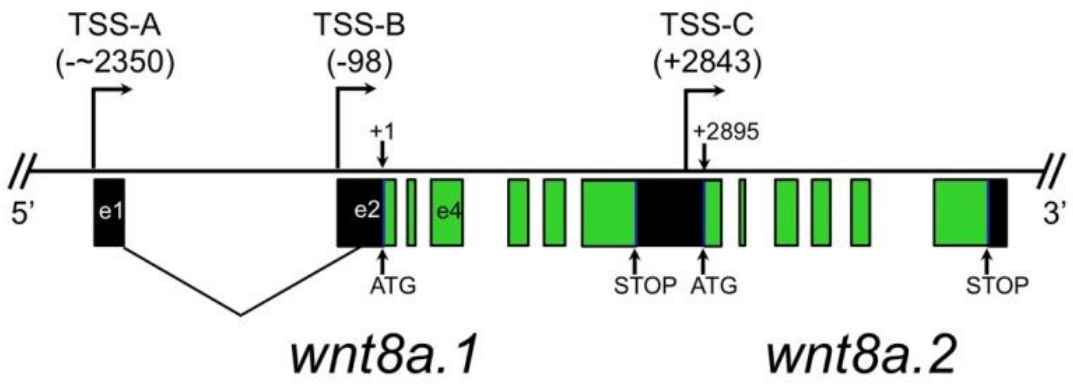

Fig.11. Complex promoter structure of the zebrafish *wnt8a* locus. Schematic map (not to scale) of the experimentally determined transcription start sites (TSS) for *wnt8a.1* and *wnt8a.2*. Numbers are coordinates relative to the translation initiation codon for *wnt8a.1*. Boxes represent exons; black boxes represent untranslated regions, green indicates coding sequence. Note that exon1 (e1) of transcripts originating from TSS-A splice to a position in exon 2 (e2) that is 35 bp downstream of TSS-B (at position -63), as indicated by the thin line.

Cis-regulatory analysis reveals multiple *wnt8a.1* regulatory regions

We previously generated a *wnt8aPAC:EGFP* transgenic line in which bright EGFP labeling of several mesodermally derived organs, including somites, YSL, pronephros, heart and blood, is observed despite the absence of transcripts at that stage (Narayanan et al., 2011). Thus, EGFP fluorescence in these tissues at 24 hpf, especially in somites and YSL, is a convenient assay to determine whether reporter constructs are active in the

precursors of these tissues. We exploited this to test *wnt8a.1* upstream cis-elements for transcriptional regulatory activity.

We first tested a fragment extending 4.8 kb upstream from the *wnt8a.1* translation start site in transient and stable transgenic reporter assays, and observed robust EGFP expression in somites, YSL and other mesodermal tissues consistent with *wnt8a.1* expression (data not shown). We observed a similar pattern of transient reporter expression driven by a smaller 2.8 kb fragment (p-*2.8wnt8a:EGFP*; Fig. 12A,Ba, Table 1), therefore we used this as a starting point to construct a series of ten reporter plasmids representing subportions of this 2.8 kb (Fig. 12A). For these ten constructs, we first characterized the relative intensity of EGFP fluorescence in the somites and YSL of 24 hpf embryos that had been injected with each construct at the one-cell stage (Table 1).

Transient expression assays with constructs 1-5, comprising nested deletions from the distal end of the 2.8 kb regulatory region (Fig. 12A, Table 1), led to four notable outcomes. First, constructs 1 and 2, but not 3, produced fluorescence in the YSL, identifying the ~500 bp distal portion of construct 2 as the site of a YSL enhancer (Fig. 12A, yellow box). Second, constructs 1-4 produced expression in the somites (to different degrees) but construct 5 did not (Table 1 and data not shown), identifying a mesoderm enhancer within constructs 3 and 4. Third, constructs 2-4 demonstrated that TSS-B is sufficient for reporter expression, and thus the intronic region upstream of the *wnt8a.1* translation initiation codon can be considered a regulatory unit, which we designated the Proximal Regulatory Region (PRR, Fig. 12A). Fourth, construct 5

produced very little EGFP fluorescence that was observed in scattered cells, not in somites or the YSL; thus, construct 5 has a functional basal promoter (i.e. TSS-B), but depends on additional enhancers for patterned expression.

We confirmed the position of the YSL enhancer with a deletion in the 2.8 kb fragment (construct 6, Fig. 12A, Table 1). As expected, this construct produced somitic but not YSL expression in transient assays (Fig. 12Be; Table 1). We then confirmed the position of the mesoderm enhancer by deleting a 1 kb fragment proximal to TSS-B; transient expression from construct 7 was observed strongly in the YSL but not in any mesodermal tissues (Fig. 12Bf, Table 1). We noted that the intronic region between TSS-A and TSS-B spans two predicted conserved elements (UCSC Genome Browser PhastCons prediction, Zv8 genome assembly) that overlap our experimentally identified YSL and mesoderm enhancers (not shown). We also noted a concentration of consensus binding sites for the transcription factors Ntl and FoxH1, an effector of Nodal signaling (Osada et al., 2000; Pogoda et al., 2000), within a ~500 bp stretch of the mesoderm enhancer (asterisks and dots, respectively, above line in Fig. 12A;). Because previous studies established Nodal and Ntl as upstream regulators of *wnt8a*, we deleted this ~500 bp and found that this removed almost all mesodermal enhancer activity within the 2.8kb regulatory region (construct 8, Table 1, Fig. 12Bg). A ~800 bp fragment spanning all Ntl and FoxH1 consensus sites was sufficient in combination with an extended *wnt8a* basal promoter to drive expression in somites (construct 9, Table 1, Fig. 12Bh) as was the

shorter ~500 bp mesoderm enhancer in combination with the c-fos minimal promoter (construct 10, Table 1, Fig. 12Bi).

We recovered stable transgenic lines for six of the ten constructs and examined EGFP fluorescence at 24 hpf. Consistently, we found that fluorescence in stable lines closely recapitulated the transient assays, validating the enhancer locations. Additionally, the stable transgenic lines allowed us to identify and confirm subtle but important differences in reporter expression patterns driven by different mesoderm enhancer constructs. First, fluorescence in the heart correlates with the mesoderm enhancer, but fluorescence in the pronephros does not (data not shown). Second, mesodermal fluorescence in *Tg(-2.8wnt8a.1:EGFP)/+* embryos (hereafter referred to as Tg(-2.8) embryos) extends anteriorly in the embryo to a position ventral to the eye, while expression in other lines terminates at approximately the level of the posterior hindbrain (cf. Fig. 12Ba,b); we consistently observed this pattern in transient expression assays as well. Third, the 550 bp mesoderm enhancer deletion removes most, but not all, somitic fluorescence (Fig. 12Bg), while the larger deletion in Tg(-2.8Δ1176/169) removes all mesodermal expression (Fig. 12Bf). This difference correlates with a total of 515 bp that

Table 1. Reporter construct expression in 24hpf transient assays

Construct number	Construct Name	Expression in YSL[a]	Expression in Somites[a]
1	-2.8wnt8a.1:EGFP	+++	+++
2	-1.8wnt8a.1:EGFP	+++	+++
3	-1.3wnt8a.1:EGFP	-	+++
4	-.8wnt8a.1:EGFP	-	++
5	-.3wnt8a.1:EGFP[b]	-	-
6	-2.8Δ1838/1270wnt8a.1:EGFP	-	+++
7	-2.8Δ 1176/169wnt8a.1:EGFP	+++	-
8	-2.8Δ 989/497wnt8a.1:EGFP	+++	+
9	-1.3Δ470/300wnt8a.1:EGFP	-	+++
10	570meswnt8a.1-cfos:EGFP	-	+++

a. +/- indicate strength/distribution of expression
b. Expression in scattered cells was observed in this construct

includes a single consensus Ntl binding site. Finally, the somitic expression in Tg(-2.8) embryos is far less uniform than that observed for either the Tg(-1.3) (Fig. 12Bc) or Tg(-1.3Δ470/300) (Fig. 12Bh) lines. In all lines, however, expression is observed to be a gradient brightest at the tip of the tail (Fig. 12Ba,b,h).

The observed difference in the anterior expression limit in Tg(-2.8) and Tg(-1.8) embryos correlates with the presence of TSS-A, exon 1 and an additional ~400 bp upstream, in which we also identified a consensus binding site for Zbtb4 (Fig. 12A, diamond symbol; Yao et al., 2010). This difference in reporter expression suggests that the region upstream of the YSL enhancer also contains important regulatory function; accordingly, we designated this region the Distal Regulatory Region (DRR; Fig. 12A). Thus, we identified two regulatory regions comprising multiple enhancers and two alternative *wnt8a* promoters. Further, transgenic reporters show qualitative differences in expression that correlate with the presence of consensus binding sites for known *wnt8a* transcriptional regulators.

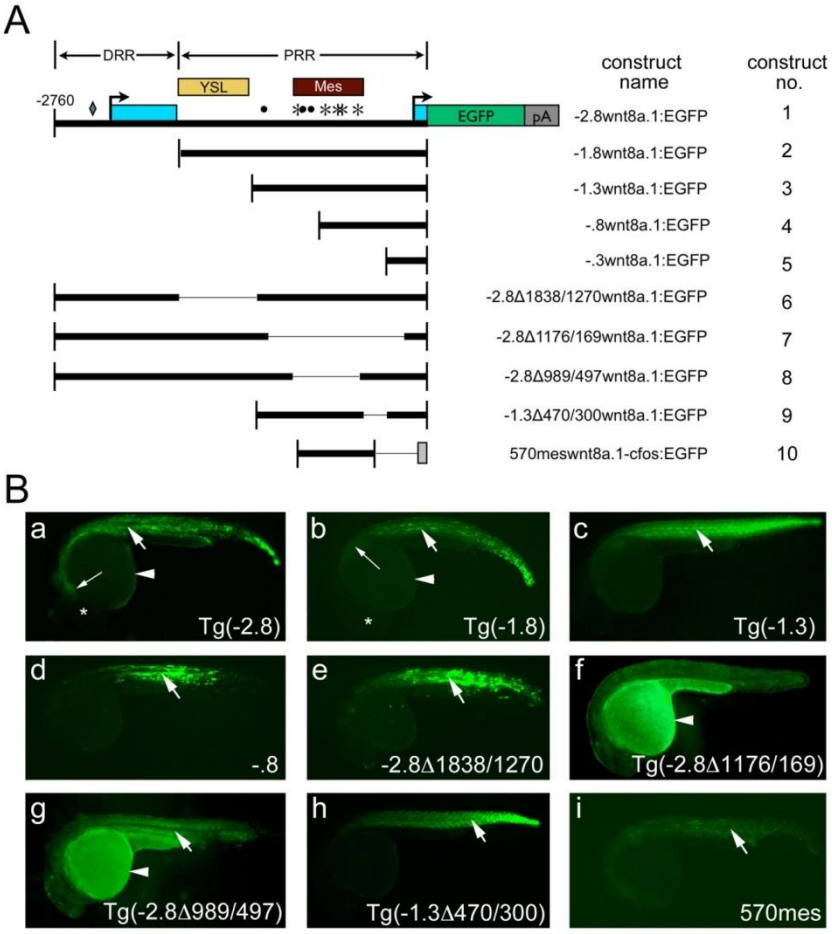

Fig.12. Dissection of *wnt8a* cis-regulatory regions. A) Schematic diagram of reporter constructs used in this study, drawn to scale. Construct 1 (-2.8wnt8a.1:EGFP) contains the fragment extending 2760 bp upstream of the *wnt8a.1* translation initiation codon (-2760). Salient functional features are indicated on construct 1. Light blue boxes are exons, arrows indicate transcription start sites. The diamond, asterisks and dots indicate Zbtb4, FoxH1 and Ntl consensus binding sequences, respectively. Yellow box: YSL enhancer. Maroon box: mesoderm enhancer. DRR: Distal Regulatory Region. PRR: Proximal Regulatory Region. Thick bars indicate the regulatory region included in each construct. Thin lines indicate deleted regions. The grey box in construct 10 indicates the c-fos minimal promoter. B) EGFP reporter expression in stable or transient assays at 24 hpf. All images: lateral views, anterior to left. Reporter construct is indicated in lower right. (a,b,c,f,g,h) Stable transgenic embryos. (d,e,i) Transient expression assays. Arrows indicate somitic expression, arrowheads indicate YSL expression. Note that YSL fluorescence in stable lines is dimmer than that observed in transient assays, likely owing to stable integration of single-copy transposons. At 24 hpf, YSL fluorescence in stable lines is observed as an outline of the yolk that is slightly brighter than yolk autofluorescence. Note that images in f,g are longer exposures than the other images. (a,b) Long arrows indicate anterior limit of reporter fluorescence, asterisks indicate position of the eye.

***wnt8a* transcriptional regulation in the margin requires both the DRR and PRR**

We previously identified distinct temporal regulatory mechanisms for *wnt8a* (Narayanan et al., 2011) from which we infer two phases of *wnt8a.1* regulation: an initial Nodal-dependent phase at *wnt8a* expression onset (phase I: 30% epiboly-60% epiboly), and a Ntl-dependent phase beginning in the mid to late gastrula (phase II: 70% epiboly and later). To determine whether our transgenic reporters recapitulated these phases of *wnt8a* regulation, we analyzed their expression at early (shield stage, 6 hpf; phase I) and late (80% epiboly, 8 hpf; phase II) gastrula stages. We found that Tg(-2.8) reporter expression recapitulated *wnt8a* phase I in the margin (Fig. 13A,F, arrows) and YSL (Fig. 13A, asterisk; *wnt8a* YSL expression is below level of detection). In contrast, Tg(-1.8) embryos did not express EGFP in the margin prior to 60-70% epiboly, although we did observe YSL expression (Fig. 13B, asterisk; YSL expression confirmed by fluorescence microscopy, see Fig. 12Bb). This indicates that the DRR is necessary for phase I expression in the margin, but not in the YSL, a conclusion further supported by Tg(-2.8Δ1176/169), Tg(-2.8Δ989/497) and Tg(-1.3Δ470/300) expression (Fig. 13C-E). Importantly, the DRR is not sufficient for phase I expression, as staining was absent from the margin in shield stage Tg(-2.8Δ1176/169) and Tg(-2.8Δ989/497) embryos (Fig. 13C,D). As these transgenes lack the mesoderm enhancer, this shows that phase I expression requires both the DRR and the mesoderm enhancer.

In contrast to phase I regulation, Tg(-2.8) does not fully recapitulate phase II expression. While *wnt8a* is expressed in a continuous band in the margin at 80% epiboly (Fig. 13L,

arrow), Tg(-2.8) expression is observed in scattered cells dispersed from the margin toward the anterior embryo (Fig. 13G, arrow, arrowheads), a pattern also observed in Tg(-4.8) embryos (data not shown). We determined that these cells represent mostly ingressing mesoderm progenitors with some endoderm progenitors, as their number was decreased only slightly after knockdown of *casanova*, which blocks endoderm formation (data not shown; Kikuchi et al., 2001). The dispersed staining could represent persistent transcriptional activity in ingressing progenitors or perduring EGFP transcripts. We favor the latter possibility, as this pattern is not observed when the Tg(-2.8) EGFP reporter also has the *wnt8a* 3' UTR (Butler and Lekven, unpublished). A continuous band of reporter expression was observed, however, in Tg(-1.8) embryos (Fig. 13H, arrow), indicating that the PRR is sufficient to recapitulate the phase II pattern, a conclusion supported by Tg(-1.3Δ470/300) (Fig. 13K). Phase II expression requires the mesoderm enhancer, as no margin staining was observed in either Tg(-2.8Δ1176/169) or Tg(-2.8Δ989/497) embryos (Fig. 13I,J). Thus, the PRR is sufficient for phase II expression. This depends upon the mesoderm enhancer, and the DRR appears to interfere with the PRR's ability to recapitulate the endogenous *wnt8a* pattern.

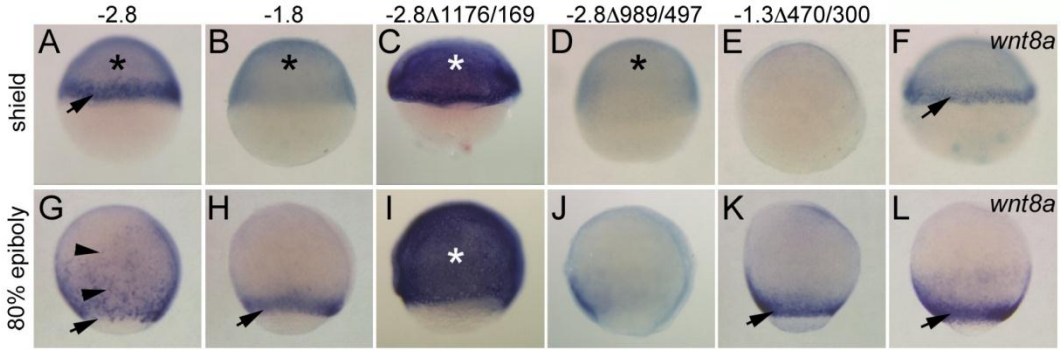

Fig.13. Phase I and Phase II *wnt8a* reporter expression. All images: in situ hybridizations for EGFP transcripts in embryos from stable transgenic lines, except F,L: *wnt8a* expression in wild-type. Lateral view, dorsal right. (A-F) Shield stage. (G-L) 80% epiboly. Reporter transgene is indicated above each row. Arrows indicate expression in the margin. Asterisks indicate YSL expression, which at this stage manifests as a general blue stain underneath the epiblast layer of the embryo, except in line Tg(-2.8Δ1176/169) (C,I) in which YSL expression is very robust perhaps due to copy number, positional effects or deletion of a negative regulatory element. YSL staining was confirmed by YSL fluorescence observed in live embryos. Arrowheads in G indicate scattered ingressing cells. Note qualitative difference between pattern in G from that in H and K and the *wnt8a* pattern in L.

This analysis also illustrates the independence of the YSL and mesoderm enhancers. For example, Tg(-2.8Δ1176/169), lacking the mesoderm enhancer, is expressed only in the YSL (Fig. 13C,I, asterisks), a pattern that persists through 24 hpf (see Fig. 12Bf). Similarly, Tg(-1.3Δ470/300), lacking the YSL enhancer, is expressed only in the margin (Fig. 13K; see also Tg(-1.3), Fig. 12Bc and data not shown).

Initial *wnt8a* transcription requires Nodal activation of the mesoderm enhancer and Zbtb4

wnt8a phase I expression is recapitulated in Tg(-2.8) embryos, and this requires the mesoderm enhancer. As Nodal signaling is upstream of *wnt8a*, the presence of

consensus FoxH1 and Smad binding sites in the mesoderm enhancer suggests Nodal may directly regulate *wnt8a*. To test this, we performed gain and loss of function assays in transgenic reporter embryos.

We treated Tg(-2.8) embryos from the one-cell stage with the Nodal antagonist SB-431542 and assayed EGFP transcripts at shield stage (Fig. 14A-D). Treated embryos show a significant decrease in reporter transcripts in the margin so that only few staining cells are observed (Fig 14A,B; 32/32 heterozygous embryos affected; controls: no change, n=30). In situ hybridization for *ntl* confirmed that the treatment induced a Nodal signaling null phenotype (Fig. 14C,D; 60/60 embryos affected as shown; Feldman et al., 1998). Thus, the Tg(-2.8) reporter recapitulates the Nodal requirement observed for *Tg(wnt8aPAC:EGFP)* and *wnt8a*. To test whether Nodal is sufficient to activate the *wnt8a* reporters, we overexpressed Taram-D, a constitutively active form of the Activin receptor *acvr1b* (Renucci et al., 1996), through mRNA injection at the one-cell stage. In Tg(-2.8) embryos, we found that Taram-D could efficiently induce ectopic EGFP transcription (Fig. 14E; 68/70 heterozygous embryos affected, image shows predominant phenotype) and also *wnt8a* (Fig. 14F; 19/20 embryos affected). As a control, we assayed induction of *ntla* by Taram-D. Consistent with the known regulation of *ntla* by Nodal (e.g. Harvey et al., 2010), Taram-D overexpression strongly activates *ntla* transcription (Fig. 14G; 32/32 embryos affected). Thus, Nodal signaling is necessary and sufficient for transcriptional activation from the -2.8 kb DRR/PRR regulatory region.

We next tested whether the PRR could respond to Taram-D, even though the PRR is insufficient for phase I expression (Fig. 13B). We injected Taram-D mRNA into 1-cell stage Tg(-1.8) outcross embryos and assayed EGFP transcripts at shield stage (Expect 50% heterozygous offspring; see materials and methods). While 0/21 embryos in a control clutch expressed EGFP at shield stage (expect 10 heterozygotes), 38/79 embryos from the injected group expressed EGFP (Fig. 14H; expect 39 heterozygous embryos; $p<0.001$). Thus, the PRR is sufficient to respond to ectopic Nodal signaling. In contrast, Taram-D did not induce EGFP in Tg(-2.8Δ989/497) embryos (Fig. 14I; 0/57 embryos in clutch express EGFP; expect 28 heterozygotes), indicating that the mesoderm enhancer is essential for TGF-β induction of the reporter. Therefore, the mesoderm enhancer is essential to reporter activation in the early gastrula, Nodal signaling stimulates reporter expression and the mesoderm enhancer is essential for Nodal stimulation of the reporter. These findings are consistent with the hypothesis that Nodal signaling activates reporter expression through the mesoderm enhancer.

Recently, the zinc-finger protein Zbtb4 was shown to directly activate *wnt8a* potentially through three binding sites in the *wnt8a* promoter region (Yao et al., 2010). We identified a single Zbtb4 consensus binding site in our constructs upstream of TSS-A, but the previous study of Yao et al. had not determined whether this site is a functional binding site in vivo. Thus, we tested whether Zbtb4 is essential for activation of our reporter transgenes. We injected a published *zbtb4* morpholino (MO; Yao et al., 2010) into Tg(-2.8) outcross embryos, then assayed EGFP transcripts at shield stage (phase I

expression). We found that only 1/51 injected embryos showed EGFP expression (Fig. 14J; expect 25 expressing heterozygotes, $p<0.001$), indicating a requirement for Zbtb4 in Tg(-2.8) activation. We then tested whether Zbtb4 activity is required for Nodal dependent transgene activation. We co-injected Tg(-2.8) outcross embryos with both *zbtb4* MO and Taram-D mRNA, then assayed EGFP transcripts at shield stage. Whereas Taram-D can efficiently induce Tg(-2.8) expression (see above), only 2/63 of the co-injected embryos expressed EGFP (Fig. 14K; expect 31 expressing embryos; $p<0.001$). Thus, Zbtb4 and Nodal signaling must be integrated for phase I expression.

We repeated the co-injection experiment in the Tg(-1.8) line, in which the reporter lacks the DRR and its Zbtb4 binding site. Tg(-1.8) embryos respond to ectopic Taram-D with precocious reporter expression at shield stage (see Fig. 14H). Similarly, 26/57 *zbtb4* MO/Taram-D co-injected Tg(-1.8) outcross embryos showed precocious reporter expression in the margin (Fig. 14L; expect 28 heterozygous embryos). Thus, Zbtb4 knockdown did not inhibit the precocious induction of Tg(-1.8) expression by Taram-D. These results suggest that the DRR is required for Zbtb4-dependent reporter regulation and that the DRR alters the response of the PRR to Nodal activity.

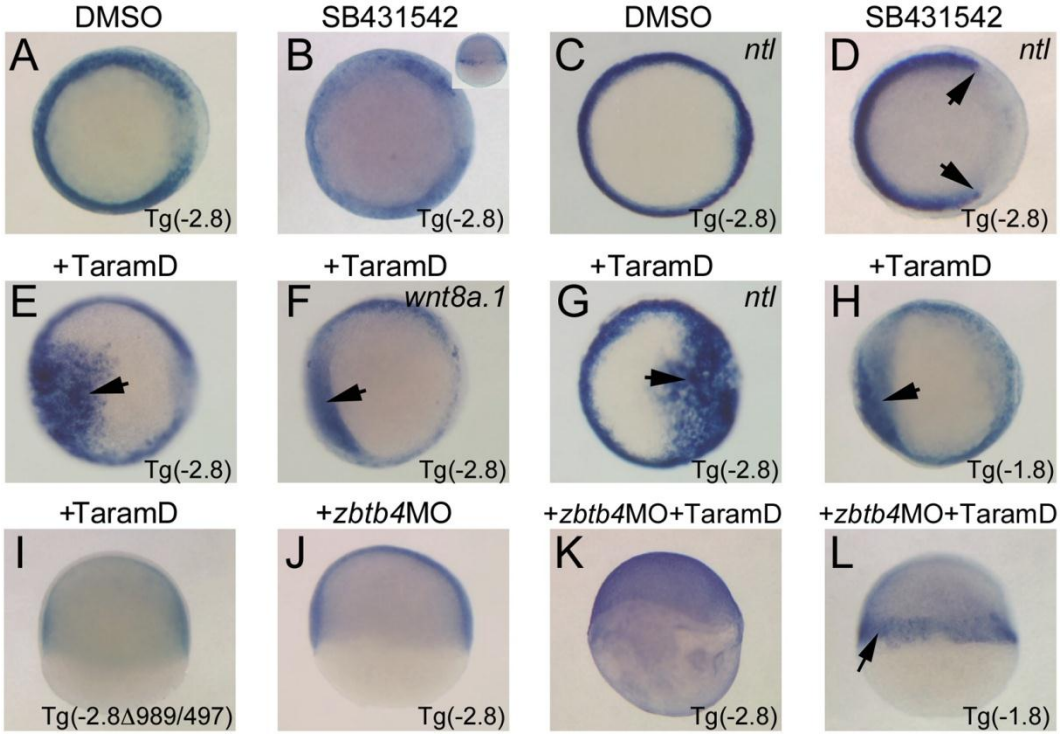

Fig.14. Phase I expression requires a Nodal response by the mesoderm enhancer and Zbtb4. (A,B) In situ hybridizations for EGFP transcripts in shield stage Tg(-2.8) embryos, animal pole views, dorsal right. (A) Control DMSO treated embryo. (B) SB431542 treated embryo. Note significant reduction in transcripts. Inset: lateral view to show patchy expression in the margin. (C,D) In situ hybridizations for *ntla* transcripts to confirm Nodal loss of function. (C) Control DMSO treated embryo. (D) SB431542 treated embryo. Note loss of dorsal mesoderm indicative of strong reduction in Nodal signaling (arrows). (E-I) In situ hybridizations on embryos injected with Taram-D mRNA at the 1-cell stage. (E) EGFP transcripts in injected Tg(-2.8) embryo. Arrow indicates ectopic EGFP reporter expression. (F) *wnt8a.1* expression in injected Tg(-2.8) embryo. Note induction of ectopic expression, though not as robust as the transgene. (G) *ntla* expression in injected Tg(-2.8) embryo. Note strong induction of *ntla*, arrow. (H) EGFP expression in injected Tg(-1.8) embryo. Note ectopic expression, arrow. (I) EGFP expression in injected embryo. Note absence of a reporter response to Taram-D when mesoderm enhancer is deleted. (J-L) Zbtb4 is required for Nodal-dependent phase I expression. EGFP in situ hybridizations. (J) Embryo injected with Zbtb4 morpholino. Note absence of reporter expression in the margin. (K) Embryo injected with Zbtb4 morpholino and Taram-D mRNA. Note absence of reporter expression. (L) Embryo injected with Zbtb4 morpholino and Taram-D mRNA. Note that the Tg(-1.8) reporter lacks the DRR, and that reporter expression is activated prematurely (arrow, compare to Fig. 13B).

Phase II *wnt8a* expression requires Ntl regulation of the mesoderm enhancer

The above results suggest that phase I expression requires both the DRR and the mesoderm enhancer, and that activation occurs through a Nodal and Zbtb4-dependent mechanism; however, this mechanism is not responsible for phase II expression. As several lines of evidence suggest phase II expression requires Ntl, we tested the response of our reporters to alterations in Ntla and Ntlb levels.

We confirmed that knockdown of Ntl does not reduce the phase I Tg(-2.8) expression as expected (Fig. 15A,B; 40/77 outcross embryos injected with *ntl* MO express EGFP, expect 38 to express; Martin and Kimelman, 2008; Narayanan et al., 2011). To test the requirement for *ntl* in phase II reporter expression, we injected Tg(-2.8) outcross embryos with *ntl* MOs and assayed EGFP transcripts at 80% epiboly. Reporter expression in late gastrula Tg(-2.8) embryos was severely diminished after *ntl* knockdown, with only a few scattered stained cells observed (Fig. 15C,D, arrows; 53/55 identified heterozygous embryos affected as shown). We observed a complete loss of EGFP expression upon *ntl* knockdown in Tg(-1.8) embryos, which do not express EGFP during phase I (Fig. 15E,F; only 1/117 injected outcross embryos stained, expect 58 embryos to stain; $p<0.001$). From this result, we infer that the few stained cells in injected Tg(-2.8) embryos may represent progenitors that express the reporter in phase I. Thus, Ntl activity is required for reporter expression beginning in late gastrula stages, i.e. phase II.

To determine whether Ntl is sufficient to activate the reporters, we used a heat-shock promoter to drive *ntla* expression ectopically in three reporter lines. Tg(-2.8) outcross embryos were injected at the one-cell stage with a plasmid encoding *ntla* driven by the hsp70 promoter (Halloran et al., 2000), aged to 4.5 hpf, heat shocked for one hour at 37^{o}C, fixed and stained for EGFP transcripts. Treated embryos showed reporter transcript distribution that closely resembled *wnt8a* transcript distribution after ectopic *ntla* expression (Fig. 15H; 38/40 heterozygous embryos affected; compare to Fig. 7D in (Martin and Kimelman, 2008). We then tested whether Tg(-2.8) embryos would respond similarly if heat shocked at 7 hpf, and found that this protocol resulted in a consistent but less pronounced activation (Fig. 15I,J; 28/30 heterozygous embryos affected). To determine whether Ntl-dependent activation of the reporter requires the mesoderm enhancer, we repeated the experiment in Tg(-2.8Δ989/497) embryos. In this case, activation of *ntla* did not induce reporter expression at shield stage (Fig. 15K,L; 0/63 embryos stained; expect 31 heterozygous embryos), confirming that the *ntla*-dependent response requires the mesoderm enhancer and consistent with the finding that Ntl associates with chromatin spanning the mesoderm enhancer (Morley et al., 2009). Finally, we tested whether the Tg(-1.8) reporter, which lacks the DRR, could be activated by ectopic *ntla*. For this experiment, we performed late gastrula heat shocks on injected Tg(-1.8) outcross embryos and found activation of the reporter in ectopic locations, although the response was less robust than that observed in Tg(-2.8) embryos (Fig. 15M,N; 18/23 transgenic embryos showed comparable phenotype to that shown).

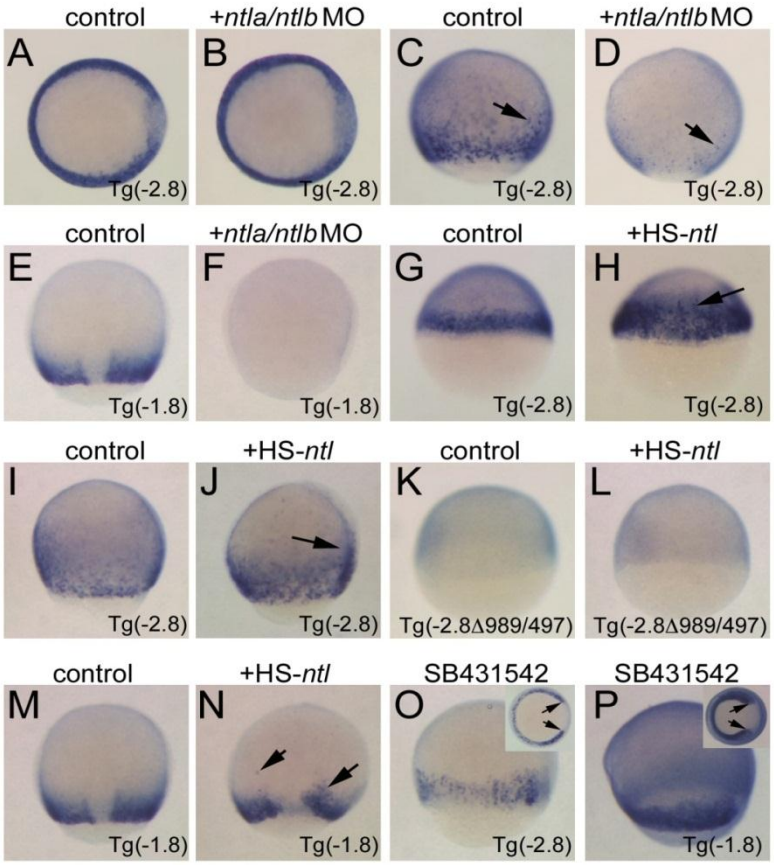

Fig.15. Phase II expression requires Ntl stimulation of the mesoderm enhancer. All images: in situ hybridizations for EGFP transcripts. Embryo genotypes indicated in the lower right corner. Treatment is indicated above each panel. (A,B) Shield stage, animal pole view, dorsal right. Note expression is unchanged in B compared to control. (C,D) 80% epiboly stage, lateral views, dorsal right. Note absence of expression in the margin in D; only scattered staining cells are observed (arrows). (E,F) 80% epiboly, dorsal view, anterior up. No staining is observed in MO injected embryos bearing the PRR-only reporter (F). (G,H) Shield stage, lateral views, dorsal right. Ectopic *ntla* is sufficient to induce the Tg(-2.8) reporter at an early stage (H, arrow). (I,J) 80% epiboly, lateral view, dorsal right. Ectopic *ntla* is sufficient to induce the Tg(-2.8) reporter in the late gastrula (J, arrow). (K,L) Shield stage, lateral view, dorsal right. Ectopic *ntla* cannot activate the reporter lacking the mesoderm enhancer (L). (M,N) 80% epiboly, dorsal view, anterior up. Ectopic *ntla* can weakly activate PRR-only reporters in the late gastrula (N, arrows). (O,P) 80% epiboly, lateral views, dorsal right. Insets: vegetal views; arrows indicate expanded dorsal clearing indicative of Nodal inhibition. Tg(-2.8) embryos show phase II reporter expression (O), and PRR-only reporters are not inhibited by Nodal antagonism (P).

These results show that Ntl regulation occurs through the mesoderm enhancer, similar to Nodal-dependent activation in phase I. This raises the question of whether the Nodal-dependent and Ntl-dependent regulatory mechanisms function independently. We previously noted that reporter expression in late gastrula Tg(-2.8) embryos appears qualitatively different from that in lines with transgenes lacking the DRR (e.g. Tg(-1.8), Tg(-1.3); see Fig. 13G,H,K). Therefore we tested whether blocking Nodal signaling in Tg(-2.8) embryos from the one-cell stage (i.e. blocking phase I expression) would lead to an alteration in reporter expression in late gastrula stages (i.e. phase II effect). We treated Tg(-2.8) and Tg(-1.8) outcross embryos with SB-431542, then assayed EGFP transcripts at 70-80% epiboly (Fig. 15O,P). Nodal inhibition in Tg(-2.8) embryos resulted in mosaic reporter expression in the margin at late gastrulation (Fig. 15O; 29/29 heterozygous embryos as shown). and eliminated the scattered stained cells observed in untreated or *ntl* MO injected Tg(-2.8) embryos (e.g. Fig. 15C,D, arrows). In contrast, reporter expression in SB431542 treated Tg(-1.8) embryos is observed as a uniform band of staining in the margin (Fig. 15P; 32/32 heterozygous embryos as shown). Thus, Nodal-dependent phase I expression and Ntl-dependent phase II expression are independently established through the mesoderm enhancer, but the presence of the DRR prevents uniform reporter expression in ventrolateral mesoderm progenitors in the margin of the late gastrula.

wnt8a mesoderm enhancer properties are conserved in teleosts

Nodal-dependent mesoderm induction, Brachyury-dependent mesoderm maintenance and *wnt8a* expression in mesoderm progenitors are conserved features of vertebrate development. However, *wnt8a* is bicistronic in teleosts but not tetrapods (Ramel et al., 2004; Lekven, unpublished), raising the question of whether the mechanism of *wnt8a* regulation through a single mesoderm enhancer is a conserved feature of teleosts. To address this, we analyzed putative regulatory regions from the three-spined stickleback *wnt8a* locus in zebrafish transient expression assays (Fig. 16).

We used a combination of BLAT and visual analysis to identify the stickleback *wnt8a* exons and the location of the conserved portion of the mesoderm enhancer (Fig. 16A; note that the absence of cDNA information precludes determining whether stickleback *wnt8a.1* also has two upstream promoters). The conserved mesoderm enhancer element in stickleback is located upstream of the *wnt8a.1* translation initiation codon, a similar relative position as in zebrafish (Fig. 16A, red box). ClustalW analysis shows considerable identity between the *Tetraodon*, stickleback and zebrafish elements, including two conserved Ntl consensus binding sequences (Fig. 16B, red boxes). We scanned the stickleback upstream region for additional Ntl and FoxH1 consensus binding sequences, and we identified two additional Ntl consensus sites adjacent to the conserved element but no FoxH1 sites within 4 kb of the start codon (not shown). However, we identified a region downstream of the *wnt8a* locus with a concentration of five potential Ntl and two FoxH1 sites and one high quality Zbtb4 site. To test their

transcriptional regulatory activity, we amplified the 2 kb region upstream of the *wnt8a.1* translation initiation site, and a 2 kb downstream fragment spanning the potential Ntl, FoxH1 and Zbtb4 binding sites (Fig. 16A, " 5' " and " 3' ", respectively). As a control we amplified a 2 kb fragment further upstream of *wnt8a* (Fig 16A, "control"). Stickleback reporter plasmids were injected into one-cell stage zebrafish embryos, and EGFP fluorescence was assayed at 24 hpf (Fig. 16C-F). Strikingly, we observed robust somitic expression from both stickleback constructs that closely resembled transient expression from zebrafish reporters. We also noted consistent and reproducible differences in the anterior limits of EGFP fluorescence mirroring differences produced by zebrafish reporters. For example, expression from the 5' fragment, which contains Ntl but not FoxH1 or Zbtb4 consensus binding sequences, was observed in somites extending to a position approximately even with the posterior hindbrain (Fig. 16C, arrow; 28/30 injected embryos). This pattern matches that observed from zebrafish reporters lacking the DRR (Fig. 16F; Supplemental Fig. 2; 18/20 injected embryos). In contrast, expression from the stickleback 3' fragment, which contains a Zbtb4 site, Ntl, and FoxH1 sites, produces reporter fluorescence along the anteroposterior length of the embryo (Fig. 16D, arrows; 22/25 injected embryos), similar to that observed from the -2.8wnt8a:EGFP reporter that contains the DRR (Fig. 16E, arrows; 20/22 injected embryos). We did not observe any somitic expression from the control fragment (not

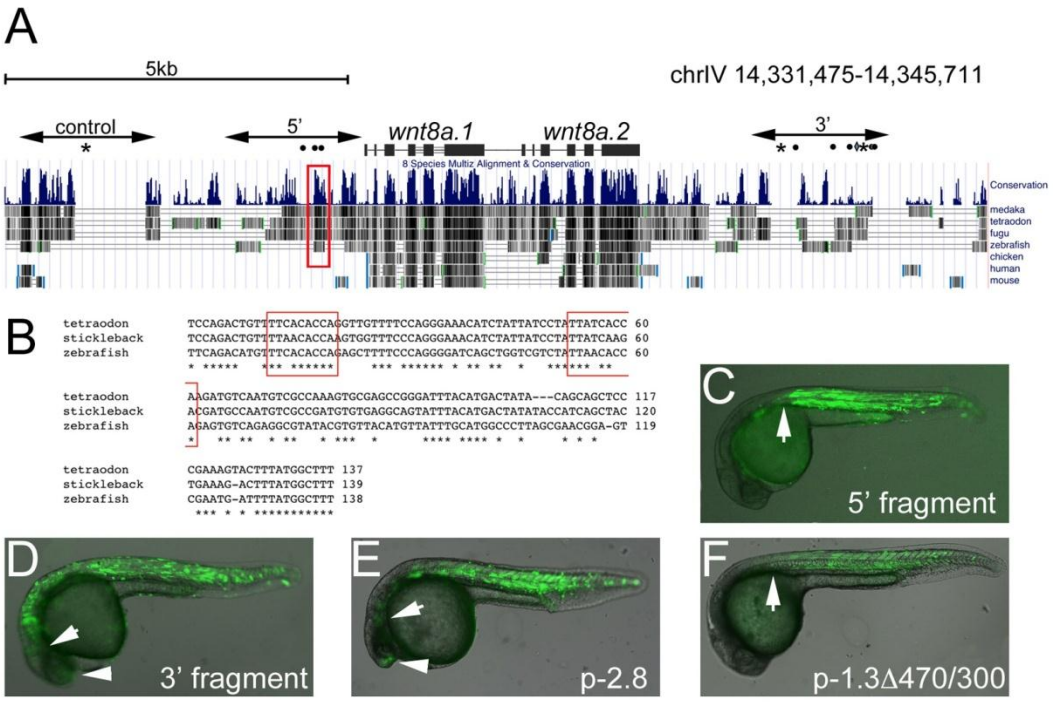

Fig.16. Identification of stickleback *wnt8a* mesoderm enhancers. (A) Schematic diagram of stickleback chromosome IV spanning the *wnt8a* locus with the conservation tracks from the UCSC genome browser shown. The positions of the *wnt8a.1* and *wnt8a.2* coding regions are indicated. Control, 5' and 3' indicate the fragments tested in zebrafish transient expression assays. Dots, asterisks and the diamond symbol above the conservation tracks indicate Ntl, FoxH1 and Zbtb4 consensus binding sequences, respectively. The red box outlines the conserved mesoderm enhancer, shown in B. (B) Alignment of the conserved mesoderm enhancer. Consensus Ntl binding sequences are boxed in red. (C-F) Transient expression assays in zebrafish, 24 hpf embryos, lateral view, anterior left. (C) Expression from the stickleback 5' fragment extends anteriorly as far as the posterior hindbrain (arrow). Compare to the anterior limit of expression from the zebrafish -1.3Δ470/300 construct (PRR-only construct) shown in (F). (D) Expression from the stickleback 3' fragment extends anteriorly as far as the position of the midbrain (arrow) and some expression is observed in the telencephalon (arrowhead). Compare to transient expression from the p-2.8*wnt8a.1:EGFP* construct (E).

shown; n=27). These results show that the stickleback *wnt8a* locus comprises at least two mesoderm enhancers with different compositions, and suggests that their regulation may mirror the regulation of zebrafish constructs with and without the DRR.

DISCUSSION

In this study, we analyzed the cis-regulatory regions of zebrafish *wnt8a.1* to better understand transcriptional mechanisms of vertebrate mesoderm development. We identified three transcriptional start sites within the locus, two of which are responsible for producing the characteristic *wnt8a* bicistronic mRNA. The two transcriptional start sites are contained within separable cis-regulatory modules we have called the Proximal and Distal Regulatory Regions. Interactions between the PRR and DRR are required to recapitulate the biphasic expression pattern of *wnt8a*.

Phase I *wnt8a* expression: integration of Nodal and Zbtb4

The first regulatory phase of zebrafish *wnt8a* expression comprises the period from 30% epiboly (~4.6 hpf), when transcripts are first detectable by in situ hybridization in the ventrolateral margin and YSL, through 70% epiboly (~7 hpf), when transcripts are observed in the ventrolateral margin only. This pattern was recapitulated by the Tg(-2.8) reporter, suggesting that all essential elements for phase I are contained within -2.8 kb of the *wnt8a.1* translation start site. Nodal signaling is an essential input that functions through the mesoderm enhancer to drive phase I expression (Fig. 17). While the five consensus FoxH1 binding sites suggest a direct response to Nodal, the mesoderm enhancer is not sufficient in the absence of the DRR for phase I expression. Thus, phase I expression requires a Nodal signal to be integrated by the mesoderm enhancer and the DRR, an interaction that may be mediated by the transcription factor Zbtb4. Yet to be determined is whether phase I expression requires TSS-A or whether Nodal and Zbtb4

could function through the mesoderm enhancer and TSS-B to activate phase I transcription.

Phase II *wnt8a* expression: Ntl/Bra regulation of the mesoderm enhancer.

The second regulatory phase of expression begins at ~70% epiboly (~7 hpf), when transcription is observed in the ventrolateral margin and continues in the tailbud after gastrulation is complete. This pattern was recapitulated in Tg(-1.8) embryos, indicating that the PRR is sufficient for phase II expression. Importantly, phase II expression depends on Ntl regulation of the mesoderm enhancer and is independent of Nodal signaling. Curiously, the Tg(-2.8) reporter, comprising both the DRR and PRR, is not expressed broadly in mesoderm progenitors in late gastrula stages (phase II), even though the PRR in isolation is sufficient for this expression pattern. This observation indicates that the DRR interferes with the response of the PRR to Ntl, and implies the existence of an additional mechanism that counteracts the influence of the DRR on PRR-driven phase II expression (Fig. 17).

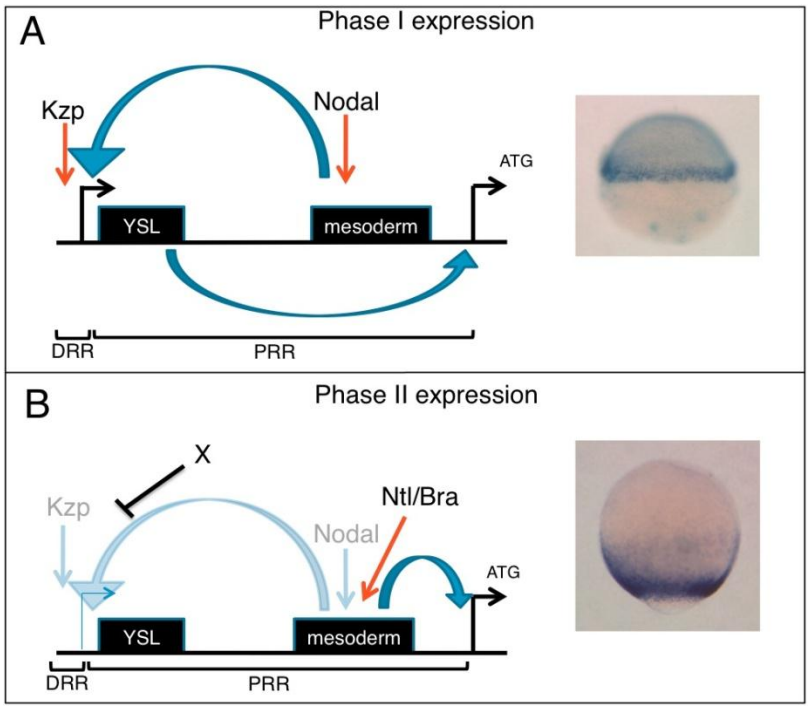

Fig.17. A model for biphasic *wnt8a.1* regulation. (A) During phase I expression, Nodal signaling induces expression through the mesoderm enhancer in concert with Zbtb4, likely inducing transcription from TSS-A. At the same time, the YSL enhancer drives expression, likely from TSS-B. (B) During phase II expression, Ntl binds to the mesoderm enhancer and drives transcription from TSS-B. Our data suggest that an additional mechanism, "X", ensures that the DRR does not interfere with Ntl dependent regulation.

***wnt8a* expression in the context of mesoderm formation pathways**.

Recent studies in the zebrafish are beginning to uncover the transcriptional basis of vertebrate mesoderm development. Cis-regulatory analysis of *nodal related 1* (*ndr1*, aka *squint*) showed its expression to be regulated by two enhancers: an intronic enhancer comprising a Nodal response element (NRE) that drives expression in marginal blastomeres, and an upstream enhancer that drives expression in the extra-embryonic enveloping layer and YSL (Fan et al., 2007). The NRE contains two putative FoxH1

binding sites that are required for the Nodal response and expression in marginal blastomeres (Fan et al., 2007; Osada et al., 2000). FoxH1 is a key effector of Nodal signaling and mediates most early Nodal signaling in zebrafish (Osada et al., 2000; Pogoda et al., 2000). The FoxH1 consensus binding site is highly conserved across taxa, and several studies have shown that FoxH1 consensus binding sites in diverse genes mediate a Nodal response (e.g., Labbe et al., 1998; Zhou et al., 1998). Interestingly, *ndr1* expression driven by the Nodal-responsive intron enhancer initiates shortly after the midblastula transition and declines in the margin by 6 hpf (Fan et al., 2007). Thus, *ndr1* expression in marginal blastomeres is governed by a transient autoregulatory mechanism that is active during Nodal-dependent phase I *wnt8a* expression.

Crucial targets of Nodal signaling that are essential to mesoderm formation are the *brachyury* paralogs *no tail a* and *no tail b* (Chen and Schier, 2001; Latinkic et al., 1997). In zebrafish, *ntla* is controlled by two separate enhancers that respond to different signaling inputs. Enhancer E2 drives Nodal-dependent expression while enhancer E1, located upstream of E2, drives expression in response to Wnt and BMP signaling (Harvey et al., 2010), suggesting that zebrafish *ntla* is a downstream target of Wnt signaling as has been shown for *Xenopus Xbra* (Vonica and Gumbiner, 2002). The presence of two *ntla* enhancers explains Nodal-independent *ntla* expression and the generation of tail mesoderm observed in Nodal mutants (Harvey et al., 2010). Wnt8a is the most likely Wnt factor responsible for early *ntla* regulation (Harvey et al., 2010).

Therefore, in the pre- and early gastrula, Nodal, BMP and Wnt8a signaling converge on dual enhancers to generate the expression of *ntl* and *bra*.

In contrast, in the late gastrula *wnt8a* likely functions downstream of Ntl to promote posterior mesoderm formation (Martin and Kimelman, 2008). Consistent with their finding that *wnt8a* is a direct target of Ntl, Morley et al identified *wnt8a* in a ChIP-based screen for Ntl target genes (Morley et al., 2009). Interestingly, the *wnt8a* regions enriched by Ntl ChIP overlap with our identified mesoderm enhancer (data not shown). Martin and Kimelman (2008) also identified a requirement for Wnt signaling in late *ntl* regulation, thus *wnt8a* and *ntl* form a feedback network in the late gastrula responsible for maintaining this gene network that promotes posterior mesoderm development. An interesting question is why Ntl is insufficient to activate the PRR *wnt8a* reporters in the early gastrula (i.e. phase I expression) when *ntla* transcripts and protein are known to be present (Schulte-Merker et al., 1992). Several alternative possibilities can be imagined, including hindrance of Ntl binding by FoxH1 or other factors, or a requirement for post-translational activation of Ntl that only occurs after 70% epiboly.

The YSL: a function for *wnt8a* expression?

The YSL is an extra-embryonic tissue that is important for both mesoderm induction and gastrulation (Carvalho and Heisenberg, 2010), and several studies have highlighted the role of YSL factors in mediating the patterning activities of this tissue (Fan et al., 2007; Hong et al., 2011; Ober and Schulte-Merker, 1999). The YSL enhancer we identified

functions independently of the mesoderm enhancer, raising the question of what role Wnt8a plays in the YSL. The fact that there is evolutionary conservation of sequences within the YSL enhancer suggests that this may be a conserved feature of *wnt8a*, however we have not tested the regulative ability of putative YSL enhancers from other species. One possible role for YSL-*wnt8a* consistent with published reports is participation in stimulating *ntla* expression through enhancer E1 (Harvey et al., 2010). Recently, Mxtx2 was found to be required in the YSL and non-autonomously synergizes with Nodal signaling to stimulate *ntla* expression in ventrolateral mesoderm progenitors (Hong et al., 2011). An intriguing possibility would be that Mxtx2 stimulates *wnt8a* expression in the YSL, which then acts in concert with Bmp ligands to activate *ntla* expression via enhancer E1.

Evolutionary conservation of the teleost *wnt8a* locus

The *wnt8a* tandem duplication is conserved in diverse teleosts (Ramel et al., 2004; this study), and our findings are consistent with teleost *wnt8a* expression being dictated by a conserved mechanism requiring Ntl, Nodal and Zbtb4. Curiously, the anteroposterior limits of stickleback mesoderm enhancer activity correlate with the presence or absence of FoxH1 and Zbtb4 binding sites, similar to the PRR and DRR interaction in zebrafish, suggesting that *wnt8a* transcriptional regulation in zebrafish may serve as a general model for teleost *wnt8a*. However, the fact that the stickleback 5' enhancer functions similarly to the zebrafish PRR and the 3' enhancer functions similarly to the zebrafish DRR/PRR suggests that the relative positions of enhancer elements in the two separate

lineages may have been rearranged. Alternatively, it is possible that the two mesoderm enhancers identified in stickleback represent “shadow enhancers” (Frankel et al., 2010; Hong et al., 2008) to confer expression pattern robustness. Further analysis of the stickleback and zebrafish mesoderm enhancers may allow the resolution of this question, and the reporter lines generated in this study will be useful reagents for continued dissection of *wnt8a* transcriptional regulation.

CHAPTER IV

SUMMARY OF EXPERIMENTS AND DISCUSSION

SUMMARY OF FINDINGS

In the vertebrate model organism, zebrafish, *wnt8a* occupies a central position in axis patterning and coordinates with other genes in posterior mesoderm development. Transcription of *wnt8a* in mesoderm progenitors is critical to its patterning function and interactions with other pathways. Thus, understanding how *wnt8a* transcription is regulated is important for unraveling how its interactions with other pathways control axis patterning. The purpose of this study was to understand the transcriptional regulation of *wnt8a*. In order to facilitate this study, we have undertaken an in-depth transgenic analysis to dissect the cis-regulatory regions surrounding the *wnt8a* locus.

Chapter II describes a new transgenic zebrafish line in which EGFP expression faithfully recapitulates *wnt8a* expression. This transgenic line was created through homologous recombination in a *wnt8a* PAC clone comprising ~80 kb spanning the *wnt8a* locus. In situ hybridization results show that the transcription pattern of the reporter matches that of *wnt8a*, including maternal expression, transcription in the embryonic margin & yolk syncytial layer and exclusion from the dorsal margin. EGFP fluorescence from this reporter serves as an excellent lineage tracer for all mesodermal derivatives of the ventrolateral embryonic margin, including heart, somites and pronephros. This property of the reporter was used to show that Nodal signaling is an early input (up to 6hpf) into *wnt8a* activation, and *wnt8a* expression labels the tail mesoderm that forms in the

absence of Nodal signaling. Further experiments show that No tail/Brachyury transcription factors regulate later *wnt8a* expression (after 6hpf). This reflects predominantly regulation in paraxial mesoderm, not intermediate mesoderm, indicating that Ntl/Bra regulation of *wnt8a* is localized to specific mesodermal domains. Thus, *wnt8a* expression is under biphasic control: by Nodal at the early stage and by Ntl/Bra in the late stages.

Chapter III discusses further dissection of the cis-regulatory elements upstream of the zebrafish *wnt8a* locus. The aim was to narrow down a minimal cis-regulatory region sufficient to recapitulate endogenous *wnt8a* expression. This led to the discovery of two alternate promoters upstream of the first Wnt8a coding region (‘*wnt8a.1’;* teleost *wnt8a* loci are bicistronic) and I focused on identifying regulatory elements in this genomic interval. A series of reporter constructs was generated that recapitulate different aspects of the *wnt8a* expression pattern in transient and stable transgenic assays. Through this, two separable enhancers that control expression were discovered: one active in the extra-embryonic yolk syncytial layer (YSL), and the other in the embryonic margin. These independent enhancers lie in the first intron, situated between the two alternate promoters. These constructs identified two functional cis-regulatory units, each with one of the promoters, which are referred to as the distal regulatory region (DRR) and the proximal regulatory region (PRR). Curiously, the enhancer that is active in the margin (which is referred to as the mesoderm enhancer) is necessary for both phase I (referred in Chapter II as early expression) and phase II expression (referred in Chapter II as late

expression), but through different cis-interactions. Phase I expression requires the mesoderm enhancer, which has several consensus binding sites for FoxH1, Smads and No Tail, to be in the context of a larger 2.8 kb regulatory region spanning both the DRR and PRR. Besides the promoter in DRR, there is also a consensus binding site for the maternal zinc-finger protein Zbtb4. Knockdown of Zbtb4 has revealed that it is essential for phase I expression. Phase II expression requires the mesoderm enhancer to be in the context of only the PRR; the presence of the DRR interferes with expression from the PRR during phase II. Nodal and No tail/Brachyury both act through the mesoderm enhancer. Thus, higher order transcriptional mechanisms seem to be at play to generate the seemingly simple *wnt8a* expression pattern. The mechanisms of *wnt8a* regulation and mesoderm development seem to be conserved. Reporter constructs that are generated from the three-spine stickleback *wnt8a* locus spanning combinations of FoxH1, No Tail and Zbtb4 binding sites show that two separate regions function as enhancers that recapitulate the expression patterns generated by the zebrafish transgenes. This finding suggests that the mechanism existing in zebrafish will likely apply broadly to teleosts and perhaps tetrapods.

POSSIBLE MODELS FOR NTL/BRA BEING REGULATORS OF *wnt8a* EXPRESSION ONLY IN PHASE II AND NOT IN PHASE I

The cis-regulatory analysis of the *wnt8a* locus has revealed a temporal control of expression by different sets of genes. Phase I expression seems to be regulated by Zbtb4 and Nodal signaling through the mesoderm enhancer. Phase II expression of *wnt8a* is

predominantly regulated by Ntl and Bra, again through the mesoderm enhancer (Martin and Kimelman, 2008, Chapter II, Chapter III). Our studies indicate that the modulators of early expression may have to be shut down for proper late expression (Chapter III). *ntl* transcripts are present in the entire mesoderm at an early epiboly stage (Ramel et al., 2005; Harvey et al., 2010); yet the regulatory effect of Ntl on *wnt8a* occurs only after shield stage. *bra* transcripts are seen only in the dorsal margin at early epiboly and extend the entire margin only at shield stage (Martin and Kimelman, 2008), suggesting that bra has no early role in *wnt8a* regulation. Ntl and Bra exert a combinatorial regulatory effect on *wnt8a* expression (Martin and Kimelman, 2008). The putative sites for Ntl/Bra and mediators of Nodal signaling (FoxH1) are concentrated in a common mesoderm enhancer region upstream of promoter B. The build up of these T Box proteins during early epiboly and gastrulation may lead to a level, sufficient enough to direct the transcription machinery to promoter B and initiate phase II expression. Upon binding to the enhancer elements within the conserved enhancer region, Ntl/Bra may be altering the binding affinity of FoxH1 or replacing them. This may be true because, it appears that Nodal signaling may be playing a significant role in early expression of *wnt8a*, but not late expression. This may suggest that Nodal signaling may have to be switched off after the early phase for proper late expression. Thus, Ntl/Bra may be doing a dual role: shutting down early expression and regulating late expression.

Another alternative model is that the Ntl/Bra may be working with another protein that gets enriched in the *wnt8a* promoter region only after shield stage. This protein may be

essential for Ntl/Bra to regulate transcription of *wnt8a*. The reason why we do not observe any *wnt8a* expression after shield stage in the Ntl/Bra knockdown may be due to the fact that this gene may also be regulated by Ntl/Bra primarily after shield stage.

DOES NODAL SIGNALING PLAY A ROLE IN EXCLUSION OF *wnt8a* EXPRESSION FROM THE DORSAL MARGIN?

wnt8a expression is seen in the ventrolateral margin and is excluded from the dorsal margin (Kelly et al., 1995, Chapter II). The Tg *(wnt8a:PAC)* and Tg (-2.8) lines recap this expression. This study has already shown the necessity of the mesoderm enhancer upstream of the *wnt8a* locus which contains putative FoxH1 sites to drive *EGFP* expression (Chapter III). The presence of putative FoxH1 binding sites in the conserved mesoderm enhancer region raises an interesting possibility. FoxH1 is a mediator of the Nodal signaling pathway. It has been shown to positively regulate expression of *sqt*, *lefty* and *pitx2* (Schier, 2003; Fan et al., 2007). FoxH1 has also been shown to negatively regulate expression of some target genes, for example- *mixl1* expression in mouse. Goosecoid (Gsc), a transcriptional repressor, can bind to FoxH1 protein and recruit HDACs to repress gene expression. Gsc does not have to directly bind to DNA in order to repress genes (Izzi et al., 2007). Gsc is a direct repressor of *Xenopus wnt8a (Xwnt8a)* and Xenopus *brachyury* (Xbra) (Latinkic et al., 1997; Danilov et al., 1998; Yao and Kessler, 2001). Hence, Goosecoid may repress *wnt8a* expression directly or indirectly through binding with FoxH1.

DIFFERENTIAL REGULATION OF MESODERMAL PROGENITOR SPECIFICATION MAY BE ACHIEVED THROUGH *wnt8a* REGULATION

Fate map studies have revealed that mesodermal precursor cells are arranged in a specific pattern along the D-V axis at shield stage (Schier and Talbot, 2005). These cells give rise to different organs during the course of development. Transgenic lines described in Chapter II and Chapter III reveals evidence for differential regulation of *wnt8a* in the mesodermal cells. Perdurance of EGFP in mesodermal derivatives may reflect earlier expression in undifferentiated mesoderm progenitors. For example, In situ hybridization for *wnt8a* transcripts or for *egfp* transcripts in the Tg (*wnt8a*:*PAC*) line reveals the staining of cells in the pronephros domain at bud stage. In a bud stage embryo, in a posterior view with dorsal facing up, the cells that label the pronephros are seen in a U-shaped domain outside the paraxial mesoderm. The T box genes, *ntl* and *bra* have been shown to regulate *wnt8a* and *egfp* expression at stages later than shield stage. However, not all mesodermal domains respond equally to the loss of Ntl/Bra. The staining in a bud stage embryo reveals persistence of transcripts in the pronephros domain in the Ntl/Bra morphants (Chapter II, 9F arrow). Strong reporter fluorescence is also noticed in a 24hpf embryo in the pronephros domain consistent with the transcripts observed in bud stage (Chapter II, 9J arrowheads). In these embryos, in-situ staining is severely reduced or gone in cells that label other mesodermal progenitors, such as paraxial mesoderm, which gives rise to somites (Chapter II, 9F asterisk). This is consistent with the reduction or absence of reporter fluorescence at 24hpf in somites (Chapter II, 9J arrow). These results suggest that additional inputs may be responsible

for specifying pronephros progenitors besides Ntl/Bra, while paraxial mesoderm may be regulated predominantly or almost entirely by Ntl/Bra. This result is further substantiated by the bud stage expression and EGFP reporter expression at 24hpf (unpublished observations) of another transgenic line which does not have the mesoderm enhancer characterized in chapter III. This transgenic line has a deletion which removes the ~550 bp mesoderm enhancer region. This deletion eliminates 2 of the 3 Ntl binding sites. In situ hybridization shows no *egfp* transcripts in shield stage. However at bud stage, transcripts are seen in the U shaped domain that labels pronephros domain (unpublished data). EGFP fluorescence at 24 hpf shows a drastic reduction of expression in somitic cells (Chapter III, Fig 12), but shows reporter fluorescence in pronephros (unpublished data). This result is strikingly similar to the Ntl/Bra morphants in Tg *wnt8a:*PAC line. Thus, the enhancer region responsible for regulating pronephros expression seems to lie outside the mesoderm enhancer. Thus, two domains of the mesoderm-paraxial and pronephros are differentially regulated mainly through the mesoderm enhancer.

Reporter fluorescence in other transgenic lines has further revealed a possible explanation for differential regulation of mesodermal progenitors. The following observations are primarily based on the EGFP fluorescence at 24hpf that labels different mesodermal lineages. As mentioned earlier, none of these transgenic lines transcribe EGFP in these tissues at 24hpf. Hence the fluorescence in mesodermally derived tissues reflects earlier transcription. The Tg (-2.8) line shows EGFP fluorescence in multiple

mesodermal lineages including heart, pronephros and somites. The Tg (-1.8) line, which has the mesoderm enhancer but not the region upstream that has a binding site for Zbtb4 and promoter A, shows expression in heart and somites but not in pronephros. These results strongly suggest that Ntl/Bra regulates expression in heart and somites through the mesoderm enhancer, but not in the pronephros. The region that is deleted in Tg (-1.8) seems to be necessary for pronephros domain expression. Tg (-2.8 Δ 989/497), which lacks the mesoderm enhancer, shows no *egfp* transcripts at shield stage. However, transcripts appear in the U shaped pronephros domain at bud stage. At 24hpf, EGFP fluorescence is seen in pronephros and a few somitic cells but not in the heart. The pronephros domain expression at bud stage embryos and EGFP fluorescence in pronephros at 24hpf in Tg (-2.8 Δ 989/497) reveal the presence of regulatory elements that lie outside the mesoderm enhancer. Thus, the transcripts seen in the pronephros domain at bud stage could be a result of the regulatory effects of the DRR. This could mean that separable enhancer regions within 2.8kb may drive expression in different mesodermally derived organs such as pronephros, somites and heart.

MESODERM ENHANCER MAY BE FUNCTIONING AS A TEMPORAL SWITCH TO FACILITATE PROMOTER SWITCHING IN *wnt8a* EXPRESSION

RACE reactions have revealed the presence of transcripts from promoter A as well as B from the *wnt8a* locus (Chapter III, Fig 11). We have also identified a biphasic mechanism of *wnt8a* regulation. Our results indicate a temporal control of *wnt8a* transcription. Phase I expression of *wnt8a* is controlled by Nodal and a non-Nodal factor.

Phase II expression after shield stage is controlled primarily by Ntl/Bra, although these genes exert some degree of differential regulation on mesodermal progenitors (Chapter II, Figure 10). We have also identified that the mesoderm enhancer is necessary for Phase I and II expression (Chapter III). As mentioned earlier, there are transcripts originating from the activity of two promoters. This raises that the possibility that mesoderm enhancer could be acting as a temporal switch between promoters facilitating the biphasic mechanism. A similar mode of temporal switching has been seen in Drosophila during primary sex determination (Gonzalez et al., 2008). Based on the results, it could be hypothesized that promoter A along with the Zbtb4 protein and mesoderm enhancer may be regulating early expression to enable Phase II expression. The activity of Promoter A may have to be shut down after early expression. Ntl/Bra regulates late expression through the mesoderm enhancer by directing the transcription machinery to Promoter B (Chapter III, Fig 17). Further study has to be done to determine whether the promoter activity switches between A and B to facilitate early and late phase expression of *wnt8a*. The functional consequences of promoter switching in the context of biphasic regulation of *wnt8a* may reveal important aspects regarding the gene regulatory networks underlying mesoderm induction and patterning. Genes with alternate promoters are common in mammalian genomes. The alternate promoters are selectively used to regulate differential transcription. Use of alternate promoters enables differential transcription regulation from a single locus and may contribute to the control of gene expression in various cell lineages, tissue types and developmental stages (Baek et al., 2007). mRNA isoforms originating from the alternate promoters can give rise to

the distribution and protein coding potential which may be expressed during different stages of development and in different types of tissues. Well characterized examples of genes with alternate promoter usage are *TP53* tumor protein p53 and *LEF1*, lymphoid enhancer binding factor 1. Abberant use of alternate promoters in these genes has been noticed in disease formation (Reviewed in Davuluri et al., 2008).

POSSIBLE FUNCTIONAL ROLES FOR *wnt8a* IN YSL

It has been shown that *wnt8a* is expressed in the YSL (Ho et al, 1999). This extra-embryonic tissue plays essential roles in mesoderm induction (Chen and Kimelman, 2000). The role of *wnt8a* in this tissue is unexplored. We have identified a ~550 bp region that is necessary for *wnt8a* expression in the YSL (Chapter III, Fig 12). This enhancer functions independently of the mesoderm enhancer (Chapter III). Recently, Mxtx2 was found to be required in the YSL and functions with Nodal signaling to stimulate *ntl* expression in ventrolateral mesoderm progenitors (Hong et al., 2011). *wnt8a* has been shown to regulate *ntl* expression (Harvey et al., 2010, Martin and Kimelman, 2008). This raises the possibility of interactions between *wnt8a* and Mxtx2 in the YSL that may be crucial for regulating ventrolateral mesoderm. Further studies may reveal the genetic inputs that regulate this enhancer and drive *wnt8a* expression in the YSL.

CONCLUSION AND FUTURE DIRECTIONS

This dissertation focuses on the transcription regulation of *wnt8a* and the general mechanisms involved in vertebrate mesoderm development. The identification of cis-regulatory regions important for *wnt8a* has revealed the complex interactions involved in regulating the gene. Future goals of this research will be to identify whether the enhancer elements in the conserved mesoderm enhancer act as direct targets in regulating *wnt8a* expression. The conserved nature of the enhancers indicates that these mechanisms may be conserved in other teleosts and possibly tetrapods.

Immediate study should focus on identifying direct roles of Nodal and Ntl/Bra in regulating *wnt8a* expression in the context of mesoderm enhancer. It has been shown that Ntl and Bra are direct regulators of *wnt8a* (Martin and Kimelman, 2008). The mesoderm enhancer has putative T box binding sites suggesting that Ntl/Bra may be regulating *wnt8a* through this region. This region has shown to respond positively to *ntl* overexpression and Ntl/Bra loss of expression. It would be very important to identify if these sites function as direct regulators for *wnt8a*. Within the mesoderm enhancer, there are also five putative FoxH1 binding sites. These sites may indicate that Nodal could be directly regulating *wnt8a* expression. Further studies have to be done to confirm the importance of these sites. Site directed mutagenesis, ChIP and other in-vitro assays can be performed to identify their roles as direct regulators of *wnt8a*.

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