THE ROLE OF NODAL SIGNALING IN PATTERNING THE ZEBRAFISH EMBRYO

by

ENGDA HAGOS
(Under the direction of Scott Dougan, PhD.)

ABSTRACT

Gastrulation is a critical event that leads the formation of the three embryonic germ layers ectoderm, mesoderm and endoderm. During this time, an impressive series of events needs to be coordinated, leading to the spatial positioning of the three germ layers along the body axis and their patterning. *nodal-related* genes comprise a highly conserved subclass of the transforming growth factor-beta (TGF-β) superfamily of secreted proteins that regulate key events in vertebrate embryogenesis. Members of this gene family are essential to instruct cells to adopt mesodermal, endodermal and ventral neural-ectodermal cell fates in all vertebrates. In addition, they are required to pattern all three germ layers and to establish the left-right body axis.

There are three known *nodal-related* genes in zebrafish, *squint* (*sqt*), *cyclops* (*cyc*) and *southpaw* (*spaw*). The two *nodal-related* genes, *squint* and *cyclops* are necessary and sufficient to instruct cells to adopt mesodermal and endodermal cell types. The third *nodal-related* genes*,* *spaw* is expressed late in development and is involved in establishing the left-right asymmetry. In zebrafish signals from the extraembryonic yolk syncytial layer (YSL) are required to pattern the embryo. Both *sqt* and *cyc* are expressed in the extraembryonic YSL and in future mesoderm and endoderm (mesendoderm).

The present has three aims: (I) (Nodal-related signals are required to specify different cell types at different times) (II) (*squint* and *cyclops* from the YSL are required to pattern the zebrafish embryo) and (III) (the role of maternal TGF-β signals including *squint*, Activin and Vg1 in zebrafish development).

The results presented here establish how Nodal-related proteins act during normal development to instruct cell fates. My data demonstrates that cells respond to Nodal-related proteins by a novel time-dependent mechanism that is not yet fully characterized. I also showed that Nodal signals from the extraembryonic tissues are required to pattern embryonic tissues. Lastly, maternal TGF-β signals are required after the mid-blastula transition for mesoderm and endoderm formation and patterning.

INDEX WORDS: Gastrulation, transformation growth factor beta (TGF-β), *nodal-related* genes, Squint, Cyclops, ectoderm, mesoderm and endoderm, extraembryonic yolk syncytial layer (YSL), Activin-like signals.

THE ROLE OF NODAL SIGNALING IN PATTERNING THE ZEBRAFISH EMBRYO

by

ENGDA HAGOS

B.S., University of Illinois at Urbana-Champaign, 1998

M.S., Northeastern Illinois University, 2001

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2007

THE ROLE OF NODAL SIGNALING IN PATTERNING THE ZEBRAFISH EMBRYO

by

ENGDA HAGOS

Major Professor:	Scott T. Dougan
Committee:	Edward T. Kipreos James D. Lauderdale Nancy Manley

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2007

DEDICATION

This dissertation is dedicated to my loving family: My amazing parents, my brothers, my sisters, my lovely wife, my daughter for selfless love and for inspiring me to become a scientist and supporting me in that great endeavor.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to a great number of people that played a part in the completion of this work.

I am grateful to my advisor Scott Dougan for all the support he has gave me throughout my tenure in his lab. Scott has given me great scientific guidance and advice throughout my graduate career as a graduate student. He has helped me grow as a thinker and especially for teaching me to ask the right scientific question.

I would like to thank the member of my committee (Dr. James Lauderdale, Dr. Nancy Manley and Dr. Edward Kipreos) for all of their helpful suggestions and research advice. In addition to this role as a committee member, special thank must go to Dr. James Lauderdale, who helped me with any questions I have throughout this long journey.

To my parent, brother Mulugeta and sister Genet for their support and love that gave me courage to see this long journey to its completion. I specially thank my brother Mulugeta for always encourage me not to gave up.

To my dear wife, Aster Dinku for supporting me in every way imaginable, Aster, your tireless love and support made this accomplishment possible. You have been a source of strength and motivation and has assisted me in more ways than can be expressed in this brief forum. It is a joy and a blessing to share my life with you, and so I thank you for all this and much more.

To my especially daughter Abigail Abebe Hagos for make me happy when I see your face. Abi, you are the new joy in my life and I cannot imagine life without you. Life would be meaningless without you and I am blessed I have you in my life.

To member of my lab, Danielle Webster and Dr. Xiang Fan for their help throughout my PhD time stayed in the lab. Special thank for Danielle for endless support and cheer me up with her beautiful smile. She made my graduate life much easier.

To lab members of Dr. Lauderdale, Dr. Jiha Kim, Dr. Anirban Majumder, and Jorn Lakowski for their stimulation and challenging discussions during lab meeting.

To the good friends I made during my time at UGA, Sam O Dell, Neeraj Sharma, Ben Parrott, Paul Griffin, Jerrod Bryson, Adrienne Cottrell, Keriayn Smith and Ariel Lane.

To Dr. Charles Keith and Dr. Marcus Fechheimer whose encouragement of teaching and service endeavors.

To Dr. Kojo Mensa-Wilmot occasionally sharing his time and giving helpful suggestions.

To cellular Biology staff, Marian Thomas, Genia King, Kathy Vision, Beth Brewer, Brett Rudolf, and Andrea Leach for all their assistance throughout my graduate school.

Finally, I would like to thank God for making all things possible.

TABLE OF CONTENTS

LIST OF FIGURES

Page

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

During embryonic development, a single cell generates the diverse array of cell types that comprise the adult body plan. Cells differentiate according to their position in the embryo in a highly reproducible fashion. Patterning is fundamental to establishing the spatial organization of the developing embryo (Heasman, 1997), ensuring that all the parts of the body are generated and that they form in exactly the right places. Although there has been great progress in recent years in the field of embryonic patterning, the cellular and genetic basis for patterning early embryonic development is not fully understood, particularly in vertebrates (Yasuo and Lemaire, 2001). My doctoral research centers on understanding the molecular network underlying pattern formation and cell type specification during early zebrafish embryonic development. I have been particularly interested in the establishment of the primary body axes and formation of the three germ layers.

Segregation of cells into three germ layers is one of the earliest events in the establishment of cell fate in the embryo. The ectoderm generates the outer layer of the embryo and contributes to the epidermis (skin) and the nervous system (Warga and Kimmel, 1990). The endoderm becomes the innermost layer of the embryo and gives rise to the epithelial lining of the gastrointestinal and respiratory tracts (Tam et al., 2003). The mesoderm becomes the middle embryonic germ layer, lying between the ectoderm and endoderm. It generates the heart, blood, kidney, bone, muscle and gonads (Kimelman, 2006).

Mesoderm induction in *Xenopus* embryo

Induction is the process whereby one group of cells signals to a second group of cells to specify or influence the fate of the second group of cells. In order for this process to occur, an inducer has to produce the signal (such as a secreted factor) and the responder cells must have competence to receive the inducing signal. Fate mapping in frogs has shown that the top half or animal hemisphere of blastula is fated to become ectodermal tissues, while the bottom half or vegetal hemisphere forms endodermal tissues, and the mesoderm is formed at the equator (Kimelman, 2006).

Using recombinants of blastula endodermal and ectodermal explants, it has been shown that the mesoderm is generated via inductive signals originating in the vegetal hemisphere (Harland and Gerhart, 1997; Kimelman and Griffin, 2000). In these experiments, when the animal and vegetal explants are cultured separately, vegetal pole cells become endoderm. The animal pole cells become ectoderm; however, no mesoderm forms. By contrast, when animal caps are grafted onto vegetal explants, the animal caps form both ectoderm and mesoderm. This demonstrated that signals from the vegetal part of the embryo are required to induce mesoderm in the animal caps (Harland and Gerhart, 1997).

Interestingly, when the animal caps were cultured with ventral or dorsal vegetal pole, they gave rise to different types of mesoderm (Harland and Gerhart, 1997; Kimelman and Griffin, 2000). When animal caps were cultured with ventral vegetal cells, they produced ventral mesoderm, including blood precursors. Dorsal vegetal cells have induced dorsal mesoderm derivatives such as notochord (Harland and Gerhart, 1997). These data demonstrated that dorsal vegetal cells could induce animal cells to become dorsal mesoderm cell types.

The formation of embryonic axes

In *Xenopus*, the animal-vegetal axis is formed during oogenesis. The dorsal-ventral axis is formed after fertilization (Harland and Gerhart, 1997; Heasman, 1997). More than 82 years ago, experiments conducted by Spemann and Mangold served to revolutionize experimental embryology and opened up an intense area of interest for several generations of developmental biologists. They showed that the dorsal blastopore lip of a gastrula-stage amphibian embryo has the ability to induce a secondary body axis when transplanted to the ventral side of another embryo (Spemann H, 1924). Additionally, the grafted tissue induced neural tissue from ectoderm of the host embryo (Harland and Gerhart, 1997). These results demonstrated that the piece of grafted tissue contained an organizing center that was capable of instructing and directing the developmental fates of surrounding tissue. While the original work was done in amphibians, studies of body axis formation led to discovery of similar signaling centers in other vertebrates including, the node in mice, the Henson's node in chickens, and the embryonic shield in zebrafish and other teleosts (Beddington, 1994; Bielinska et al., 1999; Harland and Gerhart, 1997; Oppenheimer, 1934; Schier and Talbot, 2005).

Organizer induction

The current model of organizer formation in amphibians involves two inductive steps. The sperm entry and subsequent cortical rotation determines the side that will become dorsal. The translocation of dorsal factors, including Dishevelled (Dsh) and β-catenin is the result of the cortical rotation and requires microtubule assembly (Rowning et al., 1997). A high β-catenin concentration arises at a position displaced from the vegetal pole. This forms the so-called Nieuwkoop center (De Robertis et al., 2000). The Nieuwkoop center subsequently induces the organizer (De Robertis et al., 2000; Harland and Gerhart, 1997). In embryos treated with nocodazole (a drug that prevents microtubule polymerization) or UV irradiation that inhibits cortical rotation, dorsal axis formation is abolished (De Robertis et al., 2000).

The β-catenin pathway

The Wnts are a secreted family of glycoproteins which signal through a receptor of the Frizzled class of seven transmembrane domain receptor (Wodarz and Nusse, 1998). Signaling by Wnt proteins through the Frizzled receptor causes the cytoplasmic protein Dishevelled (Dsh) to inhibit the kinase activity of glycogen synthase kinase 3 (GSK-3). This results in the subsequent stabilization and nuclear translocation of β-catenin protein. β-catenin in the nucleus regulates target gene expression through partnerships with the T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family of transcription factors (Mikels and Nusse, 2006; Wodarz and Nusse, 1998).

There are several lines of evidence that β-catenin acts as a dorsal determinant. In *Xenopus*, dorsal mesoderm formation is prevented in Frizzled-depleted embryos by antisense oligonucleotides (Sumanas et al., 2000). In addition, overexpression of Dsh causes axis duplication (Sokol, 1996). In both *Xenopus* and zebrafish, inhibition of GSK-3 activity by dominant-negative and overexpression of β-catenin results in axis duplication (Dougan et al., 2003; Kimelman and Pierce, 1996). On the other hand, depletion of maternal β-catenin results in ventralized embryos lacking dorsal axes (Heasman et al., 2000). β-catenin activates expression of several zygotic genes including *squint*, a *nodal-related* gene. (Dougan et al., 2003; Schier and Talbot, 2005).

Mesoderm-inducing factors

As discussed above, in the animal cap explants experiments, the vegetal cells are a source of mesoderm inducing signals. There are several candidates for these factors including Activin, Vg1, and Nodal-related proteins, which all encode members of the transforming growth factor (TGF) β family (Harland and Gerhart, 1997; Kimelman, 2006; Schier and Talbot, 2005). In overexpression studies, *nodal-related* genes induce mesoderm in explants and whole embryos (Erter et al., 1998; Jones et al., 1995; Rebagliati et al., 1998a). Conversely, zebrafish *nodal* mutant embryos lack all derivatives of head and trunk mesoderm (Feldman et al., 1998). Mouse *nodal* mutants have a similar phenotype, lacks the primitive streak and failing to form mesodermal derivatives (Conlon et al., 1994). These experiments support a role of *nodal* function in inducing mesoderm.

Mesoderm-inducing factor, Activin as Morphogen

A morphogen is a secreted molecule that determines the fate of different cells at a distance in a concentration-dependent manner (Gurdon and Bourillot, 2001; McDowell et al., 2001). A morphogen gradient is one model for how mesoderm cell fates are specified in the embryo. Members of secreted proteins including the Wnt, Hedgehog, and transforming growth factor-β (TGF-β) families behave as morphogen to specify different cell types in a concentration dependent manner (Gurdon and Bourillot, 2001; McDowell et al., 2001).

In overexpression assays, the zebrafish Nodal related protein Squint acts as a morphogen to specify different mesodermal fates in a concentration-dependent manner (Chen and Schier, 2001). However, significant questions still remain about how a morphogen gradient is formed and how cells interpret a morphogen concentration gradient. In the current models, it is proposed that cells would have to recognize different threshold concentrations of the morphogen through their receptor and transduce the information to the nucleus and respond accordingly (Gurdon and Bourillot, 2001).

The mesoderm inducing factor Activin can spread over many cell diameters and activate at least two responding genes at distance in a concentration dependent manner (Gurdon and Bourillot, 2001). Higher levels induce expression of the dorsal mesodermal marker *goosecoid (gsc)*, a homeobox gene, while lower concentration levels induce *Xenopus* pan-mesodermal marker *brachyury (Xbra)*, a T-box gene (Gurdon et al., 1995). Furthermore, in *Xenopus* animal cap explant, Dyson and Gurdon demonstrated that the absolute number of receptors need to be occupied, 100 for X*bra* and 300 for *gsc,* (Dyson and Gurdon, 1998), per cell determines to activate these two different genes. Thus, *gsc* requires a threefold occupancy of Activin for its activation (Dyson and Gurdon, 1998). This suggests that the absolute number of receptors occupied by the ligands determines cell fates.

Fate map of zebrafish embryo

A fate map shows which tissue and organs are likely to develop from different regions of the embryo at later stages of development (Schier and Talbot, 2005). In zebrafish, cells that make the embryo sit on the top of the yolk, which does not undergo cleavage. The precursors of different germ layers are arranged along the animal-vegetal axis. The endoderm and mesoderm are intermingled in the most marginal zone closest to the yolk (Fig. 1.1A). Cells further from the margin only adopt mesodermal fate. Cells furthest from the margin, which are located animally, adopt an ectodermal fate (Fig. 1.1A). Precursors of different mesodermal cell types are also arranged along the dorsal-ventral axis. For example, cells in the dorsal region of the embryo where the embryonic shield is located give rise to the prechordal plate and notochord (Fig. 1.1). Cells located on the ventral side become the blood, pronephros and tail somites. The heart and trunk somites are located more laterally (Keegan et al., 2004; Kimelman, 2006; Schier and Talbot, 2005).

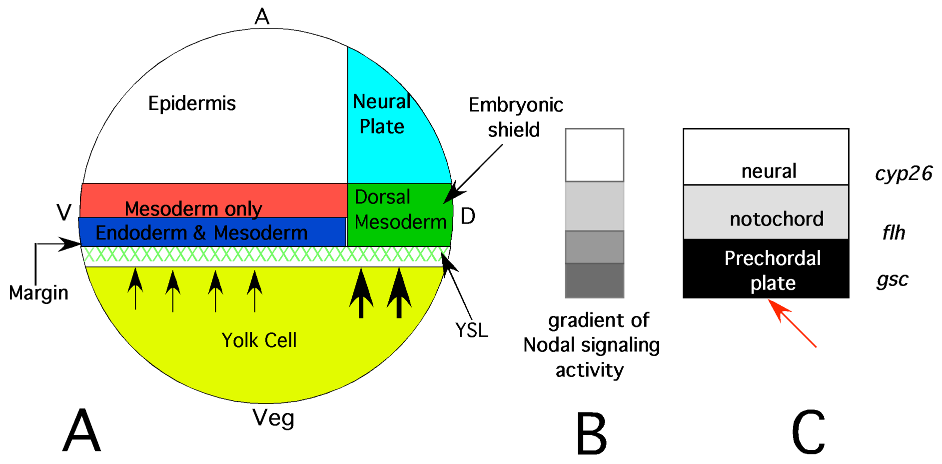


Figure 1.1. (A-C) Model for Animal-Vegetal patterning of dorsal mesoderm. (A). Schematic diagram of embryo before gastrulation. Black arrows on the margin represent direction of Nodal signals. Two arrows in the most right represent higher expression of Nodal signals. Cells closest to the margin adopt to both mesodermal and endodermal fates. Cells further from the margin only adopt mesodermal fate. Cell furthest from the margin adopt an ectodermal fate. Yellow = yolk cell, X = YSL, Red = presumptive ventrolateral mesoderm, Blue = presumptive endoderm and mesoderm, Green = embryonic shield; cells at this potion adopt dorsal mesoderm fates such as prechordal plate and notochord. Aqua = neural plate, White = epidermis, An = animal pole, Veg = vegetal pole, V = ventral side, D = dorsal side. (B,C) show the gradient of Nodal signals and types of dorsal mesodermal cell adopt at different concentration of Nodal signals. Prechordal plate (high Nodal signaling), notochord (low Nodal signaling) and neural tissue (no Nodal signaling). The red arrow on (C) indicates highest concentration of Nodal signals.

The yolk syncytial layer (YSL) in zebrafish

The dorsal yolk syncytial layer (YSL) has been suggested to be the zebrafish site corresponding to the amphibian Nieuwkoop center (Bischof and Driever, 2004; mizuno, 1996; Solnica-Krezel, 1999). The YSL is an extraembryonic structure unique to teleosts, formed at the mid-blastula stage, about 3 hours postfertilization (hpf), when cells at the marginal fuse with each other and release their contents into the underlying yolk (Fig. 1.1A) (Kimmel et al., 1995; Solnica-Krezel, 1999). Several experiments showed that the YSL signals the blastomere to induce mesoderm and endoderm (mesendoderm) and induce the embryonic shield (Chen and Kimelman, 2000; Mizuno, 1996). In these experiments, when the yolk, including the YSL, was grafted onto the animal region of a host zebrafish embryo, it induced ectopic expression of dorsal markers in the host animal pole (Mizuno, 1996). Mesendoderm fails to form when signals from the yolk are depleted by RNAse injection, indicating that these signals are essential for normal development (Chen and Kimelman, 2000). Together, these experiments suggest that signals from the YSL are necessary and sufficient to induce mesendoderm. The zebrafish *nodal- related* gene *squint* is expressed in the YSL, but the role of *squint* in the YSL is unknown (Erter et al., 1998; Feldman et al., 1998).

Nodal ligands

nodal-related genes have been identified in several vertebrate species. A single *nodal-related gene* is found in human, mouse, and chick embryos (Conlon et al., 1994; Shen, 2007). However, there are three known *nodal-related* genes in zebrafish, *squint (sqt)*, *cyclops (cyc)*, and *southpaw (spaw)* (Feldman et al., 1998; Long et al., 2003; Rebagliati et al., 1998b; Sampath et al., 1998), and six *nodal-related* genes in *Xenopus*, *xnr*1-6 (Joseph and Melton, 1997; Shen, 2007; Takahashi et al., 2000). Newly synthesized Nodal proteins precursors are secreted as homodimers with a hydrophobic leader and N-terminal prodomain (Schier, 2003). Inactive forms of the Nodal proteins, C-terminal mature ligands, can be activated by proteases. Removal of the prodomain through proteolytic processing is necessary for their function to initiate the signal transduction (Schier, 2003)).

Nodal-related signals required for germ layer formation

Members of the TGF-β superfamily of polypeptide growth factors are responsible for the proper formation of the vertebrate body plan. These crucial signals instruct cells to become mesodermal and endodermal cell types during early embryogenesis, as well as establishing important molecular patterns within this germ layer, such as germ layer patterning and left-right asymmetry (Kimelman, 2006; Schier, 2003). Nodal-related proteins are among the earliest acting zygotic signals in vertebrate development and are required for mesoderm and endoderm (mesendoderm) formation (Feldman et al., 1998). In zebrafish, *sqt;cyc* double mutants lack all derivatives of endoderm and head and trunk mesoderm including heart, blood, somites, pronephros, notochord and gut (Sampath et al., 1998). The third *nodal-related* gene *spaw* is expressed after gastrulation and is involved in regulating the left-right asymmetry (Long et al., 2003). Nodal-related signals are required in other organisms for the formation of mesoderm and endoderm. For example, mouse *nodal* mutants lack the primitive streak and fail to form mesodermal derivatives (Conlon et al., 1994). Overexpression of *Xenopus* Nodal-related proteins in ectodermal explants induces mesendoderm tissue (Jones et al., 1995; Joseph and Melton, 1997). Both mutant and overexpression studies support a role for nodal function in inducing the germ layers (Schier, 2001; Schier and Talbot, 2001; Shen and Schier, 2000).

Dose dependent requirement of Sqt and Cyc

Graded amount of *sqt* and *cyc* have been shown to specify various endodermal and mesodermal cell types; with higher levels specifying endoderm or the dorsal marginal cells and lower levels specifying mesoderm (Dougan et al., 2003). At the molecular level, high levels of Nodal-signaling are required for prechordal plate specification as noted by expression of the dorsal mesodermal marker *goosecoid* (*gsc*), whereas lower levels of *sqt* and *cyc* are essential for the notochord specification as noted by the notochord marker *floating head* (*flh*) (Gritsman et al., 2000). Furthermore, the dorsal marginal cells adopt a neural cell fate when *sqt* and *cyc* are eliminated. (Fig. 1.3B,C). These results suggest that cells adopt fates depending on their position within a gradient of Nodal-related protein.

Nodal signaling pathway

nodal-related genes form a highly conserved subclass of TGF-β superfamily use a mechanism similar to that of other members of the TGF-β superfamily to initiate signal transduction. The first step in this signaling pathway is the binding of the Nodal ligand to the transmembrane receptors (Schier, 2003). The current model of the Nodal-signaling pathway is illustrated in figure 1.2. According to this model, signaling begins when a specific ligand such as *sqt* or *cyc* binds to its cognate type II receptor (ActRIIB), a serine/threonine kinase, which then hetrodimerizes with and phosphorylates a distinct serine/threonine kinase, the type I receptor Activin-like kinase (ALK4) (Massague, 1998). This pathway also requires the coreceptor epidermal growth factor-Cripto-FRL1-Cryptic (EGF-CFC) protein, One-eye-pinhead (Oep) (Massague, 1998; Schier, 2003). The ligand, receptors and coreceptor form a complex and are internalized by the cell (Shen, 2007).

Activated type I receptor phosphorylates cytoplasmically localized Smad2 and Smad3 or both (Chen et al., 1996; Graff et al., 1996). Phosphorylated Smad2/3 forms a complex with Smad4 and is translocated into the nuclease, where it interacts with other transcription factors including FoxH1 or Mixer to regulate the expression of specific genes such as *nodal* and *lefty* (Kunwar et al., 2003; Schier, 2003). The Lefty protein in turn antagonizes Nodal signaling by preventing formation of Nodal/EGF-CFC complexes (Branford and Yost, 2004). Lefty proteins can also physically interact with Nodal ligands to block the Nodal signal transduction (Chen and Shen, 2004).

In *sqt; cyc* double mutant and maternal-zygotic one-eye-pinhead (*Mzoep*) embryos, expression of *nodal-related* genes is initiated but disappears after a short period of time (Meno et al., 1999). This shows that Nodal signals are required to maintain their own expression by positive feedback. Additionally, *Mzoep* mutants phenocopy the *sqt; cyc* double mutants and they are unresponsive to overexpression of Nodal signals (Gritsman et al., 1999).

A negative feedback, which is mediated through Lefty proteins, prevents excessive spreading of Nodal signals (Chen and Schier, 2002; Feldman et al., 2002; Meno et al., 1999). Overexpression of lefty blocks the activity of Nodal signaling and produces an embryonic phenotype that is similar to that of *sqt;cyc* or *Mzoep* mutants (Thisse et al., 2000). Furthermore, loss of lefty using morpholinos enhanced the activity of Nodal signals (Agathon et al., 2001; Chen and Schier, 2002; Feldman et al., 2002).

Mesoderm patterning along the dorsal-ventral axis

Mesoderm progenitors that are located in different regions of the dorsal-ventral axis have different fates in the late blastula (Kimelman and Griffin, 2000). The ventral cell fate is controlled by bone morphogenic protein (BMP), another TGF-β molecule that inhibits the formation of dorsal cell types. By contrast, Nodal signals at the high level induce dorsal cell fates. For example, cells in the dorsal side contributed to the dorsal mesoderm, giving rise to prechordal plate and notochord, lateral mesoderm give rise to the heart and somites, and the blood precursors come from the most ventral region of the embryo (Warga and Nusslein-Volhard, 1999).

Loss of maternal b-catenin function disrupts the expression of dorsal specific genes and eliminates the embryonic shield, which is the equivalent of Spemann's Organizer (Schier, 2003; Schier and Talbot, 2005). β-catenin induces the expression of BMP antagonist genes such as Chordin and Noggin in the dorsal region; as a result, BMP signaling is excluded from the dorsal and restricted in the ventral region of the embryo (Schulte-Merker et al., 1997). Additionally, β-catenin induces the expression of *sqt* and the transcriptional repressor Bozozok (Ryu et al., 2001), which directly represses *bmb2b* gene expression dorsally (Leung et al., 2003). Both *sqt* and Dharma are required for the formation of the organizer (Sirotkin et al., 2000). At mid-blastula transition, the ventral region of the embryo responds to ventral signals by activating zygotic transcription factors such as *vox* and *vent*, which directs ventral cell type differentiation (Melby et al., 2000). While Bozozok and Chordin promote dorsal development indirectly by antagonizing ventral factors such as BMP and *vent*, *sqt* promotes dorsal mesoderm directly by activating downstream genes such as *goosecoid* and *floating head* through the Nodal signaling pathway (Schier and Talbot, 2005) (Fig.1.3A). Two downstream target genes of BMP, *vox* and *vent* act as transcriptional repressors to repress chordin and bozozok transcription directly at the lateral and ventral regions of the embryo to promote ventral fate (Schier, 2001) (Fig.1.3A).

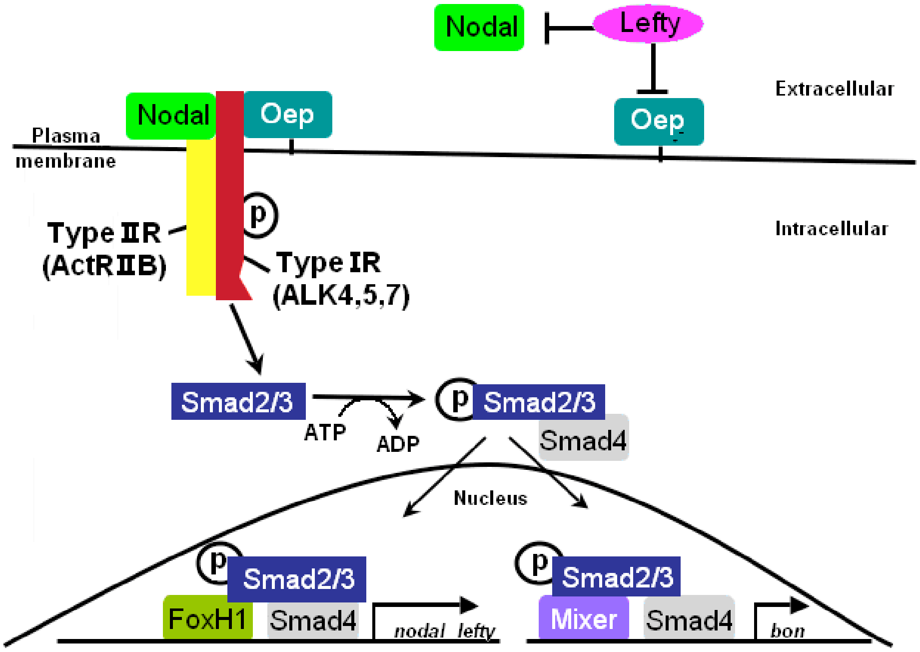


Figure 1.2. Schematic diagram of Nodal Signaling pathway. Nodal binds bipartite receptor in a manner requiring the essential coreceptor Oep transmembrane protein. When Nodal binds to type II receptor, type II phosphorylated a serine/threonine on type I receptor, thereby activated it. The activated type I receptor can now phosphorylate the transcriptional factor called Smad2/3. Smad2/3 dimerizes with Smad4 and translocated into the nucleus where it binds to regulatory DNA sequences in combination with specific transcriptional factors such as FoxH1 and Mixer, leading to transcription of nodal responsive genes including *nodal* and *lefty*. Lefty, in turn secreted and can serve as an extracellular negative regulator of Nodal signaling. (Schier and Talbot, 2005; Shen, 2007).

Molecular pathways leading to endoderm induction

In zebrafish, the endoderm originates from the most marginal blastomeres closest to the yolk (Warga and Nusslein-Volhard, 1999). In the cascade of genes controlling zebrafish endoderm development, Nodal signals induce expression of *gata5*, *bon* and *mezzo* (Alexander and Stainier, 1999)(Fig.1.3B). These three-transcription factors together controls *casanova* (*cas*) expression, a gene requires for the formation of endoderm (Alexander et al., 1999; Reiter et al., 1999) (Fig.1.3B). *cas* mutants do not form any endoderm and fail to express *sox17*, a marker of differentiated endoderm (Alexander and Stainier, 1999). Gene expression analysis and misexpression experiments in wildtype and mutant embryos have revealed that these four transcription factors are upstream of *sox17* (Alexander and Stainier, 1999) (Fig.1.3B). *mezzo*, *gata5* and *bon* expression are completely lost in *sqt;cyc* double mutants, demonstrating that their expression is regulated by Nodal signaling (Alexander and Stainier, 1999; Poulain and Lepage, 2002). This also demonstrated that these factors are downstream targets of Nodal signaling during endoderm induction in zebrafish.

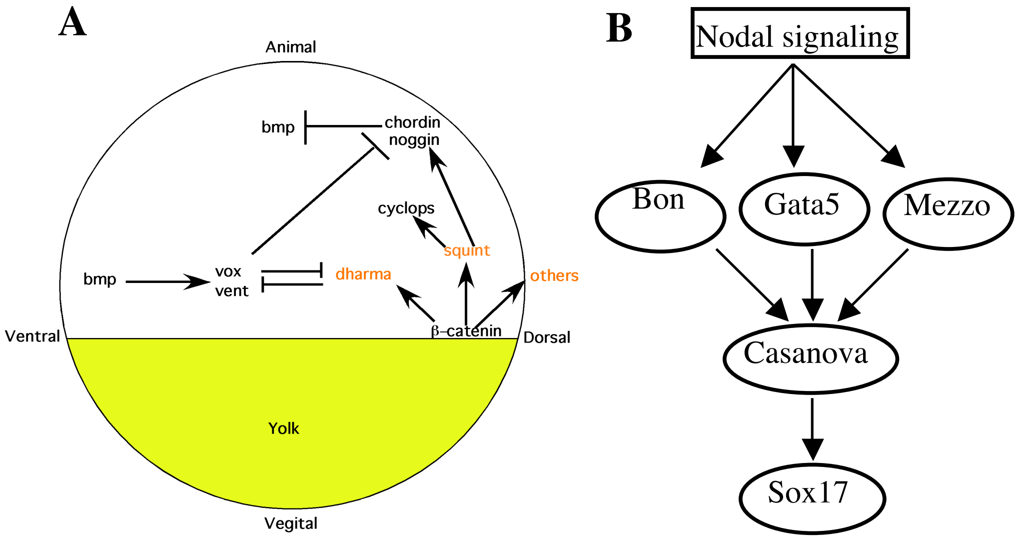


Figure 1.3. (A-B). Mesoderm and endoderm induction in zebrafish. (A). Model for dorsal-ventral patterning of mesoderm. β-catenin, *sqt* and *cyc* directly promote dorsal development. Dharma, chordin and noggin promote dorsal development indirectly by antagonizing BMP, *vox* and *vent*. BMP on the ventral side of the embryo activates *vox* and *vent* promotes ventral development indirectly by repressing dharma, chordin and noggin. (B) Molecular pathway leading to endoderm formation. Nodal signaling is required for the specification of endoderm by turning on transcription factors mezzo, Gata-5, and Bon. Casonova is dependent on these tancription factors to turn on *sox17*.

REFERENCES

Agathon, A., B. Thisse, and C. Thisse. 2001. Morpholino knock-down of antivin1 and antivin2 upregulates nodal signaling. *Genesis*. 30:178-82.

Alexander, J., M. Rothenberg, G.L. Henry, and D.Y. Stainier. 1999. casanova plays an early and essential role in endoderm formation in zebrafish. *Dev Biol*. 215:343-57.

Alexander, J., and D.Y. Stainier. 1999. A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol*. 9:1147-57.

Beddington, R.S. 1994. Induction of a second neural axis by the mouse node. *Development*. 120:613-20.

Bielinska, M., N. Narita, and D.B. Wilson. 1999. Distinct roles for visceral endoderm during embryonic mouse development. *Int J Dev Biol*. 43:183-205.

Bischof, J., and W. Driever. 2004. Regulation of hhex expression in the yolk syncytial layer, the potential Nieuwkoop center homolog in zebrafish. *Dev Biol*. 276:552-62.

Branford, W.W., and H.J. Yost. 2004. Nodal signaling: CrypticLefty mechanism of antagonism decoded. *Curr Biol*. 14:R341-3.

Chen, C., and M.M. Shen. 2004. Two modes by which Lefty proteins inhibit nodal signaling. *Curr Biol*. 14:618-24.

Chen, S., and D. Kimelman. 2000. The role of the yolk syncytial layer in germ layer patterning in zebrafish. *Development*. 127:4681-9.

Chen, X., M.J. Rubock, and M. Whitman. 1996. A transcriptional partner for MAD proteins in TGF-beta signalling. *Nature*. 383:691-6.

Chen, Y., and A.F. Schier. 2001. The zebrafish Nodal signal Squint functions as a morphogen. *Nature*. 411:607-10.

Chen, Y., and A.F. Schier. 2002. Lefty proteins are long-range inhibitors of squint-mediated nodal signaling. *Curr Biol*. 12:2124-8.

Conlon, F.L., K.M. Lyons, N. Takaesu, K.S. Barth, A. Kispert, B. Herrmann, and E.J. Robertson. 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development*. 120:1919-28.

De Robertis, E.M., J. Larrain, M. Oelgeschlager, and O. Wessely. 2000. The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat Rev Genet*. 1:171-81.

Dougan, S.T., R.M. Warga, D.A. Kane, A.F. Schier, and W.S. Talbot. 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development*. 130:1837-51.

Dyson, S., and J.B. Gurdon. 1998. The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell*. 93:557-68.

Erter, C.E., L. Solnica-Krezel, and C.V. Wright. 1998. Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev Biol*. 204:361-72.

Feldman, B., M.L. Concha, L. Saude, M.J. Parsons, R.J. Adams, S.W. Wilson, and D.L. Stemple. 2002. Lefty antagonism of Squint is essential for normal gastrulation. *Curr Biol*. 12:2129-35.

Feldman, B., M.A. Gates, E.S. Egan, S.T. Dougan, G. Rennebeck, H.I. Sirotkin, A.F. Schier, and W.S. Talbot. 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature*. 395:181-5.

Graff, J.M., A. Bansal, and D.A. Melton. 1996. Xenopus Mad proteins transduce distinct subsets of signals for the TGF beta superfamily. *Cell*. 85:479-87.

Gritsman, K., W.S. Talbot, and A.F. Schier. 2000. Nodal signaling patterns the organizer. *Development*. 127:921-32.

Gritsman, K., J. Zhang, S. Cheng, E. Heckscher, W.S. Talbot, and A.F. Schier. 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell*. 97:121-32.

Gurdon, J.B., and P.Y. Bourillot. 2001. Morphogen gradient interpretation. *Nature*. 413:797-803.

Gurdon, J.B., A. Mitchell, and D. Mahony. 1995. Direct and continuous assessment by cells of their position in a morphogen gradient. *Nature*. 376:520-1.

Harland, R., and J. Gerhart. 1997. Formation and function of Spemann's organizer. *Annu Rev Cell Dev Biol*. 13:611-67.

Heasman, J. 1997. Patterning the Xenopus blastula. *Development*. 124:4179-91.

Heasman, J., M. Kofron, and C. Wylie. 2000. Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev Biol*. 222:124-34.

Jones, C.M., M.R. Kuehn, B.L. Hogan, J.C. Smith, and C.V. Wright. 1995. Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development*. 121:3651-62.

Joseph, E.M., and D.A. Melton. 1997. Xnr4: a Xenopus nodal-related gene expressed in the Spemann organizer. *Dev Biol*. 184:367-72.

Keegan, B.R., D. Meyer, and D. Yelon. 2004. Organization of cardiac chamber progenitors in the zebrafish blastula. *Development*. 131:3081-91.

Kimelman, D. 2006. Mesoderm induction: from caps to chips. *Nat Rev Genet*. 7:360-72.

Kimelman, D., and K.J. Griffin. 2000. Vertebrate mesendoderm induction and patterning. *Curr Opin Genet Dev*. 10:350-6.

Kimelman, D., and S.B. Pierce. 1996. Regulation of dorsal-ventral axis formation in Xenopus by intercellular and intracellular signalling. *Biochem Soc Symp*. 62:13-23.

Kimmel, C.B., W.W. Ballard, S.R. Kimmel, B. Ullmann, and T.F. Schilling. 1995. Stages of embryonic development of the zebrafish. *Dev Dyn*. 203:253-310.

Kunwar, P.S., S. Zimmerman, J.T. Bennett, Y. Chen, M. Whitman, and A.F. Schier. 2003. Mixer/Bon and FoxH1/Sur have overlapping and divergent roles in Nodal signaling and mesendoderm induction. *Development*. 130:5589-99.

Leung, T., J. Bischof, I. Soll, D. Niessing, D. Zhang, J. Ma, H. Jackle, and W. Driever. 2003. bozozok directly represses bmp2b transcription and mediates the earliest dorsoventral asymmetry of bmp2b expression in zebrafish. *Development*. 130:3639-49.

Long, S., N. Ahmad, and M. Rebagliati. 2003. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development*. 130:2303-16.

Massague, J. 1998. TGF-beta signal transduction. *Annu Rev Biochem*. 67:753-91.

McDowell, N., J.B. Gurdon, and D.J. Grainger. 2001. Formation of a functional morphogen gradient by a passive process in tissue from the early Xenopus embryo. *Int J Dev Biol*. 45:199-207.

Melby, A.E., C. Beach, M. Mullins, and D. Kimelman. 2000. Patterning the early zebrafish by the opposing actions of bozozok and vox/vent. *Dev Biol*. 224:275-85.

Meno, C., K. Gritsman, S. Ohishi, Y. Ohfuji, E. Heckscher, K. Mochida, A. Shimono, H. Kondoh, W.S. Talbot, E.J. Robertson, A.F. Schier, and H. Hamada. 1999. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell*. 4:287-98.

Mikels, A.J., and R. Nusse. 2006. Wnts as ligands: processing, secretion and reception. *Oncogene*. 25:7461-8.

mizuno. 1996. mesoderm induction in zebrafish. *nature*. 383.

Mizuno, T., Yamaha, E., Wakahara, M., Kuroiwa, A., Takede., H.,. 1996. Mesoderm induction in zebrafish. *Nature*. 383:2.

Oppenheimer, J.M. 1934. Experiments on Early Developing Stages of Fundulus. *Proc Natl Acad Sci U S A*. 20:536-8.

Poulain, M., and T. Lepage. 2002. Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. *Development*. 129:4901-14.

Rebagliati, M.R., R. Toyama, C. Fricke, P. Haffter, and I.B. Dawid. 1998a. Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev Biol*. 199:261-72.

Rebagliati, M.R., R. Toyama, P. Haffter, and I.B. Dawid. 1998b. cyclops encodes a nodal-related factor involved in midline signaling. *Proc Natl Acad Sci U S A*. 95:9932-7.

Reiter, J.F., J. Alexander, A. Rodaway, D. Yelon, R. Patient, N. Holder, and D.Y. Stainier. 1999. Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev*. 13:2983-95.

Rowning, B.A., J. Wells, M. Wu, J.C. Gerhart, R.T. Moon, and C.A. Larabell. 1997. Microtubule-mediated transport of organelles and localization of beta-catenin to the future dorsal side of Xenopus eggs. *Proc Natl Acad Sci U S A*. 94:1224-9.

Ryu, S.L., R. Fujii, Y. Yamanaka, T. Shimizu, T. Yabe, T. Hirata, M. Hibi, and T. Hirano. 2001. Regulation of dharma/bozozok by the Wnt pathway. *Dev Biol*. 231:397-409.

Sampath, K., A.L. Rubinstein, A.M. Cheng, J.O. Liang, K. Fekany, L. Solnica-Krezel, V. Korzh, M.E. Halpern, and C.V. Wright. 1998. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature*. 395:185-9.

Schier, A.F. 2001. Axis formation and patterning in zebrafish. *Curr Opin Genet Dev*. 11:393-404.

Schier, A.F. 2003. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol*. 19:589-621.

Schier, A.F., and W.S. Talbot. 2001. Nodal signaling and the zebrafish organizer. *Int J Dev Biol*. 45:289-97.

Schier, A.F., and W.S. Talbot. 2005. Molecular genetics of axis formation in zebrafish. *Annu Rev Genet*. 39:561-613.

Schulte-Merker, S., K.J. Lee, A.P. McMahon, and M. Hammerschmidt. 1997. The zebrafish organizer requires chordino. *Nature*. 387:862-3.

Shen, M.M. 2007. Nodal signaling: developmental roles and regulation. *Development*. 134:1023-34.

Shen, M.M., and A.F. Schier. 2000. The EGF-CFC gene family in vertebrate development. *Trends Genet*. 16:303-9.

Sirotkin, H.I., S.T. Dougan, A.F. Schier, and W.S. Talbot. 2000. bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. *Development*. 127:2583-92.

Sokol, S.Y. 1996. Analysis of Dishevelled signalling pathways during Xenopus development. *Curr Biol*. 6:1456-67.

Solnica-Krezel, L. 1999. Pattern formation in zebrafish--fruitful liaisons between embryology and genetics. *Curr Top Dev Biol*. 41:1-35.

Spemann H, M.H. 1924 Über Induktion von Embryonanlagen durch Implantation artfremder Organisatoren. *Wilhelm Roux' Arch. f. Entw. Mech*. 100:38.

Sumanas, S., P. Strege, J. Heasman, and S.C. Ekker. 2000. The putative wnt receptor Xenopus frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. *Development*. 127:1981-90.

Takahashi, S., C. Yokota, K. Takano, K. Tanegashima, Y. Onuma, J. Goto, and M. Asashima. 2000. Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. *Development*. 127:5319-29.

Tam, P.P., M. Kanai-Azuma, and Y. Kanai. 2003. Early endoderm development in vertebrates: lineage differentiation and morphogenetic function. *Curr Opin Genet Dev*. 13:393-400.

Thisse, B., C.V. Wright, and C. Thisse. 2000. Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature*. 403:425-8.

Warga, R.M., and C.B. Kimmel. 1990. Cell movements during epiboly and gastrulation in zebrafish. *Development*. 108:569-80.

Warga, R.M., and C. Nusslein-Volhard. 1999. Origin and development of the zebrafish endoderm. *Development*. 126:827-38.

Wodarz, A., and R. Nusse. 1998. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol*. 14:59-88.

Yasuo, H., and P. Lemaire. 2001. Generation of the germ layers along the animal-vegetal axis in Xenopus laevis. *Int J Dev Biol*. 45:229-35.

Chapter 2

TIME-DEPENDENT PATTERNING OF THE MESODERM AND ENDODERM BY NODAL SIGNALS IN ZEBRAFISH

---

[1] Engda G Hagos and Scott T Dougan. *BMC Dev Biol*. 2007; 7:22
Reprinted here with permission of publisher.

ABSTRACT

Background

The vertebrate body plan is generated during gastrulation with the formation of the three germ layers. Members of the Nodal-related subclass of the TGF-b superfamily induce and pattern the mesoderm and endoderm in all vertebrates. In zebrafish, two *nodal-related* genes, called *squint* and *cyclops*, are required in a dosage-dependent manner for the formation of all derivatives of the mesoderm and endoderm. These genes are expressed dynamically during the blastula stages and may have different roles at different times. This question has been difficult to address because conditions that alter the timing of *nodal-related* gene expression also change Nodal levels. We utilized a pharmacological approach to conditionally inactivate the ALK 4, 5 and 7 receptors during the blastula stages without disturbing earlier signaling activity. This permitted us to directly examine when Nodal signals specify cell types independently of dosage effects.

Results

We show that two drugs, SB-431542 and SB-505124, completely block the response to Nodal signals when added to embryos after the mid-blastula transition. By blocking Nodal receptor activity at later stages, we demonstrate that Nodal signaling is required from the mid-to-late blastula period to specify sequentially, the somites, notochord, blood, Kupffer's vesicle, hatching gland, heart, and endoderm. Blocking Nodal signaling at late times prevents specification of cell types derived from the embryo margin, but not those from more animal regions. This suggests a linkage between cell fate and length of exposure to Nodal signals. Confirming this, cells exposed to a uniform Nodal dose adopt progressively more marginal fates with increasing lengths of exposure. Finally, cell fate specification is delayed in *squint* mutants and accelerated when Nodal levels are elevated.

Conclusions

We conclude that (1) Nodal signals are most active during the mid-to-late blastula stages, when *nodal-related* gene expression and the movement of responding cells are at their most dynamic; (2) Nodal signals specify cell fates along the animal-vegetal axis in a time-dependent manner; (3) cells respond to the total cumulative dose of Nodal signals to which they are exposed, as a function of distance from the source and duration of exposure.

BACKGROUND

During vertebrate development, cells become irreversibly committed to particular fates after a series of inductive events. The first step of this process is completed during gastrulation, when cells are allocated to the three germ layers. Fate maps of vertebrate embryos show considerable organization before gastrulation, since different mesodermal and endodermal structures are derived from distinct positions along the major body axes (Dale and Slack, 1987; Hatada and Stern, 1994; Lawson et al., 1991). In zebrafish late blastula stage embryos, for example, endoderm progenitors are restricted to the four rows of cells closest to the yolk, known as the margin, while mesoderm precursors extend further towards the animal pole (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). The germ layers are also patterned along the dorsoventral axis, such that the notochord is derived from dorsal mesoderm, the heart comes from lateral mesoderm and blood comes from ventral mesoderm (Keegan et al., 2004; Melby et al., 1996). TGF-β signals of the Nodal-related subclass are required to induce and pattern the germ layers in vertebrates (Schier, 2003). Nodal signaling is mediated by a receptor complex containing the TGF-β Type I receptor, ALK4, the Type II receptor, ActR-IIB, and the Cripto/One-Eyed-pinhead (Oep) co-receptor (Gritsman et al., 1999; Reissmann et al., 2001). The Nodal receptors can also be activated by other TGF-β ligands, including Activin and Vg1 (Cheng et al., 2003; Reissmann et al., 2001). For this reason, the Nodal-related proteins, Activin and Vg1 are collectively termed Activin-like signals.

The requirement for Nodal-related proteins to induce mesoderm and endoderm is conserved throughout the vertebrate lineage (Schier, 2003). There are three *nodal-related* genes in zebrafish, but only two, *squint* (*sqt/ndr1*) and *cyclops* (*cyc/ndr2*), have overlapping roles in mesendoderm formation (Schier and Talbot, 2005). The third *nodal-related* gene, *southpaw* (*spaw/ndr3*), is only expressed after gastrulation and is involved in establishing the left-right body axis (Long et al., 2003). In *cyc* single mutants, defects in mesendoderm are first detected at mid-gastrulation and the embryos lack floorplate and ventral diencephalon at later stages (Hatta et al., 1991; Sampath et al., 1998; Thisse et al., 1994). *sqt* single mutants have severe deficits in dorsal mesodermal derivatives at early stages, but the embryos recover and many survive to adulthood (Dougan et al., 2003; Heisenberg and Nusslein-Volhard, 1997). This recovery depends on *cyc* function, since *sqt; cyc* double mutants lack all derivatives of the mesoderm and endoderm in the head and trunk, including the skeletal muscle, heart, pronephros, blood and gut (Feldman et al., 1998).

Both gain-and loss-of-function studies indicate that Activin-like signals act in a concentration-dependent manner to specify cell fates (Agius et al., 2000; Jones et al., 1995; Smith et al., 1988; Thisse et al., 2000). In explants, high doses induce marginal cell types, such as prechordal plate and endoderm, whereas lower doses induce notochord and muscle (Erter et al., 1998). Conversely, endoderm and prechordal plate are more sensitive to reductions in Nodal levels than are notochord and muscle (Dougan et al., 2003; Thisse et al., 2000). Zebrafish Sqt behaves like a morphogen, acting directly on cells at a distance to specify fates in a concentration dependent manner (Chen and Schier, 2001; Chen and Schier, 2002). These results and other data have led to the suggestion that cells adopt fates depending on their position within a gradient of Nodal-related protein (Solnica-Krezel, 2003).

A spatial gradient model of Nodal signaling, however, does not account for two key observations. For example, in the animal region of the mesoderm territory in pre-gastrula stage embryos, somite precursors are intermingled with neurectoderm progenitors, which are specified in the absence of Nodal function (Dougan et al., 2003; Kimmel et al., 1990). Near the margin, by contrast, somite precursors are intermingled with endoderm precursors, which require high levels of Nodal (Dougan et al., 2003; Warga and Nusslein-Volhard, 1999). This raises the question of how adjacent cells could be exposed to different Nodal doses. Secondly, Cyc can fully compensate for loss of the Sqt morphogen despite the fact that it only acts over a short range (Aoki et al., 2002b; Chen and Schier, 2001; Dougan et al., 2003). This indicates that the long-range action of Nodal signals is not necessary for correct induction and patterning of the mesoderm and endoderm.

Experiments suggest that the role of Nodal signaling is quite dynamic, but it has been difficult to determine what are the functions of Nodal signals at different times. The expression pattern of *nodal-related* genes changes rapidly during the blastula stages in frogs, fish and mice (Erter et al., 1998; Jones et al., 1995; Rebagliati et al., 1998; Varlet et al., 1997). Efforts to determine when Nodal signals specify distinct mesodermal and endodermal cell types have been hampered by the fact that conditions which alter the timing of Nodal signaling also change the levels of *nodal-related* gene expression. For example, levels of Nodal decrease in zygotic *oep* mutants as maternally supplied Oep mRNA and protein decay and eventually disappear (Gritsman et al., 2000; Zhang et al., 1998). Similarly, *cyc* expression is both reduced and delayed in *sqt* mutants (Dougan et al., 2003). Thus, it has not been possible to determine whether the fate changes observed in these mutants are due to altered timing of Nodal signaling or to the reduction in Nodal activity.

Experiments in frogs and fish have suggested two mechanisms by which Nodal signals may act to specify different tissues at different times. When *Xenopus* animal cap cells are exposed to Activin-soaked beads for different lengths of time, the responding cells exhibit a stepwise progression of cell fate specification as a concentration gradient of Activin is established within the explant (Gurdon et al., 1995). These results suggested that cells constantly monitor ligand levels and "ratchet-up" their response when the concentration exceeds certain threshold levels. In this view, cell fates are determined by the absolute number of receptors occupied by the ligand rather than by how long cells are exposed to the ligand (Bourillot et al., 2002; Dyson and Gurdon, 1998). By contrast, experiments in zebrafish with a conditional allele of *cyc* determined that cells need to be exposed to Nodal signals during a two-hour window in order to become floorplate (Tian et al., 2003). This raised the possibility that cells respond differently to Nodal signals depending on when they are exposed. In this view, cells have intrinsically defined periods during which they are able to adopt particular fates if exposed to the proper Nodal dose.

We have utilized a pharmacological approach to determine when Nodal signals specify the different mesodermal and endodermal cell types in the zebrafish. For the first time, we have been able to block the activity of Nodal receptors during discrete blastula stages by treatment with the small molecules SB-431542 or SB-505124 and without disrupting signaling at earlier stages or altering endogenous Nodal levels (DaCosta Byfield et al., 2004; Inman et al., 2002). We find that Nodal signals specify most mesodermal and endodermal cell types between the mid-blastula (3 h) and late blastula (5 h) stages. By examining embryos with increased or decreased levels of Sqt and Cyc signals, we show that the Nodal dose controls the timing of cell fate specification. This rules out the idea that cells adopt different mesoderm and endoderm fates depending on when they are exposed to Nodal signals. We also show that embryonic cells respond to a uniform, high dose by adopting progressively more marginal fates with longer exposures to Nodal signals. This time-dependent transformation of cell fates is inconsistent with some aspects of the ratchet model. We conclude that cells respond to the total cumulative dose of Nodal signals to which they are exposed, as a function of distance from the source and duration of exposure.

RESULTS

Drug treatment at MBT prevents the response to zygotic Nodal signals in embryos

To determine when Sqt and Cyc signals induce and pattern the germ layers, we developed a drug-based strategy that permits us to block endogenous Nodal signals at different stages after the mid-blastula transition (MBT). SB-431542 binds competitively to the ATP binding sites of the ALK 4, 5 and 7 receptors, preventing their kinase activity (Callahan et al., 2002). This drug has been used previously on zebrafish embryos during the cleavage stages, but did not completely block Nodal signals when added after MBT (Ho et al., 2006; Sun et al., 2006). Therefore, we developed a protocol to use SB-431542 to block zygotic Nodal signals in whole embryos between MBT (2.75 h) and the onset of gastrulation (6 h) (see Methods). Control embryos had a normal morphology at 24 h, indicating that our manipulations did not affect early embryogenesis (Fig. 2.1A-C). By contrast, embryos treated with 800µM SB-431542 display severe cyclopia and lack all derivatives mesoderm and endoderm in the head and trunk, including the somites, notochord, blood, heart and Kupffer's vesicle (Fig. 2.1D-F). These defects strongly resemble those previously described for *sqt; cyc* double mutants

(Feldman et al., 1998). Like *sqt; cyc* double mutants, SB-431542 treated embryos lack axial expression of the pan-mesendodermal marker *no-tail* (*ntl*) and the notochord marker *floating head* (*flh*) (Fig. 2.1J, L, O, Q). Interestingly, *flh* expression in the neurectoderm is greatly expanded in drug treated embryos, suggesting an expanded epiphysis (Fig. 2.1Q) (Liang et al., 2000). Drug treated embryos also lack *MyoD* expression at 14 h (Fig. 2.1K, P). Since tail somites do not form until later stages, this indicates that trunk somites are missing (Szeto and Kimelman, 2006). The prechordal plate and pronephros are also missing in these embryos, as indicated by the lack of *goosecoid* (*gsc*) and *pax2.1* expression, respectively (Fig. 2.1R, M; Fig. 2.3C, D). Drug treated embryos also lack expression of *sonic hedgehog b* (*shhb*), indicating the absence of floorplate (Fig. 2.1N, S). Because high concentrations of the drug were necessary to produce these defects, we next asked if we could achieve similar results with SB-505124, a more potent and bioactive inhibitor of the ALK 4/ 5/7 receptors than SB-431542 (DaCosta Byfield et al., 2004). 30-50µM of SB-505124 is sufficient to phenocopy *sqt; cyc* mutants when added at MBT (Fig. 2.1G-I). The ability of both drugs to phenocopy *sqt; cyc* mutants when added to 2.75 h embryos indicates that they reduce ALK 4/5/7 receptor activity to levels as low as that in zygotic mutants null for *nodal-related* gene function. Subsequent experiments were performed with SB-431542 and confirmed with SB-505124 as indicated.

To determine how quickly we could observe the effects of the drug, we examined the expression of the Nodal target gene *lefty1* in a time course of embryos treated with SB-431542 at dome stage (4.3hpf) (Thisse and Thisse, 1999). We found that transcription of Nodal target genes is normal 15 minutes after treatment (Fig. 2.2A, D; N=11), but is severely reduced after 30 minutes (Fig. 2.2B, E; N=24). No transcripts are detected 45 minutes after treatment (Fig. 2.2C, F; N=21). Therefore, transcription of Nodal-dependent genes is rapidly blocked after drug treatment and the decrease in mRNA levels is apparent within 15-30 minutes.

We next asked if SB-431542 could prevent the response to a mutated and constitutively-activated receptor that is active even in the absence of ligand, such as *TARAM-D* (Callahan et al., 2002). *TARAM-D* acts in a cell-autonomous manner to induce expression of Nodal target genes, resulting in dorsalized embryos and expanded *gsc* expression (Fig. 2.3E, G; N=30) (Aoki et al., 2002b; Renucci et al., 1996). In most cases, SB-431542 completely suppresses the response to *TARAM-D*, consistent with its proposed mode of action (data not shown). In the course of our experiment, however, occasional embryos received higher doses of the activated receptor and displayed a milder phenotype than their siblings. These embryos have cyclopia and reduced or absent mesodermal tissues, including trunk somites and notochord (Fig. 2.3F). *gsc* expression is dramatically reduced in these embryos (Fig. 2.3G, H; N=20). Thus, high levels of activated receptor can rescue the defects caused by the drug. This demonstrates the specificity of the drug, since the activated Nodal receptor would not rescue defects caused by blocking receptors for other signaling pathways. SB-431542 also blocks the response to ubiquitously expressed Sqt (Fig. 2.3I-L). Thus, the drug is able to effectively penetrate and act within the entire embryo. In these experiments, we injected embryos with *sqt* or *TARAM-D* mRNA at the 1-4 cell stage (1 h) and treated with the drug at 2.75 h. Therefore, SB-431542 can block the response to receptors already present during the cleavage stages. Because the drug is effective at blocking Nodal signaling when applied as late as 2.75h, this suggests that maternally supplied Activin-like ligands normally act after MBT to effect specification of cell fates.

Nodal signals specify mesodermal tissues at different times within a three-hour period

To determine when Nodal signals specify the various mesodermal cell types, we treated embryos with SB-431542 at successively later time points during the blastula stages and scored mesodermal tissues by morphology and marker gene expression. No mesodermal derivatives are present in the head and trunk when embryos are treated with the drug at MBT (Fig. 2.1). By contrast, embryos treated with SB-431542 at 6 h, when *cyc* expression predominates, produced a phenocopy of *cyc* single mutants (Fig. 2.4E1-8). These embryos display severe cyclopia, have a ventrally curved body axis and lack the floorplate, as indicated by the absence of *shhb* expression (Fig. 2.4E1, 2, 8) (Hatta et al., 1991; Tian et al., 2003). Thus, SB-431542 treatment in early gastrulation reduces ALK 4/5/7 activity to levels at or below those in *cyc* null mutants. A number of mesodermal cell types are present in embryos treated at this stage, including somites, notochord, heart, blood, pronephros, and hatching gland (Fig. 2.4E3-7; Fig. 2.5). This defines a three-hour time window beginning at MBT during which Nodal signals are required to specify mesodermal tissues. Embryos treated after mid-gastrulation contain floorplate as revealed by *shhb* expression (data not shown), confirming earlier temperature shift experiments using a temperature sensitive allele of *cyc* (Tian et al., 2003).

We next treated embryos with SB-431542 at different times between 2.75 and 6 h post-fertilization. Embryos treated with SB-431542 at 3.7 h contain a small number of trunk somites, but we detected no other mesodermal tissues in the trunk (Fig. 2.4A1; Fig. 2.5). *ntl* was expressed in a truncated axial domain and only a small number of disorganized trunk somites are apparent, as indicated by *MyoD* expression (Fig. 2.4A4, A5). *flh* was expressed in two bilateral domains within the ectoderm, but not at the midline (Fig. 2.4A6), consistent with the lack of notochord tissue in these embryos (Fig. 2.4A1, Fig. 2.5). The expression of the pan-mesodermal marker, *ntl*, but not notochord marker, *flh,* at the midline suggests that these cells are specified to be dorsal mesoderm, but are unable to complete the differentiation program (Schulte-Merker et al., 1994; Talbot et al., 1995). The lack of *pax2.1* expression in the intermediate mesoderm indicates that the pronephros was not specified at this time point (Fig. 4A7). Therefore, only trunk somites were specified following the shortest exposure time to Nodal signals.

Embryos treated with SB-431542 at later time points contain a more diverse array of mesodermal tissues (Fig. 2.5). Small amounts of notochord are detected in embryos treated at 4 h (Fig. 2.4B1, arrow; Fig. 2.5). Red blood cells are also apparent in live embryos examined at 48 h (Fig. 2.5). *flh* expressing cells populate the midline, but do not induce expression of *MyoD* in adaxial cells within the segmented mesoderm (Fig. 2.4B4-6). *MyoD* is still expressed in adaxial cells in the presomitic mesoderm (Fig. 2.4B5). *pax2.1* expression is also apparent in embryos treated at 4 h (Fig. 2.4B7). The hatching gland and Kupffer's vesicle are first visible in embryos treated with the drug at 4.3 h (Fig. 2.4C3, arrowhead; Fig. 2.5). Although we observed beating hearts in embryos treated at 4.7 h, a functioning circulatory system was only established in embryos treated at 5 h (Fig. 2.5). Since blood is specified before the heart, we attribute the delay in circulation to the time required to specify the cells comprising the vasculature, although we have not directly examined these cell types. Tissues were specified in the same temporal order in a time course using SB-505124 (data not shown).

The total amount of mesoderm increases as embryos are treated at successively later stages. Embryos treated at 3.7 h have between 5-7 trunk somites (Fig. 2.4A1, 5). By contrast, embryos treated at the onset of gastrulation contain the normal complement of somites (Fig.

2.4E1, 5). Thus, new somite tissue is induced throughout the blastula period. Similarly, a truncated notochord forms in embryos treated at 4 h (Fig. 2.4B1, 6, arrow), but notochord tissue extends more anteriorly when later stage embryos are treated (Fig. 2.4C1-E1; C6-E6). We were unable to detect a difference in the length of notochord in embryos treated at 5 h and 6 h (Fig. 2.4D6, E6). Expression of *flh* in the neurectoderm diminishes concomitantly with its expansion along the midline, indicating that signals from the mesoderm inhibit the differentiation of some neural tissues (Fig. 2.4A6-C6). *pax2.1* expression is weak when Nodal signaling is blocked at 4 h (Fig. 2.4B7), but intensifies when Nodal signaling is blocked at later stages (Fig. 2.4C7-E7). This demonstrates that after 4 h, Nodal signals act to specify the somites, notochord and pronephros, simultaneously. This argues against, but does not completely exclude, a model in which Nodal signals specify different mesoderm and endodermal cell types during distinct time-windows.

Nodal signals pattern the animal-vegetal axis in a time-dependent manner

Somite progenitors extend to the most animal region of the mesoderm territory in the pre-gastrula stage embryo, while progenitors of the hatching gland are restricted to the margin (Dougan et al., 2003; Kimmel et al., 1990; Melby et al., 1996). This suggests a general trend in which animal cell types are specified by shorter periods of Nodal signaling than marginal cell types. To test this, we asked when Nodal signals are required to specify the neural plate, notochord, prechordal plate and endoderm, which are marked by expression of *cyp26*, *flh*, *gsc* and *sox17*, respectively (Fig. 2.6A1-C1, E1). Embryos treated at MBT do not express *flh*, *gsc* or *sox17* (Fig. 2.6B2, C2, E2). *cyp26* is expressed at the margin, consistent with fate mapping studies showing that marginal cells adopt neural cell fates in the absence of Nodal signaling (Fig. 2.6A2) (Schier and Talbot, 2005). This domain shifts toward the animal pole with later treatments, reaching its normal location in embryos treated at 5 h (Fig. 2.6A2-5).

flh is first observed at the margin in embryos treated at 3.7 h (Fig. 2.6B3), but these cells do not differentiate into notochord (Fig. 2.4A6, N=20/21). *flh* is expressed at higher levels in embryos treated at later stages and the cells do become notochord (Fig. 2.6B2-6; Fig. 2.4B6-E6). *gsc* is not observed in embryos treated at 3.7 h (N=17), but we detect a small number of *gsc* expressing cells in embryos treated at 4.3 h (Fig. 2.6C3, C4; N=9/22). *gsc* is expressed at normal levels in all embryos treated at 5 h (Fig. 2.6C5; N=20). This indicates that Nodal signals are required between 4.3 h and 5 h to specify the prechordal plate. Kupffer's vesicles are also specified at this time, as indicated by our analysis of live embryos (Fig. 2.4C3; Fig. 2.5) and of *sox17* expression in the dorsal forerunner cells (Fig. 2.6E4, arrowhead; N=17). *sox17* is expressed in endoderm progenitors in embryos treated at 5 h (Fig. 2.6E5; N=15). The paired-box transcription factor *mezzo* acts upstream of *sox17*, and is expressed along the same time course (Fig. 2.6D1-D5) (Poulain and Lepage, 2002). On the dorsal side of the embryo, therefore, specification of marginal cell types, but not more animal cell types, is inhibited by late drug treatments.

We next asked if Nodal signals act similarly in the ventrolateral margin. The teleost heart is composed of two chambers, the atrium and ventricle, which express *atrial myosin heavy chain* (*amhc*) and *ventricular myosin heavy chain* (*vmhc*), respectively (Fig. 2.7B1, C1) (Yelon et al., 1999). Both chambers express *cardiac myosin light chain 2* (*cmlc2*) (Fig. 2.7A1). Despite a large region of overlap, atrial myocardial precursors can be found in more animal locations and ventricular myocardial precursors are located closer to the margin. We found that *cmlc2* and *amhc* expression are first detected when Nodal signaling is blocked at 4 h (Fig. 2.7A2, B2 arrows; N=16/20 and N=18/21, respectively). *vmhc* expression is never observed at this time point, indicating that specification of atrial myocardium precedes specification of ventricular myocardium (Fig. 2.7C2; N=0/19). *vmhc* expression is first observed in embryos treated at 4.3 h (Fig. 2.7C3, N=24/27). The short delay between specification of atrial myocardial precursors and ventricular myocardium is consistent with the small number of atrial progenitors located in animal cells where ventricle myocardial is not found (Keegan et al., 2004). The bilateral expression of heart myocardial genes in embryos treated at 4.3 h is consistent with the lack of endoderm at this stage (Fig. 2.7A3-C3) (Yelon, 2001). Myocardial precursors fuse into a tube at the midline when Nodal signals are blocked at 4.7 h, when we first observe beating hearts in live embryos (Fig. 2.7A4-C4; Fig. 2.5). We conclude that within the presumptive mesoderm and endoderm, marginal cell types require longer periods of Nodal signaling than other cell types.

Nodal levels control when cell fates are specified

We have shown that Nodal signals specify different cell types within the mesoderm and endoderm at different times, although there is a period during which they specify multiple tissues simultaneously. This could be explained if the responding cells have fixed time windows during which they need to be exposed to Nodal signals in order to adopt particular fates. If so, then mesoderm and endodermal cell fates will be specified at the same time as wild type even when the Nodal dose is reduced. To test this, we asked when cell fates are specified in *sqt* mutants, which have reduced levels of Nodal signaling. We found that *flh* expression at the midline was only observed when Nodal signaling was blocked at 5 h in *sqt* mutants (Fig. 2.8A5; N=15), as opposed to 4 hr in wild type (Fig. 2.4B6). Thus, notochord specification is delayed by an hour in *sqt* mutants. Specification of the prechordal plate and endoderm are also delayed in *sqt* mutants. *gsc* expression is only apparent in *sqt* mutants treated at the onset of gastrulation (6 h) (Fig. 2.8B6, N=21; compare with Fig. 2.6C4), and *sox17* expression is first apparent in embryos treated at 7 h (Fig. 2.8C7, N=16/20; compare with Fig. 2.6E5). We also observed a delay in specification of ventrolateral cell types in *sqt* mutants, since *cmlc2* expression is only apparent in embryos treated at 4.7 h (Fig. 2.8D4, arrowhead, N=12/16; compare with Fig. 2.7A2). These results rule out the possibility that presumptive mesoderm and endodermal cells have discrete windows of competence that determine their response to Nodal signals.

The delay in cell fate specification in *sqt* mutants suggests that Nodal levels control when cells fates are specified. If so, then specification of mesodermal and endodermal cell types should be accelerated when Nodal levels are increased. To test this, we examined *flh*, *gsc* and *sox17* expression in embryos injected with *sqt* mRNA and treated with SB-431542 at different time points after MBT. *flh* expression was not detected in control embryos (Fig. 2.9A1), but *gsc* and *sox17* were both expressed ubiquitously (Fig. 2.9B1, C1). Expression of all three genes was inhibited when we blocked Nodal receptor activity at MBT (Fig. 2.9A2-C2). *flh* was broadly expressed in embryos treated at 3.7 h (N=14), but gaps are often apparent at the animal pole. This indicates that the notochord is specified earlier in embryos with elevated Nodal signals than in wild type (Fig. 2.9A3, compare with Fig. 4B6). Similarly, specification of both prechordal plate and endoderm occur earlier in embryos with elevated Sqt. *gsc* is first detected in embryos treated at 3.7 h, as opposed to 4.3 h in wild type (Fig. 2.9B3, N=10/19; compare with Fig. 2.6C4), and is ubiquitously expressed in all embryos treated at 4.3 h (Fig. 2.9B4; N=12). This indicates that specification of prechordal plate is greatly accelerated when Nodal signaling is elevated. *sox17* is first observed in embryos treated at 4.3 h instead of 5 h in wild type, representing a slight acceleration in endoderm specification as compared to wild type (Fig. 2.9C4, N=10/12; Fig. 2.6E5). These results show that the level of Nodal signaling determines when mesoderm and endodermal cell fates are specified.

According to the "ratchet-model", cells generate a response appropriate to the highest dose to which they are exposed independently of the duration of exposure (Gurdon et al., 1995). If true, then cells should always adopt the most marginal fate when they are exposed to a uniformly high Nodal dose, regardless of how long the exposure lasts. In contrast to this prediction, however, we found that cells in Sqt-injected embryos are transiently specified to the more animal *flh* expressing fate (Fig. 2.9A4). As the duration of exposure increases, *flh* expression gradually diminishes (Fig. 2.9A4-A7), and *gsc* and *sox17* expression increase concomitantly (Fig. 2.9B4-C7). This demonstrates that cells adopt progressively more marginal identities in response to increasing exposure times to Nodal signals. These results rule out the possibility that presumptive mesoderm and endodermal cells respond to Nodal signals by a ratcheting-type mechanism.

DISCUSSION

Chemical inhibitors of ALK 4/5/7 are new tools for dissecting the roles of Nodal signals

In this study, we addressed the question of when members of the Nodal-related subclass of the TGF-b superfamily act to pattern the mesoderm and endoderm. We took a pharmacological approach to inactivate Nodal signaling at different times, and examined the resulting cell fates by an extensive analysis of gene expression and morphology. Three lines of evidence show that we were able to inhibit zygotically expressed Nodal signals. Firstly, we generated a phenocopy of *sqt; cyc* double mutants by treating embryos with 800µM SB-431542 at the mid-blastula stage, when zygotic expression of *sqt* and *cyc* initiates (Fig. 2.1). Secondly, we could phenocopy *cyc* single mutants by treating embryos at the onset of gastrulation, when *cyc* expression predominates (Fig. 2.4). These two experiments demonstrate that our treatment reduces receptor activity to at least the levels in the respective mutants. We confirmed our results with a second drug, SB-505124, which is more potent and soluble than SB-431542 (Fig. 2.1; data not shown), which rules out possible artefacts due to the high dose of SB-431542. Finally, drug treatment in the late blastula stages inhibited expression of a Nodal target gene within 30 minutes (Fig. 2.2).

Our results differ markedly from those of earlier studies, in which 50μM SB-431542 was unable to reproduce the *sqt; cyc* phenotype when added to embryos older than the 8-cell stage (Ho et al., 2006; Sun et al., 2006). Two technical aspects of our treatment protocol may account for our different results. First, we used a much higher dose of SB-431542 (800μM) than the other groups. Secondly, we perforated the embryos to ensure the drug fully penetrated the embryos. Perforation was not necessary with SB-505124, which was also effective at a much lower dose (50μM). We conclude that the milder effects of the drug reported by others are due to the poor ability of SB-431542 to penetrate the embryo as the number of cells increases during the cleavage stages. Even though multiple ligands can activate the ALK4/5 and 7 receptors, our phenotypes all resemble those resulting from reductions of *nodal-related* gene function (Dougan et al., 2003; Thisse et al., 2000). This indicates that the other Activin-like ligands are either not expressed during the stages we examined or act downstream of Nodal signals.

Time-dependent patterning of the animal-vegetal axis by Nodal signals

Previous attempts to determine when Nodal signals specify different mesoderm and endoderm cell types have focused on the analysis of *oep* mutant embryos. In *Zoep* mutants, late Nodal signaling is blocked due to the absence of an essential co-receptor, and prechordal plate and endoderm do not form (Schier et al., 1997; Zhang et al., 1998). It is not clear, however, whether these defects are due to the absence of late Nodal signaling activity, or to the reduction of signaling levels caused by the decay of maternally supplied Oep protein. In an alternate approach to determine the role of Nodal signals at different times, *oep* function was restored to *MZoep* mutants at different stages, rescuing the ability of mutant cells to respond to Nodal signals (Aoki et al., 2002a; Gritsman et al., 2000). In these experiments, restoring Nodal signaling at early stages completely rescued *MZoep* mutants. By contrast, prechordal plate and endoderm was missing when Nodal signaling was restored at later stages. Although these results are apparently consistent with our findings, we found that *sqt* and *cyc* expression are expressed at very low levels when *oep* function is supplied at late stages (4 h; Hagos and Dougan, submitted). Since the defects in late-rescued *MZoep* mutants result from aberrant *nodal-related* gene expression, these experiments do not address the question of when Nodal signals are required to specify cell fates.

By conditionally inactivating the Nodal receptors, we were able to determine the specification state of the presumptive mesoderm and endoderm at different embryonic stages. We found a time-dependent progression of cell fate specification along the animal-vegetal axis, consistent with earlier studies demonstrating that Nodal signals pattern the animal-vegetal axis, but not the dorsoventral axis (Dougan et al., 2003). Blocking Nodal signals at late stages inhibits formation of tissues derived from the margin, such as prechordal plate and endoderm, but not from more animal regions, such as notochord or somites (Figs. 2.4-6). Previous studies have determined that endoderm and prechordal plate require higher doses of Nodal signals than somites (Dougan et al., 2003; Gritsman et al., 2000). This suggests a linkage between Nodal dosage and the length of exposure.

Nodal levels control when cells are specified to become mesoderm and endoderm

Our results place Nodal signals at the top of a developmental program that determines the fates of responding cells and controls when these fates are specified. We considered the possibility that Nodal signals pattern the mesoderm and endoderm by acting in fixed time windows to specify different cell types. When Nodal levels are low, as in *sqt* mutants, specification of endoderm does not begin until early gastrulation (2.7 h; Fig. 2.8C7). By contrast, when Nodal levels are high, specification of endoderm begins 1.7 h earlier (Fig. 2.9C4). We conclude that cell identities are specified at different times depending on the Nodal dosage (Figs. 2.8-9). These results exclude the possibility that cells have fixed time windows during which they can adopt particular mesoderm and endodermal fates in response to Nodal signals. To the contrary, the level of Nodal signalling determines when cells are specified to adopt particular mesoderm and endodermal identities.

Previous cell transplant experiments defined a broad window of competence during which cells can respond to mesoderm and endoderm inducing signals, which we now know to be the Nodal-related proteins (David and Rosa, 2001; Ho et al., 1999). Experiments in *Xenopus* animal caps demonstrated that this window of competence is controlled by an intrinsic timing mechanism and ends by mid-gastrulation (Gurdon et al., 1993). Our results show that within this broad window, cells have a considerable degree of flexibility as to when they can become mesoderm and endoderm that depends on the levels of Nodal signals. At the molecular level, the loss of the ability to respond to Nodal signals could reflect the Nodal-dependent induction of a feedback inhibitor of the pathway. Consistent with this idea, expression of the secreted Nodal antagonist Lefty is under the control of Nodal signaling (Meno et al., 1999; Thisse et al., 2000). Thus, one role of Lefty could be to place a temporal limit on when cells can respond to Nodal signals. In support of this, Nodal signals persist well into gastrulation when lefty function is depleted and act during this time to convert ectoderm into mesoderm and endoderm (X. Fan and S. Dougan, unpublished data) (Chen and Schier, 2002; Feldman et al., 2002).

The length of exposure to Nodal signals determines cell fate choices

Cells are exquisitely sensitive to the dose of Activin-like signals. Experiments with dissociated *Xenopus* animal cap cells showed that as few as 100 molecules of Activin induce expression of the pan-mesodermal marker, *Xbra*, whereas 300 bound molecules induce *gsc* expression (Dyson and Gurdon, 1998). In these experiments, cells were exposed to different doses of Activin for 10 minutes and changes in cell fate were assessed hours later. The results supported the view that cells "ratchet-up" their response as a morphogen gradient is established and the dose of Activin crosses particular threshold levels (Fig. 2.10A) (Gurdon et al., 1995). Because the length of exposure was constant, these experiments did not address the effects of prolonged exposure to Activin on cell fate decisions. In our experiments, by contrast, we examined the response to the endogenous mesoderm and endoderm inducing signals in whole zebrafish embryos. Marginal cells were continuously exposed to Nodal signals until we blocked the response by drug treatment. Our results emphasize the importance of the length of exposure in determining the overall dose and consequent fate choice. Importantly, all mesoderm and endodermal cell types are present in *sqt* mutants, but are specified at later times than in wild type. The only Nodal-related protein in these embryos is Cyc, which has a shorter range than Sqt and is expressed at reduced levels in *sqt* mutants (Chen and Schier, 2001; Dougan et al., 2003). Thus, prolonged exposure to low doses of a signal compensates for the overall reduction of levels. Furthermore, the long-range action of a secreted factor is not essential for normal development of the two germ layers in zebrafish.

Generating a response to a cumulative Nodal dose

We found that cells respond to the cumulative dose of Nodal signals to which they are exposed. In embryos exposed to a uniform, high Nodal dose, cells exhibit a time-dependent transformation towards more marginal fates as the length of exposure increases (Fig. 2.9). This means that cells must have a mechanism to record the duration of their exposure to Nodal signaling and to generate a response to the cumulative dose. Although this regulation may occur at many different levels, the ultimate readout is at the level of gene transcription. Of the marker genes we analyzed, *gsc* is a likely direct target of the Nodal pathway (McKendry et al., 1998). *gsc* expression initiates at 4 h in the absence of both *sqt* and *cyc* function, but quickly decreases (Dougan et al., 2003). This indicates that Nodal signals are required for maintenance, but not for the induction of *gsc* expression. In this study, we showed that *gsc* expression is lost when Nodal signaling is inactivated at 4.3 h, but continues when Nodal signaling is blocked at 5 h (Fig. 2.6). Thus, Nodal input is required for about an hour in order to maintain *gsc* expression. After this transient maintenance phase, *gsc* expression continues independently of Nodal, under the control of unknown factors. In *sqt* mutants, it takes a longer period of time for the *gsc* promoter to transit to the Nodal independent phase, whereas the *gsc* promoter reaches this state more rapidly when Sqt is overexpressed. Other genes have been shown to undergo similar phases of gene regulation, most notably the *Drosophila engrailed* gene (Heemskerk et al., 1991), but this is the first case to our knowledge in which the levels of a secreted factor control the length of the maintenance phase of a target gene.

A spatio-temporal gradient model for patterning by Nodal signals

Any model for how Nodal signals act to pattern the mesoderm and endoderm must account for four observations. First, the model must explain how adjacent cells become exposed to different levels of Nodal signals. Fate mapping studies show that precursors of cell types that require different levels of Nodal signaling, such as somites and endoderm, are juxtaposed in the pre-gastrula stage embryo (Dougan et al., 2003; Kimmel et al., 1990). Second, the model must account for our observation that the blastomeres are highly dynamic during the period they respond to Nodal signals. We found that Nodal signals act primarily before 5 h (40% epiboly) (Figs. 2.4-6), a period in which cells divide rapidly and frequently change positions with respect to each other (Warga and Kimmel, 1990). This presents a particular challenge to classic morphogen gradient models, which generally assume a static field of responding cells. Third, the model must explain how a short-range signal, like Cyc, can specify the same cell types as a long-range signal, like Sqt. Finally, the model must account for our observation that cells respond to the cumulative dose of Nodal signals.

We propose that the total Nodal dose is a function of both the length of time a cell is exposed to Nodal signals and the distance of a cell from the Nodal source (Fig. 2.10B). Key predictions of this model remain to be tested, but it accounts for all these observations. In this view, cells that remain near the Nodal source for an extended period would receive a high dose and adopt a marginal cell fate, such as prechordal plate or definitive endoderm. Conversely, cells that move away from the source after a short time would receive a lower dose and become somites. Specification of mesoderm and endoderm is delayed in *sqt* mutants because it takes longer than in wild type for cells to accumulate the necessary Nodal dosage. Because the gradient of positional information is influenced by the length of time responding cells are exposed to the signaling source and their distance from the source, we call this the spatio-temporal gradient model. In other species, Nodal signals also pattern tissues comprised of dynamic cell populations, such as the node and primitive streak in mice and Hensen's node in the chicken (Joubin and Stern, 1999; Kinder et al., 2001). Thus, cell movements could provide a general mechanism for generating a gradient of exposure to Nodal signals during mesoderm patterning in all vertebrates.

Our model predicts that a stable source of Nodal signals exists in the embryo that is independent of the dynamic cell movements of the responding cell population. We propose that the extraembryonic yolk syncytial layer (YSL) acts as this source. Sqt is normally expressed in this tissue and can induce fate changes in overlying blastomeres when overexpressed in the YSL (Erter et al., 1998; Feldman et al., 1998). We suggest that Nodal signals in the YSL act to induce and/or maintain *nodal-related* gene expression in the overlying blastomeres via the autoregulatory pathway. If a cell that is initially close to the YSL moves away, it will lose expression of *sqt* and *cyc*. Conversely, *sqt* and *cyc* expression will be induced in a cell as it moves closer to the YSL. Thus, the autoregulatory pathway provides a mechanism by which a stable zone of Nodal signaling can be imposed upon the dynamic, intermixing population of cells at the embryo margin.

CONCLUSIONS

Our data indicate that Nodal signals act in a time-dependent manner to pattern the mesoderm and endoderm. Three lines of evidence support the idea that cells respond to the cumulative dose of Nodal signals. First, marginal cell types, which are specified by the highest Nodal dose, require the longest exposure to Nodal signals. Second, cell fate specification is delayed when Nodal levels are reduced, and accelerated when Nodal levels are increased. Finally, in response to a uniform, high Nodal dose, cell fates transform toward progressively more marginal identities as the length of exposure increases. These results rule out the possibility that Nodal signals act during discrete time windows to specify different mesodermal and endodermal cell types. They are also inconsistent with the "ratcheting-up" model, in which the absolute number of occupied receptors determines cell fates, not the duration of exposure.

We conclude that cells respond to modifications to the cumulative Nodal dose, which we suggest is a product of the distance of the responding cell from the signaling centre and the length of exposure.

MATERIALS AND METHODS

Zebrafish strains and staging

We used the WIK strain to obtain wild type embryos. Embryos homozygous for the *sqt*^{cz35} null allele were obtained from crossing mutant adults. *oep*^{tz57} mutant adults were obtained by mRNA injection, as previously described (Gritsman et al., 1999). In all experiments, the embryonic stages were determined by morphology and are reported as hours post-fertilization (h) at 28.5˚C, according to Kimmel et al. (1995).

Drug treatment

SB-431542-(4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl-1*H*-imidazol-2-yl]benzamide), was obtained from Tocris (Ellisville, MO) and stored as a 100mM stock in DMSO at -20˚C. SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) was a kind gift from GlaxoSmithKline (King of Prussia, PA) and is stored at 10mM in DMSO at 4˚C. For the drug time course studies shown in Figs. 2.1 and 4, approximately 1000 embryos equivalently staged embryos from 3-4 single pair matings were pooled, split into 10 dishes at a density of 100 embryos/dish, and raised in an incubator at 28.5˚C. For drug treatment, embryos from one dish were removed at the desired stage, perforated near the margin with a pulled capillary tube, and split into glass dishes containing the drug in 5 ml embryo medium, at a density of 25 embryos/dish. Embryos were fixed at 10h and split into three groups for analysis of *ntl*, *flh* or *shhb* expression, or fixed at 14h and split into two groups for analysis of *MyoD* or *pax2.1*. Time courses depicted in other figures followed the same protocol, but embryos were fixed at the stages indicated for analysis of marker gene expression. In each figure, representative images are shown, and all embryos were treated on the same day. Embryos damaged by the perforation were discarded. Embryos treated with SB-505124 did not require perforation. In all experiments, some embryos in each experiment were allowed to develop until 24 h and examined morphologically to verify the efficacy of the treatment. All experiments were performed at least two times. The effective dose on 2.75 h embryos SB-431542 was determined in a titration of 5µM-1mM SB-431542 or 3µM-75µM SB-505124. SB-431542 treatment was always associated with the formation of a dark precipitate in the solution. At 800µM, all embryos resembled *sqt; cyc* mutants, whereas lower doses generated milder phenotypes similar to *Zoep* mutants (Schier et al., 1997). This milder phenotype is also observed by treating cleavage stage embryos with 50µM SB-431542 (data not shown) (Ho et al., 2006; Sun et al., 2006). The previously described toxic effects of SB-431542 in cell culture are apparent at doses above 800µM on blastula stage embryos and above 100µM on cleavage stage embryos (data not shown) (DaCosta Byfield et al., 2004). For SB-505124, the lowest dose that produced the *sqt; cyc* phenotype ranged from 30-50µM, depending on the age of the drug.

Microinjections and whole-mount *in situ* hybridization

The *sOep, sqt* and *TARAM-D* cDNAs were described previously (Feldman et al., 1998; Renucci et al., 1996; Zhang et al., 1998). Sense transcripts were synthesized using the Message Machine kit (Ambion, Inc., Austin TX). We injected 10pg *sqt*, *TARAM-D* or *b-galactosidase* mRNA into chorionated embryos at the 1-4 cell stage. 100pg *sOep* mRNA was co-injected into the YSL of *MZoep* mutants with the Oregon Green 488 lineage tracer dye (Invitrogen, Inc., Carlsbad, CA) to verify the targeting of the injection, as described

(Gritsman et al., 2000). *In situ* hybridizations were performed as in Dougan, et al., 2003. We used the following probes: *sqt*, *cyc*, *gsc*, *ntl*, *flh*, *MyoD*, *pax2.1*, *shhb*, *sox17*, *mezzo*, *cyp26*, *cmlc2*, *amhc* and *vmhc* (Alexander and Stainier, 1999; Berdougo et al., 2003; Feldman et al., 1998; Krauss et al., 1993; Krauss et al., 1991; Poulain and Lepage, 2002; Sampath et al., 1998; Schulte-Merker et al., 1994; Stachel et al., 1993; Talbot et al., 1995; Thisse et al., 2000; Weinberg et al., 1996; White et al., 1996; Yelon et al., 1999).

Acknowledgements

We thank Benjamin Feldman, Jim Lauderdale, Walter Schmidt, Howard Sirotkin, Will Talbot and an anonymous for valuable comments on the manuscript; Steve Stice for suggesting the use of SB-431542; Nicholas J. Laping for advice on the use of SB-505124; Xiang Fan, Katie Holcombe and Chari Jefferson for fish care; Steve Dalton and members of the Dougan and Lauderdale laboratories for helpful discussions. S. Dougan is a Georgia Cancer Coalition Distinguished Investigator. This work was supported by the Georgia Cancer Coalition and a grant from the University of Georgia Research Foundation.

REFERENCES

Agius, E., M. Oelgeschlager, O. Wessely, C. Kemp, and E.M. De Robertis. 2000. Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development*. 127:1173-83.

Alexander, J., and D.Y. Stainier. 1999. A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol*. 9:1147-57.

Aoki, T.O., N.B. David, G. Minchiotti, L. Saint-Etienne, T. Dickmeis, G.M. Persico, U. Strahle, P. Mourrain, and F.M. Rosa. 2002a. Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. *Development*. 129:275-86.

Aoki, T.O., J. Mathieu, L. Saint-Etienne, M.R. Rebagliati, N. Peyrieras, and F.M. Rosa. 2002b. Regulation of nodal signalling and mesendoderm formation by TARAM-A, a TGFbeta-related type I receptor. *Dev Biol*. 241:273-88.

Berdougo, E., H. Coleman, D.H. Lee, D.Y. Stainier, and D. Yelon. 2003. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development*. 130:6121-9.

Bourillot, P.Y., N. Garrett, and J.B. Gurdon. 2002. A changing morphogen gradient is interpreted by continuous transduction flow. *Development*. 129:2167-80.

Callahan, J.F., J.L. Burgess, J.A. Fornwald, L.M. Gaster, J.D. Harling, F.P. Harrington, J. Heer, C. Kwon, R. Lehr, A. Mathur, B.A. Olson, J. Weinstock, and N.J. Laping. 2002. Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5). *J Med Chem*. 45:999-1001.

Chen, Y., and A.F. Schier. 2001. The zebrafish Nodal signal Squint functions as a morphogen. *Nature*. 411:607-10.

Chen, Y., and A.F. Schier. 2002. Lefty proteins are long-range inhibitors of squint-mediated nodal signaling. *Curr Biol*. 12:2124-8.

Cheng, S.K., F. Olale, J.T. Bennett, A.H. Brivanlou, and A.F. Schier. 2003. EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. *Genes Dev*. 17:31-6.

DaCosta Byfield, S., C. Major, N.J. Laping, and A.B. Roberts. 2004. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 65:744-52.

Dale, L., and J.M. Slack. 1987. Fate map for the 32-cell stage of Xenopus laevis.*Development*. 99:527-51.

David, N.B., and F.M. Rosa. 2001. Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. *Development*. 128:3937-47.

Dougan, S.T., R.M. Warga, D.A. Kane, A.F. Schier, and W.S. Talbot. 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development*. 130:1837-1851.

Dyson, S., and J.B. Gurdon. 1998. The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell*. 93:557-68.

Erter, C.E., L. Solnica-Krezel, and C.V. Wright. 1998. Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev Biol*. 204:361-72.

Feldman, B., M.L. Concha, L. Saude, M.J. Parsons, R.J. Adams, S.W. Wilson, and D.L. Stemple. 2002. Lefty antagonism of squint is essential for normal gastrulation. *Curr Biol*. 12:2129-35.

Feldman, B., M.A. Gates, E.S. Egan, S.T. Dougan, G. Rennebeck, H.I. Sirotkin, A.F. Schier, and W.S. Talbot. 1998. Zebrafish organizer development and germ-layer formation require nodal- related signals. *Nature*. 395:181-5.

Gritsman, K., W.S. Talbot, and A.F. Schier. 2000. Nodal signaling patterns the organizer. *Development*. 127:921-32.

Gritsman, K., J. Zhang, S. Cheng, E. Heckscher, W.S. Talbot, and A.F. Schier. 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell*. 97:121-32.

Gurdon, J.B., P. Lemaire, and K. Kato. 1993. Community effects and related phenomena in development. *Cell*. 75:831-4.

Gurdon, J.B., A. Mitchell, and D. Mahony. 1995. Direct and continuous assessment by cells of their position in a morphogen gradient. *Nature*. 376:520-1.

Hatada, Y., and C.D. Stern. 1994. A fate map of the epiblast of the early chick embryo. *Development*. 120:2879-89.

Hatta, K., C.B. Kimmel, R.K. Ho, and C. Walker. 1991. The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature*. 350:339-41.

Heemskerk, J., S. DiNardo, R. Kostriken, and P.H. O'Farrell. 1991. Multiple modes of engrailed regulation in the progression towards cell fate determination. *Nature*. 352:404-10.

Heisenberg, C.P., and C. Nusslein-Volhard. 1997. The function of silberblick in the positioning of the eye anlage in the zebrafish embryo. *Dev Biol*. 184:85-94.

Ho, C.Y., C. Houart, S.W. Wilson, and D.Y. Stainier. 1999. A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr Biol*. 9:1131-4.

Ho, D.M., J. Chan, P. Bayliss, and M. Whitman. 2006. Inhibitor-resistant type I receptors reveal specific requirements for TGF-beta signaling in vivo. *Dev Biol*. 295:730-42.

Inman, G.J., F.J. Nicolas, J.F. Callahan, J.D. Harling, L.M. Gaster, A.D. Reith, N.J. Laping, and C.S. Hill. 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 62:65-74.

Jones, C.M., M.R. Kuehn, B.L. Hogan, J.C. Smith, and C.V. Wright. 1995. Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development*. 121:3651-62.

Joubin, K., and C.D. Stern. 1999. Molecular interactions continuously define the organizer during the cell movements of gastrulation. *Cell*. 98:559-71.

Keegan, B.R., D. Meyer, and D. Yelon. 2004. Organization of cardiac chamber progenitors in the zebrafish blastula. *Development*. 131:3081-91.

Kimmel, C.B., R.M. Warga, and T.F. Schilling. 1990. Origin and organization of the zebrafish fate map. *Development*. 108:581-94.

Kinder, S.J., T.E. Tsang, M. Wakamiya, H. Sasaki, R.R. Behringer, A. Nagy, and P.P. Tam. 2001. The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development*. 128:3623-34.

Krauss, S., J.P. Concordet, and P.W. Ingham. 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell*. 75:1431-44.

Krauss, S., T. Johansen, V. Korzh, and A. Fjose. 1991. Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. *Development*. 113:1193-206.

Lawson, K.A., J.J. Meneses, and R.A. Pedersen. 1991. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development*. 113:891-911.

Liang, J.O., A. Etheridge, L. Hantsoo, A.L. Rubinstein, S.J. Nowak, J.C. Izpisua Belmonte, and M.E. Halpern. 2000. Asymmetric nodal signaling in the zebrafish diencephalon positions the pineal organ. *Development*. 127:5101-12.

Long, S., N. Ahmad, and M. Rebagliati. 2003. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development*. 130:2303-16.

McKendry, R., R.M. Harland, and S.E. Stachel. 1998. Activin-induced factors maintain goosecoid transcription through a paired homeodomain binding site. *Dev Biol*. 204:172-86.

Melby, A.E., R.M. Warga, and C.B. Kimmel. 1996. Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development*. 122:2225-37.

Meno, C., K. Gritsman, S. Ohishi, Y. Ohfuji, E. Heckscher, K. Mochida, A. Shimono, H. Kondoh, W.S. Talbot, E.J. Robertson, A.F. Schier, and H. Hamada. 1999. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell*. 4:287-98.

Poulain, M., and T. Lepage. 2002. Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. *Development*. 129:4901-14.

Rebagliati, M.R., R. Toyama, C. Fricke, P. Haffter, and I.B. Dawid. 1998. Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev Biol*. 199:261-72.

Reissmann, E., H. Jornvall, A. Blokzijl, O. Andersson, C. Chang, G. Minchiotti, M.G. Persico, C.F. Ibanez, and A.H. Brivanlou. 2001. The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev*. 15:2010-22.

Renucci, A., V. Lemarchandel, and F. Rosa. 1996. An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling pathway involved in fish head organiser formation. *Development*. 122:3735-43.

Sampath, K., A.L. Rubinstein, A.M. Cheng, J.O. Liang, K. Fekany, L. Solnica-Krezel, V. Korzh, M.E. Halpern, and C.V. Wright. 1998. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature*. 395:185-9.

Schier, A. 2003. Nodal Signaling in Vertebrate Development. *Annu. Rev. Cell Dev. BIol.* 19:589-621.

Schier, A.F., S.C. Neuhauss, K.A. Helde, W.S. Talbot, and W. Driever. 1997. The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development*. 124:327-42.

Schier, A.F., and W.S. Talbot. 2005. Molecular Genetics of Axis Formation in Zebrafish. *Annu Rev Genet*.

Schulte-Merker, S., F.J. van Eeden, M.E. Halpern, C.B. Kimmel, and C. Nusslein-Volhard. 1994. no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development*. 120:1009-15.

Smith, J.C., M. Yaqoob, and K. Symes. 1988. Purification, partial characterization and biological effects of the XTC mesoderm-inducing factor. *Development*. 103:591-600.

Solnica-Krezel, L. 2003. Vertebrate development: taming the nodal waves. *Curr Biol*. 13:R7-9.

Stachel, S.E., D.J. Grunwald, and P.Z. Myers. 1993. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development*. 117:1261-74.

Sun, Z., P. Jin, T. Tian, Y. Gu, Y.G. Chen, and A. Meng. 2006. Activation and roles of ALK4/ALK7-mediated maternal TGFbeta signals in zebrafish embryo. *Biochem Biophys Res Commun*. 345:694-703.

Szeto, D.P., and D. Kimelman. 2006. The regulation of mesodermal progenitor cell commitment to somitogenesis subdivides the zebrafish body musculature into distinct domains. *Genes Dev*. 20:1923-32.

Talbot, W.S., B. Trevarrow, M.E. Halpern, A.E. Melby, G. Farr, J.H. Postlethwait, T. Jowett, C.B. Kimmel, and D. Kimelman. 1995. A homeobox gene essential for zebrafish notochord development. *Nature*. 378:150-7.

Thisse, B., C.V. Wright, and C. Thisse. 2000. Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature*. 403:425-8.

Thisse, C., and B. Thisse. 1999. Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development*. 126:229-40.

Thisse, C., B. Thisse, M.E. Halpern, and J.H. Postlethwait. 1994. Goosecoid expression in neurectoderm and mesendoderm is disrupted in zebrafish cyclops gastrulas. *Dev Biol*. 164:420-9.

Tian, J., C. Yam, G. Balasundaram, H. Wang, A. Gore, and K. Sampath. 2003. A temperature-sensitive mutation in the nodal-related gene cyclops reveals that the floor plate is induced during gastrulation in zebrafish. *Development*. 130:3331-42.

Varlet, I., J. Collignon, and E.J. Robertson. 1997. nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development*. 124:1033-44.

Warga, R.M., and C.B. Kimmel. 1990. Cell movements during epiboly and gastrulation in zebrafish. *Development*. 108:569-80.

Warga, R.M., and C. Nusslein-Volhard. 1999. Origin and development of the zebrafish endoderm. *Development*. 126:827-38.

Weinberg, E.S., M.L. Allende, C.S. Kelly, A. Abdelhamid, T. Murakami, P. Andermann, O.G. Doerre, D.J. Grunwald, and B. Riggleman. 1996. Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development*. 122:271-80.

White, J.A., Y.D. Guo, K. Baetz, B. Beckett-Jones, J. Bonasoro, K.E. Hsu, F.J. Dilworth, G. Jones, and M. Petkovich. 1996. Identification of the retinoic acid-inducible all-trans-retinoic acid 4- hydroxylase. *J Biol Chem*. 271:29922-7.

Yelon, D. 2001. Cardiac patterning and morphogenesis in zebrafish. *Dev Dyn*. 222:552-63.

Yelon, D., S.A. Horne, and D.Y. Stainier. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev Biol*. 214:23-37.

Zhang, J., W.S. Talbot, and A.F. Schier. 1998. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. *Cell*. 92:241-51.

FIGURE LEGENDS

Figure 2.1. Treatment with 800µM SB-431542 or 50µM SB-505124 at MBT prevents formation of mesoderm and endoderm. (A-F) Images of live embryos at 24hpf treated at 2.75 h with DMSO (A-C), SB-431542 (D-F; J-S), or SB-505124 (G-I). Embryos treated with SB-431542 (D-F) or SB-505124 (G-I) lack derivatives of the mesoderm and endoderm in the head and trunk, display severe cyclopia and lack Kupffer's vesicle. (J-P) Images of embryos treated with DMSO (J-N) or SB-431542 at MBT (O-S) and processed to reveal expression of markers for derivatives of dorsal mesoderm (*ntl*: J, O; *flh*: L, Q), paraxial mesoderm (*myoD*: K, P), intermediate mesoderm (*pax2.1*: M, R), and ventral neurectoderm (*shhb*: N, S). Dorsal views of embryos fixed at 10 h (J, L, N, P, Q, S) or 14 h (K, M, P, R). Arrowhead in (C) is the Kupffer's vesicle.

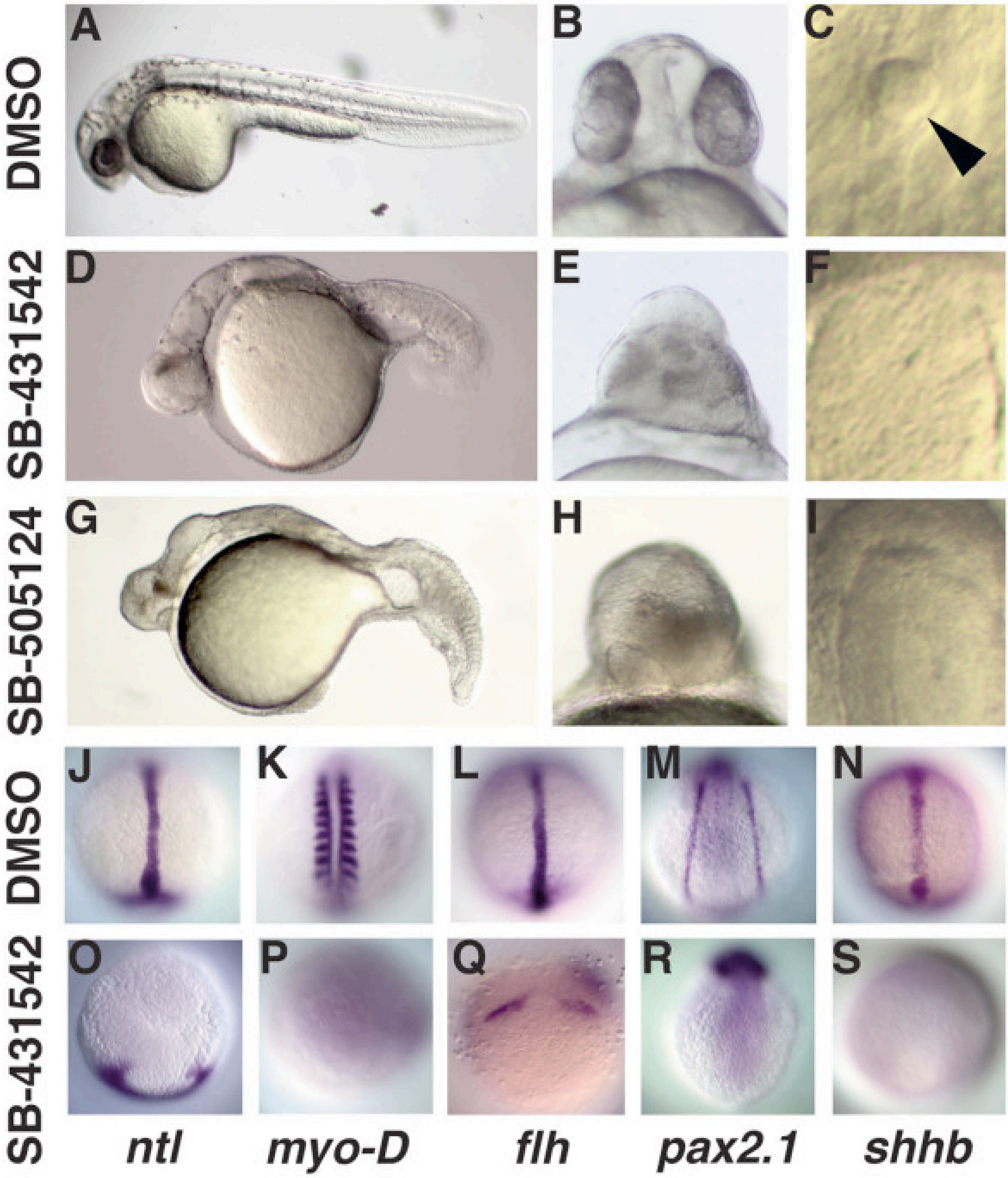

DMSO
SB-431542
SB-505124
DMSO
SB-431542
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
ntl
myo-D
flh
pax2.1
shhb

Figure 2.2. SB-431542 rapidly blocks transcription of Nodal target genes. *lefty1* expression in embryos treated with DMSO (A-C) or SB-431542 (D-F) at 4.3 h (dome stage), and fixed after 15 minutes (A, D), 30 minutes (B, E) or 45 minutes (D, F).

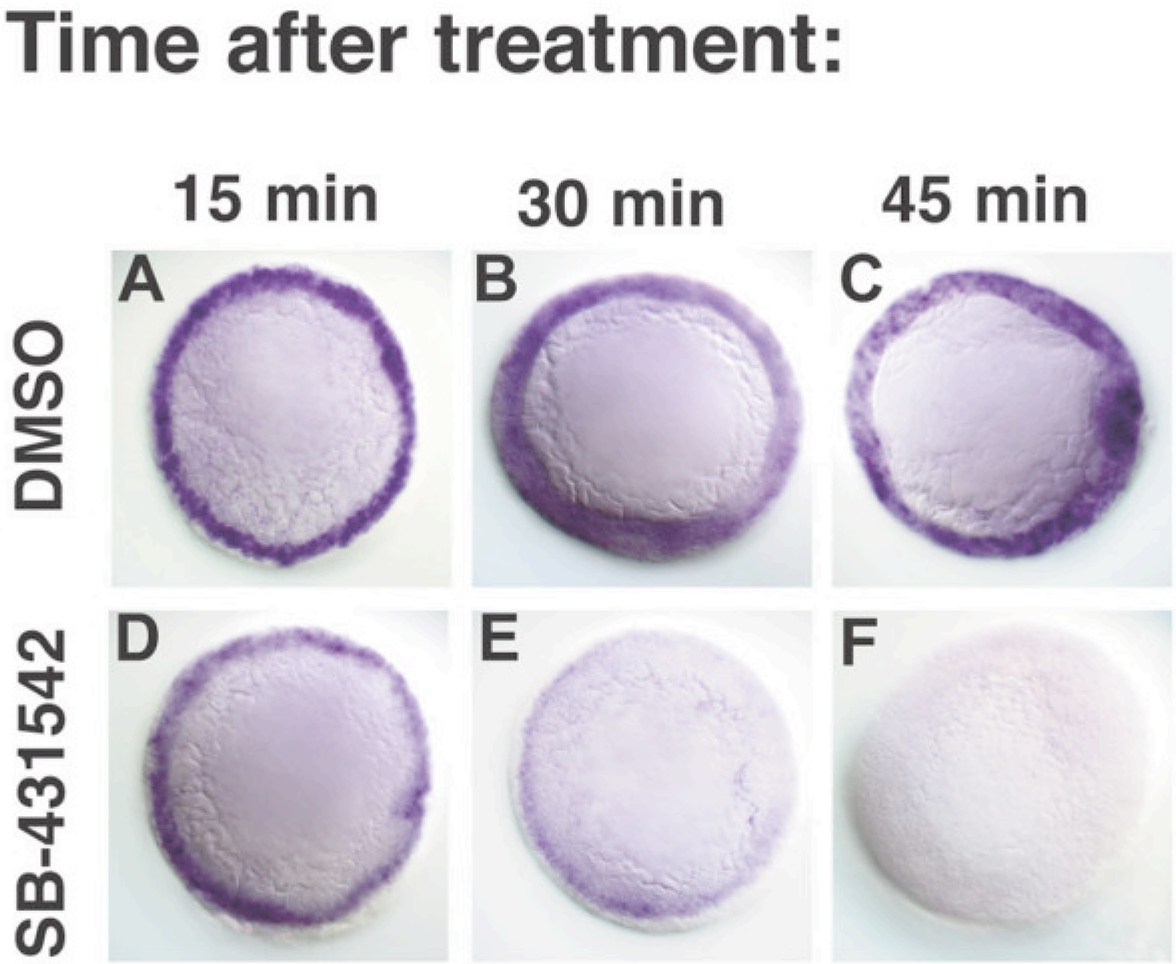

Time after treatment:
15 min
30 min
45 min
DMSO
SB-431542
A
B
C
D
E
F

Figure 2.3. Treatment at MBT blocks the response to receptors activated during the cleavage stages. Embryos injected at the 1-4 cell stage with 10pg *b-galactosidase* (A-D), *TARAM-D* (E-H), or *sqt* (I-L) mRNA, and treated at 2.75 h with DMSO (A, E, I, C, G, K) or SB-431542 (B, F, J, D, H, L). (E, G) *TARAM-D* induces ectopic body axes and *gsc* expression. (F, H) The effects of *TARAM-D* are suppressed by treatment with SB-431542. (I, K) *sqt* overexpression arrests epiboly and induces ubiquitous expression of *gsc*. (J, L) The response to ubiquitous Sqt is blocked by treatment with SB-431542. Images of live embryos at 30 h, anterior to the left (A, B, E, F, I, J). Animal pole views of fixed embryos at 5 h, dorsal to the right (C, D, G, H, K, L).

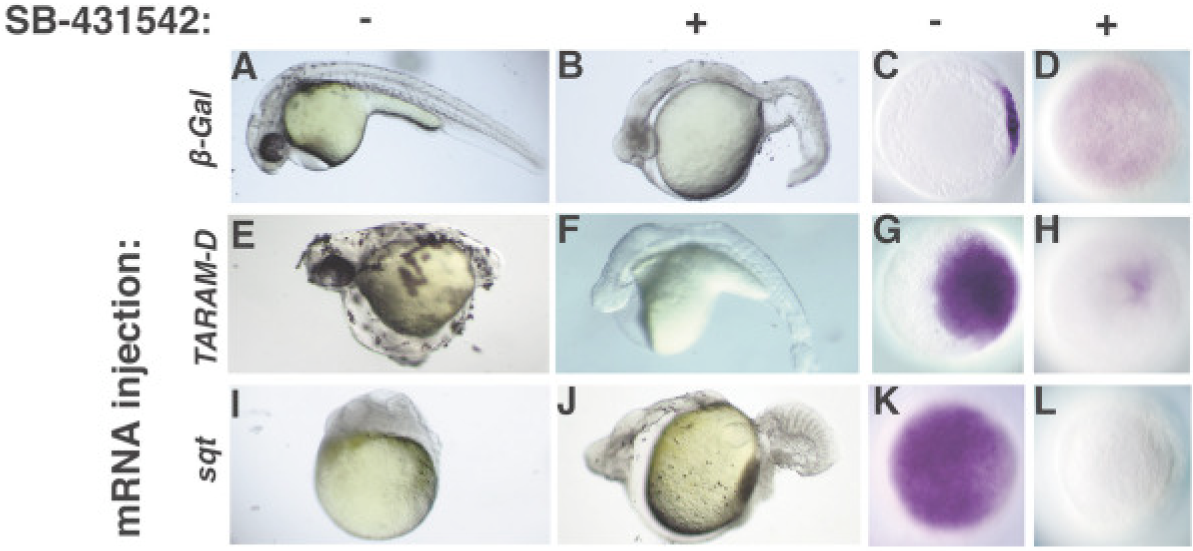

SB-431542:
-
+
-
+
mRNA injection:
β-Gal
TARAM-D
sqt
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2.4. Nodal signals pattern the mesoderm during a three-hour time-window. (A1-A8) Trunk somites form in embryos treated at 3.7 h (A1, 5), but *flh* is expressed in four ectodermal domains (A6). (B1-B8) Embryos treated at 4 h contain more somites (B5). *flh* is expressed at the midline and small amounts of notochord tissue are observed in live embryos (B1, arrow). *pax2.1* expression is also observed (B7). At later time points, embryos have progressively more somites and more notochord (C1-E7). *flh* expression extends further up the midline and *pax2.1* is expressed more strongly. Kupffer's vesicle forms in embryos treated 4.3 h (C3, arrowhead). *shhb* is not expressed in embryos treated before gastrulation (A8-E8). Images of live embryos at 24 h, anterior to the left (A1-E1; A2-E2) or 14hpf (A3-E3); dorsal views of fixed embryos at 10 h (A4-E4; A6-E6; A8-D8) or 14 h (A5-E5; A7-E7). Control embryos are depicted in Fig. 1J-S, which are from the same experiment.

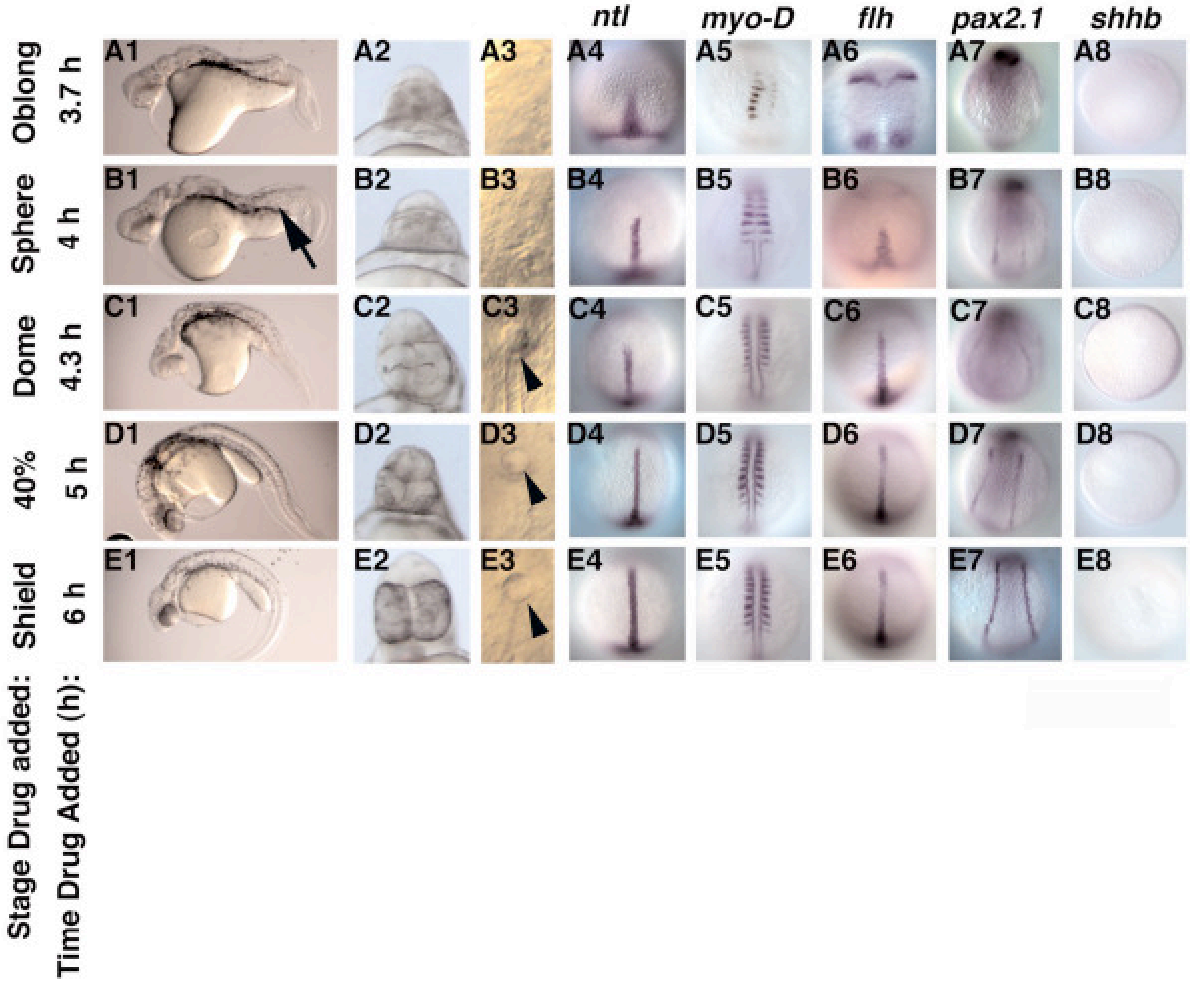

ntl
myo-D
flh
pax2.1
shhb
Oblong
3.7 h
Sphere
4 h
Dome
4.3 h
40%
5 h
Shield
6 h
Stage Drug added:
Time Drug Added (h):
A1
A2
A3
A4
A5
A6
A7
A8
B1
B2
B3
B4
B5
B6
B7
B8
C1
C2
C3
C4
C5
C6
C7
C8
D1
D2
D3
D4
D5
D6
D7
D8
E1
E2
E3
E4
E5
E6
E7
E8

Figure 2.5. Time-dependent specification of mesodermal tissues by Nodal signals. Embryos were treated with SB-431542 at different stages of development between the mid-blastula transition and the onset of gastrulation, and were examined morphologically at 14, 24 and 48 h for visible trunk somites, notochord, blood, Kupffer's vesicle, hatching gland, a beating heart and a functional circulatory system. The steep curves for somites, hatching gland, Kupffer's vesicle, and beating hearts indicates that specification of these tissues occurs rapidly and in a stepwise fashion. Specification of notochord and blood occurs over a longer period. The number of embryos examined at each time point is indicated.

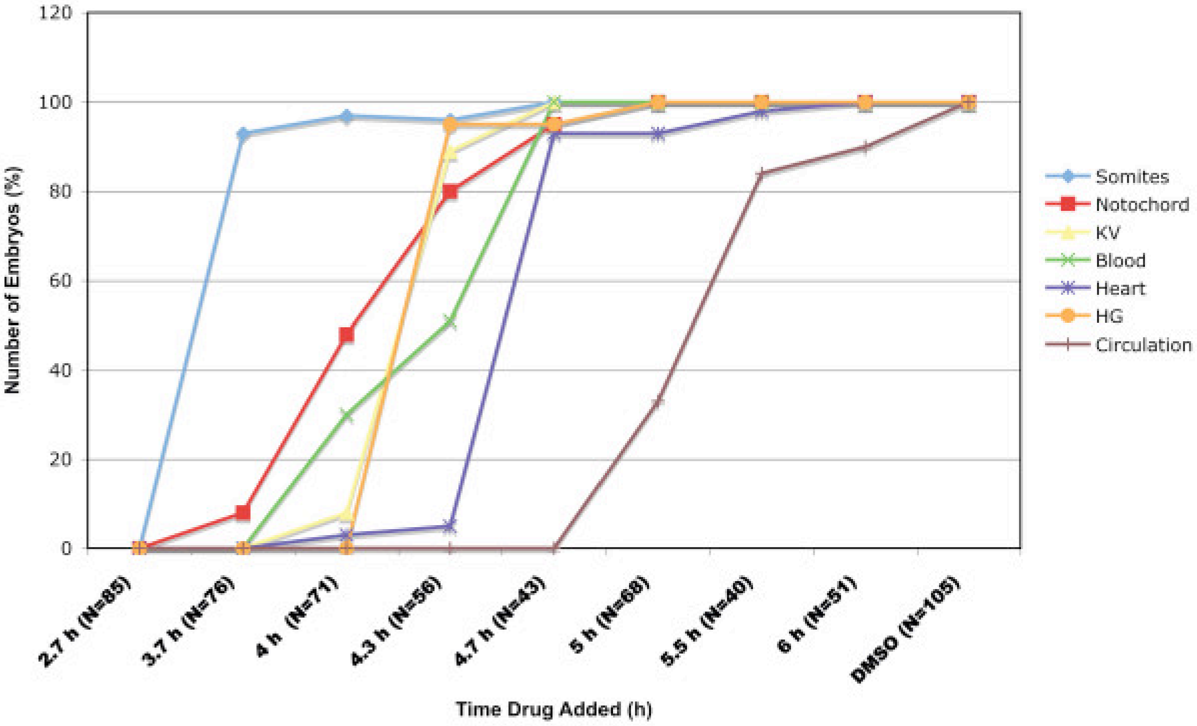

120
100
80
60
40
20
0
Number of Embryos (%)
Somites
Notochord
KV
Blood
Heart
HG
Circulation
2.7 h (N=85)
3.7 h (N=76)
4 h (N=71)
4.3 h (N=56)
4.7 h (N=43)
5 h (N=68)
5.5 h (N=40)
6 h (N=51)
DMSO (N=105)
Time Drug Added (h)

Figure 2.6. Nodal signals pattern the dorsal mesoderm and endoderm along the animal-vegetal axis in a time-dependent manner. Dorsal cell fates were examined in embryos treated with DMSO (A1-E1), or with SB-431542 at various time points. (A2-5) *cyp26* expression was expressed at the margin in embryos treated at MBT, but is expressed in more animal locations at later time points. (B2-5) *flh* is first detected in embryos treated at 3.7 h. (C2-5) *gsc* is first observed in embryos treated at 4.3 h (dome stage), but is expressed at normal levels in embryos treated after 5 h (40% epiboly). *mezzo* transcripts are observed in embryos treated after 5 h (40% epiboly) (D5), but not at earlier stages (D2-4). *sox17* is expressed in the dorsal forerunner cells in embryos treated 4.3 h (dome stage) (E4, arrowhead), but is first detected in endoderm progenitors in embryos treated at 5 h (40% epiboly) (E5). Lateral views of embryos at 10 h are depicted in A1-5, dorsal to the right. Dorsal views of embryos at 7 h (60% epiboly) (B1-C5), 5.5 h (germ ring) (D1-5) and 8 h (80% epiboly) (E1-5) are depicted. Arrowheads (E1, 4, 5) indicate *sox17* in dorsal forerunner cells. All embryos are siblings.

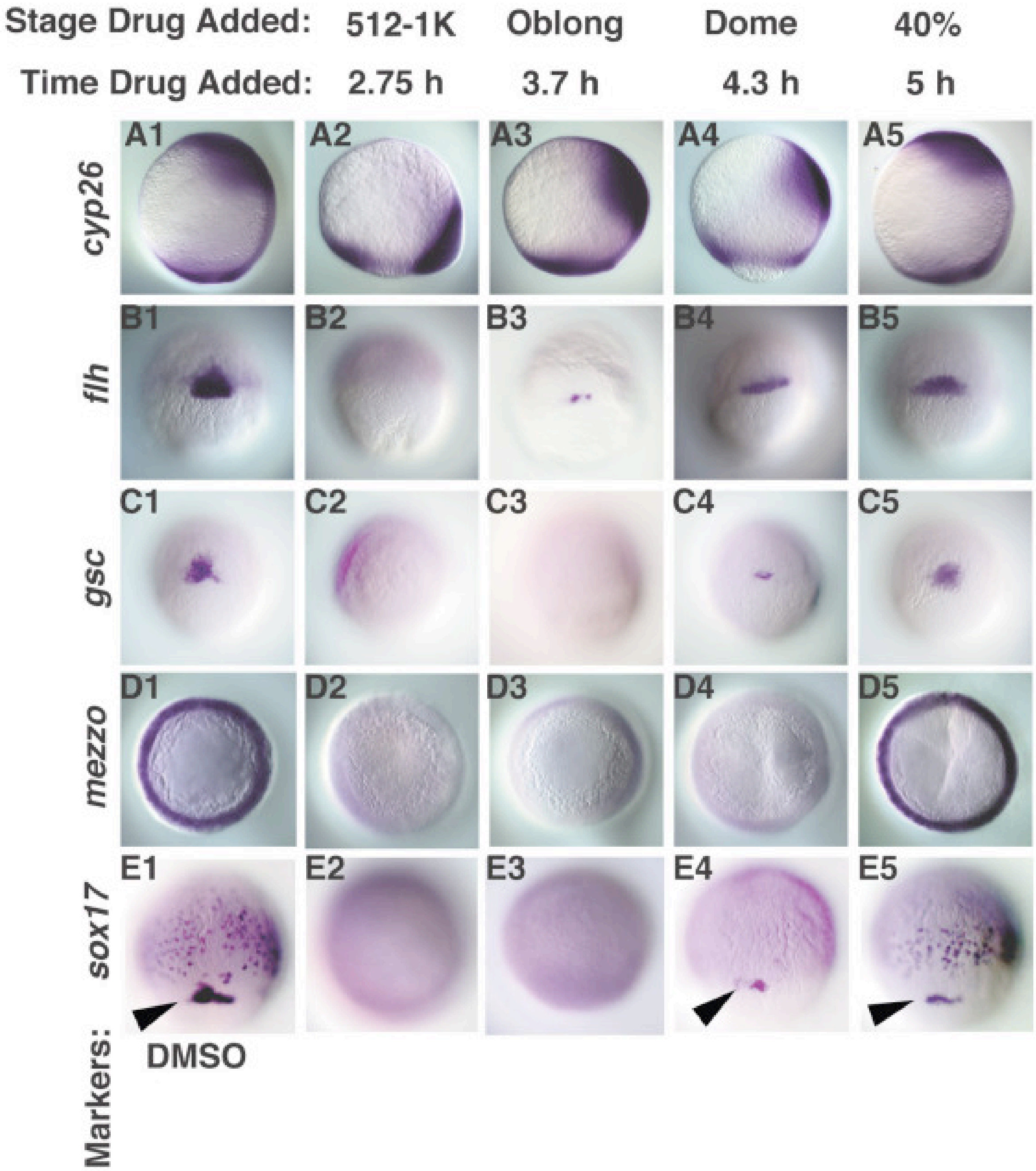

Stage Drug Added:
512-1K
Oblong
Dome
40%
Time Drug Added:
2.75 h
3.7 h
4.3 h
5 h
cyp26
A1
A2
A3
A4
A5
flh
B1
B2
B3
B4
B5
gsc
C1
C2
C3
C4
C5
mezzo
D1
D2
D3
D4
D5
sox17
E1
E2
E3
E4
E5
DMSO
Markers:

Figure 2.7. Nodal signals pattern the ventrolateral mesoderm along the animal-vegetal axis in a time-dependent manner. Heart myocardial cell fates were examined in embryos treated with DMSO (A1-C1), or with SB-431542 at various time points (A2-C5). (A2-C2) Embryos treated at 4 h, express small amounts of *amhc* and *cmlc2*, but not *vmhc* (arrows). (A3-3) *cmlc2*, *amhc* and *vmhc* are bilaterally expressed in embryos treated at 4.3 h. (A4-C5) All heart markers are expressed at the midline in embryos treated at 4.7 h. Images are dorsal views at 24 h, anterior to the left.

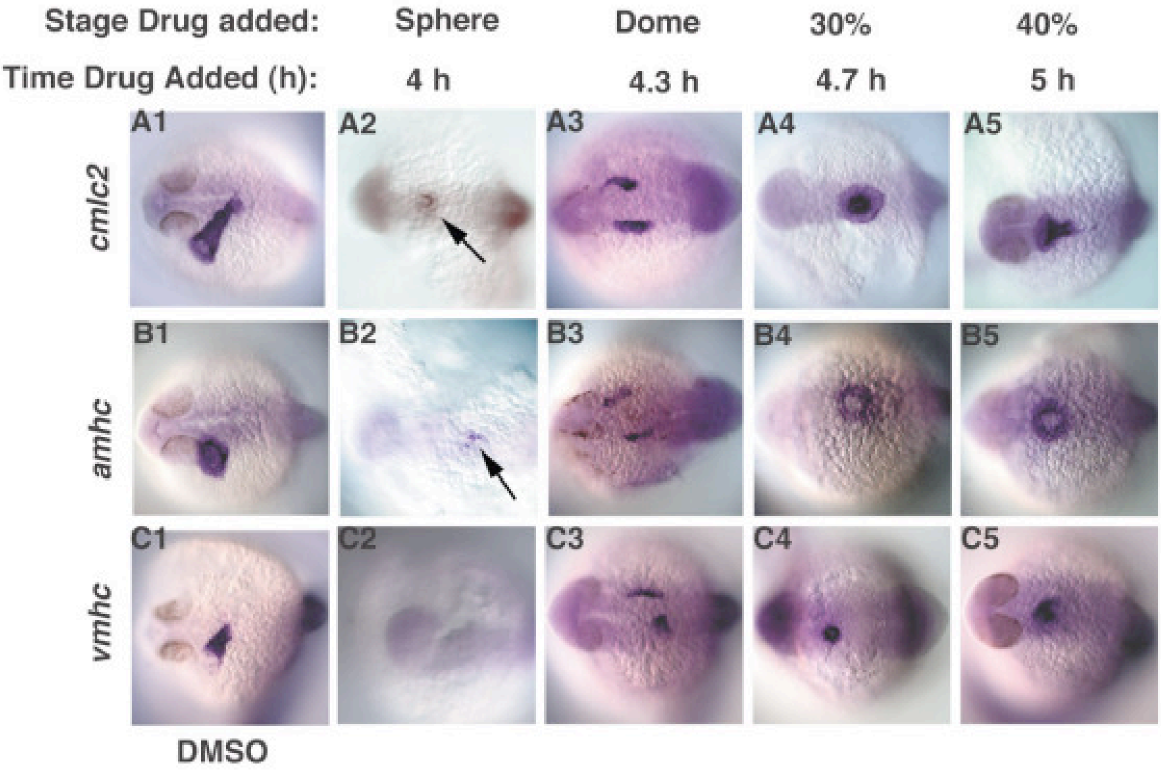

Stage Drug added:
Sphere
Dome
30%
40%
Time Drug Added (h):
4 h
4.3 h
4.7 h
5 h
A1
A2
A3
A4
A5
cmlc2
B1
B2
B3
B4
B5
amhc
C1
C2
C3
C4
C5
vmhc
DMSO

Figure 2.8. Cell fate specification is delayed *squint* mutants. Cell fates were examined in *sqt* mutant embryos treated with DMSO (A1-D1), or with SB-431542 at various time points. (A1-7) *flh* was first expressed at the midline in embryos treated at 5 h (A5). (B2-7) *gsc* expression is first detected in embryos treated at 6 h (B6). (C2-7) *sox17* expression is first detected when embryos are treated at 7 h (C7). (D1-7) *cmlc2* expression was first detected in embryos treated 4.7 h (D4, arrowhead). Dorsal views of 10 h (A1-B7), 8 h (C1-C7) or 24 h (D1-D6). In D1-D6, anterior is up. The embryos in Figs. 8 and 9 are from the same clutch and were treated in parallel, along with wild type controls (not shown).

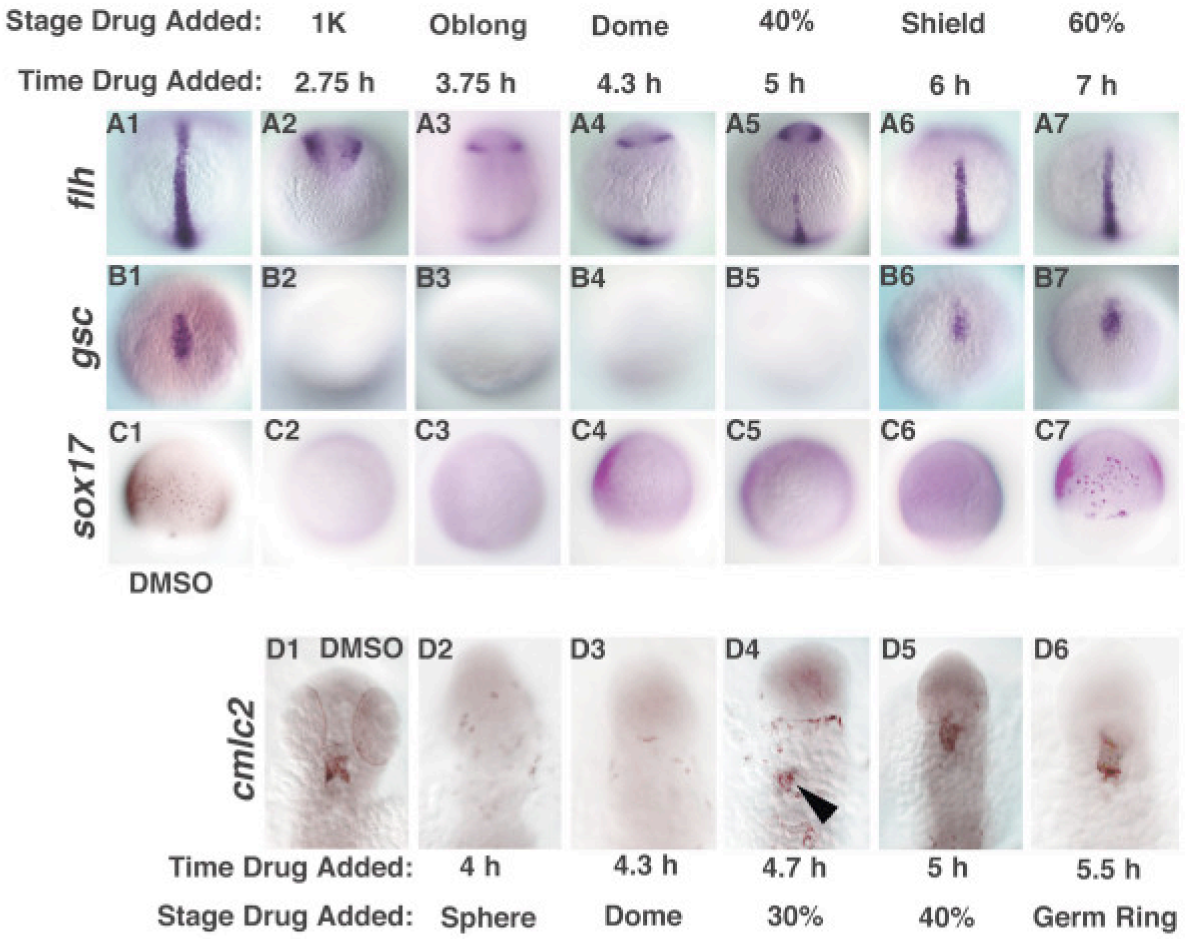

Stage Drug Added: 1K Oblong Dome 40% Shield 60%
Time Drug Added: 2.75 h 3.75 h 4.3 h 5 h 6 h 7 h
flh
A1 A2 A3 A4 A5 A6 A7
gsc
B1 B2 B3 B4 B5 B6 B7
sox17
C1 C2 C3 C4 C5 C6 C7
DMSO
cmlc2
D1 DMSO D2 D3 D4 D5 D6
Time Drug Added: 4 h 4.3 h 4.7 h 5 h 5.5 h
Stage Drug Added: Sphere Dome 30% 40% Germ Ring

Figure 2.9. Cell fate specification is accelerated when Nodal levels are increased. Embryos were injected with *sqt* mRNA at the 1-4 cell stage and treated with DMSO (A1-C1) or SB-431542 at various time points (A2-C7). Sqt induces ubiquitous expression of *gsc* and *sox17* (B1, C1) but not *flh* (A1). (A2-C2) SB-431542 treatment at MBT blocks expression of each of these markers. *flh* is strongly expressed in embryos treated at 3.7 h (A3), but fades at later time points (A4-7). *gsc* expression is first detected in embryos treated at 3.7 h (B3), and expands at later time points (B4-7). *sox17* expressing cells are first detected when embryos are treated at 4.3 h (C4), and expands at later time points (C5-7). Animal pole views at 10 h.

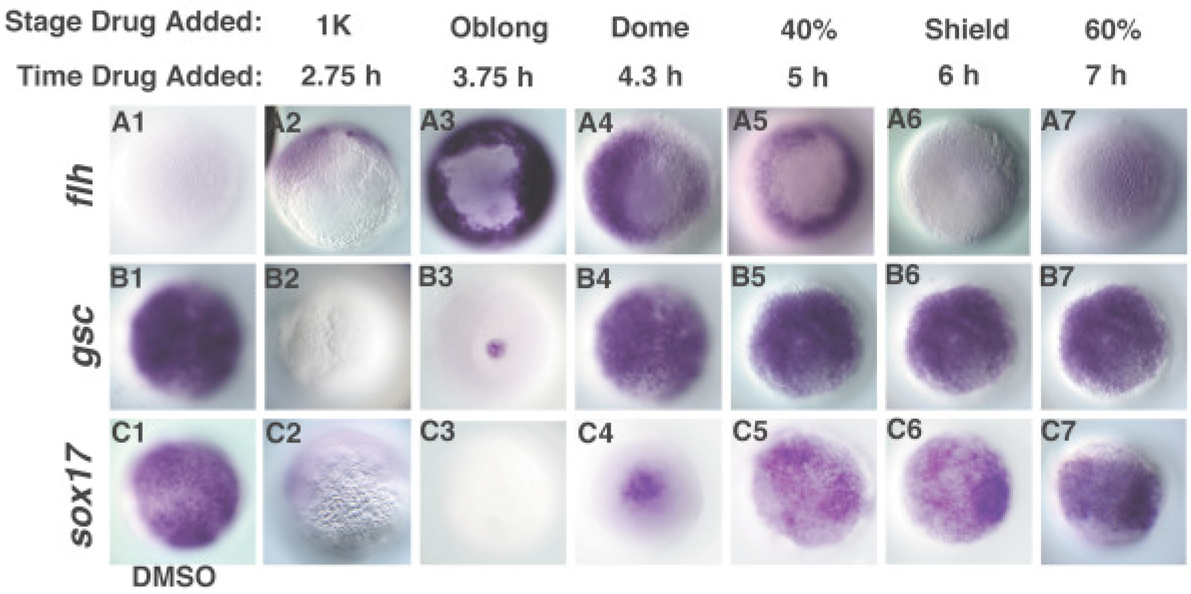
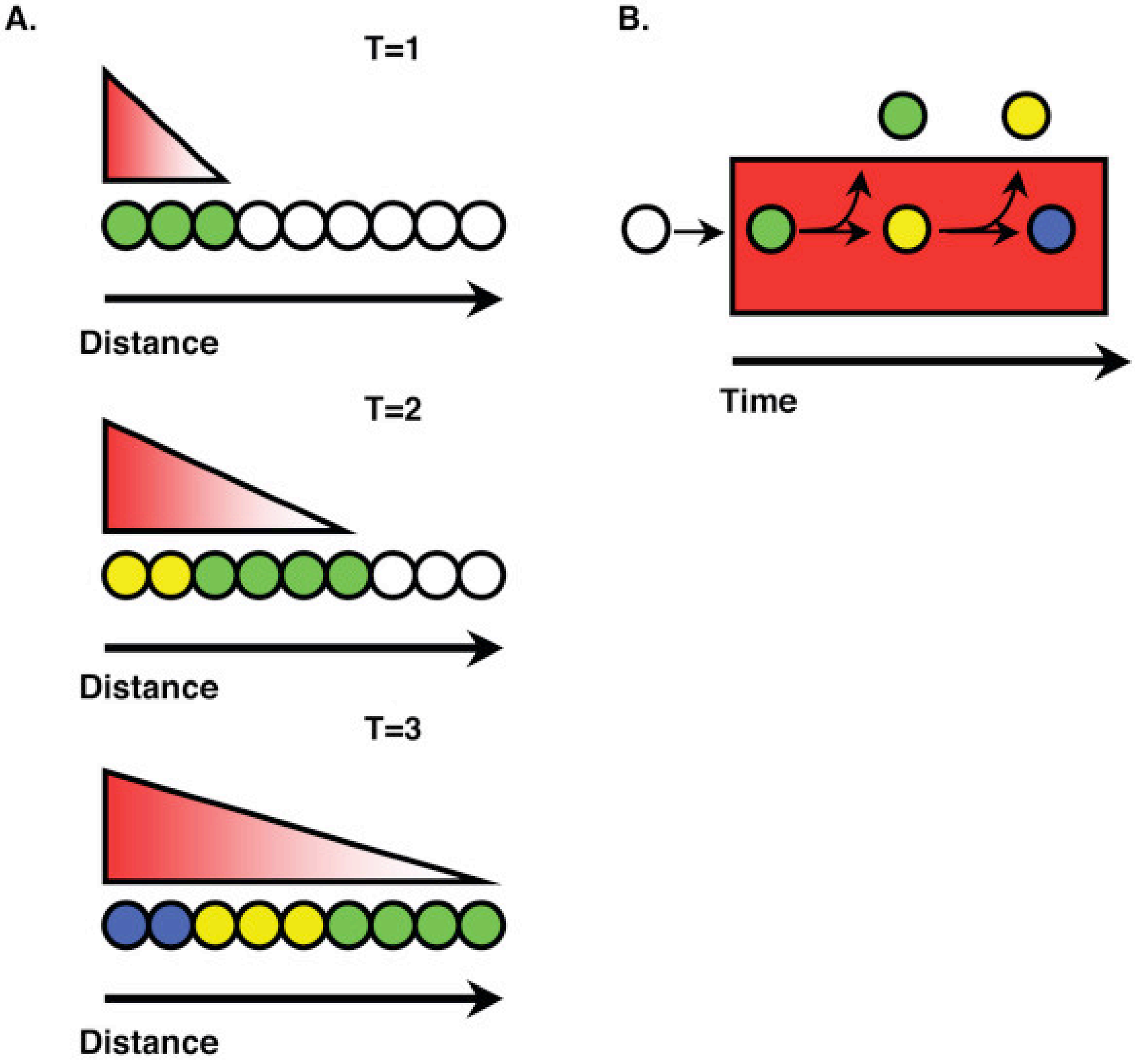

Stage Drug Added:
1K
Oblong
Dome
40%
Shield
60%
Time Drug Added:
2.75 h
3.75 h
4.3 h
5 h
6 h
7 h
flh
gsc
sox17
A1
A2
A3
A4
A5
A6
A7
B1
B2
B3
B4
B5
B6
B7
C1
C2
C3
C4
C5
C6
C7
DMSO

Figure 2.10. Models for forming a gradient of Nodal signals over time. In the ratchet model (A), the morphogen spreads through a tissue over time and cells receive different Nodal doses at different times. Cells respond to the highest dose to which they are exposed. In the spatio-temporal gradient model (B), cells freely move into (not depicted) and out of (arrows) a zone of signaling activity (red rectangle). A short residence time in this zone determines a low cumulative dose of Nodal signals (green circle), whereas longer residence times determine higher cumulative Nodal dose (yellow and blue circles).


A.
T=1
Distance
T=2
Distance
T=3
Distance
B.
Time

Chapter 3

THE ROLE OF NODAL SIGNALS IN THE YOLK SYNCYTIAL LAYER IN ZEBRAFISH

---

[1] Engda G Hagos and Scott T Dougan. This will comprise part of a manuscript to be submitted to the Proceedings of the National Academy of Sciences.

ABSTRACT

Mesoderm and endoderm (mesendoderm) induction and its dorsoventral specification are important process of vertebrate early development. *nodal-related* genes are required to form the germ layers, and specify different cell types with each germ layer. In zebrafish, signals from the extraembryonic tissue yolk syncytial layer (YSL) are both necessary and sufficient to induce and pattern the germ layers. However, the molecular nature of these signals is not known. Several genes involved in embryonic development are expressed in the YSL including *squint* (*sqt*). To test the requirement of *nodal-related* genes in this tissue, *sqt* and *cyc* morpholinos were co-injected into the yolk closest to the margin after the YSL is formed at 3hours post fertilization (hpf). I have analyzed zebrafish embryos lacking both *sqt* and *cyc* in the YSL. My results show that embryos lacking Nodal signals in the YSL lose endoderm and dorsal mesoderm. Furthermore, the expression of *sqt* and *cyc* in the blastoderm is greatly reduced. This indicates that *sqt* and *cyc* from the YSL are required to induce or maintain *nodal-related* genes in the blastoderm. This data suggests that YSL is the functional equivalent of anterior visceral endoderm of mouse.

INTRODUCTION

In vertebrates, *nodal-related* genes comprise a highly conserved subclass of the TGF-β superfamily of secreted proteins (Schier, 2003; Whitman and Mercola, 2001). Members of this gene family are required to instruct cells to adopt mesodermal, endodermal and ventral neural-ectoderm cell fates in all vertebrates (Conlon et al., 1994; Erter et al., 1998; Feldman et al., 1998; Joseph and Melton, 1997; Takahashi et al., 2000). In addition, they are required to pattern all three germ layers and to establish the left-right body axis (Feldman et al., 1998; Jones et al., 1995; Long et al., 2003; Rebagliati et al., 1998b; Sampath et al., 1998).

Genetic studies with mouse and zebrafish have demonstrated that Nodal signaling in whole embryos is required to pattern vertebrate embryos (Conlon et al., 1994; Feldman et al., 1998). The mouse embryo consists of epiblast, extraembryonic ectoderm and visceral endoderm (Rodriguez et al., 2005). The epiblast induces the anterior visceral endoderm (AVE) from the visceral endoderm (Brennan et al., 2001). The extraembryonic structures have been implicated in axis formation during mouse development (Beddington and Robertson, 1999). Embryological experiments indicate that signals from the extraembryonic AVE play critical roles in specification of anterior-posterior axis (Bielinska et al., 1999; Thomas and Beddington, 1996). In the mouse, surgical removal of AVE leads to reduce expression of anterior neural markers (Thomas and Beddington, 1996; Varlet et al., 1997). Additionally, a chimeric embryo approach has been used to show that Nodal expression in the AVE is necessary for normal development of forebrain (Varlet et al., 1997). In these experiments, they injected wildtype ES cells into *nodal-/-* blastocysts that give rise to chimeric embryos in which the visceral endoderm is composed of Nodal deficient cells resulting in embryos that fail to develop neural structures such as the forebrain. These results demonstrate that the AVE is required for normal formation of forebrain.

Gene expression studies have shown that the AVE expresses a specific set of genes that are required for embryonic patterning (Beddington and Robertson, 1999). Recent data on marker gene expression indicates that many marker genes including, *nodal* and homeobox gene *Hex* expressed in the mouse AVE have homologues that are similarly expressed in zebrafish extraembryonic yolk syncytial layer (YSL) (Beddington and Robertson, 1999; Ho et al., 1999). Nodal activity in the epiblast is required for specification of the AVE (Brennan et al., 2001).

Like the AVE, an extraembryonic yolk syncytial layer (YSL) performs a pivotal role during zebrafish embryogenesis to pattern the embryo.

The YSL forms at the mid-blastula transition (3hpf) when marginal blastomeres fuse with each other and release their contents into the yolk (Kimmel and Law, 1985). In zebrafish, YSL plays important roles in mesoderm and endoderm (mesendoderm) induction (Chen and Kimelman, 2000; Mizuno, 1996). When the yolk, including the YSL, was transplanted onto the animal-pole region of host blastula embryos, it induced ectopic mesoderm and endoderm in the host animal pole (Mizuno, 1996). Furthermore, mesendoderm fails to form when signals from the yolk are depleted by RNAse injection, indicating that these signals are essential for normal development (Chen and Kimelman, 2000). Collectively, these results demonstrate that YSL is an important source of patterning signals, however, little is known about the molecular nature of these signals. In the present study, I establish the essential role of the extraembryonic tissue YSL, in patterning the zebrafish embryo.

The zebrafish *nodal-related* genes, *sqt* and *cyc* are co-expressed in all marginal cells at 5hpf with *sqt* being expressed in the YSL (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a). Overexpression of *sqt* in the YSL of wildtype embryos is sufficient to induce dorsal mesoderm in the overlying blastomeres (Feldman et al., 1998), suggesting an involvement of extraembryonic tissue in patterning of the zebrafish embryo. However, it is not known if *sqt* is required in YSL.

sqt and *cyc* have overlapping roles for mesoderm formation. Defects in *sqt* and *cyc* single mutants are confined to dorsal axial structures at 24h, including prechordal plate mesoderm and ventral neural ectoderm (Feldman et al., 1998; Sampath et al., 1998). In *sqt* mutant, embryos have defects in early dorsal mesoderm formation as noted by the absence of the zebrafish organizer. In contrast to *sqt* mutants, *cyc* mutants have no defect in early mesoderm formation but lack a floorplate, and the ventral neural tube (Feldman et al., 1998; Sampath et al., 1998). However, *sqt; cyc* double mutants lack all derivatives of the mesoderm and endoderm in the head and trunk, including the blood, heart, pronephros, skeletal muscle and gut (Feldman et al., 1998).

To test whether *nodal-related* genes are required in the YSL, I examined cell fates in embryos lacking *sqt* and *cyc* function using endodermal (*sox17* and *mezzo*), mesodermal (g*sc, flh*, *ntl*, *pax2.1, cmcl2* and *myoD*) and expression of *sqt* and *cyc* markers. I show that embryos co-injected with *sqt;cyc*- morpholino oligonucleotides (MO) lack marginal cell types closest to the yolk such as endoderm and dorsal mesoderm. Furthermore, the expression of *sqt* and *cyc* in the YSL is greatly reduced. This suggests that *sqt* and *cyc* from the YSL induce and/or maintain *nodal-related* genes in the blastoderm to pattern the embryo.

MATERIALS AND METHODS

Zebrafish strain and genotype analyses

Wild-type embryos were obtained from natural crosses of Wik fish. Collected embryos were maintained at 28.5°C and staged according to morphological criteria (Kimmel et al., 1995). *sqt* and *cyc* were obtained after crossing fish heterozygous for sqt^{cz35} and cyc^{m294} mutation respectively. The genotype analysis for *sqt* and *cyc* alleles was determined by PCR as previously described (Feldman et al., 1998; Sampath et al., 1998).

In situ hybridization

Whole-mount *in situ* hybridization was performed using digoxigenin labeled antisense RNA as previously describe (Dougan et al., 2003). *In situ* probes used: *flh* (Talbot et al., 1995), *ntl* (Schulte-Merker et al., 1994), *gsc* (Stachel et al., 1993), *myo-D* (Weinberg et al., 1996), *pax2.1* (Krauss et al., 1992), *clcm2* (Yelon et al., 1999), *mezzo* (alPoulain and Lepage, 2002), *sox17* (Alexander and Stainier, 1999), *sqt* (Erter et al., 1998), *cyc* (Rebagliati et al., 1998b).

Injection of antisense morpholino oligonucleotides (MO) into the YSL

I used *sqt*-MO and *cyc*-MO tagged with rhodamine (RH) and fluorescent (FL) respectively. All MOs were dissolved in distilled water at a concentration of 30mg/ml and stored at –20^0C. The working for each MO was diluted to 8ng in 0.2M KCL prior to injection. Phenol red was added to the solution in order to trace the injection. To rule out the possibility that the phenotype was not nonspecific, I designed 5 base pairs mismatched (mis) MOs for each gene. Embryos were co-injected with 8ng of each MO into the yolk cell, near the margin, after YSL forms at 3hours post fertilization. Injected embryos were monitored under epifluorescent microscope two hours after injection. Embryos with weak fluorescence, with MOs in the vegetal yolk or MOs located in the blastomeres were discarded. As a control, both mismatched or either mismatched with *sqt*-MO or *cyc*-MO were injected at the same concentration. Furthermore, to examine the function *sqt* alone in the YSL embryos were injected with a mixture of 8ng of functional *sqt*-MO and 8ng of mismatched *cyc*-MO. A mixture of 8ng of functional *cyc*-MO and 8ng of mismatched *sqt*-MO were also injected to determine the function of *cyc* in the YSL. Cell fates were assessed in live embryos and by *in situ* hybridization of different marker genes.
Four different combination of antisense morpholino (MOs) were obtained from (Gene Tool, Inc., Philomath, OR) for *sqt*, *cyc* and their mismatched controls were designed as follow. Lower case letters indicates change in *sqt* and *cyc* MOs sequence.

Sqt MO-RH: 5’-ATGTCAAATCAAGGTAATAATCCAC-3’

Sqt MO-mis: 5’-ATcTgAAAATgAAGcTAATAATgCAC-3’

Cyc MO-FL: 5’-GCGACTCCGAGCGTGTGCATGATG-3’

Cyc-MO-mis: 5'-GCcACTgCGAGaGTGTGgATcATG-3'

RESULTS

Morpholino injection targets *sqt* and *cyc* in the YSL

To knockdown extraembryonic *nodal-related* gene function in the YSL I used previously described morpholino oligonucleotides (MOs) (Feldman and Stemple, 2001; Karlen and Rebagliati, 2001). To follow localization of morpholino in the embryos I used a fluorescein-tagged *sqt*-MO and a rhodamine-tagged *cyc*-MO. I injected *sqt;cyc*-MOs into zebrafish embryos at 1-cell stage (0.75hpf) and into the YSL at 1,000-cell stage (3hpf). In embryos injected into the margin closest to the yolk at 3hpf, the signal was restricted to only the YSL (Fig. 3.1D-F) and do not diffuse into the embryonic cells even 24h post-injection (data not shown). When I injected the same morpholino at 0.75hpf, the fluorescein and rhodamine signal was distributed throughout the entire embryo but excluded from yolk (Fig. 3.1A-C). These experiments demonstrate that injection of MO into the YSL could be an effective method to knockdown *sq*t and *cyc* function in the YSL.

sqt and *cyc* are required in the YSL for mesoderm and endoderm formation

To investigate the function of Nodal signals in the YSL, I examined phenotype of embryos lacking *sqt* and *cyc* in the YSL. I found that embryos injected with both *sqt*;*cyc*-MOs at 3hpf fail to form the organizer. At 24 hpf these embryos show severe cyclopia and have defects in dorsal mesoderm such as the prechordal plate and heart (N=28/35; Fig. 3.2J-L). By contrast, embryos injected with the control *sqt;cyc* mismatch-MOs into the YSL do not display any abnormal phenotype (N=10/10; Fig.3.2A-C). These results suggest that *sqt* and *cyc* in the YSL are required for the formation of the organizer and normal development. Since *sqt* and *cyc* have overlapping roles in mesoderm and endoderm formation (Feldman et al., 1998), I asked if the two genes also have overlapping functions in the YSL. Embryos injected with *sqt*-MO alone showed reduced embryonic shield but exhibited normal morphology at 24hpf (N=21/30; 3.2D-F). Knocking down *cyc* function in the YSL did not affect embryonic patterning (N=26/26; 3.2G-I). By contrast, simultaneously knocking down both *sqt* and *cyc* function in the YSL causes severe defects, including the elimination or reduction of derivatives of endoderm and dorsal mesoderm including the prechordal plate, notochord and heart (N=28/35; Fig. 3.2J-L; Fig. 3.3). These results indicate s*qt* and *cyc* have overlapping function in the YSL and are able to compensate for each other during early development for proper formation of the germ layers.

To determine if mesoderm and endoderm formation is affected in *sqt*;*cyc*-MOs injected embryos in the YSL, I examined cell fates by an extensive analysis of gene expression. Loss of *sqt* and *cyc* from the YSL disrupts prechordal plate formation as indicated by lack of *goosecoid (gsc*) expression (N=20/28; Fig. 3.3A,B). Endoderm markers, *mezzo* (N=16/16) and *sox17* (N=21/47) are absent or severely reduced upon *sqt*;*cyc*-MO injection (Fig. 3.3G-J). These embryos display a dorsal gap similar to *sqt;cyc* double mutants, in *no-tail (ntl)* expression, a pan-mesendodermal marker (N=5/24; Fig. 3.3F) (Feldman et al., 1998). I also show that expression of several tissue specific mesodermal markers such as, *floating head (flh)* that marks the notochord (N=29/29; Fig. 3.3D); *myo-D,* (11/11; Fig. 3.3N) which marks the developing somite, *pax2.1* (N=7/7; Fig. 3.3L) in the pronephros, and *cmlc2* (N=10/16; Fig. 3.5P) in differentiating heart muscle are reduced compare to control mismatch-MO (Fig. 3.3C,K,M,O). These results indicate that *sqt* and *cyc* in the YSL are required for the formation of derivatives of endoderm and dorsal mesoderm, including prechordal plate, notochord, heart and somites.

Nodal signals from the YSL is required to maintain *sqt* and *cyc* in the blastomeres

It has been shown that Nodal signals are autoregulated and are able to turn on their own expression in the blastoderm (Meno et al., 1999; Schier, 2003). Therefore to investigate whether *sqt* and *cyc* in the YSL are required to maintain *nodal-related* genes in the blastoderm, I analyzed the expression of both *sqt* and *cyc* in the blastomeres at 5hpf in *sqt*;*cyc*-MOs injected embryos (Fig. 3.4). The ventral-lateral expression of *sqt* (N=15/30) and *cyc* (N=7/17) is severely reduced in *sqt* and *cyc* MOs compared to the control injected embryos (N=22/22; Fig. 3.4A-D) and the dorsal expression of both genes is eliminated (N=10/35; Fig. 3.4B, D). Taking together these results, it appears that the maintenance of *nodal* expression in the blastomeres is partially mediated by Nodal signaling from the YSL.

DISCUSSION

My data indicates that *sqt* and *cyc* from the YSL regulates *nodal-related* genes in the blastoderm for proper formation of mesoderm and endoderm. I have also shown that Nodal signals from the YSL are required for the organizer induction.

MOs injection at 1000-cell stage specifically targets *sqt* and *cyc* in the YSL

To generate *nodal* loss of function in the YSL we used previously designed MOs against the initiation of translation of *sqt* and *cyc* genes and co-injected them into the margin just after the YSL forms at 3hpf (Feldman and Stemple, 2001; Karlen and Rebagliati, 2001). The MO localized at the margin in embryos injected with both *sqt*;*cyc*-MOs at 3hpf into the YSL at 5hpf and 24hpf (Fig. 3.1D-F; data not shown). These results demonstrate that the MOs injected into YSL at 3hpf are not transferred from the YSL to overlying blastomeres but instead are efficiently targeted and specifically knockdown *sqt* and *cyc* function in the YSL. I have presented evidence that injection of *sqt;cyc* MOs are specifically knocking down *nodal* genes in the YSL because injection of a 5 bp mismatches *sqt;cyc* MOs did not cause any phenotype when injected into the YSL (Fig. 3.2; 3.3).

Nodal signals from the YSL are required to regulate *nodal* genes in the blastoderm

Expression of both *sqt* and *cyc* is maintained by an autoregulation feedback loop through the winged helix transcription factor, FoxH1 (Meno et al., 1999; Schier, 2001). *sqt* expression is normal in *cyc* mutant but expression of *cyc* in dorsal cells is dependent upon *sqt* function (Dougan et al., 2003). To investigate if the expression of both *sqt* and *cyc* in the blastomeres is dependent upon the *nodal* genes in the YSL, I examined the expression of *sqt* and *cyc* in the blastomeres. I asked whether loss of *nodal* genes in the YSL alters the expression of *sqt* and *cyc* in the blastomeres. When Nodal signals were blocked in the YSL by injecting *sqt;cyc*-MOs, the expression of *sqt* and *cyc* was severely reduced in the blastomeres (Fig. 3.4B-D). By contrast, none of the control mismatch-MO-injected embryos exhibited reduction in *sqt* and *cyc* expression in the blastomeres (Fig. 3.4A, C). These results provide convincing support for the idea that *nodal* signals from the YSL are necessary to maintain *sqt* and *cyc* in the blastoderm for normal development.

Genetic studies in zebrafish have revealed that Nodal signal transduction requires One eye pinhead (Oep), an EGF-CFC, as co-receptor (Gritsman et al., 1999; Shen and Schier, 2000).

Like *sqt;cyc* double mutants, embryos lacking both maternal and zygotic *oep* (MZ*oep*) function lack all endoderm and mesoderm in the head and trunk (Feldman et al., 1998; Schier, 2003). To determine if the YSL is receiving signals from the blastoderm, we collaborated with (Burdine, Rebecca lab from Princeton University). As part of collaborative effort, they injected *oep*-MO into the YSL to knockdown Oep function and I analyzed *sqt* and *cyc* expression at 5hpf (unpublished data). I found that the expression of *nodal-related* genes is reduced in embryos injected with *oep*-MO compared to control embryos (data not shown). This indicates that the YSL has the ability to respond to signals from the blastomeres.

The YSL of zebrafish might have equivalent function to visceral endoderm of mouse

Previous studies have proposed that the extraembryonic tissue visceral endoderm in mice has similar function like the zebrafish extraembryonic yolk syncytial layer (Beddington and Robertson, 1998; Ho et al., 1999). Nodal function is required in the epiblast for proper formation and patterning of the anterior visceral endoderm (AVE) (Brennan et al., 2001). The AVE, in turn, is required for specification of anterior structures such as neural tissue in the overlying epiblast cells (Beddington and Robertson, 1999). Consequently, embryos lacking *nodal* function fail to specify the anterior visceral endoderm (AVE) (Brennan et al., 2001; Varlet et al., 1997). These results indicate that reciprocal cell-cell interaction is necessary between the epiblast and the extraembryonic tissues for proper formation of the anterior-posterior polarity (Brennan et al., 2001). Collectively, these studies have demonstrated that the important role of extraembryonic tissue in patterning the developing embryo. To further support this idea, I found that the extraembryonic tissue, YSL in zebrafish has similar function as the anterior visceral endoderm of mice in patterning the embryo.

The results present here support the proposed hypothesis that the AVE and YSL, extra embryonic tissues have similar function in early development to pattern the developing embryos based on the following three experimental results. First like AVE in mice, YSL is required to pattern the zebrafish embryo. I found that eliminating extraembryonic *nodal* gene function causes severe defects including the elimination or reduction of derivatives of endoderm or dorsal mesoderm, such as prechordal plate (Fig.3.2, 3). Second, I showed that Nodal signals from the extraembryonic tissue are required to maintain *nodal* genes in the blastoderm (Fig. 3.4). Third, similar to the AVE that received signals from the embryo proper (Beddington and Robertson, 1999) the *oep*-MO experiment suggests that there is reciprocal interaction between the YSL and blastomeres. My data, in combination with other results, suggest that the YSL is structural and functional equivalent of the AVE in mouse.

REFERENCES

Alexander, J., and D.Y. Stainier. 1999. A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol*. 9:1147-57.

Beddington, R.S., and E.J. Robertson. 1998. Anterior patterning in mouse. *Trends Genet*. 14:277-84.

Beddington, R.S., and E.J. Robertson. 1999. Axis development and early asymmetry in mammals. *Cell*. 96:195-209.

Bielinska, M., N. Narita, and D.B. Wilson. 1999. Distinct roles for visceral endoderm during embryonic mouse development. *Int J Dev Biol*. 43:183-205.

Brennan, J., C.C. Lu, D.P. Norris, T.A. Rodriguez, R.S. Beddington, and E.J. Robertson. 2001. Nodal signalling in the epiblast patterns the early mouse embryo. *Nature*. 411:965-9.

Chen, S., and D. Kimelman. 2000. The role of the yolk syncytial layer in germ layer patterning in zebrafish. *Development*. 127:4681-9.

Conlon, F.L., K.M. Lyons, N. Takaesu, K.S. Barth, A. Kispert, B. Herrmann, and E.J. Robertson. 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development*. 120:1919-28.

Dougan, S.T., R.M. Warga, D.A. Kane, A.F. Schier, and W.S. Talbot. 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development*. 130:1837-51.

Erter, C.E., L. Solnica-Krezel, and C.V. Wright. 1998. Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev Biol*. 204:361-72.

Feldman, B., M.A. Gates, E.S. Egan, S.T. Dougan, G. Rennebeck, H.I. Sirotkin, A.F. Schier, and W.S. Talbot. 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature*. 395:181-5.

Feldman, B., and D.L. Stemple. 2001. Morpholino phenocopies of sqt, oep, and ntl mutations. *Genesis*. 30:175-7.

Gritsman, K., J. Zhang, S. Cheng, E. Heckscher, W.S. Talbot, and A.F. Schier. 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell*. 97:121-32.

Ho, C.Y., C. Houart, S.W. Wilson, and D.Y. Stainier. 1999. A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr Biol*. 9:1131-4.

Jones, C.M., M.R. Kuehn, B.L. Hogan, J.C. Smith, and C.V. Wright. 1995. Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development*. 121:3651-62.

Joseph, E.M., and D.A. Melton. 1997. Xnr4: a Xenopus nodal-related gene expressed in the Spemann organizer. *Dev Biol*. 184:367-72.

Karlen, S., and M. Rebagliati. 2001. A morpholino phenocopy of the cyclops mutation. *Genesis*. 30:126-8.

Kimmel, C.B., and R.D. Law. 1985. Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. *Dev Biol*. 108:78-85.

Krauss, S., M. Maden, N. Holder, and S.W. Wilson. 1992. Zebrafish pax[b] is involved in the formation of the midbrain-hindbrain boundary. *Nature*. 360:87-9.

Long, S., N. Ahmad, and M. Rebagliati. 2003. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development*. 130:2303-16.

Meno, C., K. Gritsman, S. Ohishi, Y. Ohfuji, E. Heckscher, K. Mochida, A. Shimono, H. Kondoh, W.S. Talbot, E.J. Robertson, A.F. Schier, and H. Hamada. 1999. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell*. 4:287-98.

Mizuno, T., Yamaha, E., Wakahara, M., Kuroiwa, A., Takede., H., . 1996. Mesoderm induction in zebrafish. *Nature*. 383:2.

Poulain, M., and T. Lepage. 2002. Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. *Development*. 129:4901-14.

Rebagliati, M.R., R. Toyama, C. Fricke, P. Haffter, and I.B. Dawid. 1998a. Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev Biol*. 199:261-72.

Rebagliati, M.R., R. Toyama, P. Haffter, and I.B. Dawid. 1998b. cyclops encodes a nodal-related factor involved in midline signaling. *Proc Natl Acad Sci U S A*. 95:9932-7.

Rodriguez, T.A., S. Srinivas, M.P. Clements, J.C. Smith, and R.S. Beddington. 2005. Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. *Development*. 132:2513-20.

Sampath, K., A.L. Rubinstein, A.M. Cheng, J.O. Liang, K. Fekany, L. Solnica-Krezel, V. Korzh, M.E. Halpern, and C.V. Wright. 1998. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature*. 395:185-9.

Schier, A.F. 2001. Axis formation and patterning in zebrafish. *Curr Opin Genet Dev*. 11:393-404.

Schier, A.F. 2003. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol*. 19:589-621.

Schulte-Merker, S., F.J. van Eeden, M.E. Halpern, C.B. Kimmel, and C. Nusslein-Volhard. 1994. no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development*. 120:1009-15.

Shen, M.M., and A.F. Schier. 2000. The EGF-CFC gene family in vertebrate development. *Trends Genet*. 16:303-9.

Stachel, S.E., D.J. Grunwald, and P.Z. Myers. 1993. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development*. 117:1261-74.

Takahashi, S., C. Yokota, K. Takano, K. Tanegashima, Y. Onuma, J. Goto, and M. Asashima. 2000. Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. *Development*. 127:5319-29.

Talbot, W.S., B. Trevarrow, M.E. Halpern, A.E. Melby, G. Farr, J.H. Postlethwait, T. Jowett, C.B. Kimmel, and D. Kimelman. 1995. A homeobox gene essential for zebrafish notochord development. *Nature*. 378:150-7.

Thomas, P., and R. Beddington. 1996. Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr Biol*. 6:1487-96.

Varlet, I., J. Collignon, and E.J. Robertson. 1997. nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development*. 124:1033-44.

Weinberg, E.S., M.L. Allende, C.S. Kelly, A. Abdelhamid, T. Murakami, P. Andermann, O.G. Doerre, D.J. Grunwald, and B. Riggleman. 1996. Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development*. 122:271-80.

Whitman, M., and M. Mercola. 2001. TGF-beta superfamily signaling and left-right asymmetry. *Sci STKE*. 2001:RE1.

Yelon, D., S.A. Horne, and D.Y. Stainier. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev Biol*. 214:23-37.

FIGURE LEGENDS

Figure 3.1. Morpholino injection targets *sqt* and *cyc* in the YSL. (A,D) rhodamine-tagged *sqt*-MO; (B,E) fluorescent-tagged *cyc*-MO; (C,F) overlap of both MOs. (A,B,C) MO injected at 1 to 4-cell stage showed MO is in the blastomere but not in the yolk. (D,E,F) showed localization of MO in the YSL two hours post injection. Rhodamine-tagged *sqt*-MO and fluorescent-tagged *cyc*-MO are efficiently targeted signals in the YSL and do not diffuse to blastomere.

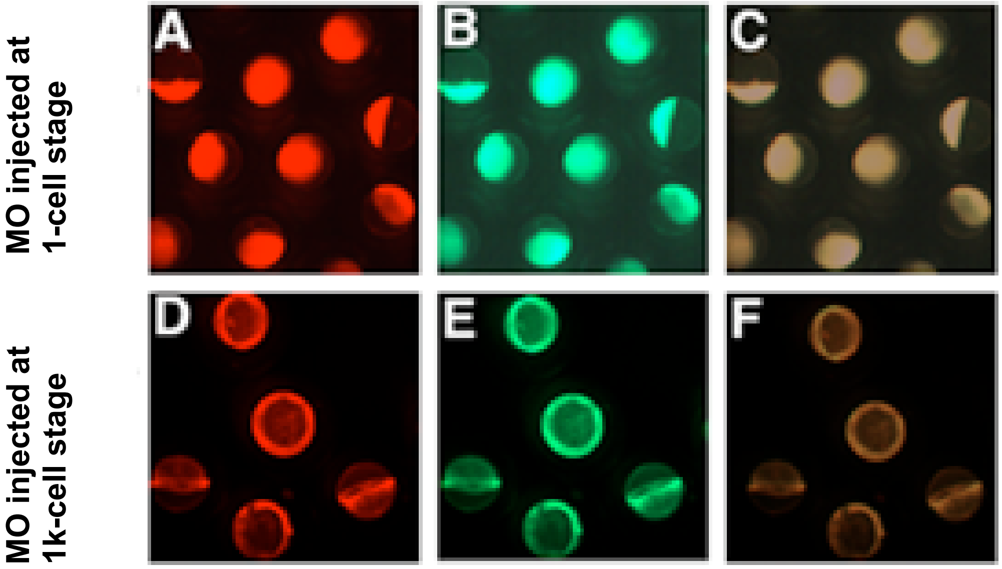

MO injected at 1-cell stage
MO injected at 1k-cell stage
A
B
C
D
E
F

Figure 3.2. Nodal signals in the YSL are required to pattern the embryo. (A-D) Animal view, of live embryos at 6hpf, dorsal is to the right (E-H) later view of live embryos at 24 hpf, anterior is to the left and dorsal up. The four rows represent embryos that were injected with control mismatch-MOs, *sqt*-MO-*cyc*-mismatch, *cyc*-MO-*sqt*-mismatch and *sqt* and *cyc*-MOs. The embryos develop normal at 24 hpf and the embryonic shield is formed at the future dorsal side of control and embryos injected with either morpholinos (A-F), whereas in these embryos injected with sqt and cyc morpholino lack the embryonic shield and have defect in dorsal mesoderm (G,H).

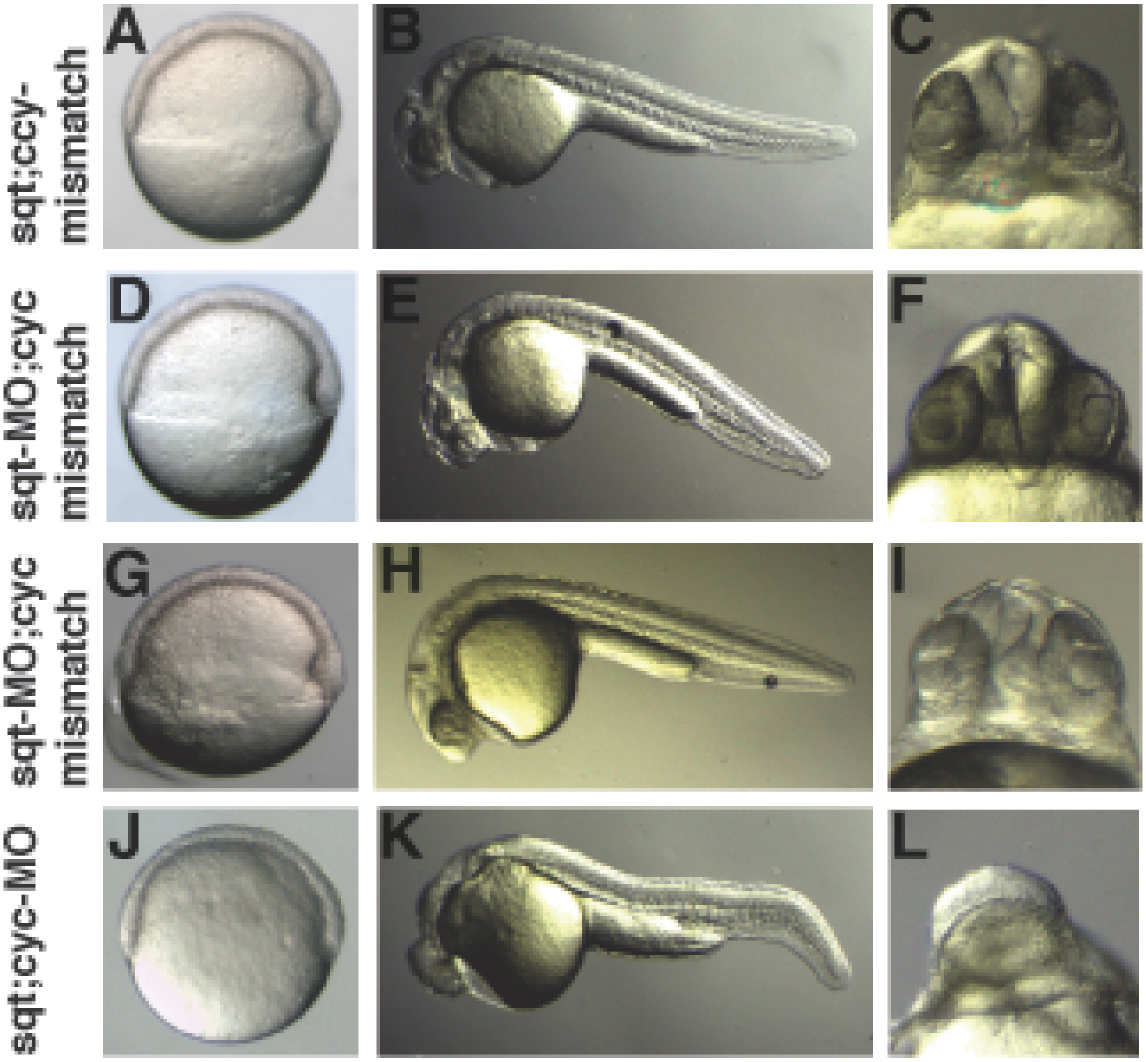

sqt;ccy-
mismatch
sqt-MO;cyc
mismatch
sqt-MO;cyc
mismatch
sqt;cyc-MO
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.3. Analysis of markers for endoderm and mesoderm in *sqt;cyc*-MOs. Endoderm and mesoderm cell fates were examined in embryos injected with *sqt* and *cyc*-MOs (B,D,F,H,J,L,N,P), or control mismatched-MOs (A,C,E,G,I,K,M,O). (O) normal expression of *sox17* and (P)only dorsal forerunner cells are expressed (whitehead). *mezzo* (C,D), *cmlc2* (E,F), *pax2.1* (I,J) are not expressed in embryos injected with *sqt* and *cyc*-MOs. (C,D) *ntl* expression is missing from dorsal side (arrowhead). Embryos injected with *sqt* and *cyc*-MOs lack *flh* and *myo-D* expression (A,B;G,H). Animal view (A,B,C,D,K,L,M,N), dorsal to the right. Dorsal view at 8hpf (O,P), 14hpf (G,H,I,J). Lateral view of embryos at 24hpf (E,F).

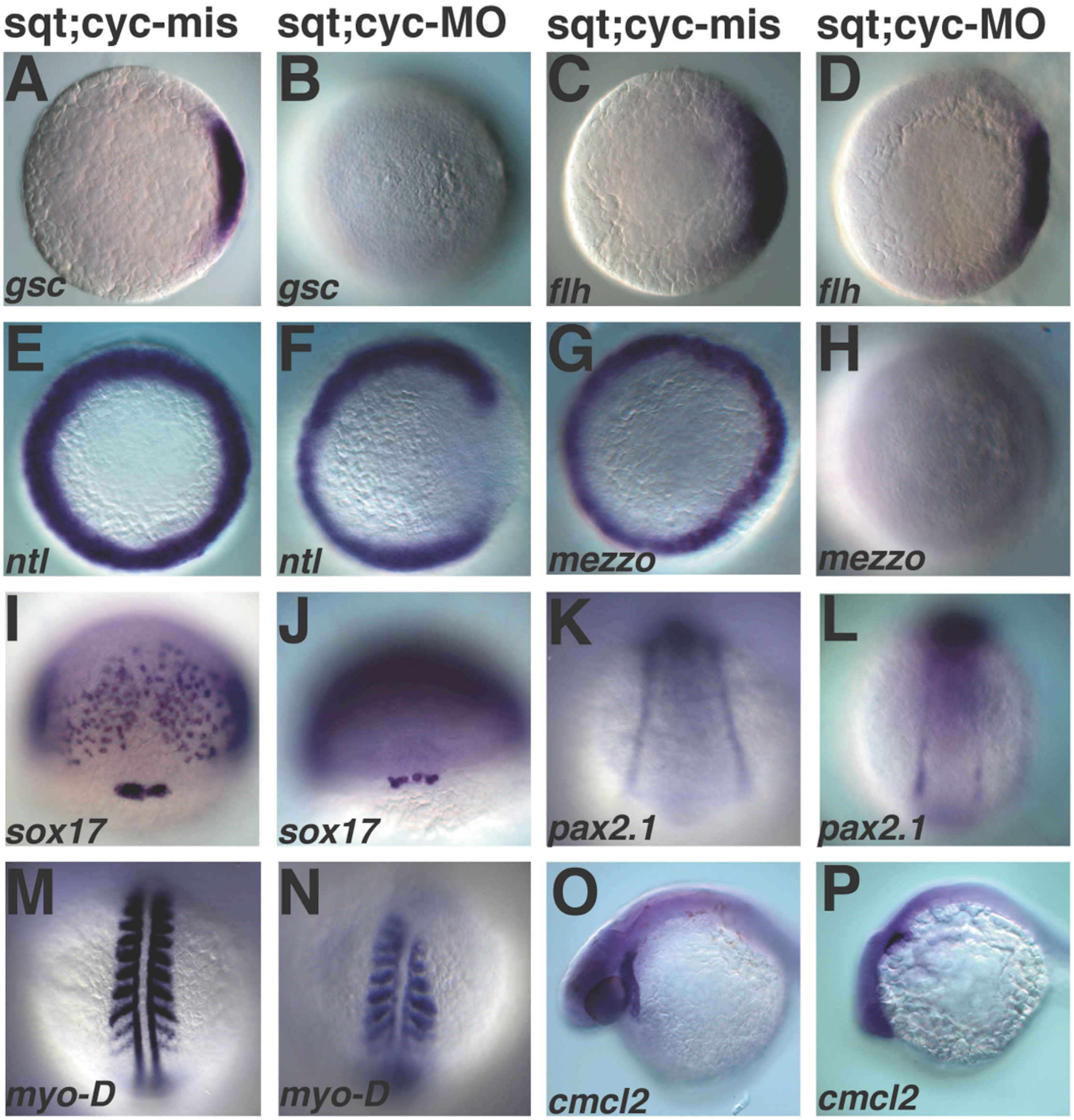
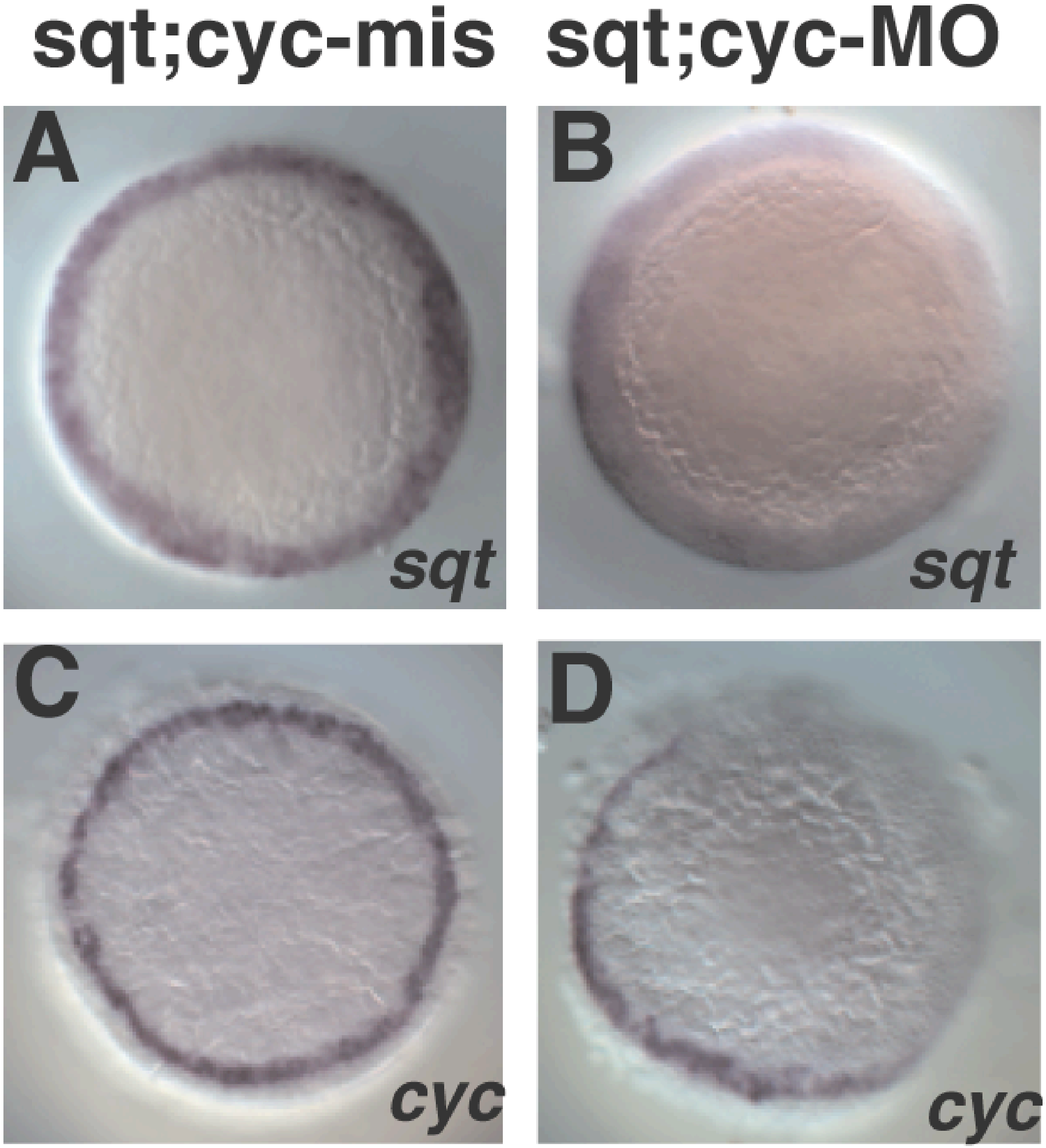

sqt;cyc-mis
sqt;cyc-MO
sqt;cyc-mis
sqt;cyc-MO
A
gsc
B
gsc
C
flh
D
flh
E
ntl
F
ntl
G
mezzo
H
mezzo
I
sox17
J
sox17
K
pax2.1
L
pax2.1
M
myo-D
N
myo-D
O
cmcl2
P
cmcl2

Figure 3.4. Analysis of *sqt* and *cyc* expression. (A, C) control injected embryos. (B, D) embryos injected with *sqt;cyc*-MOs. (A, B) expression of *sqt.* (C, D) expression of *cyc*. Expression of *sqt* and *cyc* is severely reduced in the dorsal side (B, D) compare to control injected embryos (A, C).


sqt;cyc-mis
sqt;cyc-MO
A
sqt
B
sqt
C
cyc
D
cyc

Chapter 4

MATERNAL ACTIVIN-LIKE SIGNALS ARE NOT REQUIRED FOR AXIS FORMATION IN ZEBRAFISH

---

[1] Engda G Hagos and Scott T Dougan. Submitted to *Dev Biol.*
Manuscript Number: DBIO-07-300

ABSTRACT

Maternal Activin-like proteins, a subgroup of the TGF-β superfamily, play a key role in establishing the body axes in many vertebrates, but their role in teleosts is unclear. At least two maternal Activin-like proteins are expressed in zebrafish, including the Vg1 orthologue, zDVR-1, and the *nodal-related* gene, Squint. Our analysis of embryos lacking both maternal and zygotic *squint* function revealed that maternal *squint* is required in some genetic backgrounds for the formation of dorsal and anterior tissues. We show that these defects are an indirect consequence of reduced Nodal levels. Conditional inactivation of the ALK4, 5 and 7 receptors by SB-505124 treatment during the cleavage stages ruled out a role for maternal Squint, zDVR-1, or other Activin-like ligands, as axis determinants. Furthermore, we show that maternal Activin-like signals are not required during the cleavage stages to induce zygotic *nodal-related* gene expression. *nodal-related* gene expression decreases when receptor inhibition continues past the mid-blastula transition, resulting in a progressive loss of mesoderm and endoderm. We conclude that maternally expressed Activin-like signals have a less important role in zebrafish embryonic development than in *Xenopus*.

INTRODUCTION

During vertebrate development, maternally expressed factors determine the precise location of zygotically active signaling centers that pattern the major body axes. This process is best understood in *Xenopus*. In frogs, determinants of the major axes are localized at the vegetal pole and are activated after fertilization (Black and Gerhart, 1986; Scharf and Gerhart, 1983). Rotation of the cortical cytoplasm during the first cell cycle transports components of the Wnt pathway, such as Disheveled and β-catenin, from the vegetal pole to the dorsal blastomeres (Miller et al., 1999; Rowning et al., 1997). In these cells, high levels of maternal Wnt11 trigger the accumulation of nuclear β-catenin (Kofron et al., 2007; Larabell et al., 1997; Tao et al., 2005). The resulting localized expression of Wnt target genes induces Spemann's Organizer, the major signaling center that patterns the dorsoventral axis (Harland and Gerhart, 1997; White and Heasman, 2007). Additional vegetally localized determinants include the T-box transcription factor, VegT, and the TGF-β ligand, Vg1 (White and Heasman, 2007). VegT is required for zygotic expression of the *nodal-related* genes, which encode a subclass of TGF-β proteins that induce mesodermal precursors at the margin (Kofron et al., 1999). Vg1 is required for the expression of organizer-specific genes, such as *xnr3*, *chordin*, *cerberus* and *noggin* (Birsoy et al., 2006). Thus, specification of the dorsal axis involves the coordinated activation of maternal Wnt and TGF-β signaling pathways.

In zebrafish, the function of the maternal Wnt pathway is conserved, but the role of maternal TGF-β signals is highly controversial. Yolk depletion studies demonstrated that one or more dorsal determinants are localized to the vegetal pole prior to fertilization and are transported to the blastomeres during the first cell cycle (Mizuno et al., 1999). Activation of the Wnt pathway results in the accumulation of nuclear β-catenin protein in dorsal blastomeres during the cleavage stages (2.25 h) (Dougan et al., 2003). Loss of maternal β-catenin function disrupts the expression of dorsal specific genes and eliminates the embryonic shield, which is the equivalent of Spemann's Organizer (Bellipanni et al., 2006; Kelly et al., 2000). Conversely, β-catenin induces ectopic shields when overexpressed (Dougan et al., 2003; Kelly et al., 1995). Thus, maternal activation of the Wnt pathway is necessary and sufficient to specify the dorsal axis in zebrafish, although the maternally required Wnt ligand has not been identified.

Yolk grafting experiments demonstrated that a mesendoderm-inducing signal also resides within the yolk cell and acts to pattern the animal-vegetal axis (Mizuno et al., 1996). Mesendoderm fails to form when signals from the yolk are depleted by RNAse injection, indicating that these signals are essential for normal development (Chen and Kimelman, 2000). Zebrafish contain at least two maternal transcripts encoding TGF-β proteins, which are good candidates for these signals. The Vg1 orthologue, zDVR-1, is ubiquitously expressed in oocytes and early embryos (Dohrmann et al., 1996; Helde and Grunwald, 1993). Loss-of-function experiments have not been performed on zDVR-1, but the fully processed ligand can induce mesoderm (Dohrmann et al., 1996). Maternal transcripts encoding the zebrafish Nodal-related protein Sqt, another TGF-β protein, are localized throughout the cytoplasm of stage V oocytes, but become asymmetrically distributed after fertilization (Gore et al., 2005; Gore and Sampath, 2002; Rebagliati et al., 1998a). By the 8-cell stage (1.25 h), these transcripts are localized in dorsal blastomeres, but the function of maternal *sqt* is unclear. The dorsal axis does not form when the function of the maternal *sqt* message is knocked down in oocytes by injection of antisense morpholino oligonucleotides (Gore et al., 2005). These results are contradicted, however, by an analysis of *MZsqt* embryos, which were reported to be indistinguishable from *Zsqt* mutants (Aoki et al., 2002b). These results could be reconciled if the *sqt*^{cz35} allele retains some function, despite the 2kb insertion at the 5' end of the gene (Feldman et al., 1998).

The Nodal-related proteins and Vg1 activate the same receptor complex, consisting of the TGF-_ Type I receptor, ALK4, the Type II receptor, ActR-IIB, and the Cripto/One-Eyed-pinhead (Oep) co-receptor (Cheng et al., 2003; Gritsman et al., 1999; Reissmann et al., 2001). Another TGF-β ligand, Activin, activates the receptor complex in an Oep-independent fashion. ALK4 is a Ser/Thr kinase that phosphorylates cytoplasmic Smad2 and Smad3 (Massague and

Wotton, 2000). Collectively, ligands that act through Smad2 and Smad3 are referred to as Activin-like signals. Phosphorylated-Smad2 or phosphorylated Smad3 dimerizes with Smad4, translocates to the nucleus and activates transcription of target genes, including the *nodal-related* genes themselves and the secreted Nodal antagonist, *lefty* (Massague et al., 2005; Meno et al., 1999). In zebrafish, this feedback loop perpetuates expression of *nodal-related* genes at the embryo margin until gastrulation (Chen and Schier, 2002; Feldman et al., 2002).

Studies inactivating different components of the receptor complex during the cleavage stages have led to conflicting conclusions about the requirement for maternal TGF-β signaling in zebrafish. One set of experiments utilized the drug SB-431542, an inhibitor of the ALK 4 receptor, to block activation of the pathway at different times between fertilization and the mid-blastula transition (Ho et al., 2006; Inman et al., 2002; Sun et al., 2006). Treatment at later times led to a milder phenotype than treatment at the 1-4 cell stage, suggesting a role for activation of the pathway during the cleavage stages. In addition, the phenotype of embryos unable to respond to Vg1 or Nodal-related proteins due to the absence of the Oep co-receptor (*MZoep* mutants) is enhanced by treatment with SB-431542 (Sun et al., 2006). These results suggest that one or more Activin-like proteins act during the cleavage stages to establish the body axes. A different conclusion is reached by experiments demonstrating that *MZoep* mutants can be rescued to viability when *oep* function is supplied at the onset of mid-blastula transition (Aoki et al., 2002a; Gritsman et al., 2000). These results argue against a role for maternal Vg1 or Sqt, but leave open the possibility that these or other Activin-like signals may act during the cleavage stages by an Oep-independent mechanism.

In contrast to maternal TGF-β signals, the role of zygotically expressed Activin-like signals is clear (Schier, 2003). In mouse *nodal* mutants, the primitive streak does not form and the resulting embryos lack derivatives of the mesoderm (Conlon et al., 1994; Varlet et al., 1997; Zhou et al., 1993). In fish, two zygotically expressed Nodal-related proteins, Squint and Cyclops (Cyc), cooperate to induce the mesoderm and endoderm, and to pattern the germ layers in a dosage-dependent manner (Schier and Talbot, 2005). In the absence of zygotic *sqt* function, embryos lack dorsal mesoderm at early stages (Feldman et al., 1998). These embryos recover during gastrulation, however, and by 24 hpf *sqt* mutants display only mild cyclopia or are indistinguishable from wild type (Dougan et al., 2003; Hagos and Dougan, 2007; Heisenberg and Nusslein-Volhard, 1997). Cyc signals are necessary for the recovery of *sqt* mutants, and *sqt; cyc* double mutants lack all derivatives of the mesoderm and endoderm in the head and trunk (Dougan et al., 2003; Feldman et al., 1998). Overexpression studies and genetic loss-of-function analyses in frogs and fish indicate that high Nodal levels induce endoderm and prechordal plate, whereas lower levels specify other mesodermal cell types, such as notochord, somites and pronephros (Agius et al., 2000; Dougan et al., 2003; Erter et al., 1998; Jones et al., 1995). Experiments conditionally inactivating the Nodal receptors during the blastula stages showed that cells respond to the cumulative Nodal dose to which they are exposed (Hagos and Dougan, 2007). Thus, specification of prechordal plate and endoderm requires longer periods of Nodal signaling than other mesendodermal cell types.

We adopted two approaches to determine the function of maternally expressed Activin-like signals. In our genetic analysis, we found that eliminating both maternal and zygotic *sqt* function (*MZsqt*) disrupts formation of dorsal and anterior tissues, a phenotype never observed in *Zsqt* mutants. To determine if maternal *sqt* is required during the cleavage stages, we used SB-505124 to conditionally inactivate the ALK4, 5 and 7 receptors (Callahan et al., 2002; DaCosta Byfield et al., 2004). These experiments are the first to conditionally block both Oep-dependent and –independent signaling at early stages without disrupting later receptor activity. We treated embryos with SB-505124 at 0.75 hpf and washed out the drug at successively later stages, thereby restoring receptor activity in older embryos. Embryos were normal when receptor activity was restored just after the cleavage stages, ruling out a role for maternal Activin-like signals as determinants of the body axes. When receptor activity was restored at later times during the blastula stages, marginal expression of *sqt* and *cyc* is reduced and eventually abolished, resulting in a progressive loss of cell fates. Finally, we show that in the absence of ALK 4/5/7 receptor activity, embryos are more sensitive to changes in BMP signaling levels. Thus, variation in BMP activity between embryos could account for the phenotypic variation in embryos with reduce Nodal levels. Our results indicate that maternally encoded Activin-like signals do not play a significant role during zebrafish development.

MATERIALS AND METHODS

Zebrafish strains and staging

We used the WIK strain to obtain wild type embryos. Adults homozygous for the *sqt*^{cz35} null allele were obtained by intercrossing heterozygous carrier adult fish (Feldman et al., 1998). *oep*^{tz57} mutant adults were obtained by mRNA injection, as previously described (Gritsman et al., 1999). Embryos from natural mating were staged according to Kimmel et al. (1995) and by hours post-fertilization (hpf).

Drug treatment

SB-431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl-1*H*-imidazol-2-yl]benzamide), was obtained from Tocris (Ellisville, MO) and stored as a 100mM stock in DMSO at -20˚C. SB-

505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) was a kind gift from GlaxoSmithKline (King of Prussia, PA) and is stored at 10mM in DMSO at 4˚C. 20-25 embryos were treated with 100μM for SB-431542 or 40μM for SB-505124 at 1-4-cell stage, which we denote as 0.75 hpf in the figures. Control embryos were treated with an equivalent concentration of DMSO. To relieve inhibition of the receptors, we washed the embryos in 5 changes of 300ml egg water at the indicated stage. The effective dose of SB-505124 was determined previously in Hagos and Dougan (2007). We treated *MZoep* mutants with SB-431542 as described in Sun et al. (2006). To verify that the washout yielded reproducible results for in situ hybridization, some embryos in each experiment were permitted to develop until 24 hpf, when they were examined morphologically. Live and fixed embryos after *in situ* hybridization were photographed using compound microscope.

Microinjections and whole-mount *in situ* hybridization

The *sOep,* and *chordin* cDNAs were described previously (Zhang et al., 1998; Miller-Bertoglio et al., 1999a). Sense transcripts were synthesized using the Message Machine kit (Ambion, Inc., Austin TX). We injected 25pg *chordin* or *β-galactosidase* mRNA into chorionated embryos at the 1-4 cell stage. 100pg *sOep* mRNA was co-injected into the YSL of *MZoep* mutants with the Oregon Green 488 lineage tracer dye (Invitrogen, Inc., Carlsbad, CA) to verify the targeting of the injection, as described (Gritsman et al., 2000). *In situ* hybridizations were performed as in Dougan, et al., 2003. We used the following probes: *cyc* (Sampath et al., 1998), *gsc* (Stachel et al., 1993), *flh* (Talbot et al., 1995), *MyoD* (Weinberg et al., 1996), *sox17* (Alexander and Stainier, 1999), *pax6a* (Krauss et al., 1991a), *emx1* (Morita et al., 1995), *fgf8* (Reifers et al., 1998), *krox20* (Oxtoby and Jowett, 1993).

RESULTS

A variable requirement for maternal *sqt* for dorsal and anterior neural fates

To understand the role of maternal *sqt*, we raised *sqt*$^{-/-}$ embryos and their *sqt*$^{+/-}$ siblings to adulthood and examined the phenotypes of their progeny. Although *sqt*$^{-/-}$ embryos have severe defects early in development, most mutants are indistinguishable from wild type by 24 hpf (Fig. 4.1A-D) and many mutants survive to adulthood (Aoki et al., 2002b; Dougan et al., 2003). When *sqt*$^{-/-}$ females are crossed to wild type males, the resulting progeny all lack maternal *sqt* function (*Msqt*), but nonetheless have embryonic shields at 6 hpf (N=45). At 24 hpf, the embryos contain all derivatives of the mesoderm, including the notochord and somites (Fig. 4.1E). The ventral forebrain is present, indicating that these embryos have a functional prechordal plate (Fig. 4.1F). This means that maternal *sqt* is not required for embryonic development, or alternately, that zygotic *sqt* function compensates for the loss of maternal *sqt*.

To distinguish between these possibilities, we examined the phenotypes of embryos lacking both maternal and zygotic *sqt* function (*MZsqt*), generated by crossing *sqt*$^{-/-}$ females to *sqt*$^{-/-}$ males. In all crosses, *MZsqt* embryos lack embryonic shields at 6 hpf, like zygotic *sqt* mutants (*Zsqt*) (data not shown) (Dougan et al., 2003; Feldman et al., 1998). There was considerable variation in the phenotypes apparent at 24 hpf, however. Some females consistently produced *MZsqt* embryos that were indistinguishable from wild type at 24 hpf (Class I; N=45; Fig. 4.1G, H). Other females produced a variable fraction, ranging from zero to 78%, of *MZsqt* embryos with mild cyclopia (Class II; N=91; Fig. 4.1I, J). In the cross of *sqt*$^{+/-}$ adult depicted in Figure 1, none of the *Zsqt* mutant progeny displayed cyclopia (N=25) (Fig. 4.1C, D). In other crosses, up to 60% of the *Zsqt* mutants were cyclopean (N=6/10), but typically about 4% of the mutants are cyclopic (Dougan et al., 2003; Heisenberg and Nusslein-

Volhard, 1997). Thus, loss of maternal *sqt* function does not affect the frequency or severity of cyclopia in *Zsqt* mutants. A third group of females generated *MZsqt* embryos with reduced or absent eyes and forebrain (Class III; N=57; Fig. 4.1K, L). This novel phenotype is never observed in crosses of *sqt*$^{+/-}$ adults (N=25; Fig. 4.1C, D). Class III embryos are always associated with the presence of Class II embryos, but the converse is not true. The class III phenotype disappears when females from this group are crossed to wild type males (N=45; Fig. 4.1E, F). Thus, our results demonstrate an essential role for maternal *sqt* in embryonic development, but this requirement is masked by zygotic *sqt* in most genetic backgrounds.

We encountered difficulty in our initial attempts to characterize the novel Class III phenotype in more detail because these embryos disappeared from our crosses over time. To quantify this effect, we examined the ratio of Class I, II and III embryos generated by a single *sqt*$^{-/-}$ pair that was mated repeatedly over a period of several months. We found that the frequency of mildly cyclopic class II embryos decreased only slightly during the course of our study, from 25% to 21% (Fig. 4.2). In contrast to Class II, the fraction of Class III *MZsqt* embryos decreased dramatically from 22% to 3% as the mothers' age (Fig. 4.2). The loss of Class III embryos was not distributed evenly over time, since the frequency dropped tenfold during the last two months after remaining stable for five months. This suggests that the loss of Class III embryos occurs by a stepwise mechanism, rather than by a gradual process. The decrease in Class III embryos is accompanied by a concomitant increase in the fraction of Class I embryos, which are indistinguishable from wild type (Fig. 4.2). Thus, females transition from producing Class III to Class I embryos over time. The dependence of the Class III phenotype on the genetic background and age of the *sqt*$^{-/-}$ female could explain why it was not observed in previous studies (Aoki et al., 2002b).

The loss of dorsal and anterior neural structures in Class III *MZsqt* embryos is reminiscent of the defects previously reported for depletion of maternal *sqt* (Gore et al., 2005). To determine if these defects are consistent with the lack of a dorsal determinant, we examined the expression of neural genes that mark different positions along the anterior-posterior axis at 24 hpf. To ensure the presence of Class III embryos, we crossed only $sqt^{-/-}$ females younger than eight months and we classified the embryos based on eye morphology. The posterior neural tube is normal in all three types of *MZsqt* embryos, as reflected by *Krox20* expression in rhombomeres 5 and 7, and *fgf8* expression in the midbrain/hindbrain boundary (data not shown) (Reifers et al., 1998; Woo and Fraser, 1998). By contrast, we observed aberrant expression of markers in the anterior neural tube. *emx1* is expressed in a graded fashion throughout the telencephalon, with the highest levels in the posterior (N=20; Fig. 4.3A) (Morita et al., 1995). Expression of *emx1* in the posterior telencephalon expands in Class III mutants (N=11), but not in Class I and II embryos (N =10 and N=6, respectively; Fig. 4.3A-D). This is similar to the expansion of *emx1* in *sqt; cyc* and in *MZoep* mutants (N=22; Fig. 4.3E) (Feldman et al., 2000; Gritsman et al., 1999; Sirotkin et al., 2000). *pax6a* is expressed in the retina as well as in the ventral telencephalon in wild type (N=30) and in *MZsqt* Class I and II embryos (N=15 and N=7, respectively; Fig. 4.3F-H) (Nornes et al., 1998). By contrast, the retinal expression of *pax6a* is abolished in Class III embryos (N=13; Fig. 4.3I). Thus, Class III *MZsqt* embryos are characterized by a loss of anterior neural tissue and an accompanying expansion of posterior neural tissue. This phenotype is similar to, but milder than, the previously described defects in *sqt; boz* double mutants (Shimizu et al., 2000; Sirotkin et al., 2000). Although this is consistent with a role as a dorsal determinant, we also observed that *pax6a* is expressed in the cyclopic eye of *MZoep* mutants (N=22; Fig. 4.3J). Since these embryos are completely unresponsive to

Nodal-related proteins, this suggests that the loss of eyes in Class III *MZsqt* embryos is not a direct consequence of the reduction of Nodal signaling (Gritsman et al., 1999).

Maternal Activin-like signals are not required during the cleavage stages for axis formation

Our genetic analysis demonstrates a requirement for maternal *sqt*, but does not support a role as a dorsal determinant. Two caveats could reconcile our results with previous results depleting *sqt* function from oocytes (Gore et al., 2005). First, it is formally possible that the sqt^{cz35} allele has some residual function despite the 2kb insertion into the 5' UTR (Feldman et al., 1998). Second, maternal Sqt could act in some contexts by an Oep-independent mechanism, which has not been previously described. Finally, our analysis did not address the function of other maternally expressed Activin-like signals, such as zDVR-1. To test if these ligands could act as dorsal determinants, we utilized the competitive inhibitor of the ALK 4, 5 and 7 receptors, SB-505124 (DaCosta Byfield et al., 2004). Whereas control embryos treated with DMSO develop normally (Fig. 4.4A, B), embryos treated at the mid-blastula transition (MBT; 3 hpf) with 40μM SB-505124 are severely cyclopic and lack all derivatives of the mesoderm and endoderm in the head and trunk (Fig. 4.4E, F). These embryos are indistinguishable from those treated soon after fertilization (0.75 hpf) (Fig. 4.4C-F), or from *MZoep* mutants (Gritsman et al., 1999). Thus, blocking the ALK 4/5/7 receptors during the cleavage stages does not enhance the phenotype caused by blocking the receptors at later stages.

Time-dependent loss of mesoderm and endoderm

To distinguish the contribution of potential maternal Activin-like signals from zygotically expressed signals, we treated embryos with SB-505124 at 0.75 hpf and washed out the drug at later times, activating the receptors for different lengths of time during the blastula period. When the drug is washed out at the onset of gastrulation (6 hpf), the embryos lack most derivatives of head and trunk mesoderm, except for a few somites above the yolk extension (Fig. 4.4K, arrow, L). Expression of *MyoD* at 14 hpf confirms that these are trunk somites (Fig. 4.5D), since tail somites are not apparent until later stages (Szeto and Kimelman, 2006). Consistent with the lack of notochord tissue, these embryos fail to express *flh* at the midline (Fig. 4.5H). We detect two broad domains of *flh* expression in the forebrain, which are likely due to an expanded epiphysis, and two ectopic domains in the posterior (Fig. 4.5H) (Hagos and Dougan, 2007). The prechordal plate, endoderm and dorsal forerunner cells are all missing in these embryos, as revealed by the lack of *gsc* at the midline and by the complete absence of *sox17* (Fig. 4.5L, P). This indicates that cell types specified by low Nodal doses, such as the somites, are induced even when the ALK 4/5/7 receptors are activated as late as 6 hpf.

Progressively more mesoderm and endodermal cell types are specified when the drug is washed out at earlier times, restoring receptor activity for longer periods of the blastula stages. For example, somites and notochord form normally when the drug is washed out at 4.3 hpf, but the embryos are cyclopic, indicating a loss of dorsal mesoderm (Fig. 4.4I, J). Consistent with this, *MyoD* and *flh* expression are indistinguishable from DMSO treated controls (Fig. 4.5C, G), while *gsc* expression is absent from the prechordal plate (Fig. 5K). Ectodermal expression of *gsc* is not affected. *sox17* is present in the dorsal forerunner cells but absent from the endoderm precursors, as indicated by the lack of punctate staining (Fig. 4.5O). Finally, when the drug is washed out at 3hpf, restoring receptor activity just after the onset of zygotic transcription, the embryos are indistinguishable from controls (Fig. 4.4G, H). These embryos have normal somites and notochord, as revealed by morphology of living embryos (Fig. 4.4G, H) and by expression of *MyoD* and *floating head (flh)* at earlier stages (Fig. 4.5A, B, E, F). The prechordal plate marker, *goosecoid* (*gsc*) and endodermal marker, *sox17*, are also indistinguishable from controls (Fig. 4.5I, J, M, N). These results indicate that activation of ALK4, 5 and 7 receptors, by Oep-dependent and Oep-independent mechanisms, is not required during the cleavage stages for axis formation. Furthermore, the time-dependent loss of cell fates with progressively later activation of the ALK 4/5/7 receptors is consistent with our earlier studies indicating that cells respond to the total cumulative Nodal dose to which they are exposed (Hagos and Dougan, 2007). It could also reflect, however, changes in the levels of *nodal-related* gene expression as receptor activity is restored at late stages.

Time-dependent induction of *nodal-related* gene expression

Since embryos often fully recover from early reductions in mesoderm and endoderm, examining the phenotypes of post-gastrula stage embryos did not eliminate a role for maternal Activin-like signals at earlier times. Because formation of mesoderm and endoderm is entirely dependent on the zygotic function of *nodal-related* genes, we asked if maternal Activin-like signals are required for normal expression of *sqt* and *cyc*. We found that *sqt* and *cyc* expression are dramatically affected in drug treated embryos when SB-505124 is washed out during the late blastula stages. During gastrulation, *cyc* is expressed at the midline in axial mesoderm (Fig. 4.5Q) (Rebagliati et al., 1998a; Rebagliati et al., 1998b; Sampath et al., 1998). This expression is only mildly reduced when the drug is washed out at 4.3 hpf (Fig. 4.5R, S). By contrast, axial expression of *cyc* is greatly reduced, but still apparent, when the drug is washed out at 6 hpf (Fig. 4.5T). This expression likely accounts for the induction of trunk somites in these embryos (Fig. 4.5D). The expression of *cyc* when the receptors are activated at the onset of gastrulation demonstrates that *cyc* expression in axial mesoderm is induced independently of earlier signaling by Nodal-related proteins or other Activin-like ligands.

sqt and *cyc* are co-expressed in a marginal ring in the late blastula stage (5 hpf) (Fig. 4.6A-C) (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a; Rebagliati et al., 1998b; Sampath et al., 1998). Expression of both genes is severely reduced when the drug is washed out at 4.3 hpf (*sqt*=22/24, *cyc*=9/9; Fig. 4.6H, I). This indicates that the autoregulatory pathway is required before 4.3 hpf for *sqt* and *cyc* expression to achieve normal levels at the margin. Thus, in addition to the delay in *nodal-related* gene expression caused by the drug treatment, *sqt* and *cyc* are expressed at reduced levels. The aberrant *nodal-related* gene expression likely contributes to the subsequent patterning defects we observed. At 24 hpf, the embryos have normal notochord and trunk somites, but lack derivatives of the prechordal plate and display cyclopia (Fig. 4.6G). By contrast, when the drug is washed out at 3 hpf, *sqt* and *cyc* are expressed at normal levels and in the normal distribution (*sqt*=11/21, *cyc*=10/12; Fig. 4.6E, F). This suggests that signaling through the ALK 4/5/7 receptors is not required before 3 hpf to induce zygotic *nodal-related* gene expression. A significant fraction of embryos in this experiment have reduced *sqt* and *cyc* expression, so we could not exclude a role for a maternally expressed Activin-like ligand inducing zygotic *nodal-related* gene expression. Alternately, trace amounts of drug remaining in the embryo after the wash out could dampen receptor activity and impair Nodal autoregulation, leading to the reduction in *nodal-related* gene expression.

To test if an Oep-dependent ligand is required, we supplied *oep* function to *MZoep* mutants at 3 hpf or 4.3 hpf and examined *sqt* and *cyc* expression (Cheng et al., 2003; Gritsman et al., 2000). *MZoep* embryos injected with *β-galactosidase* mRNA lack all derivatives of the mesoderm and endoderm in the head and trunk (Fig. 4.7A) (Cheng et al., 2003; Gritsman et al., 1999). When sOep is supplied at 4 hpf the embryos are severely cyclopic at 24 hpf (Fig. 4.7G), consistent with previous results showing that prechordal plate and endoderm are not specified in these embryos (Aoki et al., 2002b; Gritsman et al., 2000). At earlier stages, expression of both *cyc* (N=19/21; Fig. 7H) and *sqt* (N=23/25; Fig. 4.7I) are greatly reduced, and large gaps are frequent. Thus, the patterning defects in late-rescued *MZoep* mutants result from aberrant *sqt* and *cyc* expression, similar to the results from the drug washout experiments. By contrast, formation of mesoderm and endoderm was completely rescued in *MZoep* mutants injected with a secreted form of Oep (sOep) at 3 hpf, as previously described (Fig. 4.7A, D; N=12/14) (Aoki et al., 2002b; Gritsman et al., 2000). Both *cyc* (Fig. 4.7B, E; N=17/19) and *sqt* (Fig. 4.7C, F; N=22/24) are expressed at normal levels in these embryos, although we occasionally observe gaps due to uneven distribution of injected mRNA (Fig. 4.7F, arrow). These results demonstrate that Oep-dependent signaling is not required during the cleavage stages for zygotic expression of *sqt* and *cyc*.

Drug treatment during the blastula stages enhances the *MZoep* mutant phenotype

To test the role of Oep-independent activation of the ALK 4/5/7 receptors in embryonic development, we treated *MZoep* mutants with 100μM SB-431542 during the cleavage stages according to the protocol of Sun et al. (2006). Control *MZoep* mutants typically develop a long tail that contains somites (Fig. 4.8A) (Gritsman et al., 1999; Szeto and Kimelman, 2006). A minority, however, develop with a shortened tail that lacks somites (4/16; Fig. 4.8B). Thus, our crosses of *oep*$^{-/-}$ adults generate mutant progeny with considerable variation in tail size and morphology. This is similar to the previously reported variation in the tails of *sqt; cyc* double mutants (Erter et al., 2001). Following treatment at the 1-4-cell stage with 100µM SB-431542, all *MZoep* mutants display the short-tail phenotype (9/9; Fig. 4.8C). As previously described, this effect is dose dependent, since only 5/15 embryos have reduced tails after treatment with 40µM SB-4315432, and only 1/13 embryos are affected at 35µM (Sun et al., 2006). Thus, high concentrations of the drug increase the fraction of *MZoep* mutants with poorly developed tails. This could indicate that a maternal Activin-like signal is required for tail formation in *MZoep* mutants (Sun et al., 2006). If so, then tails should develop normally if *MZoep* mutants are treated with the drug after the onset of zygotic transcription (MBT). Following treatment with 40µM SB-505124 at 3 hpf, however, all *MZoep* mutants had short tails with no apparent somites (N=22; Fig. 4.8D). This indicates that tail formation in *MZoep* mutants is, at least in part, under the control of signals acting through the ALK 4/5/7 receptors after MBT.

DISCUSSION

Maternal Activin-like signals play an important role during *Xenopus* development, but their function in zebrafish is controversial. In this work, we examined the role of maternal Activin-like signals during zebrafish development. Our data conclusively demonstrate that maternal Sqt does not act as a dorsal determinant. We could find no evidence that Sqt, zDVR-1, or any other maternally expressed Activin-like signals are required during the cleavage stages. Maternal *sqt* is required in some genetic backgrounds, but acts after the mid-blastula transition.

Our results provide a reconciliation of contradictory results in the published literature (Aoki et al., 2002b; Gore et al., 2005; Sun et al., 2006).

Maternal Activin-like signals are not required for axis formation in zebrafish

Three lines of evidence indicate that maternally expressed Activin-like signals are not required for axis formation. First, we could not detect any defects in *Msqt* embryos (Fig. 1E). Based on the precedent of maternal β-catenin, we expected that dorsal tissues would be missing if maternal *sqt* were required to induce the dorsal axis (Kelly et al., 2000). We found, however, that the embryonic shield forms in *Msqt* mutants, and the embryos are indistinguishable from wild type at 24 hpf. This indicates that dorsal mesoderm is induced even in the absence of maternal *sqt* function. Secondly, the body axes form normally even when the ALK 4/5/7 receptors are temporarily blocked during the cleavage stages, either by treatment with SB-505124 (Fig. 4.4C-H; Fig. 4.5B, F, J, N) or by the rescue of *MZoep* mutants at 3 hpf (Fig. 4.7D). We have previously shown that constitutive activation of these receptors during the cleavage stages alone is not sufficient to induce ectopic axes (Hagos and Dougan, 2007). Thus, activation of the ALK 4/5/7 receptors during the cleavage stages is neither necessary nor sufficient for axis formation in zebrafish.

We also showed that activation of the receptors during the cleavage stages is not required to induce zygotic *nodal-related* gene expression. In most embryos, *sqt* and *cyc* are expressed at normal levels in all marginal cells when SB-505124 is washed out at 3 hpf. Although a large fraction of the embryos displayed slight reductions in *sqt* expression in some experiments, in other experiments *sqt* expression was expressed normally in the vast majority of embryos. This suggests that the reduced expression is likely due to experimental variation in the ability to completely wash out the drug. This conclusion is supported by the rescue of *MZoep* mutants at 3 hpf, in which the vast majority of embryos had normal expression of *sqt* and *cyc* in the late blastula stage (5 hpf) (Fig. 4.7E, F). Since Nodal signaling controls the specification of all mesoderm and endodermal cell types, normal *sqt* and *cyc* expression in the margin indicates that these tissues are properly specified at early stages. These experiments ruled out the possibility that blocking maternal Activin-like signals causes early defects in patterning that were not apparent in older embryos.

Our conclusions differ from a previous analysis based on the analysis of embryos treated with SB-431542, which showed that treatment with SB-431542 generated progressively milder phenotypes when added to embryos at later times during the cleavage period (Sun et al., 2006). This drug, however, forms a precipitate in aqueous solutions, indicating its poor solubility (Hagos and Dougan, 2007). We attribute the mild effects of this compound to the decreased ability of SB-431542 to penetrate cleavage stage embryos as the cell number increases. In this study, by contrast, we used SB-505124, a much more soluble and effective competitive inhibitor of the ALK 4/5/7 receptors than SB-431542 (DaCosta Byfield et al., 2004). Unlike SB-431542, treatment with SB-505124 produces a phenocopy of *sqt; cyc* double mutants when added to embryos at the 1-4-cell stage (0.75 hpf) or when added at MBT (3 hpf). This supports our conclusion that signaling through the ALK 4/5/7 receptors is not required during the cleavage stages.

The role of maternal Activin-like signals

Our genetic analysis of embryos lacking both maternal and zygotic *sqt* function revealed a requirement for maternal *sqt* function in embryonic development. A subclass of *sqt*$^{-/-}$ females produced *MZsqt* mutant embryos, termed Class III, that lack dorsal and anterior neural structures, as indicated by reduced or absent eyes and expression of *pax6a* (Fig. 4.1K, L and Fig. 4.3I). Since our drug treatment studies ruled out a requirement for maternal Activin-like signals during the cleavage stages, maternal Sqt must act after MBT. Maternal *sqt* transcripts are present for more than an hour after MBT, indicating that maternal Sqt protein can act after the onset of zygotic expression (Rebagliati et al., 1998a). It is highly unlikely, however, that maternal Sqt acts directly on neural tissue to pattern the forebrain, since the transcript is gone by the onset of gastrulation, well before specification of the forebrain, and the protein is inherently unstable (Grinblat et al., 1999; Le Good et al., 2005; Rebagliati et al., 1998a). Furthermore, *pax6a* is expressed in the cyclopic eye of *MZoep* mutants, which are unresponsive to Nodal signals (Fig. 4.3J). We conclude that the defects in Class III *MZsqt* mutants are a secondary consequence of reduced Nodal levels.

The loss of dorsal and anterior structures in Class III *MZsqt* mutants is similar to the phenotype of embryos lacking *sqt* and the dorsal transcription factor, *bozozok*. *boz; sqt* double mutants exhibit a dramatic loss of telencephalon, as indicated by the absence of *emx1* and *six3* expression (Shimizu et al., 2000; Sirotkin et al., 2000). Surprisingly, *emx1* expression is completely restored in *sqt; cyc; boz* triple mutants, which lack all mesoderm. This indicates that a factor expressed in the mesoderm under the control of *cyc*, represses anterior neural development (Sirotkin et al., 2000). One candidate for this factor is Wnt8, which is expressed in the lateral mesoderm and acts to posteriorize the neural tube (Erter et al., 2001). Therefore, we propose that synergistic signaling by maternal and zygotic *sqt* after MBT can modulate the levels of posteriorizing signals, possibly Wnt8, either directly or indirectly by controlling *cyc* expression. The loss of dorsal and anterior neural tissue is only apparent in the sensitized background generated by the absence of zygotic *sqt* function, which explains why we never obtained the Class III phenotype in wild type embryos treated with SB-505124.

Previously, the role of maternal *sqt* was examined in experiments depleting *sqt* function from oocytes by injection of anti-sense morpholino oligonucleotides (Gore et al., 2005). As in our crosses of *sqt*$^{-/-}$ adults, these embryos exhibit a range of phenotypes, including cyclopean embryos similar to our Class II *MZsqt* mutants (Fig. 4.1I, J). A minority of these embryos lack dorsal and anterior structures, like the Class III *MZsqt* mutants (Fig. 4.1K, L). Thus, the same tissues are affected in *MZsqt* mutants and in embryos from *sqt*-depleted oocytes, and the defects occur at similar frequencies. By analogy with *MZsqt* mutants, therefore, we conclude that the loss of dorsal and anterior neural tissue in some embryos from *sqt*-depleted oocytes is an indirect consequence of the loss of both maternal and zygotic Sqt signals.

Maternal age and genetic background influence the *MZsqt* phenotype

Only some *sqt*$^{-/-}$ females are able to generate Class III embryos, and these females only have this capacity while they are young (Fig. 4.1 and Fig. 4.2). This indicates that genetic background and maternal age have a strong influence on the embryonic phenotype of *MZsqt* mutants. Similar effects have been described for other embryonic mutants in zebrafish. For example, the strongly dorsalized phenotype of *vox*, *vent* double mutants is completely suppressed in some genetic backgrounds, suggesting the presence of a dominant modifier (Imai et al., 2001). Background dependent effects are common in mice, with the phenotypes of some mutations apparent only in certain strains (Erickson, 1996). In most cases, however, the modifiers have not been identified and may be the result of interactions between many genes. It is more difficult to explain how the age of the mother could affect the strength or penetrance of an embryonic phenotype. To our knowledge, this phenomenon has only been described for one other gene. The penetrance and severity of the *boz* mutant phenotype decreases so dramatically as the mother ages that *boz* homozygotes are frequently viable when derived from older females (Fekany et al., 1999). Our time-course analysis provides some insight into the mechanism by which maternal age can influence embryonic phenotypes. We showed that the frequency of Class III embryos decreases dramatically in one step, after remaining stable for a long period (Fig. 4.2). Thus, a sudden change occurs as *sqt*$^{-/-}$ females age that mitigates the Class III phenotype. This could be accomplished if oocytes produce increasing amounts of a suppressor over time, or decreasing amounts of an enhancer.

Oep-independent Activin signaling promotes tail formation during the blastula stages

Our data, and that from other labs, indicates that the ALK 4/5/7 receptors retain some activity in *MZoep* mutants (Sun et al., 2006). Most *MZoep* mutants have long tails, complete with somites, but a minority develops with short tails that lack somites (Fig. 4.8A, C) (Gritsman et al., 1999). This indicates that tail size and morphology are under the control of background mutations in our lines. These embryos strongly resemble the previously described enhanced *sqt; cyc* double mutant phenotype, observed in the TL background (Erter et al., 2001). We found that blocking ALK 4/5/7 receptor activity increases the penetrance of the short-tail phenotype in *MZoep* mutants (Fig. 4.8B), indicating that ALK 4/5/7 activity is required for tail formation. We obtained this phenotype whether we treated embryos with SB-431542 during the cleavage stages (Fig. 4.8B), or SB-505124 after MBT (Fig. 4.8D). This demonstrates that activation of the ALK 4/5/7 receptors during the blastula stages is required for tail formation. The variation in tail size in *MZoep* mutants (Fig. 4.8C), therefore, is due to fluctuations in the levels of ALK 4/5/7 activity during the blastula stages. The same mechanism probably accounts for accounts for the enhanced *sqt; cyc* phenotype (Erter et al., 2001).

Since BMP signaling is known to promote tail formation in zebrafish, the loss of tails in drug treated embryos could be explained if the drug binds to the BMP with a low-affinity and dampens their activity. Three observations indicate that this is not the case. First, in cell culture, neither SB-431542 nor SB-505124 prevent phosphorylation of Smad1, an effector of the BMP pathway (DaCosta Byfield et al., 2004; Inman et al., 2002). Secondly, BMP target genes such as Δ*Np63*, *Tbx6* and *gata2* are upregulated in embryos following treated with SB-431542 (EGH and STD, unpublished data) (Sun et al., 2006). Finally, in human embryonic stem cells, treatment with SB-431542 increases levels of phosphorylated Smad1, an effector of BMP signaling (Besser, 2004; James et al., 2005). This suggests an antagonistic relationship exists between the Nodal and BMP pathways, as has been suggested in *Xenopus* is consistent with observations in *Xenopus* (Candia et al., 1997; Yeo and Whitman, 2001). An antagonistic relationship, however, would not explain the requirement for both BMP and signaling through the ALK 4/5/7 receptors in tail formation. We found, however, that drug treatment enhances the response to low doses of *chordin* (EGH and STD, unpublished data). This indicates that interactions between the BMP and Nodal signal transduction pathways are more complex than previously suspected.

Time-dependent induction of zygotic *nodal-related* gene expression

Our results reveal that expression of *cyc* in the axial mesoderm is induced independently of earlier Nodal signaling. When the drug is washed out at 6 hpf, restoring receptor activity at the onset of gastrulation, *cyc* is expressed at low levels at the midline (Fig. 4.5T). This indicates that axial expression of *cyc* is induced independently of earlier Nodal signals, but requires the autoregulatory pathway in order to achieve normal levels. This means that zygotic *nodal-related* gene expression is induced in three independent phases. First, *sqt* is expressed in the dorsal margin under the control of maternal β-catenin (Bellipanni et al., 2006; Dougan et al., 2003). At later stages, *sqt* and *cyc* are both induced in all marginal cells by an unknown factor (Erter et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998). During this phase, Sqt and Cyc signals in the marginal-most cells activate a positive feedback loop, which stimulates *sqt* and *cyc* expression in cells farther from the margin (Solnica-Krezel, 2003). Finally, an unknown factor induces *cyc* expression in the axial mesoderm. Our results confirm earlier results indicating that there is considerable flexibility in the timing of specification of mesodermal cell types. When Nodal signals are blocked before 6 hpf, Cyc signals during gastrulation are sufficient to specify trunk somites (Fig. 4.5D). Previously we showed that somites are specified by a pulse of Nodal signaling from 3-4 hpf (Hagos and Dougan, 2007). Thus, cells remain competent to become somites throughout the blastula and early gastrula stages.

We observed a time-dependent loss of cell fates when drug treatment continued past MBT, blocking receptor activity for increasing periods. Cell types derived from the margin, such as endoderm and prechordal plate, are only specified if receptor activity is restored prior to 4.3 hpf (Fig. 4.5O). By contrast, cell types that are derived more animal regions, such as notochord and somites, are specified even when receptor activity is restored at later times (Fig. 4.5 D, G). These defects in cell fate specification can be entirely explained by changes in the zygotic expression of the *nodal-related* genes. We found that *sqt* and *cyc* are expressed at reduced levels and in an altered pattern when the drug is washed out after MBT, restoring receptor activity during the blastula period (Fig. 4.6 and Fig. 4.7). This supports our conclusion that there is no role for maternally expressed Sqt or other Activin-like signals. It is not clear whether the patterning defects in these embryos are caused by the reduced Nodal dosage, by the shortened duration of Nodal signaling. It is likely that both factors contribute to the defects in cell fate specification, since cells integrate the total cumulative Nodal dose over time to generate the appropriate response (Hagos and Dougan, 2007).

Acknowledgements

We thank Dr. Benjamin Feldman for communicating results prior to publication and for critical comments on the manuscript, Dr. Rebecca Burdine, James D. Lauderdale, Dr. Xiang Fan, Dr. Nancy Manley, Dr. Ping Shen and members of the Dougan and Lauderdale laboratories for helpful discussions and advice. We thank Dr. Nicholas J. Laping for advice on the use of SB-505124. S.T.D. is a Georgia Cancer Coalition Distinguished Investigator. This work was supported by the Georgia Cancer Coalition and a grant from the University of Georgia Research Foundation.

REFERENCES

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C., and De Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development* **127,** 1173-83.

Aoki, T. O., David, N. B., Minchiotti, G., Saint-Etienne, L., Dickmeis, T., Persico, G. M., Strahle, U., Mourrain, P., and Rosa, F. M. (2002a). Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. *Development* **129,** 275-86.

Aoki, T. O., Mathieu, J., Saint-Etienne, L., Rebagliati, M. R., Peyrieras, N., and Rosa, F. M. (2002b). Regulation of nodal signalling and mesendoderm formation by TARAM-A, a TGFbeta-related type I receptor. *Dev Biol* **241,** 273-88.

Bellipanni, G., Varga, M., Maegawa, S., Imai, Y., Kelly, C., Myers, A. P., Chu, F., Talbot, W. S., and Weinberg, E. S. (2006). Essential and opposing roles of zebrafish beta-catenins in the formation of dorsal axial structures and neurectoderm. *Development* **133,** 1299-309.

Besser, D. (2004). Expression of Nodal, Lefty-A, and Lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3. *J Biol Chem*.

Birsoy, B., Kofron, M., Schaible, K., Wylie, C., and Heasman, J. (2006). Vg1 is an essential signaling molecule in Xenopus development. *Development* **133,** 15-20.

Black, S. D., and Gerhart, J. C. (1986). High-frequency twinning of Xenopus laevis embryos from eggs centrifuged before first cleavage. *Dev Biol* **116,** 228-40.

Callahan, J. F., Burgess, J. L., Fornwald, J. A., Gaster, L. M., Harling, J. D., Harrington, F. P., Heer, J., Kwon, C., Lehr, R., Mathur, A., Olson, B. A., Weinstock, J., and Laping, N. J. (2002). Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5). *J Med Chem* **45,** 999-1001.

Candia, A. F., Watabe, T., Hawley, S. H., Onichtchouk, D., Zhang, Y., Derynck, R., Niehrs, C., and Cho, K. W. (1997). Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124,** 4467-80.

Chen, S., and Kimelman, D. (2000). The role of the yolk syncytial layer in germ layer patterning in zebrafish. *Development* **127,** 4681-9.

Chen, Y., and Schier, A. F. (2002). Lefty proteins are long-range inhibitors of squint-mediated nodal signaling. *Curr Biol* **12,** 2124-8.

Cheng, S. K., Olale, F., Bennett, J. T., Brivanlou, A. H., and Schier, A. F. (2003). EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. *Genes Dev* **17,** 31-6.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B., and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120,** 1919-28.

DaCosta Byfield, S., Major, C., Laping, N. J., and Roberts, A. B. (2004). SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **65,** 744-52.

Dohrmann, C. E., Kessler, D. S., and Melton, D. A. (1996). Induction of axial mesoderm by zDVR-1, the zebrafish orthologue of Xenopus Vg1. *Dev Biol* **175,** 108-17.

Dougan, S. T., Warga, R. M., Kane, D. A., Schier, A. F., and Talbot, W. S. (2003). The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development* **130,** 1837-1851.

Erickson, R. P. (1996). Mouse models of human genetic disease: which mouse is more like a man? *Bioessays* **18,** 993-8.

Erter, C. E., Solnica-Krezel, L., and Wright, C. V. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev Biol* **204,** 361-72.

Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V., and Solnica-Krezel, L. (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. *Development* **128,** 3571-83.

Fekany, K., Yamanaka, Y., Leung, T., Sirotkin, H. I., Topczewski, J., Gates, M. A., Hibi, M., Renucci, A., Stemple, D., Radbill, A., Schier, A. F., Driever, W., Hirano, T., Talbot, W. S., and Solnica-Krezel, L. (1999). The zebrafish bozozok locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development* **126,** 1427-38.

Feldman, B., Concha, M. L., Saude, L., Parsons, M. J., Adams, R. J., Wilson, S. W., and Stemple, D. L. (2002). Lefty antagonism of squint is essential for normal gastrulation. *Curr Biol* **12,** 2129-35.

Feldman, B., Dougan, S. T., Schier, A. F., and Talbot, W. S. (2000). Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. *Curr Biol* **10,** 531-4.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F., and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal- related signals. *Nature* **395,** 181-5.

Gore, A. V., Maegawa, S., Cheong, A., Gilligan, P. C., Weinberg, E. S., and Sampath, K. (2005). The zebrafish dorsal axis is apparent at the four-cell stage. *Nature* **438,** 1030-5.

Gore, A. V., and Sampath, K. (2002). Localization of transcripts of the Zebrafish morphogen Squint is dependent on egg activation and the microtubule cytoskeleton. *Mech Dev* **112,** 153-6.

Grinblat, Y., Lane, M. E., Sagerstrom, C., and Sive, H. (1999). Analysis of zebrafish development using explant culture assays. *Methods Cell Biol* **59,** 127-56.

Gritsman, K., Talbot, W. S., and Schier, A. F. (2000). Nodal signaling patterns the organizer. *Development* **127,** 921-32.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S., and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97,** 121-32.

Hagos, E. G., and Dougan, S. T. (2007). Time-dependent patterning of the mesoderm and endoderm by Nodal signals in zebrafish. *BMC Dev Biol* **7,** 22.

Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu Rev Cell Dev Biol* **13,** 611-67.

Heisenberg, C. P., and Nusslein-Volhard, C. (1997). The function of silberblick in the positioning of the eye anlage in the zebrafish embryo. *Dev Biol* **184,** 85-94.

Helde, K. A., and Grunwald, D. J. (1993). The DVR-1 (Vg1) transcript of zebrafish is maternally supplied and distributed throughout the embryo. *Dev Biol* **159,** 418-26.

Ho, D. M., Chan, J., Bayliss, P., and Whitman, M. (2006). Inhibitor-resistant type I receptors reveal specific requirements for TGF-beta signaling in vivo. *Dev Biol* **295,** 730-42.

Imai, Y., Gates, M. A., Melby, A. E., Kimmelman, D., Schier, A., and Talbot, W. S. (2001). The homeobox genes *vox* and *vent* are redundant repressors of dorsal fates in zebrafish. *Development* **128,** 2407-2420.

Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **62,** 65-74.

James, D., Levine, A. J., Besser, D., and Hemmati-Brivanlou, A. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* **132,** 1273-82.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C., and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121,** 3651-62.

Kelly, C., Chin, A. J., Leatherman, J. L., Kozlowski, D. J., and Weinberg, E. S. (2000). Maternally controlled (beta)-catenin-mediated signaling is required for organizer formation in the zebrafish. *Development* **127,** 3899-911.

Kelly, G. M., Erezyilmaz, D. F., and Moon, R. T. (1995). Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of beta-catenin. *Mech Dev* **53,** 261-73.

Kofron, M., Birsoy, B., Houston, D., Tao, Q., Wylie, C., and Heasman, J. (2007). Wnt11/beta-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. *Development* **134,** 503-13.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C., and Heasman, J. (1999). Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development* **126,** 5759-70.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D., and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J Cell Biol* **136,** 1123-36.

Le Good, J. A., Joubin, K., Giraldez, A. J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A. F., and Constam, D. B. (2005). Nodal stability determines signaling range. *Curr Biol* **15,** 31-6.

Massague, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. *Genes Dev* **19,** 2783-810.

Massague, J., and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. *Embo J* **19,** 1745-54.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W. S., Robertson, E. J., Schier, A. F., and Hamada, H. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell* **4,** 287-98.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L., and Moon, R. T. (1999). Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J Cell Biol* **146,** 427-37.

Mizuno, T., Yamaha, E., Kuroiwa, A., and Takeda, H. (1999). Removal of vegetal yolk causes dorsal deficencies and impairs dorsal-inducing ability of the yolk cell in zebrafish. *Mech Dev* **81,** 51-63.

Mizuno, T., Yamaha, E., Wakahara, M., Kuroiwa, A., and Takeda, H. (1996). Mesoderm induction in zebrafish. *Nature* **383,** 131-2.

Morita, T., Nitta, H., Kiyama, Y., Mori, H., and Mishina, M. (1995). Differential expression of two zebrafish emx homeoprotein mRNAs in the developing brain. *Neurosci Lett* **198,** 131-4.

Nornes, S., Clarkson, M., Mikkola, I., Pedersen, M., Bardsley, A., Martinez, J. P., Krauss, S., and Johansen, T. (1998). Zebrafish contains two pax6 genes involved in eye development. *Mech Dev* **77,** 185-96.

Rebagliati, M. R., Toyama, R., Fricke, C., Haffter, P., and Dawid, I. B. (1998a). Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev Biol* **199,** 261-72.

Rebagliati, M. R., Toyama, R., Haffter, P., and Dawid, I. B. (1998b). cyclops encodes a nodal-related factor involved in midline signaling. *Proc Natl Acad Sci U S A* **95,** 9932-7.

Reifers, F., Bohli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y., and Brand, M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125,** 2381-95.

Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M. G., Ibanez, C. F., and Brivanlou, A. H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* **15,** 2010-22.

Rowning, B. A., Wells, J., Wu, M., Gerhart, J. C., Moon, R. T., and Larabell, C. A. (1997). Microtubule-mediated transport of organelles and localization of beta- catenin to the future dorsal side of Xenopus eggs. *Proc Natl Acad Sci U S A* **94,** 1224-9.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E., and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395,** 185-9.

Scharf, S. R., and Gerhart, J. C. (1983). Axis determination in eggs of Xenopus laevis: a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation. *Dev Biol* **99,** 75-87.

Schier, A. (2003). Nodal Signaling in Vertebrate Development. *Annu. Rev. Cell Dev. BIol.* **19,** 589-621.

Schier, A. F., and Talbot, W. S. (2005). Molecular Genetics of Axis Formation in Zebrafish. *Annu Rev Genet*.

Shimizu, T., Yamanaka, Y., Ryu, S. L., Hashimoto, H., Yabe, T., Hirata, T., Bae, Y. K., Hibi, M., and Hirano, T. (2000). Cooperative roles of Bozozok/Dharma and Nodal-related proteins in the formation of the dorsal organizer in zebrafish. *Mech Dev* **91,** 293-303.

Sirotkin, H. I., Dougan, S. T., Schier, A. F., and Talbot, W. S. (2000). bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. *Development* **127,** 2583-92.

Solnica-Krezel, L. (2003). Vertebrate development: taming the nodal waves. *Curr Biol* **13,** R7-9.

Sun, Z., Jin, P., Tian, T., Gu, Y., Chen, Y. G., and Meng, A. (2006). Activation and roles of ALK4/ALK7-mediated maternal TGFbeta signals in zebrafish embryo. *Biochem Biophys Res Commun* **345,** 694-703.

Szeto, D. P., and Kimelman, D. (2006). The regulation of mesodermal progenitor cell commitment to somitogenesis subdivides the zebrafish body musculature into distinct domains. *Genes Dev* **20,** 1923-32.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X., and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell* **120,** 857-71.

Varlet, I., Collignon, J., and Robertson, E. J. (1997). nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* **124,** 1033-44.

White, J. A., and Heasman, J. (2007). Maternal control of pattern formation in Xenopus laevis. *J Exp Zoolog B Mol Dev Evol*.

Woo, K., and Fraser, S. E. (1998). Specification of the hindbrain fate in the zebrafish. *Dev Biol* **197,** 283-96.

Yeo, C., and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* **7,** 949-57.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L., and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* **361,** 543-7.

FIGURE LEGENDS

Figure 4.1. Embryos lacking maternal *sqt* function have variable defects. Lateral (A, D, C, E, G, I, K) or ventral (B, D, F, H, J, L) images of live embryos at 30 hpf. The body axis forms normally in wild type (A), *Zsqt* (C) and *Msqt* (E) embryos, and none of these embryos are cyclopic (B, D, F), although *Zsqt* mutants from some crosses display mild cyclopia. Embryos lacking both maternal and zygotic *sqt* can be classified into three groups depending on their phenotype. The frequency of the phenotypes depends on the genetic background of the mother, as described in the text. The *MZsqt* embryos depicted in G-L are siblings. Class I *MZsqt* mutants (G, H) are indistinguishable from wild type, while Class II mutants (I, J) display mild cyclopia (J), typical of the previously reported phenotype of *Zsqt* mutants (Dougan et al., 2003). Class III mutants have reduced dorsal and anterior structures, characterized by reduced (L, arrow) or absent eyes. Anterior is to the left when apparent.

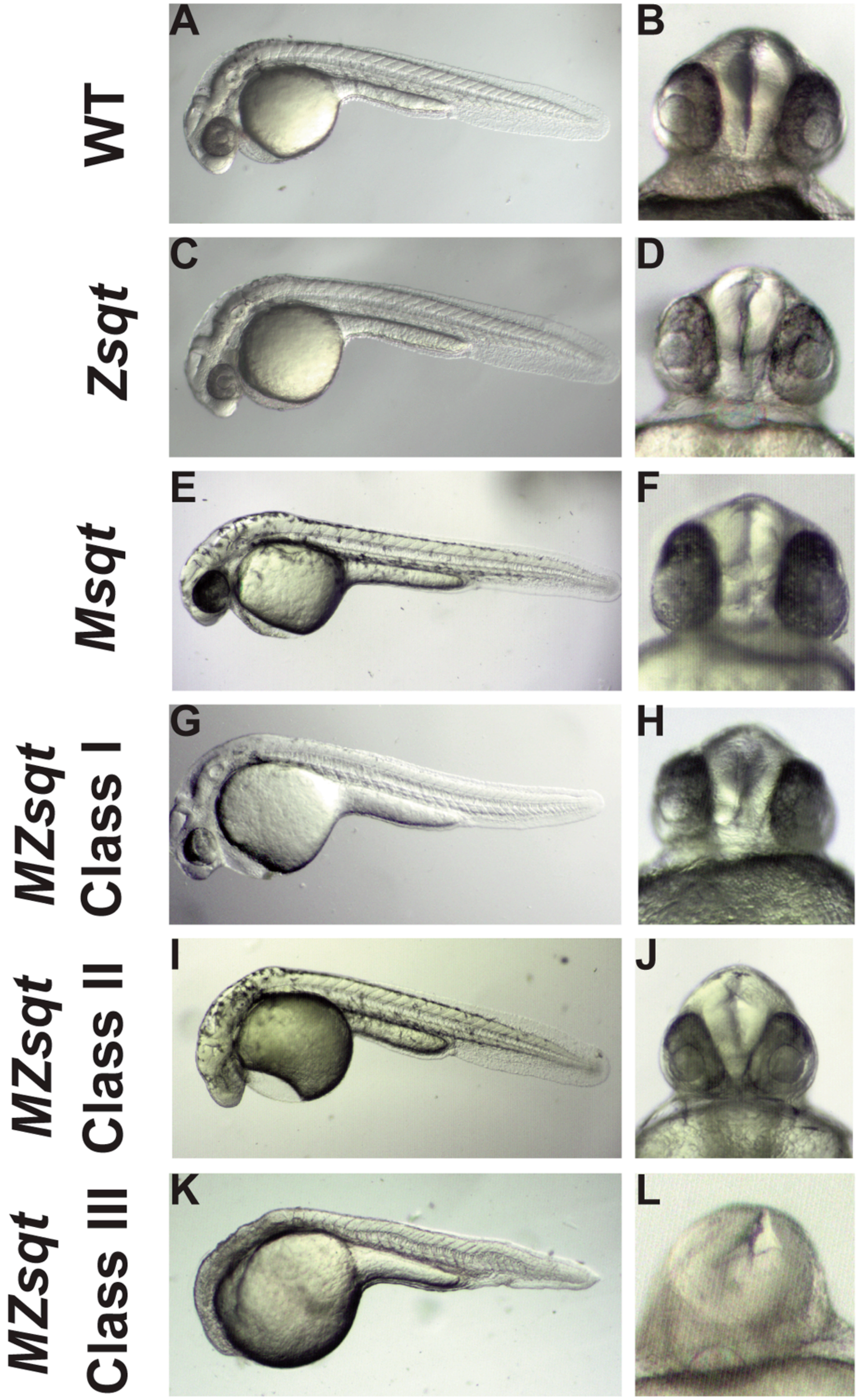
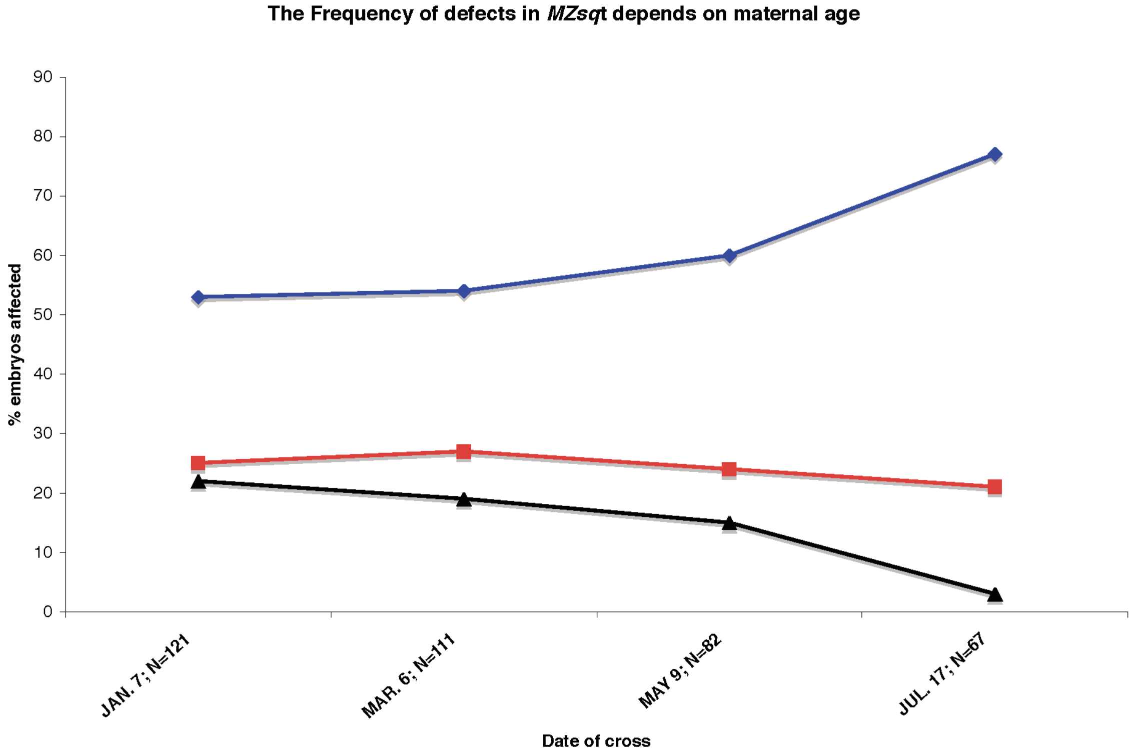

WT
Zsqt
Msqt
MZsqt Class I
MZsqt Class II
MZsqt Class III
A
B
C
D
E
F
G
H
I
J
K
L

Figure 4.2. The frequency of the Class III phenotype depends on maternal age. One pair of *sqt*$^{-/-}$ adults was repeatedly crossed over a period of seven months, and the embryos were classified according to their phenotype. The frequency of Class II embryos (red squares) stayed fairly constant, ranging from 25% to 21% with a peak at 33%. By contrast, the frequency of Class I embryos (blue diamonds) increases from 53% to 77%, with the largest increase occurring in the last two months. The frequency of Class III *MZsqt* embryos remained stable for the first five months at 22%, then decreased precipitously in the last two months to 3%.



The Frequency of defects in *MZsqt* depends on maternal age
% embryos affected
0
10
20
30
40
50
60
70
80
90
JAN. 7; N=121
MAR. 6; N=111
MAY 9; N=82
JUL. 17; N=67
Date of cross

Figure 4.3. Dorsal and anterior neural tissue is reduced or missing in Class III *MZsqt* embryos. *emx1* (A-E) or *pax6a* (F-J) expression in wild type (A, F), Class I *MZsqt* (B, G), Class II *MZsqt* (C, H), Class III *MZsqt* (D, I), or *MZoep* (E, J) embryos at 24 hpf. *emx1* expression in the posterior telencephalon is indistinguishable from wild type in Class I and Class II *MZsqt* (A-C), but is expanded in Class III *MZsqt* (D) and in *MZoep* (E). *pax6a* is expressed in the retinas of wild type and Class I *MZsqt* embryos (F, G) and in the retina of the cyclopic eyes in Class II *MZsqt* (H) and *MZoep* (J). *pax6a* is not expressed in Class III *MZsqt* embryos (I). (A-E) Lateral view with anterior to the left. (F-J) Dorsal view with anterior to the left. Arrowheads indicate retinal *pax6a* expression (F-H, J) or the absence of retinal *pax6a* expression (I).

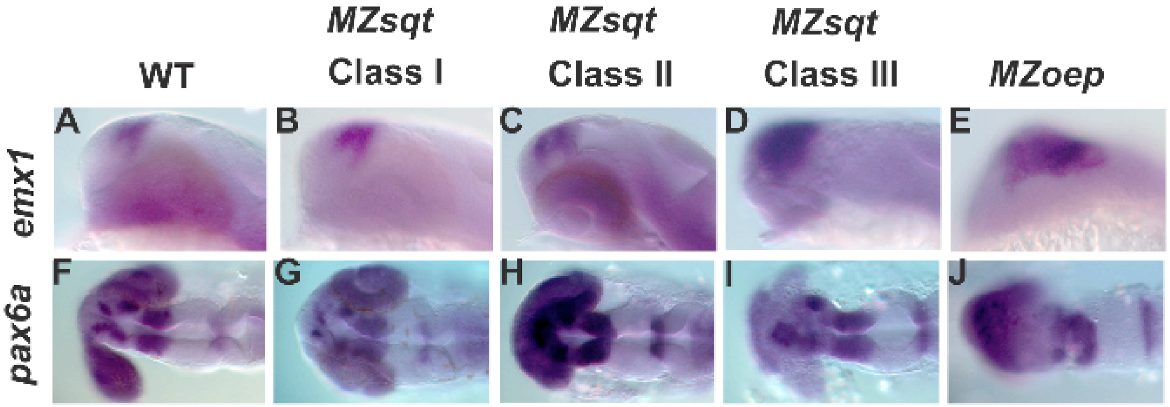

WT
MZsqt Class I
MZsqt Class II
MZsqt Class III
MZoep
emx1
pax6a
A
B
C
D
E
F
G
H
I
J

Figure 4.4 Maternal Activin-like signals are not required during the cleavage stages. Images of live embryos at 24hpf. Embryos were treated with DMSO (A, B) or with SB-505124 at 0.75 hpf (C, D, G-L) or at 3hpf (E, F). In G-L, the drug was applied at 0.75 hpf and washed out at the indicated time. Embryos treated with SB-505124 at 0.75 hpf and 3 hpf (C, E) lack all derivatives of the mesoderm and endoderm in the head and trunk, and are severely cyclopic (D, F). (G, H) When embryos are exposed to the drug only between 0.75 hpf and 3 hpf, they are indistinguishable from wild type (A, B). (I, J) When embryos are exposed to SB-505124 between 0.75 hpf and 4.3 hpf, they lack head mesoderm and endoderm, as indicated by severe cyclopia (J), but contain trunk mesoderm, as indicated by trunk somites and notochord (I). Finally, embryos exposed to SB-505124 between 0.75 hpf and 6 hpf lack notochord, but contain trunk somites (K), and are severely cyclopic (L). Lateral views, anterior is to the left (A, C, E, G, I, K) or ventral views (B, D, F, H, J, L).

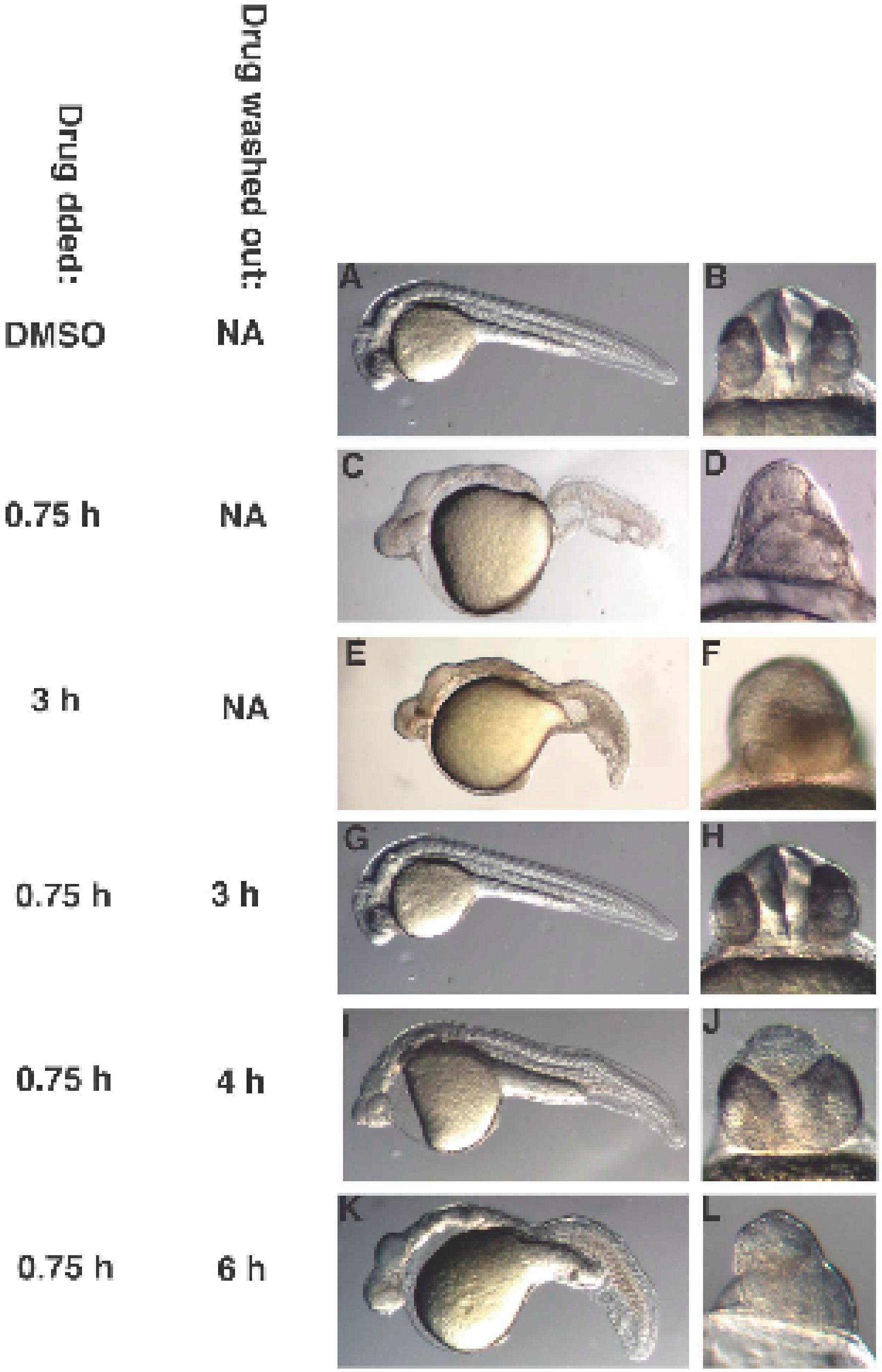

Drug dded:
Drug washed out:
DMSO
NA
A
B
0.75 h
NA
C
D
3 h
NA
E
F
0.75 h
3 h
G
H
0.75 h
4 h
I
J
0.75 h
6 h
K
L

Figure 4.5. Progressive loss of cell fates with shorter exposures to Activin-like signals. Embryos were treated with DMSO (A, E, I, M, Q) or with SB-505124 between 0.75 hpf and 3 hpf (B, F, J, N, R), 4.3 hpf (C, G, K, O, S) or 6 hpf (D, H, L, P, T). Sibling embryos were divided into five groups. One group was fixed at 14 hpf and stained for *MyoD* expression (A-D). Embryos fixed at 10 hpf were stained for either *flh* (E-H), or *gsc* (I-L). Embryos fixed at 8 hpf were stained for either *sox17* (M-P) or *cyc* (Q-T). When the drug is washed out at 3 hpf (B, F, J, N, R), expression of all markers is indistinguishable from wild type (A, E, I, M, Q). When the drug is washed out at 4.3 hpf, *MyoD* and *flh* expression is normal (C, G), but *gsc* is absent from the prechordal plate (K) and *sox17* is only expressed in the dorsal forerunner cells (O). The axial domain of *cyc* expression is reduced (S). When the drug is washed out at 6 hfp, MyoD is expressed in trunk somites (D), and *flh* is expressed in four ectodermal domains, but not in the axial mesoderm (H). *gsc* is absent from the prechordal plate (L) and *sox17* expression is abolished (P). *cyc* expression in the axial mesoderm is severely reduced, but is still present (T). In A-L, images are dorsal views, with anterior to the top of the page, In M-I, images are dorsal views with the animal pole at the top.

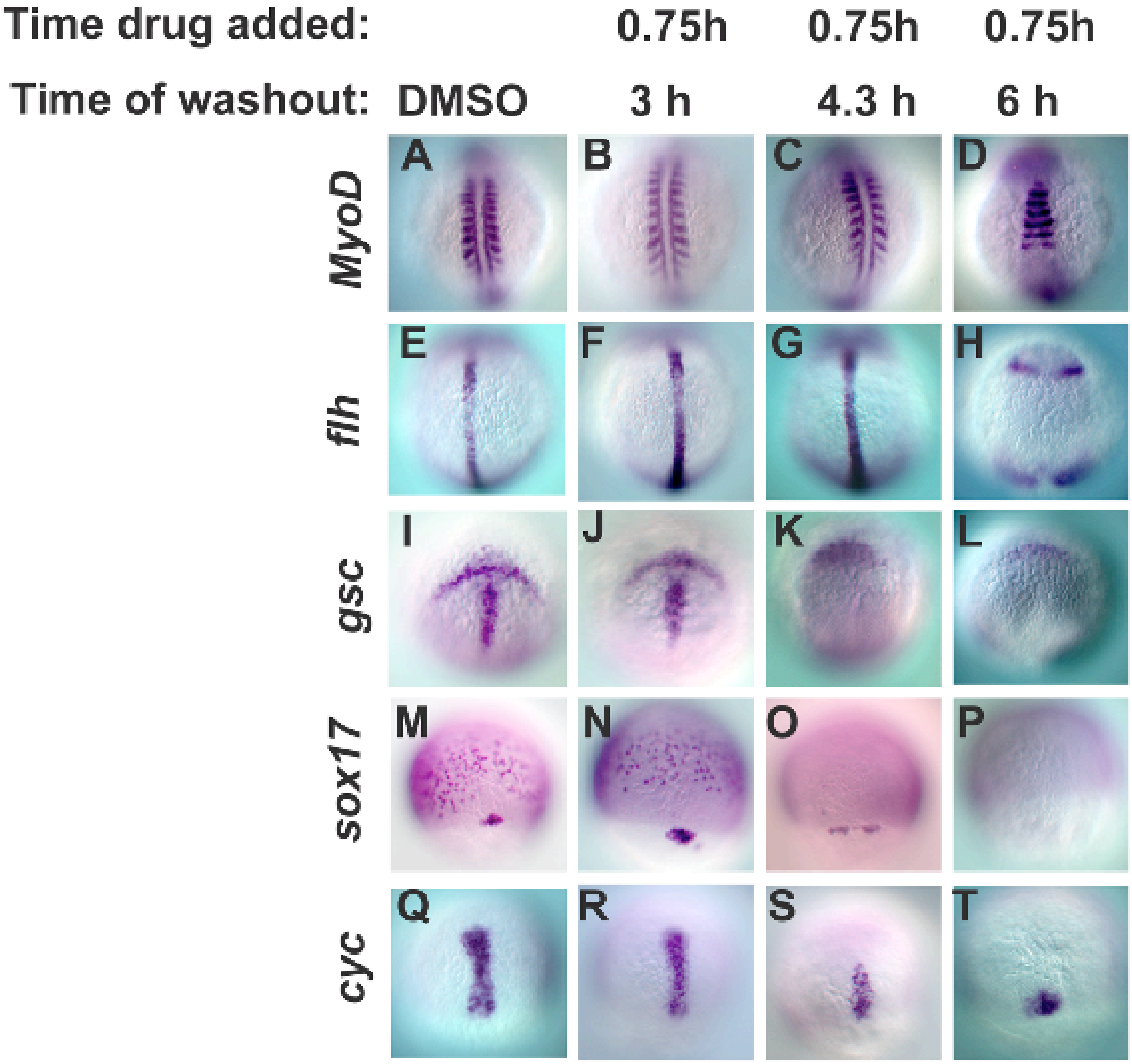

Time drug added:
0.75h
0.75h
0.75h
Time of washout:
DMSO
3 h
4.3 h
6 h
MyoD
flh
gsc
sox17
cyc
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 4.6. Signaling through the ALK 4/5/7 receptors is not required before 3 hpf to induce zygotic *nodal-related* gene expression. Sibling embryos were treated with DMSO (A-C) or SB-505124 from 0.75 hpf to 3 hpf (D-F) or to 4.3 hpf (G-I). Embryos were examined for morphology at 24 hpf (A, D, G), and for expression of *cyc* (B, E, H) and *sqt* (C, F, I) at 5 hpf. Embryos treated with SB-505124 from 0.75 hpf to 3 hpf are indistinguishable from wild type at 24 hpf (A, D). Similarly, both *cyc* (B, E) and *sqt* (C, F) are expressed at normal levels with a normal distribution. Embryos treated with SB-505124 from 0.75 hpf to 4.3 hpf are severely cyclopic (G) and *cyc* (H) and *sqt* (I) expression are both severely reduced. Animal pole views of fixed embryos at 5 h, dorsal to the right (B, C, E, F, H, I). For live embryos, anterior is to the left (A, D, G).

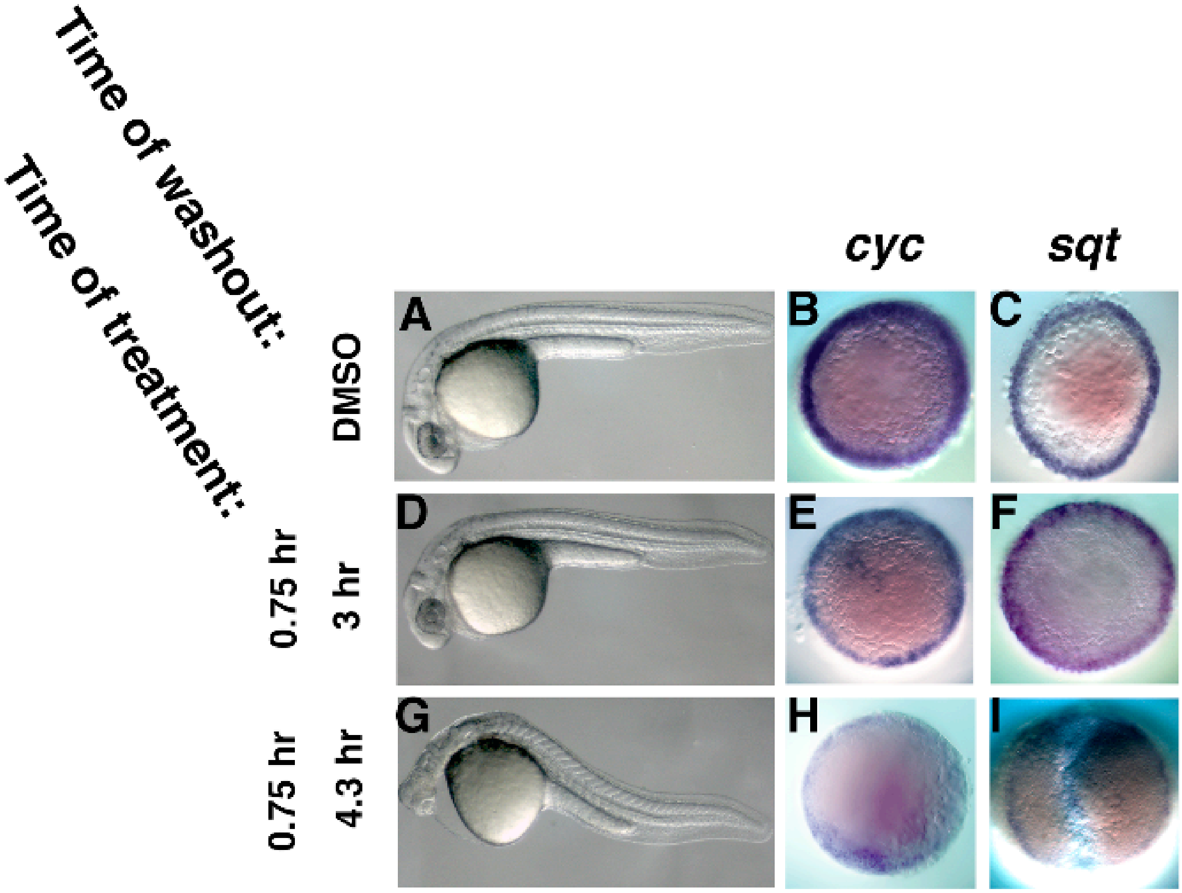

Time of washout:
Time of treatment:
cyc
sqt
A
B
C
DMSO
D
E
F
0.75 hr
3 hr
G
H
I
0.75 hr
4.3 hr

Figure 4.7. *oep* function is not required before 3 hpf. Images of uninjected *MZoep* mutants (A-C), or those injected with 100pg *sOep* mRNA into the YSL at 3 h (D-F) or 4 h (G-I). *MZoep* embryos lack all derivates of mesoderm and endoderm in the head and trunk at 24h (A), and lack expression of *cyc* (B) and *sqt* (C) at 5 hpf. Injection of *sOep* mRNA at 3 hpf restores mesoderm and endoderm (D), as well as *cyc* (E) and *sqt* (F) expression. Embryos injected at 4 h are severely cyclopic, but contain trunk somites and notochord (G). Expression of both *cyc* (H) and *sqt* (I) are severely reduced. Animal pole views, with dorsal to the right.

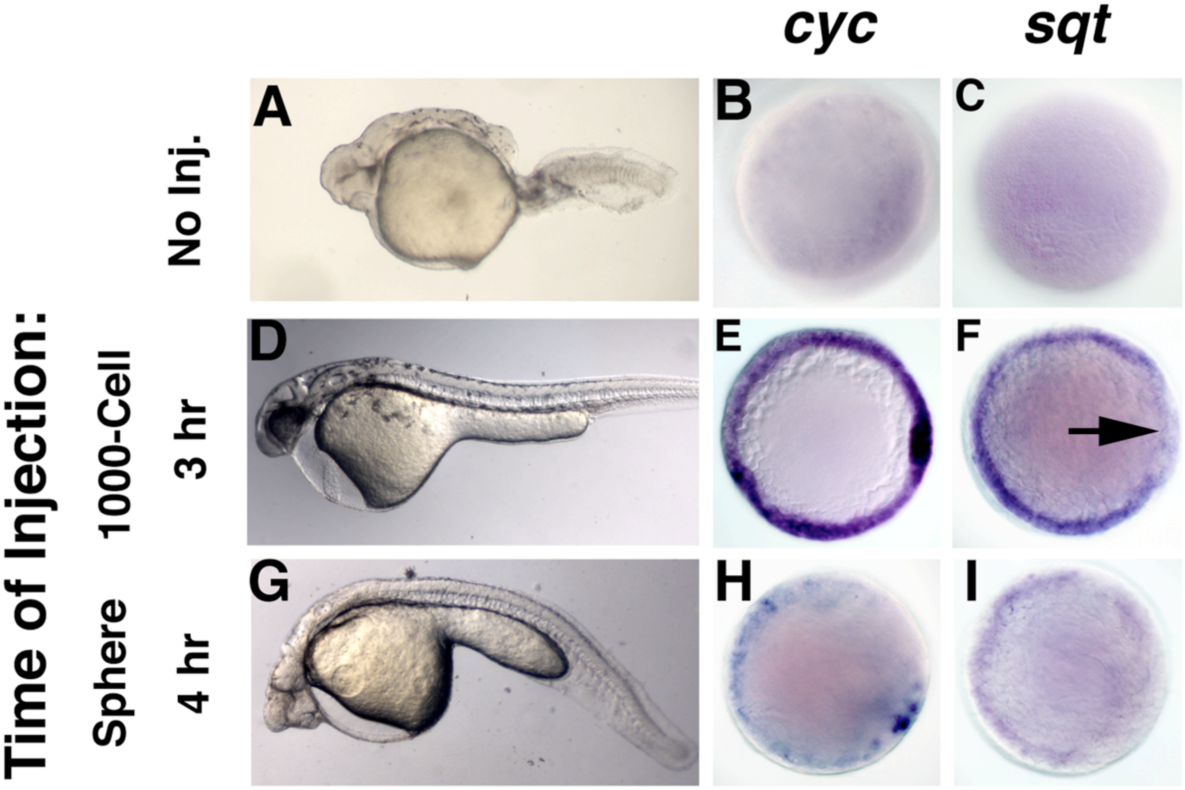
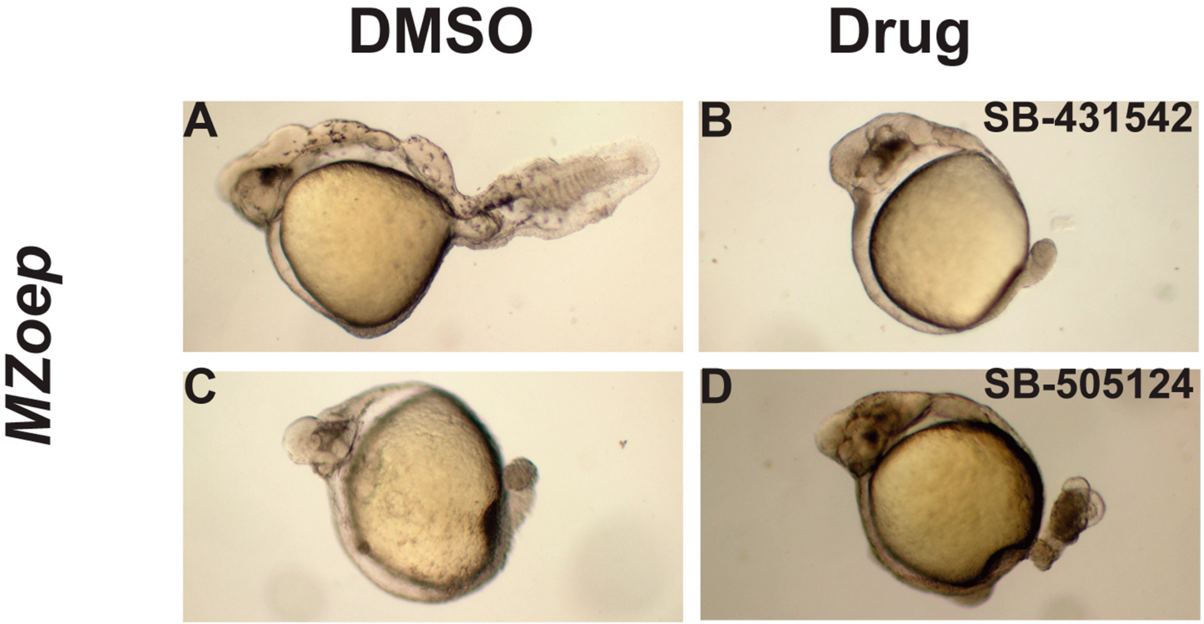

cyc
sqt
Time of Injection:
No Inj.
1000-Cell
3 hr
Sphere
4 hr
A
B
C
D
E
F
G
H
I

Figure 4.8. Changes in BMP signaling levels explain variations in the *MZoep* phenotype. MZoep mutants were treated with DMSO (A, C), SB-431542 at the 16-32-cell stage (B), or SB-505124 at 3 hpf (D). Wild type embryos were injected with 25pg *chordin* mRNA (E, G), or β-galactosidase mRNA (F) and treated with DMSO (E) or SB-431542 (F, G). The majority of *MZoep* mutants treated with DMSO develop long tails containing somites (A), but a minority has underdeveloped tails with no apparent somites (C). After treatment with SB-431542 during the cleavage stages, all *MZoep* mutants have shortened tails with no apparent somites (B). Similarly, *MZoep* mutants have short tails after treatment with SB-505124 at 3 hpf (D). Wild type embryos lack the ventral tailfin after injection with 25pg chordin mRNA (E). Embryos are mildly cyclopic after treatment with 100μM SB-431542 at the 16-32-cell stage, but have trunk notochord and somites (F). 25pg chordin mRNA strongly dorsalizes drug treated embryos, which have truncated tails and lack all derivatives of the trunk mesoderm (G). Images of live embryos at 24h, anterior to the left.


DMSO
Drug
MZoep
A
B
SB-431542
C
D
SB-505124

Chapter 5

CONCLUSION

In all organisms, cells differentiate according to their relative position in embryo, generating a highly reproducible pattern of cell fates. In vertebrates embryonic development starts upon fertilization of an oocyte, followed by rapid cell cleavage. As development proceeds, the embryo is patterned by regionalization to form the three germ layers: ectoderm, mesoderm and endoderm. Subsequently, each germ layer gives rise to specific sets of differentiated cells types. The molecular mechanisms underlying axis formation are conserved in all vertebrates including zebrafish, frog, chick and mouse (Schier, 2001; Schier, 2003). These organisms are use the same signaling pathways and transcription factors during early embryogenesis (De Robertis and Kuroda, 2004; Lu et al., 2001; Schier and Talbot, 2005). For example, in all vertebrates embryonic patterning requires several conserved inductive signals including Wnt, FGF, BMP, and Nodal signaling pathway. Activating these signals turns on different transcription factors such as homeodomain and T-box proteins that play an essential role in controlling many aspects of embryogenesis, (Kimelman, 2006; Schier and Talbot, 2005). Since many of these molecular pathways are conserved from flies to humans, results from zebrafish will likely apply to humans and can contribute to the development of new diagnostic tools and treatment of human diseases.

In addition to its role in early vertebrate development, the Nodal-signaling pathway has been implicated in numerous human cancers and is also required in human embryonic stem cells (James et al., 2005; Massague, 2000). The bone morphogen proteins (BMPs), another TGF-β signaling molecule is known to promote hESC differentiation. While the Nodal-related proteins maintain embryonic stem cell identity (James et al., 2005). Supporting this idea I found that targets of the BMP pathway were upregulated when Nodal signaling is blocked. This suggests that Nodal signals may directly antagonize BMP signals in certain tissues. Additionally, mutations in components of Nodal signals transduction machinery have been linked to birth defects such as holoprosencephaly, which is caused by abnormal development of the forebrain (Bamford et al., 2000; Hayhurst and McConnell, 2003; Muenke and Beachy, 2000). Mutation in *nodal related* genes in zebrafish leads to holoprosencephaly and cyclopia, consistent with role for Nodal signaling for ventral forebrain (Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). By furthering our knowledge of how these signals normally act and elucidate the mechanisms regulating *nodal-related* genes expression my studies may contribute to better diagnoses and treatments human diseases.

The are five conclusions drawn from this thesis: First, Nodal-related signals have essential role in early embryogenesis and are required to specify different cell types along the animal-vegetal axis in time-dependent manner. Second, previous studies indicate that Nodal signals acts in a concentration-dependent manner to specify different cell types. However, I show that specification of endoderm and mesoderm in zebrafish depends on the duration of time that cells are exposed to Nodal signals. Third, my work suggests a mechanism by which endoderm and mesoderm cell types are specified in the absence of the proposed morphogen *sqt*. These results demonstrate that cells respond to the total cumulative dose of Nodal signals to which they are exposed, as a function of distance from the source and duration of exposure. Additionally, the results presented here indicate that the competence of cells to respond to Nodal signals is not fixed by an intrinsic factor as shown in *sqt* mutant which is delayed and *sqt* injected embryos which is expressed earlier than wild-type embryos. Perhaps the competence window is determine by Nodal signals themselves. Fourth, Nodal signals from the YSL are required to pattern the embryos and *squint* and *cyclops* from the extraembryonic tissue regulate *nodal* gene expression in the blastoderm. Finally, maternally supplied Activin-like ligands including *squint*, Activin and zDVR-1 do not act as dorsal determinants, but they play an important role in embryonic patterning.

Morphogenic activity of squint is not required in the zebrafish embryo

Previous studies have been proposed that Nodal signaling acts in concentration dependent manner to specify different cell types in zebrafish embryo. According to this model, Sqt protein diffuses into the surrounding tissue, thus exposing neighboring cells to different Nodal concentrations and cells respond accordingly. This model, however, does not explain how a short-range signaling molecule like Cyc could fully compensate for loss of the Sqt morphogen (Dougan et al., 2003). This model is also unable to explain the intermingling of endoderm and mesoderm precursors in the zebrafish fate map, even though these cell types require different levels of Nodal signaling (Warga and Nusslein-Volhard, 1999). Alternatively, the temporal gradient model could explain how the graded action of Nodal signals induces and patterns mesoderm in all vertebrates (Fig. 2.10). According to this model, cells that remain near the Nodal source for an extended period would receive a high dose and adopt a marginal cell fate, such as prechordal plate or definitive endoderm. Conversely, cells that move away from the source after a short time would receive a lower dose and become somites.

Having proposed, Nodal signals pattern the zebrafish embryo by the temporal gradient model, one of the future challenges is to determine how cells receive Nodal signaling and change their morphology to acquire the position they need to be in the embryo. For example, according to our model, cells specified to become somite progenitors should exit the margin and get their position close to precursors of ectodermal fates in a more animal pole position. For this model to be valid, individual cells in the embryo must selectively change and confer the ability to migrate and acquired their position in the embryo. Thus, cell movements during normal development generate positional information composed of cells exposed to Nodal signals for different duration of time. Additionally, cells must sense how long they have been exposed to Nodal signals and translate this information into specific patterns of gene expression such as *myoD* that marks precursors of somites. This result suggests that the factors that determine the shape of different cell types have to be under the control of Nodal signaling.

Cell mixing at early stage of development is a common feature of vertebrate development. Dynamic populations of cells also comprise the node and primitive streak in mouse and chicken. Despite cell mixing a distinct mesodermal and endodermal cell types pattern properly (Joubin and Stern, 1999; Kinder et al., 2001). The temporal gradient model has also been proposed in mouse to specify cell types such as the distal limb development by Sonic Hedgehog to pattern the digits along the anterior-posterior axis (Ahn and Joyner, 2004; Harfe et al., 2004).

It is possible that Nodal signaling patterns the embryo in another vertebrate such as, amphibian by temporal gradient model. This model can be tested by developing transgenic animal embryos that contain inducible activators or by Nodal inhibitor SB-505124. The availability of the SB-505124 can make it possible to test this model since the Nodal signaling pathway is conserved in all vertebrate.

It is important to study the dynamic movements of these different cell types in responding to Nodal signals. It is possible that cells exposed at the same time to Nodal signals attract each other by local cell-cell signaling and perhaps adhesive interaction. Transgenic lines that express

GFP from a cell type specific promoter might be useful in determining whether somite cells have adopt different morphology compared to other cell types.

The extraembryonic tissue in zebrafish is functionally conserved as mouse extraembryonic

Extraembryonic tissues in mammalian play important role in the patterning of an early embryo. For example, the anterior visceral endoderm (AVE) have critical role in patterning mouse embryos. Results presented in this thesis suggest that the anterior visceral endoderm has similar function in the extraembryonic tissue yolk syncytial layer (YSL), in zebrafish. Two lines of evidences support this. First, Nodal signals from this tissue in both species are required to pattern the embryo. Second, reciprocal cell-cell interaction between the extraembryonic tissue and the embryo proper is necessary during early stage for normal development (Brennan et al., 2001; Varlet et al., 1997).

Although it is difficult to directly compare different species based on gene expression analysis and structural similarity, I argue here that the mouse and fish are more evolutionary conserved than the frog which lacks extraembryonic structure. Mouse embryos are radially symmetrical, consisting of an epiblast, extraembryonic ectoderm and visceral endoderm. This also seen in zebrafish, which also has the deep cells that form the embryo proper and extraembryonic tissues, enveloping layer and yolk syncytial layer. The use of genetic markers and structural similarities provides evidence for the evolutionary relationship between these two species. Additionally, the evolutionary conserved role of *nodal-related* genes in extraembryonic tissues suggests that Nodal signaling acts by similar mechanisms to pattern the teleost and the mammalian embryo. Since a zebrafish embryo can be manipulated easily further experiments will be needed to determine the function of extraembryonic signals. These studies will be useful in providing understanding about how embryo pattering is common among vertebrate.

Activin like signals including Squint do not function as dorsal determinant

The organizer plays a major role in setup of the dorsal axis. A long-standing hypothesis states that the dorsal determinants have to be deposited into the egg by the mother to establish the dorsal axis is established. Although the molecular nature of these determinants has not yet been elucidated, the dorsal determinants activate the Wnt pathway and thereby lead to the expression of genes that are involved in induction of the organizer. *squint* is one of the genes downstream of Wnt signaling that required to induce the dorsal organizer. However, Gore and colleagues have recently proposed that Squint serves as dorsal determinant (Gore et al., 2005).

The work presented here shows that Activin-like signals do not act as dorsal determinants to determine the dorsal axis in zebrafish. This is supported by, first, maternal-zygotic *squint* mutant embryos, which are indistinguishable from zygotic *squint* mutants and have variable defects at 24h. A significant number survive to sexual maturity, becoming fertile adults (Dougan et al., 2003). Second, I demonstrate that embryos treated with Nodal signal inhibitors, SB-431542 or SB-505124 were indistinguishable from *sqt;cyc* double mutants when treated at 1-cell or 1000-cell stage (DaCosta Byfield et al., 2004; Inman et al., 2002). Third, embryos treated with SB-505124 are normal when receptor blocked during the cleavage stages and washout during the blastula period. These results demonstrated that Activin-like signals are not required before mid-blastula transition to pattern the zebrafish embryo, and therefore this rule out the possibility of these factors as dorsal determinants.

Although significant diversity in embryonic development has emerged throughout evolution, the Wnt signaling pathway retained a crucial role during primary axis formation in vertebrate (Cadigan and Nusse, 1997). In mouse, a member of the Wnt family trigger the formation of the primitive streak (Liu et al., 1999). In *Xenopus* and zebrafish, maternal activation of the Wnt pathway is necessary and sufficient to specify the dorsal axis (Schier and Talbot, 2005). Even though, the studies in this thesis contributed to our understanding of early vertebrate development, many exiting questions remain to be answered. For example, we still do not understand the maternal determinants that initiate axis formation. However, with the zebrafish genome completed in the near future and use of microarrays it will be possible to understand the molecular and cellular mechanisms that regulate axis formation in vertebrates.

REFERENCES

Ahn, S., and A.L. Joyner. 2004. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell*. 118:505-16.

Bamford, R.N., E. Roessler, R.D. Burdine, U. Saplakoglu, J. dela Cruz, M. Splitt, J.A. Goodship, J. Towbin, P. Bowers, G.B. Ferrero, B. Marino, A.F. Schier, M.M. Shen, M. Muenke, and B. Casey. 2000. Loss-of-function mutations in the EGF-CFC gene CFC1 are associated with human left-right laterality defects. *Nat Genet*. 26:365-9.

Brennan, J., C.C. Lu, D.P. Norris, T.A. Rodriguez, R.S. Beddington, and E.J. Robertson. 2001. Nodal signalling in the epiblast patterns the early mouse embryo. *Nature*. 411:965-9.

Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev*. 11:3286-305.

DaCosta Byfield, S., C. Major, N.J. Laping, and A.B. Roberts. 2004. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 65:744-52.

De Robertis, E.M., and H. Kuroda. 2004. Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu Rev Cell Dev Biol*. 20:285-308.

Dougan, S.T., R.M. Warga, D.A. Kane, A.F. Schier, and W.S. Talbot. 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development*. 130:1837-51.

Feldman, B., M.A. Gates, E.S. Egan, S.T. Dougan, G. Rennebeck, H.I. Sirotkin, A.F. Schier, and W.S. Talbot. 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature*. 395:181-5.

Gore, A.V., S. Maegawa, A. Cheong, P.C. Gilligan, E.S. Weinberg, and K. Sampath. 2005. The zebrafish dorsal axis is apparent at the four-cell stage. *Nature*. 438:1030-5.

Harfe, B.D., P.J. Scherz, S. Nissim, H. Tian, A.P. McMahon, and C.J. Tabin. 2004. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell*. 118:517-28.

Hayhurst, M., and S.K. McConnell. 2003. Mouse models of holoprosencephaly. *Curr Opin Neurol*. 16:135-41.

Inman, G.J., F.J. Nicolas, J.F. Callahan, J.D. Harling, L.M. Gaster, A.D. Reith, N.J. Laping, and C.S. Hill. 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 62:65-74.

James, D., A.J. Levine, D. Besser, and A. Hemmati-Brivanlou. 2005. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development*. 132:1273-82.

Joubin, K., and C.D. Stern. 1999. Molecular interactions continuously define the organizer during the cell movements of gastrulation. *Cell*. 98:559-71.

Kimelman, D. 2006. Mesoderm induction: from caps to chips. *Nat Rev Genet*. 7:360-72.

Kinder, S.J., T.E. Tsang, M. Wakamiya, H. Sasaki, R.R. Behringer, A. Nagy, and P.P. Tam. 2001. The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development*. 128:3623-34.

Liu, P., M. Wakamiya, M.J. Shea, U. Albrecht, R.R. Behringer, and A. Bradley. 1999. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet*. 22:361-5.

Lu, C.C., J. Brennan, and E.J. Robertson. 2001. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev*. 11:384-92.

Massague, J. 2000. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol*. 1:169-78.

Muenke, M., and P.A. Beachy. 2000. Genetics of ventral forebrain development and holoprosencephaly. *Curr Opin Genet Dev*. 10:262-9.

Rebagliati, M.R., R. Toyama, P. Haffter, and I.B. Dawid. 1998. cyclops encodes a nodal-related factor involved in midline signaling. *Proc Natl Acad Sci U S A*. 95:9932-7.

Sampath, K., A.L. Rubinstein, A.M. Cheng, J.O. Liang, K. Fekany, L. Solnica-Krezel, V. Korzh, M.E. Halpern, and C.V. Wright. 1998. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature*. 395:185-9.

Schier, A.F. 2001. Axis formation and patterning in zebrafish. *Curr Opin Genet Dev*. 11:393-404.

Schier, A.F. 2003. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol*. 19:589-621.

Schier, A.F., and W.S. Talbot. 2005. Molecular genetics of axis formation in zebrafish. *Annu Rev Genet*. 39:561-613.

Varlet, I., J. Collignon, and E.J. Robertson. 1997. nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development*. 124:1033-44.

Warga, R.M., and C. Nusslein-Volhard. 1999. Origin and development of the zebrafish endoderm. *Development*. 126:827-38.