Temporal Spatial requirement of the Nodal induced head mesendoderm in neurulation

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An inexpensive, efficient method for regular egg collection from zebrafish in a recirculation tank

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Abstract

Chapter 2

Zebrafish in our laboratory are usually bred by removing the fish from the recirculating aquatic system and placing them into 1-2 L spawning tanks. These spawning tanks consist of a bottom reservoir, a lid, and an insert that fits in closely into the bottom reservoir. When the fish breed, the eggs fall through holes of the insert and into the reservoir, thus preventing them from being cannibalized. Because fish in these spawning tanks are not fed and do not get fresh water, they are bred only once a week. During a period where we had high demand for embryos, we instead tried breeding the fish for multiple consecutive days on the recirculating system. Fish were placed into the spawning insert as usual, but the insert was placed into the home tank instead of into the bottom reservoir. We found that there was no significant difference in the number of fertilized eggs produced between the spawning tank and home tank breeding methods. Further, the fish in the home tanks regularly produced fertile embryos over a 28-day time course, with the highest number of eggs per pair produced by the tank with only one pair of adult fish. This method is time-saving as fish bred in home tanks only require to be set up once. It is also an effective way to collect embryos over long periods from the same pair or group of fish and to more easily obtain embryos from stocks with low spawning frequency.

Chapter 3

The neural tube is the precursor to the brain and spinal cord. Failure of neural tube closure in humans is one of the most common causes of birth defects. Zebrafish with a decrease in Nodal signaling have a phenotype that is analogous to the fatal human birth defect anencephaly, which is caused by an open anterior neural tube. Previous work in our laboratory has found that Nodal signaling acts through the induction of the head mesendoderm and anterior mesoderm, which underlie the anterior neural tube. Using a pharmacological approach, we determined that Nodal signaling is required up to the late blastula stage of 4.3 hpf for a closed neural tube. This falls within the developmental period when Nodal signaling is most active in mesendoderm and mesoderm induction, supporting the model that Nodal acts through the induction of these tissues. We also found there was a strong correlation between the presence of multiple anterior mesendodermal and mesoderm tissues and neural tube closure. However, no individual tissue was required for neural tube closure. Our finding identifies a specific time window of when Nodal is required for the process of neurulation. This time occurs before the neuroectoderm starts to form, suggesting that Nodal and anterior mesendoderm/mesoderm act very early in the process of neurulation. Further, the finding that multiple mesendodermal/mesodermal tissues are involved suggests that wide region of tissue helps promote closure of the adjacent neural tube.

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I. INTRODUCTION

Feedback mechanisms exist in all biological systems including the ecosystem, the biosphere and within the cells of an organism. Within organisms, signaling pathways are driven by positive and negative feedback loop and determine the cell fates of a developing embryo. My thesis describes the spatial and temporal requirements of the Nodal signaling pathway in the development of a closed neural tube. Neural tube closure is crucial for the survival of the organism as it is the precursor to the brain and the spinal cord. Despite the developmental differences of species across phyla, evolutionarily conserved pathways including Nodal signaling have highly specific characteristics in different contexts. The signals transmitted in these pathways possess spatial and temporal accuracy allowing for precise developmental stages and tissue organization.

For instance, in the Nodal signaling pathway, the *lefty1*-Nodal negative feedback loop regulates the Nodal dose across embryonic cells. The regulation of the Nodal dose is key for ensuring differential cellular fates. There are also many other signaling pathways that are also tightly regulated by feedback mechanisms. The apoptotic pathways have many developmental roles in vertebrates, including the cell death in the Inter Digital Space to form distinct digits (Gilbert, 2006). One signal involved in apoptosis is the Bone Morphogenetic Protein (BMP), expressed across cells in a concentration gradient (Gilbert, 2006). The high concentration of BMP at the site of cell death and the low concentration of the BMP in the surrounding cells is monitored by positive and negative feedback loops driven by BMP protagonists or antagonists respectively (Lagna, 2006). The regulation BMP by the feedback loops are essential because the BMP gradient

allows for BMP binding to cell surface receptors triggering caspases downstream. These caspases are proteases with a primary role for triggering apoptosis (Lagna, 2006).

The high degree of conservation in vertebrate BMP, its homologs in invertebrates, and other apoptotic pathways has allowed for mechanisms determined in lower species such as *Caenorhabditis elegans* and *Drosophila melanogaster* to characterize apoptosis in mammalian cells. Similarly, signals important in neural tube formation are monitored by feedback loops and conserved across vertebrates. These allow for drawing our findings of the zebrafish Nodal signaling to characterize the role of Nodal signaling in mammalian neurulation.

Zebrafish Is An Established Model System For Vertebrate Development

Zebrafish (*Danio rerio*) is a tropical fish, native to streams and rivers in Northeastern India (Barman, 1991). Zebrafish possess many characteristics that make them a powerful model organism for studying mechanisms involved in vertebrate development (Rubinstein, 2003). Unlike mammals, zebrafish fertilization occurs externally, outside of the mother. This makes the zebrafish much more accessible for visual analyses of well-characterized early developmental stages (Fig. 1) (Kimmel *et al*, 1995). The optically translucent zebrafish embryos allow for easy visual analyses of organs and tissues (Rubinstein, 2003). Additionally, the development of the organs, known as organogenesis, occurs very rapidly in the zebrafish. Within the first six days after fertilization, most of the major organs are well developed within the larvae (Fig. 1) (Rubinstein, 2003). Zebrafish also have a relatively fast sexual maturity. Three months after fertilization, the fish is sexually mature (Lele and Krone, 1996). Furthermore

because of the small size (2.5 inches for adults), large numbers of zebrafish can be easily kept in a limited space (Lele and Krone, 1996). Finally, zebrafish under suitable conditions in captivity are capable of breeding all year round, usually in the first hour after light exposure in the morning (Spence and Smith, 2006; Liang *et al*, 2011). For example, a healthy female can spawn every 1-2 days and can lay up to hundreds of eggs per spawn (Eaton and Farley, 1974; Gonsar *et al*, 2012).

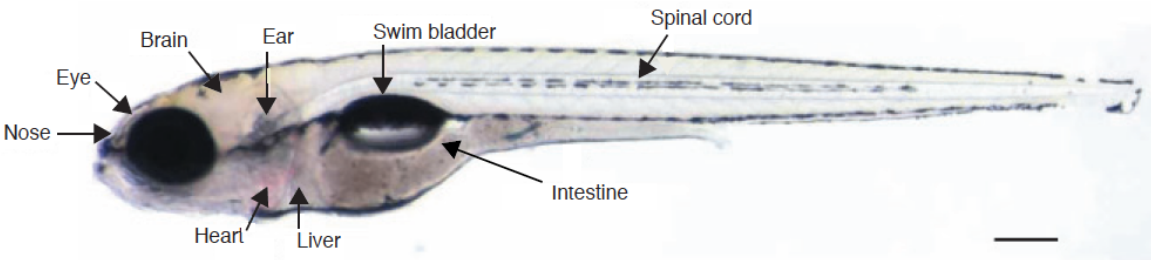


Figure 1: The zebrafish larvae at a 6 dpf display most of the major organs. Scale bars: 250 μm (Figure from Rubinstein, 2003).

The Blastula And Gastrula Stages Are Key For Zebrafish Neurulation

The Blastula Stages Initiate Zygotic Transcription

In all organisms, the fusion of an egg and a sperm results in a single, fertilized egg. This single celled organism is termed a zygote and undergoes rapid mitotic divisions, leading to the formation of a blastomere, a cleavage-stage cell (Gilbert *et al*, 2006). The blastomeres then undergo the blastula period during which the cells reach the Mid-Blastula Transition (MBT) (Kane and Kimmel, 1993). During MBT, cells undergo elongation and asynchronous cell cycles (Signoret and Lefresne, 1971; Gerhart, 1980).

In most vertebrates, including zebrafish, the MBT roughly coincides with Maternal to Zygotic Transition (MZT) (Fig. 2) (Tadros and Lipshitz, 2009). Up until the

MZT, the organism is completely controlled by the maternal mRNAs and proteins made from the maternal genome or similar for all aspects of early development. When MZT is initiated, there is degradation of a subset of maternal RNA (Fig. 2) (Tadros and Lipshitz, 2009). This is followed by the onset of zygotic transcription, when the zygote transcribes its own genome and additionally transcribes mRNA and micro RNAs that enhance the efficiency of maternal product degradation. (Fig. 2) (Tadros and Lipshitz, 2009). In zebrafish majority of the zygotic transcription is observed after 2.75 hours post-fertilization (hpf) by which time, most of the maternal transcripts are degraded. Following MZT, the embryo has control over its own development, transcribing RNA from its own genome (Tadros and Lipshitz, 2009).

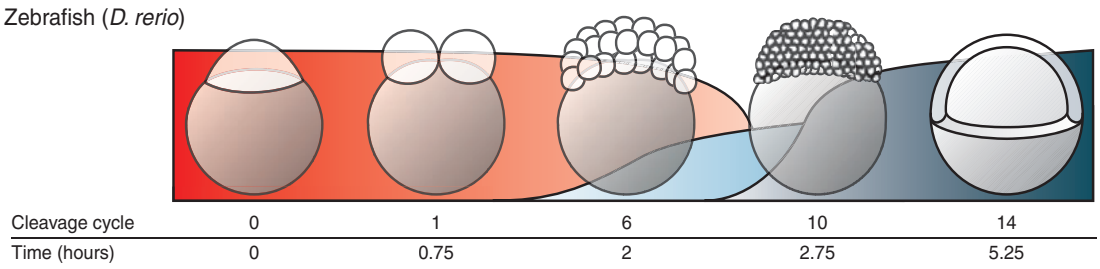


Figure 2: Illustration of the mid zygotic transition during the mid-blastula transition. Post MZT (light blue), the embryo initiates transcription. Red indicates usage of maternal transcripts. Dark blue indicates highest usage of zygotic transcripts (Figure from Tadros *et al*, 2009).

Gastrulation Results In The Formation Of The Germ Layers

The blastula period is followed by a group of cellular rearrangements called gastrulation. Gastrulation begins at the dorsal pole end of the embryo initiated by the formation of an organizer. Gastrulation is a critical stage in all early vertebrate embryogenesis when the developing embryo reorganizes from a single cell type into three distinct germ layers; ectoderm, mesoderm and endoderm (Schmidt, 2005; Gilbert *et al*,

2006). The three germ layers are precursors to all adult tissues and organs. Endoderm, the innermost of the three layers and the first cells to migrate through the organizer, forms the lining of the digestive and the respiratory tracts. The mesoderm forms the second layer, giving rise to the adult red blood cells, muscles and bone tissue (Gilbert *et al*, 2006). Finally, the ectoderm is the last germ layer to form and it gives rise to the epidermis, the brain and the Central Nervous System (CNS) (Gilbert *et al*, 2006).

Gastrulation involves four evolutionarily conserved morphogenetic steps that transform a cluster of cells into an embryo with a distinct head, trunk and tail parts (Solnica-Krezel and Sepich, 2012). These steps include internalization, epiboly and convergence and extension movements. In vertebrates, these four steps are responsible for the rearrangement, the specification and the shaping of the future body plan.

The first step is termed internalization when the mesodermal and endodermal precursors move through the blastoderm margin (in zebrafish) and position under the future ectodermal cells (Keller *et al*, 1991). Epiboly is the second step involving the spreading and thinning of the three germ layers over the entire embryo (Trinkaus and Lentz, 1967; Solnica-Krezel and Sepich, 2012). Convergence and extension (C&E) are the final two morphogenetic steps of gastrulation that occur concomitantly. In the growing embryo, extension of the germ layers occurs along the anterior-posterior axis and convergence allows the tissues to narrow along the dorsal ventral region (Solnica-Krezel and Sepich, 2012).

During gastrulation zebrafish undergo similar cellular movements to other vertebrates. At the inception of gastrulation, the zebrafish embryo exhibits a blastoderm (group of blastomeres) at the animal pole region (Kimmel *et al*, 1995). The blastoderm

consists of an outer layer and deeper cells; the latter giving rise to all future embryonic tissues. In zebrafish, the first morphological step is epiboly during which the blastoderm spreads towards the vegetal pole (Fig. 3) (Warga and Kimmel, 1990). The spreading of the blastoderm over the vegetal pole region results in the distribution of the germ layer's precursors over the yolk (Kimmel *et al*, 1990). Similar to other vertebrates zebrafish cells in the animal pole regions house the future ectodermal cells whereas the endodermal cells reside close to the blastoderm margin (Kimmel *et al*, 1990; Warga and Nusslein-Volhard, 1999). Additionally the endoderm and mesoderm progenitors are intermingled at the location close to the margin (Kimmel *et al*, 1990). This area is termed mesendoderm. The mesendoderm give rise to specific endoderm and mesoderm derivatives (Kimelman and Griffin, 2000). The endoderm derived from the mesendoderm gives rise to the presumptive gut and the mesoderm derived from the mesendoderm gives rise to the heart and muscles (Warga and Nusslein-Volhard, 1999).

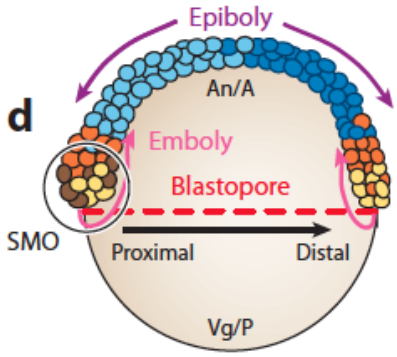


Figure 3: Gastrulation movements in the zebrafish. Lateral view of the patterns of epiboly (purple arrows) and emboly (internalization, pink arrows) in the early gastrula stage. Anterior on top, dorsal to the left. Cellular elements identified are mesoderm (orange), prechordal mesendoderm (brown), endoderm (yellow), epidermis (dark blue), neuroectoderm (light blue) and various embryonic tissues (green/brown/purple). Abbreviations: SMO, organizer; P, posterior; Vg, Vegetal; An, Animal**.** (Figure from Solnica-Krezel, 2005).

The second morphological step in zebrafish gastrulation is internalization. The mesendoderm moves through the blastopore lining beneath the future neuroectodermal on the dorsal side and the epidermis along the ventral side (Fig. 3) (Keller *et al*, 2008). After moving through the blastopore, the mesendoderm moves away from the blastopore, further up into the animal pole region simultaneously extending the tissue along the developing anterior-posterior axis (Gritsman *et al*, 2000; Sepich *et al*, 2005). Towards the middle of gastrulation (~7.5 hpf) progenitor cells of all the germ layers undergo convergence and extension movements in a spatio-temporal pattern (Concha and Adams, 1998; Yin *et al*, 2009).

The patterning of C&E are movements not well understood in the zebrafish endoderm and ectoderm. However, the C&E movements of the mesodermal tissues along the different positions along the dorsal-ventral axis are characterized (Myers *et al*, 2002a,b). During C&E, mesodermal cells intercalated on the future dorsal side do not undergo convergence but extend anteriorly allowing for cell narrowing along the dorsal midline (Keller *et al*, 2008). On the ventral side mesodermal cells are not involved in C&E but migrate towards the vegetal pole (Myers *et al*, 2002a)

Development Of The Anterior Neural Tube From Primary Neurulation

The process of neurulation results in the formation of the neural tube. Within a single vertebrate organism, the neural tube forms from two tubes that develop independent of each other. Primary neurulation drives the rostral neural tube closure. Primary neurulation is derived from epithelial cells held together by adherin proteins and tight junctions (Fig. 4) (Lowery and Sive, 2004). Secondary neurulation drives the

closure of the caudal neural tube. Secondary neurulation are derived from mesenchymal populated cells (Fig. 4) (Lowery and Sive, 2004). Mesenchymal cells are unconnected, loose cells (Gilbert, 2006).

Primary neurulation begins with the division of the ectoderm into three regions. The first region is the neural plate, which gives rise to the central nervous system. The second is the non-neural ectoderm, which gives rise to the epidermis. The last region is composed of cells bordering the non-neural ectoderm and the neural plate, which gives rise to neural crest cells (Gammill and Bronner-Fraser, 2003).

There are variations across and within vertebrates, but the basic morphological steps comprising primary neurulation are conserved (Fig. 4) (Lowery and Sive, 2004). These basic conserved steps initiate with the columnarization of ectodermal epithelial cells (Fig. 4) (Colas and Schoenwolf, 2001). The columnarization of the cells forms a flat sheet of epithelium called the neural plate (Fig. 4). As neurulation advances, the borders of the neural plate thicken and elevate forming the neural folds and a central neural groove (Fig. 4) (Colas and Schoenwolf, 2001). Finally, the neural fold tissues move towards each other and converge at the dorsal midline forming a hollow neural tube (Fig. 4) (Colas and Schoenwolf, 2001).

Zebrafish neurulation starts after the gastrulation period of the developing embryo with the formation of the neural plate (Fig. 4) (Papan and Campos-Ortega, 1994). After neural plate formation, there is thickening of the plate's lateral edges. The center of the neural plate then 'sinks' into the embryo forming the neural keel (Fig. 4) (Papan and Campos-Ortega, 1994; Schmitz *et al*, 1993; Kimmel *et al*, 1995). The keel then rounds up as the lateral edges fuse at the dorsal midline (Fig. 4). This results in the neural rod,

which is characterized by a solid mass of cells (Schmitz *et al*, 1993; Papan and Campos-Ortega, 1994, Kimmel *et al*, 1995). The keel and rod are unique features of zebrafish neurulation, although it has also been observed in part in the *Xenopus* neural tube formation (Davidson and Keller, 1999). After keel and rod formation, a lumen forms within the mass of cells starting ventrally and moving toward the dorsal end (Schmitz *et al*, 1993). The complete lumen formation results in a hollow neural tube (Fig. 4).

The difference in primary neurulation within and across vertebrates, occurs in the rolling/bending of the flat epithelial cells converging at the dorsal midline (Fig. 5) (Shum and Copp, 1996). The neural keel/rod formation before lumen formation in the zebrafish is one of the known variations of primary neurulation within vertebrates (Schmitz *et al*, 1993). Another variation is the smooth rolling of the flat neural plate observed in the *Xenopus* brain resulting in a round lumen (Fig. 5) (Davidson and Keller, 1999). The bending of the developing tube at specific hinge points is also another variation (Fig. 5). In the chick, at different positions along the anterior-posterior axis, different hinge points are apparent (Smith and Schoenwolf, 1991). The hinge points in the brain region early in development are different from the hinge points in the more posterior regions later in development, resulting in different shaped tubes within an embryo. For instance, a median hinge point is evident in the ventral midline along the anterior neural tube of the chick embryo. Meanwhile in the brain two dorso-lateral hinge points develop (Lowery and Sive, 2004). Therefore in the developing neural tube, the spinal cord region displays a slit like lumen but the brain of the chick displays a diamond shaped lumen (Smith and Schoenwolf, 1991).

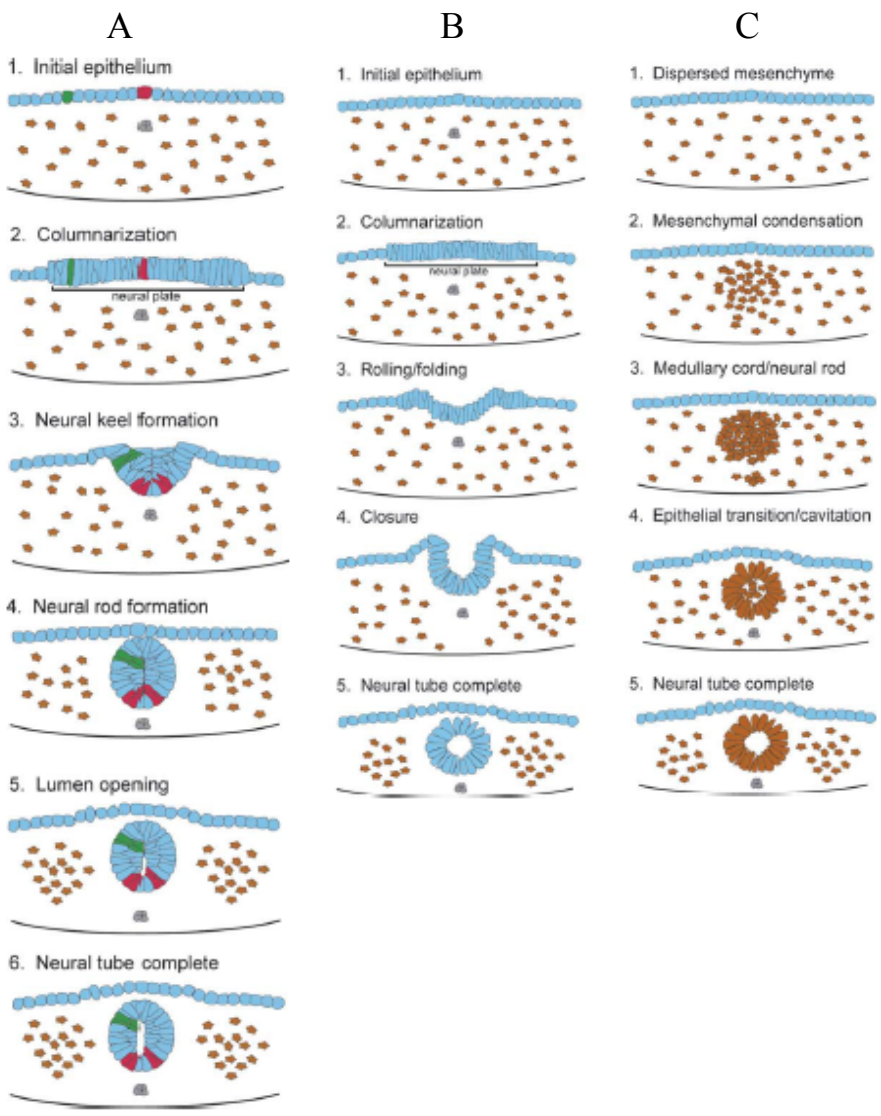


Figure 4: The neural tube develops from the primary and secondary neurulation. A) zebrafish primary neurulation. B) mammalian primary neurulation C) mammalian secondary neurulation. The components identified are epithelial cells (blue), mesenchymal cells (brown) and labeled neural plate (red and green) cells demonstrating 'sinking'. (Adapted from Lowery and Sive, 2004).

Recent work also demonstrates that zebrafish display a dorso-lateral hinge point during the neural rod stage, which is after convergence of the dorsal midline, indicating the hinge points may be important during lumen shaping (Nyholm *et al*, 2009). The final variation in primary neurulation is a solid rod of cells as opposed to the lumen observed

in all other known variations of closed neural tubes (Fig. 5). As described previously, this solid rod of cells known as the neural keel is transiently apparent in parts of the *Xenopus* and in the neural tube of the zebrafish (Fig. 5) (Davidson and Keller; Lowery and Sive, 2004).

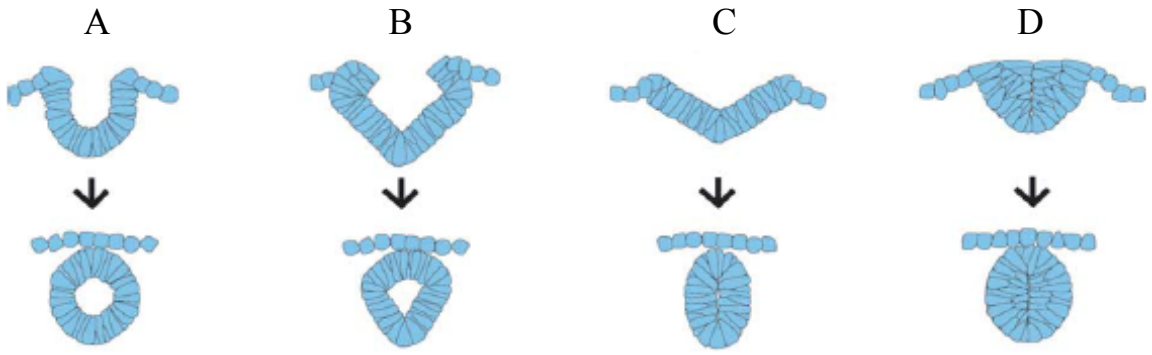


Figure 5: Variations of primary neurulation. A-D) The neural tube is displayed in the open stage (top row) and in the closed stage (bottom row). The neuroepithelial cells can A) roll, B) form multiple hinge points, C) form a single hinge point, or D) form a solid rod of cells. (Adapted from Lowery and Sive, 2004).

Molecular Candidates For Driving A Closed Neural Tube

Despite some differences in the tissue movements, the molecular mechanisms driving neurulation are largely conserved across all vertebrates (Lowery and Sive, 2004). For instance, studies of various vertebrates demonstrate a number of candidate genes/molecules that are responsible for cell adhesion, also have a strong association with primary neurulation (Copp *et al*, 2003; Colas and Schoenwolf, 2001). One such protein is N-cadherin (N-cad). Detailed analysis of N-cad in the zebrafish demonstrates that this adhesion molecule regulates cell convergence and the maintenance of the neural plate morphology during neurulation (Lele *et al*, 2002). Additionally, zebrafish *N-cad* mutants exhibit Neural tube defects including an open neural tube (Lele *et al*, 2002, Aquilina-Beck *et al*, 2007).

In other vertebrates, N-cad expression is similar to zebrafish, but loss of N-cad does not result in an open neural tube phenotype. In mice, N-cad is expressed during gastrulation and neurulation and null *N-cad* mutants display an undulated neural tube (Radice *et al*, 1997). However, unlike zebrafish, mice *N-cad* mutants display a closed neural tube (Radice *et al*, 1997). N-cads come from a family of Ca^{2+} dependent cell adhesion molecules called cadherins and this family is crucial for multiple steps in early embryonic development (Tepass *et al*, 2000). The less severe phenotype observed in mice *N-cad* mutants maybe attributed to the plasticity and the abundance of cadherins in mammals. Mammals possess over 80 known cadherins, with around 30 expressed in the nervous system alone (Hatta and Takeichi, 1986; Miyatani *et al*, 1989; Redies, 2000; Yagi and Takeichi, 2000). Several studies in mammals show that some of these cadherins can substitute for each other. For instance E-cadherin can rescue the heart phenotype in N-cad null mice mutants (Luo *et al*, 2001). Hence, it is possible that the mice mutant displaying a closed neural tube phenotype may be a result of another cadherin substituting for the absent N-cad. Additionally, studies in other model systems such as chick and the Xenopus suggest that N-cad appears to be a key player in the early step of neural plate formation (Nakagawa and Takeichi, 1995; Nandadasa *et al*, 2009).

Dorsal-Ventral Patterning Of The Neural Tube

In vertebrates, the roofplate and the floorplate of the neural tube are two signaling centers that control the specification of the dorsal-ventral axis of the neural tube (Fig. 6) (Blader and Strähle, 2000; Gilbert *et al*, 2006). The BMP from the roof plate, signals for the dorsal neural tube formation. During neural patterning, the BMP2 and BMP4

signaling from the epidermis induce the roof plate in the dorsal region. The BMP proteins are secreted in the highest concentration in the dorsal neural tube as compared to ventral neural cells (Fig. 6). (Nguyen *et al*, 2000).

The Sonic Hedgehog (shh) signaling from the floor plate signal for ventral neural tube formation (Blader and Strähle, 2000). The Nodal signaling is upstream of shh expression and studies in zebrafish suggest Nodal signaling is essential for floor plate and ventral brain development (Sampath *et al*, 1998; Blader and Strähle, 2000). In developing vertebrates, the floor plate secretes shh, which then signals to other cells within the neural tube, with the highest concentration of shh in the ventral region (Fig. 6) (Roelink *et al*, 1995; Briscoe *et al*, 1999).

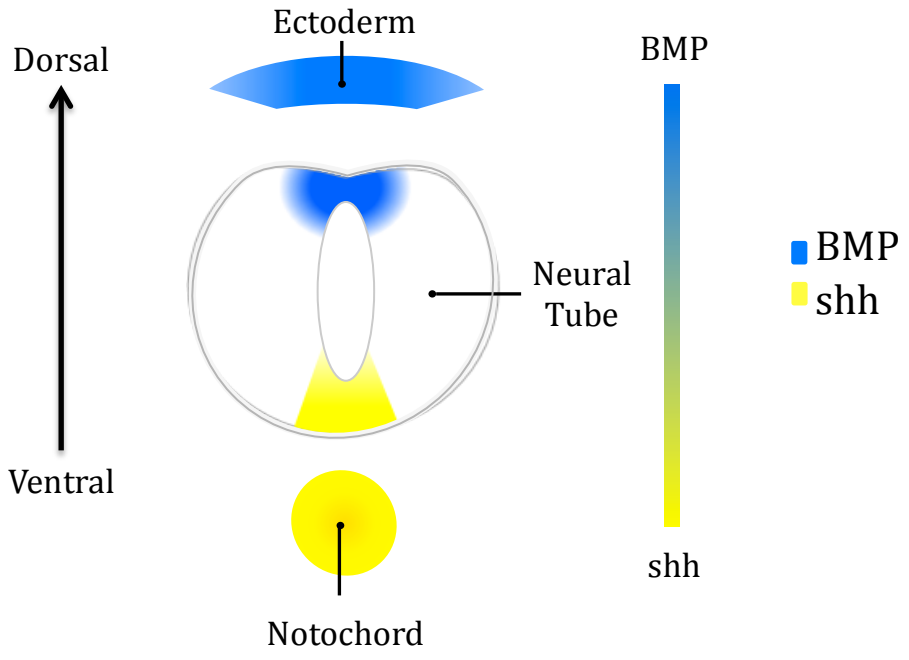


Figure 6: The dorsal-ventral patterning of the vertebrate embryo. Cross sectional illustration of the anterior neural tube. Patterning is driven by the concentration gradients of the sonic hedgehog and bone morphogenetic protein across the neural tube. Highest Sonic Hedgehog concentration in the ventral neural tube and highest BMP concentration in the dorsal neural tube. (Figure from Gilbert *et al*, 2006).

Additionally Nodal signaling mutants including the *cyc*, *cyc;sqt and MZoep* lack ventral brain and floor plate, suggesting a potential role for Nodal signaling in the ventral neural tube development (Hatta *et al*, 1991; Sampath *et al*, 1998, Blader and Strähle, 2000). For instance, the *cyc* mutants display a cyclopic phenotype but also lack medial floor plate formation in addition to ventral brain development (Hatta *et al*, 1991; Sampath *et al*, 1998). However, *cyc* mutants have a closed neural tube suggesting patterning of the ventral brain is not essential for neurulation.

Failure Of Neural Tube Closure Leads To Fatal Neural Tube Defects

Failure in either primary or secondary neurulation results in neural tube defects (Fig. 7) (NTDs). NTDs are among the most common human birth defects, occurring in approximately 1 in 1000 births worldwide (Detrait *et al*, 2005). The two most common types of NTDs include spina bifida and anencephaly. Spina bifida results when the caudal spinal cord fails to close. In most cases this defect can be corrected surgically, though it may result in lifelong disabilities from nerve damage (Mitchell *et al*, 2004). Anencephaly is a fatal birth defect where the cephalic neural tube fails to close, ceasing the development of the forebrain (Detrait *et al*, 2005).

Studies indicate that NTDs are driven by genetic and nutritional factors (Gittelsohn and Milham, 1962). For instance, nutritional folic acid supplementation in pregnant mothers significantly decreases the chance of NTDs. The Medical Research Council (MRC) Vitamin Research Group conducted a clinical study done on 1817 women spanning 7 countries (Wild *et al*, 1992). This study observed a decrease in NTDs

in approximately 70% cases of women on folic acid supplements whom previously had one affected embryo during pregnancy (Wild *et al*, 1992). In the 30% that were untreatable with folic acid supplements, there was a recurrence in NTDs despite an increased folic acid intake, suggesting a resistance to folic acid in certain NTDs (Cavalli *et al*, 2011). In mice with a folic acid resistance, intake of the Inositol isomer (myo-inositol and D-chiro-inositol) reduced NTDs (Cogram *et al*, 2005, 2004). The Inositol supplement was then administered along with folic acid supplements to would be human mothers with a previous NTD birth. The 12 mothers administered gave birth to 17 babies, none of who displayed NTDs, suggesting nutritional factors affect NTDs (Cavalli *et al*, 2011).

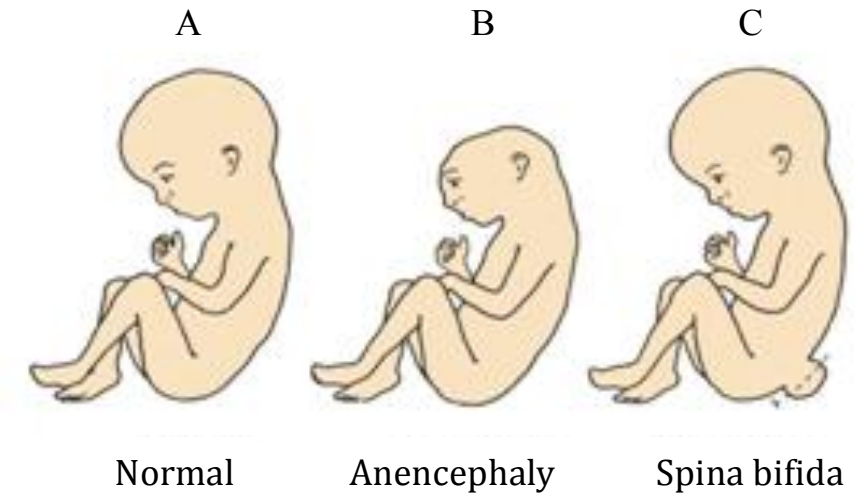


Figure 7: Variety of phenotypes can result from the failure in neural tube closure. A) Infant displays closure of the anterior and posterior neural tube. B) Displays anencephaly, which results from the failure in closing the anterior neural tube. C) Displays spina bifida, which results from failure in the closing of the posterior neural tube. (Figure adapted from Gilbert, 2006).

The Zebrafish Pineal Organ Marks The Closed Neural Tube

The neural tube closure in the zebrafish forebrain can be assayed by using the

pineal marker, orthodenticle homolog 5 (*otx5*) (Fig. 8) (Gamse *et al*, 2002; Aquilina-Beck *et al*, 2007). The pineal organ is part of the photoneuroendocrine system. In zebrafish, the pineal organ contains photoreceptor cells that function by responding to environmental light cues and conveying the photoperiod information to a circadian clock within the pineal cells (Ekström and Meissl, 1997). The information conveyed is transmitted through the modulation of neural pathways and indoleamine synthesis, most notably melatonin. Melatonin is synthesized by the pineal organ and is a hormone important for regulating the organism's internal circadian rhythm.

In zebrafish, neurulation begins around 10 hpf at the onset the segmentation period of development. At the inception of neurulation, the pineal precursors are positioned at the lateral points of the neural plate. As neurulation advances, the lateral edges of the neural plate move towards each other, simultaneously moving the pineal precursors along. As the edges of the neural plate fold and form a rod, the pineal precursors also converge. Therefore at the end of neurulation, the pineal anlage localizes to the presumptive epiphysis in the dorsal midline of the diencephalon, indicating a closed neural tube (Fig. 8) (Gamse *et al*, 2002; Aquilina-Beck *et al*, 2007). However a failure in the neural tube closure will result in a divided or an elongated pineal (Aquilina-Beck *et al*, 2007).

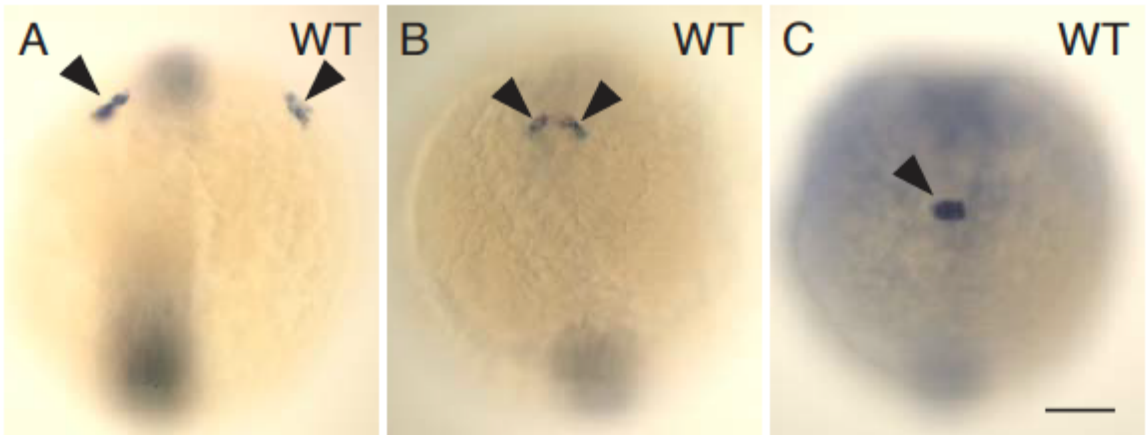


Figure 8: The pineal morphology marks the process of neurulation. WT embryos processed by in-situ hybridization using the pineal marker, *flh*. A) ~10 hpf, pineal precursors are expressed laterally at the neural plate border. B) As neurulation advances, the pineal precursors move towards each other. C) By ~14 hpf, when the neural rod has formed, the pineal precursors merge at the dorsal midline, indicating closure. (Adapted from Aquilina-Beck *et al*, 2007).

The Role Of Nodal Signaling In Neural Tube Closure

Nodal Signaling

In zebrafish, Nodal signaling mutants are marked by a divided pineal gland and exhibit NTDs analogous to anencephaly (Aquilina-Beck *et al*, 2007). Nodals are signaling proteins in the transforming growth factor beta (TGFβ) superfamily (Schier, 2003). Nodals are found in chordates including mice, frogs, lancelets and the zebrafish (Schier, 2003). However, Nodals have not been found in *Drosophila melanogaster* or *Caenorhabditis elegans*, a strong indication that Nodals may have a more specific role in vertebrate development (Schier, 2003). In zebrafish, Nodal signaling is required for a closed neural tube (Aquilina-Beck *et al*, 2007), and in all vertebrates Nodals have a role in left right asymmetry, neural patterning, and mesendoderm, mesoderm, and endoderm induction (Schier, 2003).

Zebrafish have three Nodal homologs, which include Cyclops (cyc) and Squint

(sqt) and Southpaw. These Nodal ligands interact with the Activin receptors, type I transmembrane serine/threonine kinase receptor Acvr1b (TARAM-A) along with the Activin receptor type-2B receptor Acvr2b (Fig. 9) (Schier, 2003). The interaction of the Nodal ligands with Acvr2b allows for the homodimerization of the Acvr1b and the Acvr2b, thereby phosphorylating the serine/threosine of the Acvr2b (Fig. 9). This activation of the Acvr1b phosphorylates the cytoplasmic Smad 2 and Smad 3, which interact with Smad 4, forming a Smad complex. The Smad complex translocates to the nucleus where interaction with FoxH1 initiates Nodal-mediated gene expression (Fig. 9) (Whitman, 2001).

The triggering of Nodal signals by Activin and Smad components are also shared with other TGFβ signals. However, there are proteins that are specific to the Nodal signaling pathway. This includes the EFG-CFC family coreceptor One Eyed Pinhead (oep) and the lefty inhibitors (Fig. 9) (Reissmann *et al*, 2001; Yeo and Whitman, 2001). Currently the exact mechanism for how oep acts as a co-receptor is unknown, but one hypothesis suggests that oep changes the conformation of the Acvr1B allowing Nodal interaction with the Activin receptors (Schier, 2003).

The Nodal signaling pathway also drives expression of the genes encoding the lefty1 and lefty2 inhibitors. lefty temporally and spatially restrict Nodal signaling through a negative feedback, where lefty decreases Nodal activity at distant cells (Green and Smith, 1990; Hamada *et al*, 2002). The mechanism of how lefty inhibits Nodal is unclear, but it is believed that lefty competes for common receptors preventing Nodal-receptor complex interaction (Meno *et al*, 1999). Hence if lefty is mutated, Nodal signaling is enhanced, driving more cells toward mesoderm and mesendodermal fates (Meno *et al*,

1999). On the other hand, overexpression of lefty results in embryos lacking mesoderm/mesendoderm, much like Nodal mutant embryos (Agathon *et al*, 2001).

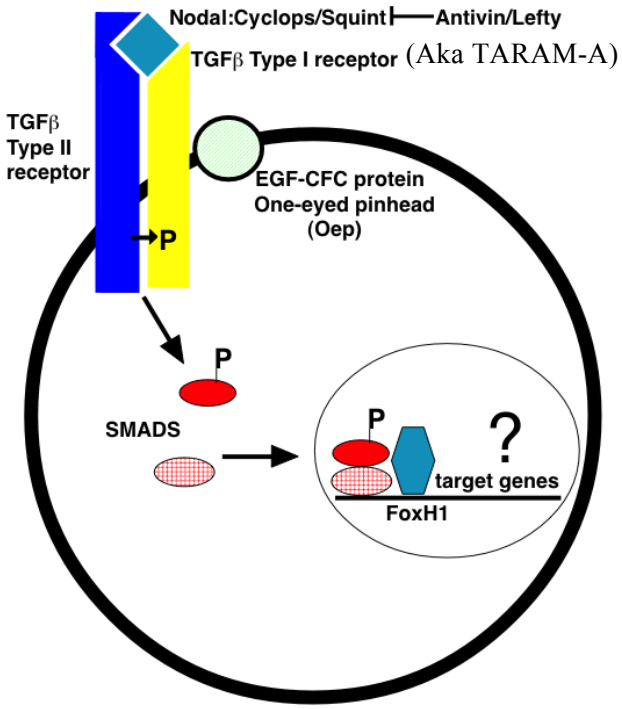


Figure 9: The Nodal signaling pathway in zebrafish. Nodal is activated by interaction with TGF receptors and EGF-CFC co-receptor, One-eyed Pinhead. The signal is transmitted within the cell by phosphorylation of the Smad complex, which interacts with transcription factor FoxHI that in turn promotes transcription of Nodal genes (Figure adapted from Liang and Rubinstein, 2003).

Strength of Nodal Signaling

In all vertebrates, Nodal signals function both as an autocrine and as a paracrine signal over a few cell diameters to induce the mesendoderm (Schier, 2003). Studies show that prior to the onset of gastrulation, induction of dorsal marginal cells (prechordal plate precursors) requires consistent Nodal signaling, while a shorter duration of Nodal signaling, such as that received by cells that are distant from the margin, causes the formation epidermal cells (Schier, 2003). Additionally, the extent of mesendoderm,

endoderm and mesoderm formation is primarily dependent on the strength of the Nodal signaling (Schier, 2003). For example, an enlarged primitive streak, a mesendodermal derivative, is observed in mice with increased Nodal signaling. Similarly, blocking lefty1 and 2 in the zebrafish resulted in an enlarged germ ring, which is a mesendodermal derivate (Agathon *et al*, 2001; Chen and Schier, 2002; Feldman, 2002). Therefore, the expansion of the mesendoderm, endoderm, and mesoderm is dependent on Nodal signaling. These studies indicate that Nodal induces different fates via a spatial gradient (Schier, 2003).

Conversely, in the absence of Nodal signaling, altered cell fates from the wild type are observed (Carmany-Rampey and Schier, 2001; Feldman *et al*, 2000). Lack of Nodal signaling results in embryos that lack almost all endoderm and mesoderm derivatives (Feldman *et al*, 1998). For example, the dorsal marginal cells normally give rise to mesodermal notochord and mesendodermal prechordal plate. However in the absence of Nodal signaling (using Nodal *cyc*:*sqt* double mutants or MZ*oep* mutants) the cells instead give rise to the hindbrain and the midbrain of the neuroectoderm (Schier, 2003).

Nodal Signaling Is Required Early For Neurulation

Nodal mutant studies suggest that Nodal signaling is required early in development for neural tube closure (Schier, 2003). Null *cyc* mutants have no ventral brain or spinal cord (Hatta, 1991; Mathieu *et al*, 2002; Rebagliati *et al*, 1998a,b; Sampath *et al*, 1998; Rohr *et al*, 2001). Furthermore *cyc* mutants have a reduced prechordal mesoderm, lack of floor plate, and display cyclopia. However, though *cyc* mutants lack

some mesodermal and mesendodermal derivatives, they always display a closed neural tube (Aquilina-Beck *et al*, 2007). Similar to *cyc*, *sqt* mutants lack mesendodermal derivatives, which include defects in the axial mesoderm, a precursor to the notochord (Schier, 2003). *sqt* mutants present a range of neural tube closure phenotypes, with the most severe *sqt* mutants displaying an open neural tube (Aquilina-Beck *et al*, 2007).

cyc is expressed during gastrulation around 6 hpf. *sqt* expression on the other hand peaks soon after MBT, when maternal squint is degraded and zygotic transcription initiates. The earlier presence of *sqt* as compared to *cyc*, and the open neural tube phenotype of the *sqt* mutants suggest that Nodal's role in neural tube closure is during the blastula stages around the MBT.

Nodal Signaling Rescues Mesendoderm And Promotes A Closed Neural Tube

The zebrafish Nodal mutants, including the Maternal-Zygotic *one eyed-pinhead* (MZ*oep*), *sqt* and *cyc*:*sqt* double mutants have an open neural tube phenotype. Studies (particularly with the *oep* mutants) suggest that the Nodal signaling involvement in neural tube closure occurs through mesendoderm induction (Aquilina-Beck *et al*, 2007). The oep protein is translated from the maternal *oep* mRNA prior to the MBT and is translated from zygotic mRNA after the MBT (Tadros and Lipshitz, 2009). The MZ*oep* mutants lack both the maternal and zygotic *oep* mRNA (Grittsman *et al*, 1999), resulting in a completely non-functional oep co-receptor. The MZ*oep* mutants phenocopy the *cyc*;*sqt* mutants, displaying an almost complete loss of mesoderm/mesendoderm tissue along with an open neural tube (Aquilina-Beck *et al*, 2007). In WT zebrafish, Nodal ligands including cyc and sqt interact with Acvr1B and

the Nodal receptor complex constituents to initiate downstream Nodal signaling (Schier, 2003).

In MZ*oep* mutants injection of TARAM-A* (a constitutively activated version of the Acvr1B mRNA which does not require ligand binding) results in the activation of Nodal signaling in the injected cell and its future daughter cells (Aquilina-Beck *et al*, 2007). In the injected MZ*oep*, Nodal signaling can cell autonomously rescue the formation of mesendodermal/mesodermal tissue (Aquilina-Beck *et al*, 2007). For instance, 68% of MZ*oep* injected with TARAM-A* had significant recovery of the mesendodermal derived hatching gland and the mesoderm derived notochord (Aquilina-beck *et al*, 2007). Within the injected MZ*oep* displaying rescue of both the hatching gland cells and notochord cells, 73% had a closed neural tube (Aquilina-beck *et al*, 2007). This finding suggests that the activity of Nodal signaling for mesoderm/ mesendoderm induction is competent for neurulation.

Research Design

An Inexpensive, Efficient Method For Regular Egg Collection From Zebrafish In A Recirculation Tank

Traditionally laboratories breed zebrafish by removing the fish from the recirculating aquatic system and placing them into 1–2 L spawning tanks. The fish placed in these spawning tanks are not fed and do not get fresh water, and hence are only bred once a week. During the time when we had a high demand of embryos, we developed a new method for zebrafish breeding allowing daily egg collection with constant access to fresh water.

Chapter 2 of this thesis proposes the new method of zebrafish egg collection. Our new method of egg collection demonstrated comparable number of fertilized eggs produced from the traditional method. Moreover as males and females were constantly kept together, this allowed the opportunity for females to spawn every day. Further, we found a zebrafish spawning behavior where fish kept in low density-tanks had higher rates of egg production as compared to fish kept in high-density tank. This work is included the manuscript Gonsar, N., Schumann, A. C., Buchard, J. N., and Liang, J. O. (2012). An Inexpensive, Efficient Method for Regular Egg Collection from Zebrafish in a Recirculating System. *Zebrafish*, *9* (1), 50-55.

Temporal And Spatial Requirements For Nodal Induced Head Mesendoderm In Anterior Neural Tube Closure

Previous work in our lab demonstrated that zebrafish with disruptions along the Nodal signaling pathway develop NTDs analogous to anencephaly (Aquilina-Beck *et al*, 2007). Nodal signaling acts indirectly in neurulation through the induction of the head mesoderm and mesendoderm (cells that give rise to a subset of both mesodermal and endodermal derivatives). Therefore the zebrafish head mesoderm/mesendoderm formation is essential for anterior neural tube closure (Aquilina-Beck *et al*, 2007). Similarly in mouse mutants, a lack of development of the head mesenchyme (a combination of neural crest and mesoderm) results in an open neural tube (Copp *et al*, 2003). Nevertheless, to date the mechanisms including the role of the head mesendoderm in neurulation are poorly understood.

Chapter 3 of this thesis examines the temporal and the spatial requirements of the

Nodal induced head mesendoderm that is important for a closed neural tube. Based on my research, we have proposed a model for Nodal signaling in neurulation. We found that a cumulative dose of Nodal before the onset of neurulation is necessary to induce a wide region of the head mesendoderm for a closed neural tube. Therefore signals necessary for the neural tube formation begin many hours prior to the neural plate formation and a wide range of tissues of the head mesendoderm are competent to promote neural tube closure. This work encompasses the complete manuscript sent for peer review: Gonsar, N., Clay, J., and Liang, J. O. (2013). Temporal and spatial requirements for Nodal induced head mesendoderm in anterior neurulation. Submitted on 7.3.2013 *Developmental Biology.*

II. AN INEXPENSIVE, EFFICIENT METHOD FOR REGULAR EGG COLLECTION FROM ZEBRAFISH IN A RECIRCULATING SYSTEM

Introduction

Zebrafish are capable of breeding all year round, with a single pair capable of producing more than a hundred embryos in one day (Hisaoka and Firlit, 1962; Eaton and Farley, 1974; Legault, 2000; Spence *et al*, 2008; Blanco-Vives and Sanchez-Vazquez, 2009). This is one of the characteristics that make the zebrafish a powerful model organism for studying the mechanisms that control vertebrate development, and has led to its growing use in the study of disease, behavior and a number of other fields. Several factors have been found to improve the rate of spawning in laboratory conditions. The frequency of spawning increases with rising temperature, with a minimum of 5 days between spawning for fish at 26°C and a minimum of 2 days at 30°C (Legault, 2000). Keeping males and females together has a positive effect on the number of eggs produced per day and is required for the release of oocytes in the female (Hisaoka and Firlit, 1962; Eaton and Farley, 1974). In addition, a depth gradient within the spawning tank increases the number of embryos produced, and there is a corresponding increase in residency of the fish at the shallow end of the tank (Sessa *et al*, 2008). These findings match well with what is known about the natural history of zebrafish, which are river fish native to India, Bangladesh, and Nepal (Spence *et al*, 2008).

A number of methods have been used to breed zebrafish in laboratory settings. One of the first methods was to include marbles in a region of the tank as a spawning site

(Legault, 1958; Westerfield, 2000; Spence *et al*, 2005; Spence *et al*, 2006) Eggs fall into the marble layer, and a siphon is used for egg collection (Westerfield, 2000). As the number of zebrafish laboratories has expanded, commercially available spawning tanks have become available. These tanks consist of a lower reservoir holding 1-2 L of water and an insert with holes in the bottom. The adult fish are placed within the insert, and the eggs fall through the holes into the bottom reservoir, protecting them from cannibalism by the parental fish. These spawning tanks do not allow continuous spawning, as there is no circulation or filtration of the water. Breeding fish are typically placed in the spawning tanks in the evening and then left in the spawning tanks through the following morning. More recently, several methods have been generated for obtaining large quantities of embryos at the same stage of development. For instance, Adatto and colleagues invented a specialized breeding vessel that enables over 100 fish to be bred at once over short periods of time (10 minutes) (Adatto *et al*, 2011). The fertilized eggs are easily collected as they fall into a narrow opening at the bottom of the vessel (Adatto *et al*, 2011).

We have built on these earlier techniques to develop a breeding method with a unique set of advantages. In this method, the inserts that come with commercially available spawning tanks are placed directly into 10 L or 3 L tanks on a recirculating aquatic system. The adult fish are placed into the insert, and embryos are collected from the bottom of the tank by pouring or using a self-starting siphon. Comparison of this method with breeding in commercial spawning tanks found no significant difference between the numbers of fertile embryos produced. Further, because the fish are maintained on the recirculating system and can be fed regularly, we found this method can be used over many days or weeks. Fish maintained in this spawning system for 28-

days continued to produce fertilized embryos every 1-3 days. Setting up the fish for breeding in the home tanks took much less time than for the commercial spawning tanks. Thus, the home tank method would be especially useful for gathering embryos to be raised, expanding a new stock, or working with sensitive fish that might be harmed by removal from the recirculating system. Since gathering embryos from 3 L home tanks took a similar amount of time to traditional spawning tanks, breeding in these smaller home tanks would be suitable for techniques that require rapid harvesting of embryos, such as embryo injections. The home tank method offers an alternative approach that can be used for many purposes and requires only equipment that is already available in most laboratories, or easily and inexpensively purchased from commercial sources.

Materials and Methods

Aquatic System

All fish were maintained in their respective 10 L and 3 L home tanks in an aquatic facility equipped with an automatic recirculation system. Water in this facility was maintained at 450-800 mS conductivity, pH 7.4, and ~28 ºC. The fish were maintained in a 14:10 hr daily light:dark cycle. Zeitgeber (ZT) was used to indicate time within this cycle, with ZT0 corresponding to when the lights turned on in the morning and ZT14 to when the lights turned off in the evening. Fish were fed on weekday mornings with live brine shrimp and on weekday afternoons and weekends with commercial flake food (a 1:1 mixture of Freshwater Aquarium Flake Food from Ocean Star International and Tetramin Tropical Flakes from Tetra, Inc.). During the breeding experiments, the fish were fed with commercial flake food or with commercial flake food and a fine powder

consisting of a 1:1 mixture of Argent Technology's Spirulina Microfine Powder and Hatchfry Encapsulon.

Breeding In Spawning Tanks

For the experiments in spawning tanks, fish were removed from their home tank into temporary 3 L tanks for sorting by gender. The fish were initially placed into one central tank and then sorted into one tank for females and another for males. Females were sorted based on their rounder, whiter abdomen. Males were identified based on their slightly more yellow coloring and their flat abdomen. The male and female fish were then evenly distributed between 2 L spawning tanks (Table 2). These spawning tanks consisted of a lower reservoir that held the water and a spawning insert with holes in the bottom that fit into this reservoir (Aquatic Habitats). These tanks were filled to approximately 2 cm from the top with aquatic system water, and plastic plants were placed into the insert to provide refuge.

The following day, the adult fish were removed from the spawning insert and placed back into their home tank on the recirculating system. The bottom reservoir was kept still for a few minutes to allow the eggs to settle to the bottom. Most of the water was then poured out slowly from one corner. When there was approximately 30 ml of water left in the reservoir, the water was agitated to suspend the eggs, and then the remaining water and eggs were poured quickly into a deep Petri dish (100 mm × 25 mm).

Breeding In Home Tanks Of The Recirculating Aquatic System

Males and female fish were sorted and set up in a 2 L spawning as described

above for breeding in spawning tanks. However, after completion of this set up, the spawning insert, the adult fish, plastic plants, and the cover to the spawning tank were placed into a 3 or 10 L tank on the recirculating system (Fig. 10). The water entering into the tank was adjusted to ~200 ml/minute (fast drips) so the eggs would not be washed out of the tank. The fish were not fed brine shrimp during the experiment, as we found it would quickly fall through the bottom of the insert and contaminate the eggs. The following day the fish, insert, plastic plants, and spawning tank cover were temporarily moved to the bottom reservoir of a spawning tank to enable the eggs to be collected (Fig. 10).

Eggs were collected from the 3 L home tank by pouring, similar to egg collection from the traditional spawning tanks (Fig. 10). For the 10 L home tanks, the eggs and all of the water in the home tank were moved into the collection tank using a self-starting siphon (Small 15/8" × 9" UltraGravelVac from Lee's Aquarium and Pet Products) (Fig. 10). The nozzle of the siphon was placed into the 10 L home tank, and the end of the siphon tubing was clamped to the inside of a collection tank that consisted of a 10 L tank and a 400 μm screen baffle (Fig. 10) (Aquatic Habitats). The collection tank was then placed on a secure surface several feet below the home tank (Fig. 10). The water flow was started by quickly moving the siphon nozzle up and down until the tubing was completely filled with water and no bubbles were present. The nozzle was kept submerged during the whole siphoning process.

Typically, there was about 1 L of water left in the home tank after water flow through the siphon had stopped. This last 1 L often contained many eggs, and so was poured manually into the 10 L collection tank. The home tank was rinsed with about 0.5

L of aquatic system water, and this rinse water was also poured into the 10 L collection tank. Before this final rinse was added to the protocol, we occasionally collected embryos that were over 24 hpf, suggesting eggs had been missed during the previous day's collection.

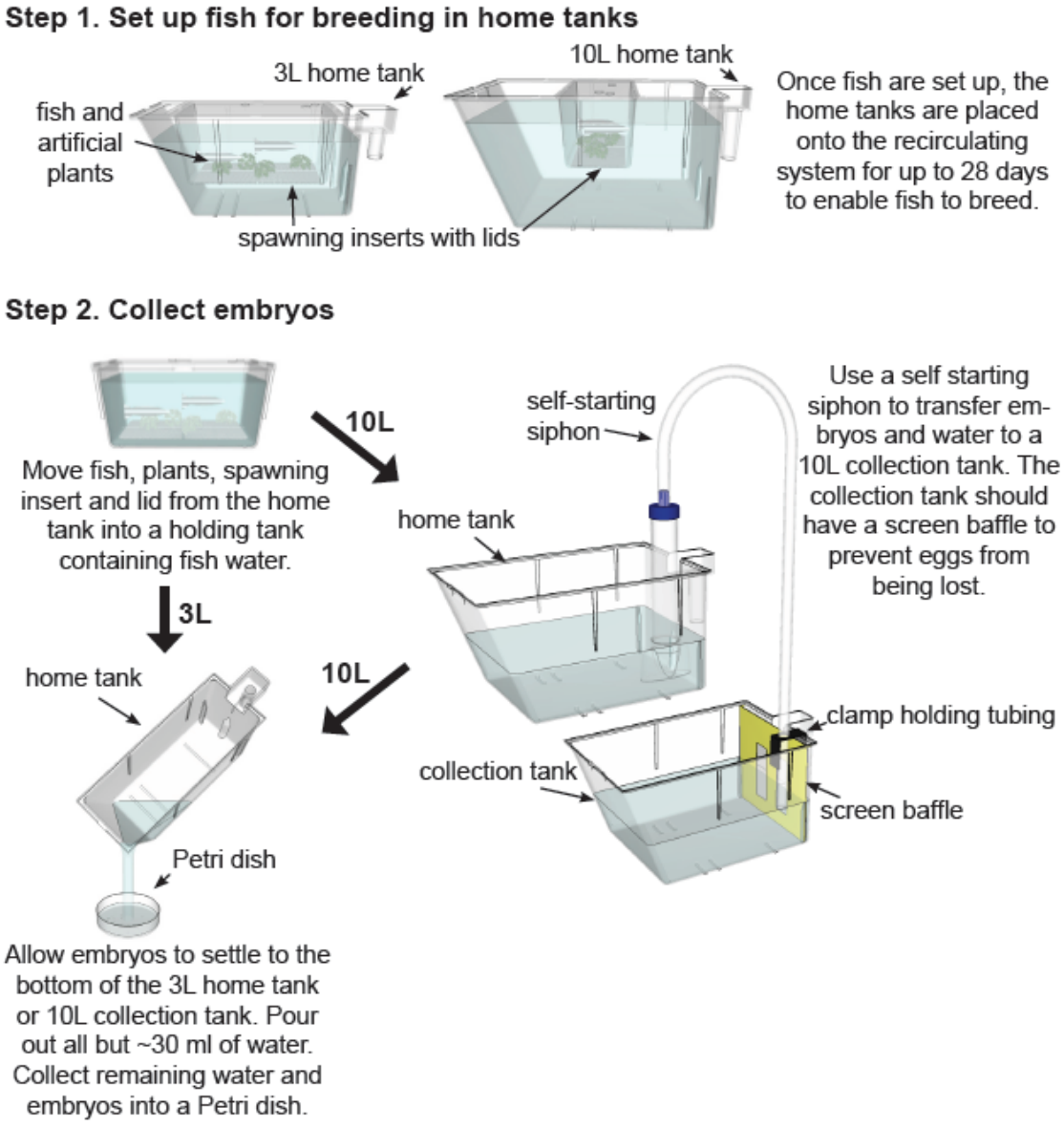


Figure 10: Outline of home tank breeding procedure.

Statistical Analysis And Modeling

Data was analyzed by Repeated Measures Analysis of Variance (MANOVA) using JMP 9.0 software. Three dimensional models were created using SketchUp 8.0.

Results

Comparable Levels Of Embryo Production When Fish Are Bred In Spawning Tanks And Home Tanks

Our first goal was to compare embryo production between fish bred within the recirculating system and fish bred in regular spawning tanks. To make this test as realistic as possible, we used the fish in four existing tanks of WT fish. These tanks each had fish of different ages, levels of prior breeding, and numbers of males and females (Table 2). The fish in each tank were set up four times: twice in spawning tanks and twice in their home tanks. In each case, the week of daily breeding started on Monday and continued to Friday, so the number of eggs produced was counted for four consecutive days (Fig. 11). The numbers of fish in each "spawning insert" were kept as constant as possible, and each tank of fish was given a week of rest between each week of breeding.

For the weeks when the fish were in spawning tanks, the males and females were put together at ZT6.5 each day from Monday-Thursday, and then left together until ZT3.5 the following day. Between ZT3.5 and ZT6.5 they were placed back into their home tank and fed once or twice with flake food before being moved back into the spawning tanks for the next round of breeding. The number of infertile embryos, fertile embryos, and their stages of development were scored between ZT4 and ZT4.5 on the day the eggs were collected.

For the weeks when fish were in their home tanks, the fish were sorted by gender and placed into spawning inserts in their home tank at ZT6.5 on Monday. The adults remained in the spawning insert in their home tank until ZT3.5 on Friday, except for short periods when they were placed into a temporary tank so that eggs could be collected. As with the breeding in spawning tanks, the eggs were collected at ZT3.5, and counted and staged between ZT4.5 and ZT5.

We found that there was a large variation in the number of fertile embryos produced each day, with a low of 0 embryos and a high of 225 (Fig. 11A). The number of non-viable embryos was similar and low for each method (n=76/1175 for spawning tanks, n=31/1306 for home tanks). These results are quite consistent with our previous experiences. There was also not a clear trend of change in the number of embryos from Tuesday-Friday, likely because there were multiple females in each breeding tank, increasing the chance of producing eggs on multiple days during the week (Fig. 11). The data were analyzed using MANOVA, which took into account that each group of breeding fish was assayed several times over the time course of the experiment. This analysis produced a P value of 0.70, suggesting that there was no significant difference in the number of eggs produced between the two methods of breeding.

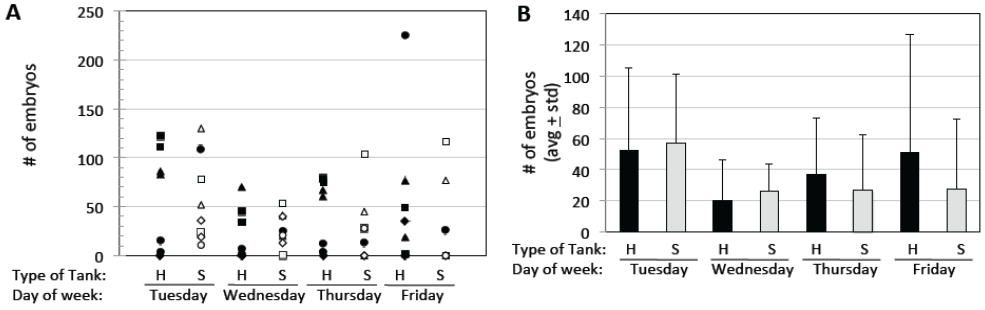


Figure 11: Comparison of embryos produced by fish in spawning tanks and home tanks. A. The number of embryos produced in the previous 24 hours is reported for each spawning of the fish in four different tanks (Table 1). Half were in spawning tanks (S) outside of the recirculating system and half in home tanks (H) within the recirculating system. **B.** Presentation of the data in A as average ± std for each time point.

Fish In Home Tanks Continued To Breed Throughout 28-day Time Course

One potential advantage of the home tank breeding method was it could be done continuously over a long period of time, as the fish do not miss any feedings and are continuously supplied with fresh water. To gain insight into long-term patterns of breeding, we kept groups of fish in spawning inserts in 3 L and 10 L home tanks for 28-days. As in the earlier experiment, the eggs were collected at ZT3.5 each day and the embryos were counted and staged between ZT4.5 and ZT5 on the day they were collected.

The highest number of embryos was typically produced during the first week (n=11/12 home tanks; Fig. 12). Interestingly, examination of the time line by day instead of by week revealed that the large number of embryos during the first week was primarily due to a large number of eggs being produced on the first day (Fig. 13). This suggests that the fish had many mature gametes ready to release right away, but that gamete production persisted for several weeks at lower levels. Statistical analysis indicated that there was a significant difference between the 10 L and 3 L tanks when 3 pairs were present ($P=0.05$) but not when one pair was present ($P=0.38$). However, when only 3 pair trials done in parallel were compared $P=0.24$, suggesting the significant difference was in part due to factors other than tank size.

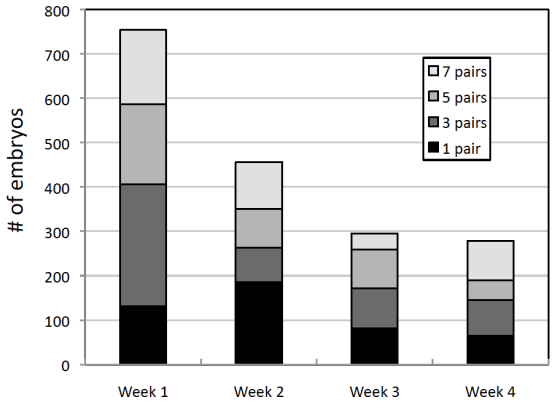


Figure 12: Embryos produced each week in 28-day time course in 10 L home breeding tanks. The number of fertile embryos produced each week by each of four tanks during a 28-day time course. The tanks containing 1, 3, 5, and 7 pairs were assayed in parallel over the same 28-day period. This experiment was repeated two times, with the first trial shown here.

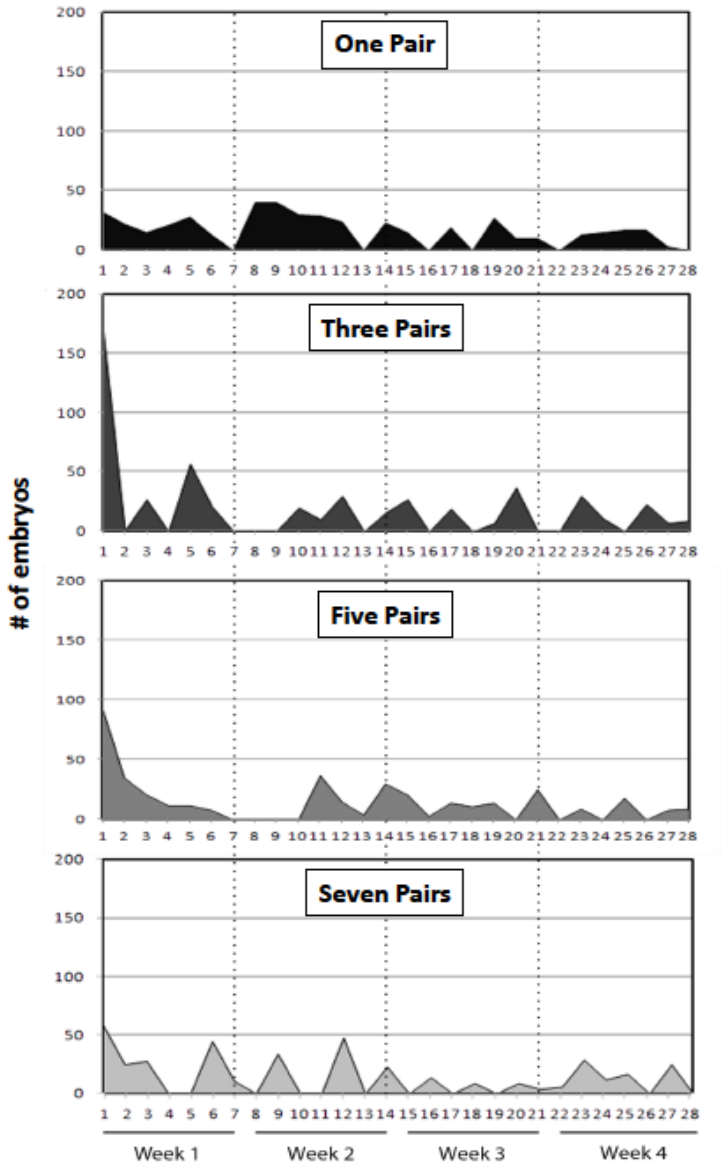


Figure 13: **Daily spawning in a 28-day time course in 10 L tanks.** Embryos collected each day for the same experiment summarized in Figure 12. Dotted lines mark embryos collected on Mondays, and each week of the experiment is marked by a set of underlined days. The combined analysis of daily spawning for this experiment and one repetition is included in Table 1.

To determine if embryo production would increase if fish were bred for fewer days at a time, we carried out 3-day experiments with a minimum of three days rest in between each trial. The fish in this less intense breeding scheme produced similar numbers of embryos per day as the fish bred continuously for 28-days, suggesting that the rests did not have a positive effect (Table 1).

Table 1. Daily Embryo Production for Breeding in Home Tanks

	Daily embryo Production (avg ± std)	*Daily embryo production per pair (avg)*
28-day time courses 10 L home tanks[1]		
1 pair	19.5 ± 19.5	19.5
3 pairs	14.9 ± 27.8	5.0
5 pairs	13.4 ± 18.2	2.7
7 pairs	12.3 ± 16.1	1.8
28-day time course in 3 L home tanks[2]		
1 pair	7.3 ± 11.5	7.3
3 pairs	7.8 ± 18.0	2.6
3-day time courses in 3 L home tanks[3]		
1 pair	16.7 ± 15.1	16.7
3 pairs	8.6 ± 17.7	4.3

[1]n=54 days; [2]n=28 days; [3]n=18 days.

As part of these experiments, we also gained insight into the effect of fish density. Each of the tanks had between one and seven male/female pairs (Table 2). Surprisingly, the number of embryos did not increase linearly with the number of pairs (Table 1). In both the 3 L and the 10 L home tanks, the single pair mating produced larger number of embryos per pair than higher fish densities (Table 1). Further, as the number of fish increased the number of embryo per pair decreased (Table 1). The difference in embryo

production between 1 pair and 3 pairs reached significance in the 3-day experiments (P=0.004), but not in the 28-day experiments (P=0.17).

Table 2. Summary of Fish Used in Breeding Experiments

Tank	*# of males*	*# of females*	*# of mating inserts*	*Ages at time of assays*
Comparison of spawning tanks and home tanks				
A	19	24	3	10–15 months
B	22[1]	23	3	5–9 months
C	7	7	2	4–7 months
D	18	15	3	10–12 months
28-time courses in 10 L home tanks				
E	1	1	1	Trial 1
F	3	3	1	3–6 months
G	5	5	1	Trial 2
H	7	7	1	5–8 months
28-day time courses in 3 L home tanks				
I, J	1	1	1	
L, M	3	3	1	5–8 months
3-day trials in 3 L home tanks				
N	1	1	1	8–9 months
O	3	3	1	8–9 months

[1]The gender of 10 of these fish was hard to determine. Although they are listed as males, they could have been thin females.

Discussion

We have developed an economical and time efficient method of breeding fish within a recirculating aquatic system. This method requires very little handling of the adult fish, makes it possible to breed the same fish continuously over a long period of time, and has similar levels of embryo production as the common method of breeding in spawning tanks. Overall, breeding in the 10 L and the 3 L home tanks took less hands-on time because the fish did not have to be netted and sorted by gender except on the first day. We suggest that breeding on the recirculating system would be very useful for producing new stocks of fish, producing eggs from the same fish multiple times in one week, breeding fish that are sensitive to being netted, and studies on spawning behavior. The biggest potential deficit is that this method could potentially take away from space needed for other purposes, as the fish in their home tanks produced embryos most

efficiently when at low densities.

Because harvesting embryos from 10 L home tanks took much longer than other methods, breeding in 10 L tanks would only be useful for experiments that require low fish densities and infrequent egg collection. In contrast, a similar amount of time was needed for collection of embryos from 3 L home tanks and 2 L spawning tanks. Therefore, breeding in 3 L home tanks could be used for single pair matings to identify fish carrying a specific mutation, crosses to produce embryos for injection, and to generate mutant and transgenic embryos for experiments. This would be especially useful for fish that are breeding poorly as they would have a longer opportunity to spawn.

We found that zebrafish spawning continued periodically during 28-days in their home tanks, with a large peak of embryo production on the morning after the fish were placed into the inserts. Our male and female fish were housed together in between breeding experiments. Based on earlier studies, this would have stimulated the constant production of oocytes by the females (Hisaoka and Firlit, 1962; Eaton and Farley, 1974). Thus, when the females were placed into the more conducive breeding environment of the spawning insert, there could have been a large store of gametes ready to be released the following morning. After this large peak of embryo production, the fish began to produce lower numbers of embryos every 1-3 days. This closely matched the periodicity of embryo production in other experiments where zebrafish had continuous opportunity to spawn (Hisaoka and Firlit, 1962).

Interestingly, our comparison of embryo production by fish at different densities found the highest number of eggs per pair occurred in the tank with only one pair of fish. Since zebrafish have complex spawning behaviors that require interactions between

males and females, it might be anticipated that embryo production would increase at higher densities. However, several other studies have also found decreasing embryo production with increasing density (Spence *et al*, 2005). Male to male aggression and competition among females for limited spawning space may limit egg production in crowded tanks (Spence *et al*, 2005).

There are several ways that this method could potentially be improved. Most of our tanks contained fish from the same clutch. As sexually mature zebrafish females have a preference to avoid siblings, improvements in embryo production could come from using a more genetically diverse group of parental fish (Gerlach *et al*, 2006). We found that using live brine shrimp was problematic in this breeding method, as the shrimp would fall through the bottom of the spawning inserts and contaminate the embryos. Thus, addition of another rich food would likely improve embryo production and the overall health of the breeding adults, and extend the time fish can kept be in this breeding scheme to several months. Finally, using different tank topologies could also bring improvements. For instance, adding a depth gradient to the spawning insert containing the adult fish has been shown to increase embryo production by approximately 20% (Sessa *et al*, 2008).

III. TEMPORAL AND SPATIAL REQUIREMENTS FOR NODAL-INDUCED HEAD MESENDODERM IN ANTERIOR NEURULATION

Introduction

The neural tube is a structure that runs along the entire anterior-posterior axis of the developing embryo. The portion of the neural tube that will give rise to the brain and the majority of the spinal cord forms by the process of primary neurulation, which converts a flat neuroepithelium into a closed tube (Lowery and Sive, 2004). If the neural tube fails to close, this leads to neural tube defects (NTDs). NTDs are among the most common human birth defects, occurring in approximately 1 in 1000 births worldwide (Detrait *et al*, 2005). One of the most severe NTDs is anencephaly in which the anterior neural tube fails to close, causing complete or partial absence of the developing cranial vault and cerebral hemisphere (Detrait *et al*, 2005). Studies indicate that NTDs are caused in part by genetic factors, although these are not yet fully understood (Wald *et al*, 1991; Juriloff and Harris, 2000).

The morphological events of primary neurulation are well characterized across many vertebrate species. Primary neurulation initiates with the columnarization of ectodermal epithelial cells forming the neural plate (Lowery and Sive, 2004). This is followed by the thickening and elevation of the neural plate borders to form the neural folds, which fuse at the dorsal midline, forming the neural tube (Colas and Schoenwolf, 2001). In zebrafish, there is an additional step. The first phase of neural tube closure in the zebrafish is the formation of a neural rod in which cells of the right and left sides of

the neural tube are in direct contact (Schmitz *et al*, 1993; Colas and Schoenwolf, 2001). Later, a lumen forms in the center of the rod to form a neural tube (Schmitz *et al*, 1993; Detrait *et al*, 2005).

The molecular mechanisms driving neurulation are also largely conserved (Lowery and Sive, 2004). For instance, in all vertebrates, adhesion molecules that mediate the formation of the neural epithelium play a crucial role in neural tube formation (Copp *et al*, 2003). In zebrafish, the major cell adhesion protein important for neural tube closure is N-cadherin. N-cadherin mutants fail to form a neural rod in the brain and posterior spinal cord (Lele *et al*, 2002; Aquilina-Beck *et al*, 2007). Cell adhesion is required for the neural tube to maintain a coherent tissue that is capable of undergoing cell movements during neurulation.

In zebrafish, Nodal signaling mutants exhibit a NTD in the anterior neural tube that is analogous to anencephaly (Aquilina-Beck *et al*, 2007; Lu *et al*, 2012). Zebrafish have three Nodal ligands, Cyclops (cyc), Squint (sqt), and Southpaw (Schier, 2003). These ligands all signal through a receptor complex containing the One Eyed Pinhead (oep) protein (Schier, 2003). *oep*, *sqt*, and *cyc*;*sqt* double mutants all exhibit open anterior neural tubes, as do embryos overexpressing the Nodal signaling inhibitor lefty1 (Aquilina-Beck *et al*, 2007; Lu *et al*, 2012). The primary defect in these embryos appears to be lack cell adhesion during the earliest steps of neurulation. The cells of the developing neural tube in Nodal signaling mutants are disorganized, and have decreased N-cadherin expression (Aquilina-Beck *et al*, 2007).

Our previous research suggested that the role for Nodal signaling is not directly in the neuroepithelium, but rather in the induction of the anterior mesendoderm and

mesoderm that underlies the developing brain (Aquilina-Beck *et al*, 2007). Embryos that completely lack Nodal signaling have no anterior mesendoderm or mesoderm, and always have an open neural tube (Aquilina-Beck *et al*, 2007). In these Nodal mutants, activation of the Nodal signaling pathway cell-autonomously rescued the formation of mesendoderm/mesodermal tissue and resulted in a closed neural tube (Aquilina-Beck *et al*, 2007). This suggests that the Nodal signaling pathway need not be activated in the neural tube cells. Rather, rescue of the mesendoderm and mesoderm is sufficient to correct the NTD.

Similar to the role of the anterior mesendoderm and mesoderm in zebrafish, the head mesenchyme in mice plays a vital role in neurulation (Chen and Behringer, 1995). In rodents, the elevation of the cranial neural folds is preceded by the expansion of the underlying head mesenchyme (Solursh and Morriss, 1977; Morriss and Solursh, 1978a,b; Tuckett and Morriss-Kay, 1986). Additionally, loss of function of various genes expressed in the head mesenchyme results in lethal NTDs, including anencephaly (Chen and Behringer, 1995). For example, the *twist* and *cart1* genes are both expressed in the mesenchyme. Knockout of either of these genes affects the expansion of the cranial mesenchyme and result in NTDs (Chen and Behringer, 1995; Zhao *et al*, 1996). This has led to the hypothesis that expansion of head mesenchyme helps drive the convergence of the left and right folds resulting in the fusion of the dorsal midline and closure of the neural tube. However, more characterization is needed to determine whether the head mesendoderm/mesoderm in zebrafish and head mesenchyme in mice have overlapping functions.

Our goal was to identify the temporal requirement for Nodal signaling in

neurulation and define the areas of the mesendoderm and mesoderm that have a role in neural tube closure. We found that Nodal signaling is required very early in development for anterior neurulation. Using a pharmacological Nodal inhibitor, the requirement for Nodal was found to occur through the blastula stages, up to 4.3 hpf. This temporal requirement falls within the time when Nodal is inducing mesoderm and mesendoderm, but is prior to the onset of neural plate formation. These results suggest that there is a specific time window during which Nodal induces the mesendodermal and mesodermal tissues involved in neurulation. Further, this timing suggests that these tissues could be involved in the earliest steps of neurulation. Using a correlative approach, we found a strong statistical correlation between the presence of head mesendoderm derivatives and a closed neural tube. However, none of the tissues assayed (hatching gland, anterior notochord, cephalic paraxial mesoderm, and head muscles) were absolutely required for a closed neural tube. These results supported a model in which Nodal signaling induces a range of anterior mesendodermal and mesodermal tissues that are all competent to promote neurulation.

Methods

Zebrafish Stocks

Zebrafish were maintained as per standard protocols at a constant temperature of 28.5°C in a 14:10 light:dark cycle (Westerfield, 2000). The stocks used were WT strain Zebrafish *Danio rerio* (ZDR), (Aquatica Tropical, Plant City, FL) and *squint* (sqt^{cz35}) mutants (Pei *et al*, 1998). Adult fish were placed in 1 L spawning tanks or in spawning inserts within tanks on the recirculation rack on the evening prior to the experiment

(Gonsar *et al*, 2012). The following morning fertile embryos were pooled; WT embryos were incubated at 28.5°C and *sqt* embryos were incubated at 33°C to increase the penetrance of the open neural tube phenotype (Lu *et al*, 2013; Hagos and Dougan, 2007). All embryonic stages were defined morphologically (Kimmel *et al*, 1995).

Drug Treatment

At the 1000-cell stage, embryos were dechorionated using 2 mg/ml Pronase (Roche) following established methods (Link *et al*, 2006, Westerfield, 2000). Embryos not fully dechorionated by the Pronase solution were dechorionated through gentle agitation with forceps. Embryos were transferred using Sigma Cote (Sigma-Aldrich) coated glass pipettes. At the desired morphological stage, embryos were exposed to 50 µM, 75 µM or 100 µM of 2-(5-benzo [1,3] dioxol-5-yl-2-terbutyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride hydrate (SB505124) (Sigma Aldrich) (Hagos and Dougan, 2007) for a duration of 20 minutes at room temperature. Post drug exposure, embryos were incubated at 28.5°C until the desired stage. Subsequently the embryos were then fixed and assayed.

Whole mount RNA in situ hybridization (WISH)

Embryos were assayed by RNA whole mount in situ hybridization using established methods (Thisse *et al*, 1993). Digoxygenin antisense RNA probes included *orthodenticle homeobox 5* (*otx5*) (Gamse *et al*, 2001), *cathepsin L 1b* (*ctsl1b*) (Vogel and Gerster, 1997), *paired box gene 2.1* (*pax2.1*) (Pfeffer *et al*, 1998), *floating head* (*flh*) (Talbot *et al*, 1995), *goosecoid* (*gsc*) (Stachel *et al*, 1993), *collagen type IX alpha 2*

(*col9a2*) (Thisse *et al*, 2001), *collagen type 2 alpha-1a* (*col2a1a*) (Yan *et al*, 1995) and *smooth muscle myosin heavy chain 2* (*smyhc2*). (Elworthy *et al*, 2008).

PCR Genotyping

sqt heterozygotes were identified by fin clip genomic DNA extraction. Polymerase Chain Reaction (PCR) amplified the DNA using sqt^{cz35} specific primers (Pei *et al*, 1998).

Photography

Bright field images were taken with a SPOT Insight Fire Wire camera mounted on a Nikon Eclipse 80i microscope. All images were processed using Adobe InDesign 5.0 or 6.0 (Adobe Systems Inc).

mRNA Injections

One to two celled stage ZDR embryos were pressure injected with 0.38-2.5 pg of *lefty1* mRNA using Harvard Apparatus PL190-nitrogen driven pico-injector. The embryos were incubated post injections at 28.5°C and assessed at ~24 hpf for phenotypes indicating Nodal deficiency (Fig. 14) (Thisse and Thisse, 1999). Uninjected sibling embryos served as controls.

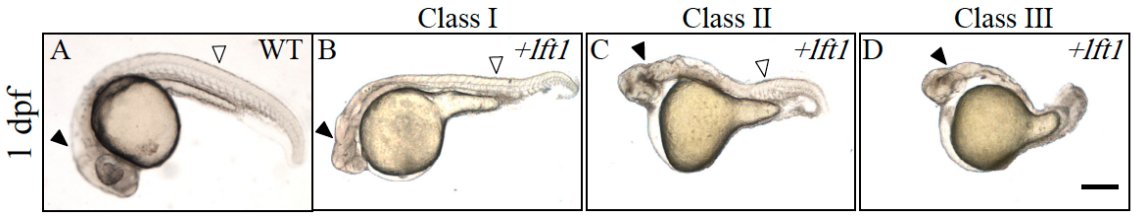


Figure 14: *lefty1* injection of WT embryos results in variable loss of mesendodermal tissue. (A-D) Lateral view of 1 dpf embryos. (A) Uninjected WT embryos display normal somite formation (white arrowheads) and have a smooth round head (closed arrowheads) with overall normal mesendoderm formation. (B-D) *lefty1* mRNA injected embryos display dose dependent loss of mesendodermal and mesodermal tissues. *lefty1* mRNA injected embryos with (A) least (B) intermediate and (C) severe mesodermal tissue loss. Scale bar: 400 μm (A-D).

Data Analyses

sqt and *lefty1* mRNA overexpressing embryos were assayed by WISH for the presence of head mesendodermal tissues and the pineal morphology. The embryos were categorized into 4 groups: mesendodermal/mesodermal tissue present and elongated or divided pineal (open NT), mesendodermal/mesodermal tissue absent and open NT, mesendodermal/mesodermal tissue present and closed NT, and mesendodermal/mesodermal tissue absent and closed NT. These groups corresponded to the four quadrants within a 2 × 2 contingency table of a one-tailed Fisher's exact test. If a statistically significant relationship was observed ($P<0.05$), the strength of the correlation between neural tube closure and mesendodermal/mesodermal tissue presence was determined through the Phi Coefficient (φ). All statistical tests were conducted using JMP 10.0 software.

Results

Temporal Overlap In Nodal Signaling's Role In Neural Tube Closure And Mesendoderm Induction

Our previous work strongly suggested that the role for Nodal signaling in neurulation is through Nodal induction of the head mesendoderm and/or anterior mesoderm (Aquilina-Beck *et al*, 2007). If this hypothesis is correct, then Nodal's temporal requirement in neurulation should occur at the same time as Nodal induction of mesendoderm/mesoderm. Studies have found that Nodal activity is required from 2.75 hpf (mid-blastula stage) to 5.0 hpf (late blastula stage) for mesendoderm, mesoderm, and endoderm induction (Hagos and Dougan, 2007). We found the requirement for Nodal signaling in neural tube closure occurs within this same time frame.

To determine the temporal requirement for Nodal signaling in neurulation, we exploited the unique properties and specificity of the Nodal inhibitor SB505124. SB505124 is a small molecule which blocks the activity of ALK 4, 5, and 7 Nodal specific receptors (Byfield *et al*, 2004). In zebrafish, SB505124 causes phenotypes similar to those in mutants with reduced Nodal signaling (Hagos and Dougan, 2007). Further, mRNA levels of Nodal regulated genes decreases within 15-30 minutes after inhibitor treatment (Hagos and Dougan, 2007).

WT embryos were treated at specific developmental time points across the blastula and gastrula stages (Fig. 15, 16). Embryos were exposed to 50 μm dose of SB505124 for 20 minutes, after which the inhibitor was removed and treated embryos were raised to 1 day post-fertilization (dpf). The treated embryos were then assayed by WISH for the pineal morphology. Previous studies demonstrated that embryos with a closed anterior

neural tube have an oval shaped pineal organ at the dorsal midline of the anterior neural tube, while embryos with an open anterior neural tube have an elongated or divided pineal (Fig. 15, 16) (Aquilina-Beck *et al*, 2007).

The majority of embryos treated with the inhibitor from 3.8 hpf (mid-blastula stage) up to 4.3 hpf (late blastula stage) had an open neural tube phenotype (Fig. 15A-C, J, 16A-C, E). After 4.3 hpf, embryos consistently had a closed neural tube (Fig. 15C-I, 16C-D). However the inhibitor remained active, as embryos exposed to the inhibitor after 4.3 hpf had severe to mild cyclopia, phenocopying a key characteristic of mutants with defective Nodal signaling (Fig. 16A-D) (Schier, 2003). This suggests that though Nodal signaling is active after the blastula stages, it is not required for neurulation. Hence, Nodal is required for neurulation only up to 4.3 hpf and matched a subset of time when Nodal functions in mesendoderm and mesoderm induction.

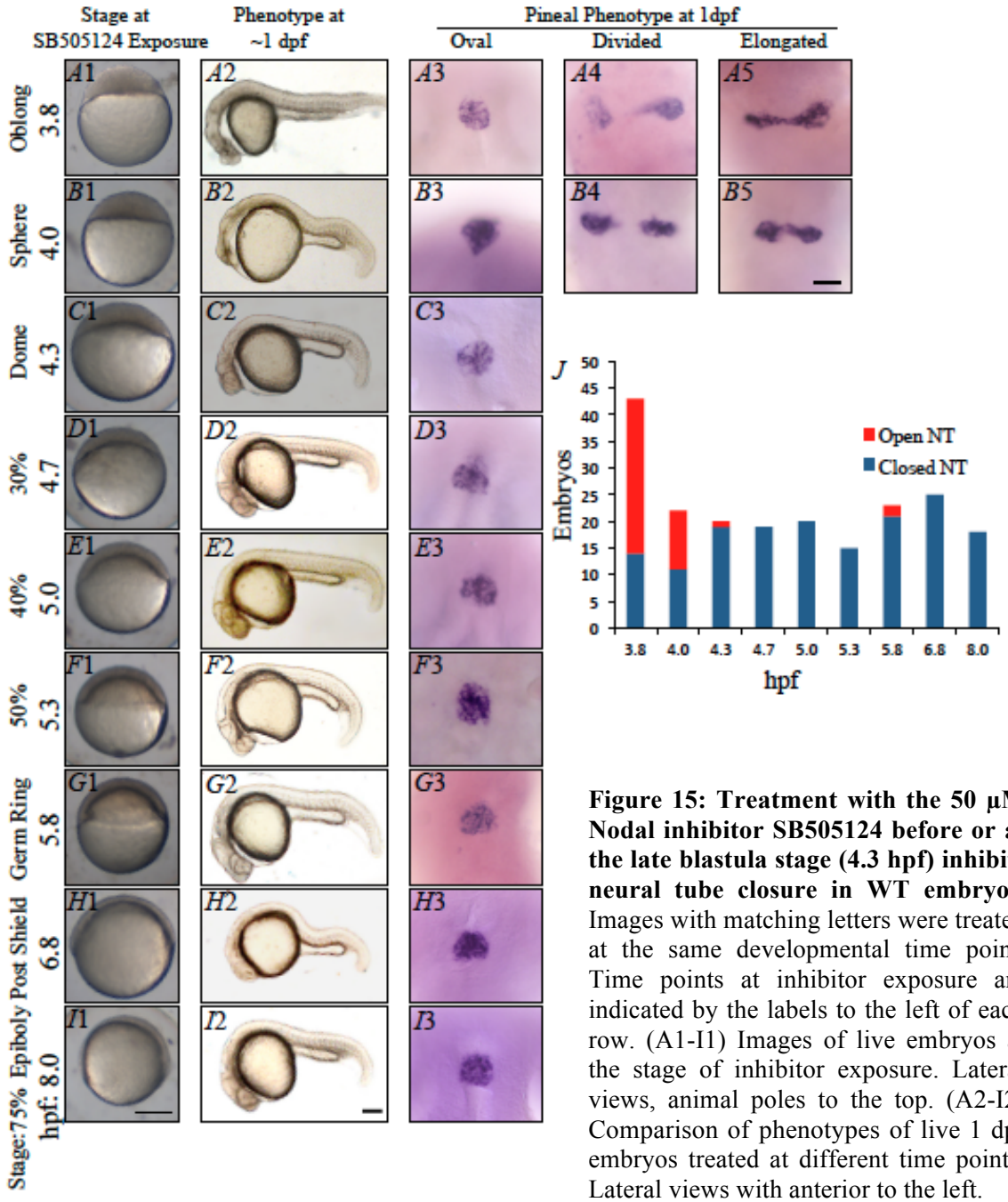


Figure 15: Treatment with the 50 µM Nodal inhibitor SB505124 before or at the late blastula stage (4.3 hpf) inhibits neural tube closure in WT embryos. Images with matching letters were treated at the same developmental time point. Time points at inhibitor exposure are indicated by the labels to the left of each row. (A1-I1) Images of live embryos at the stage of inhibitor exposure. Lateral views, animal poles to the top. (A2-I2) Comparison of phenotypes of live 1 dpf embryos treated at different time points. Lateral views with anterior to the left. (A3-B5) WT embryos assayed for *otx5* expression in the pineal gland. Dorsal views with anterior to the top. Oval pineal indicates a closed neural tube. Elongated and divided pineals indicate an open neural tube. (J) Graph of the total number of open and closed neural tubes in WT embryos at each stage of inhibitor exposure. Even WT embryos occasionally have unexplained developmental defects. This is likely the cause of open neural tube phenotype in two embryos treated at 5.8 hpf. Scale bars: 250 µm (A1-I2), 50 µm (A3-B5).

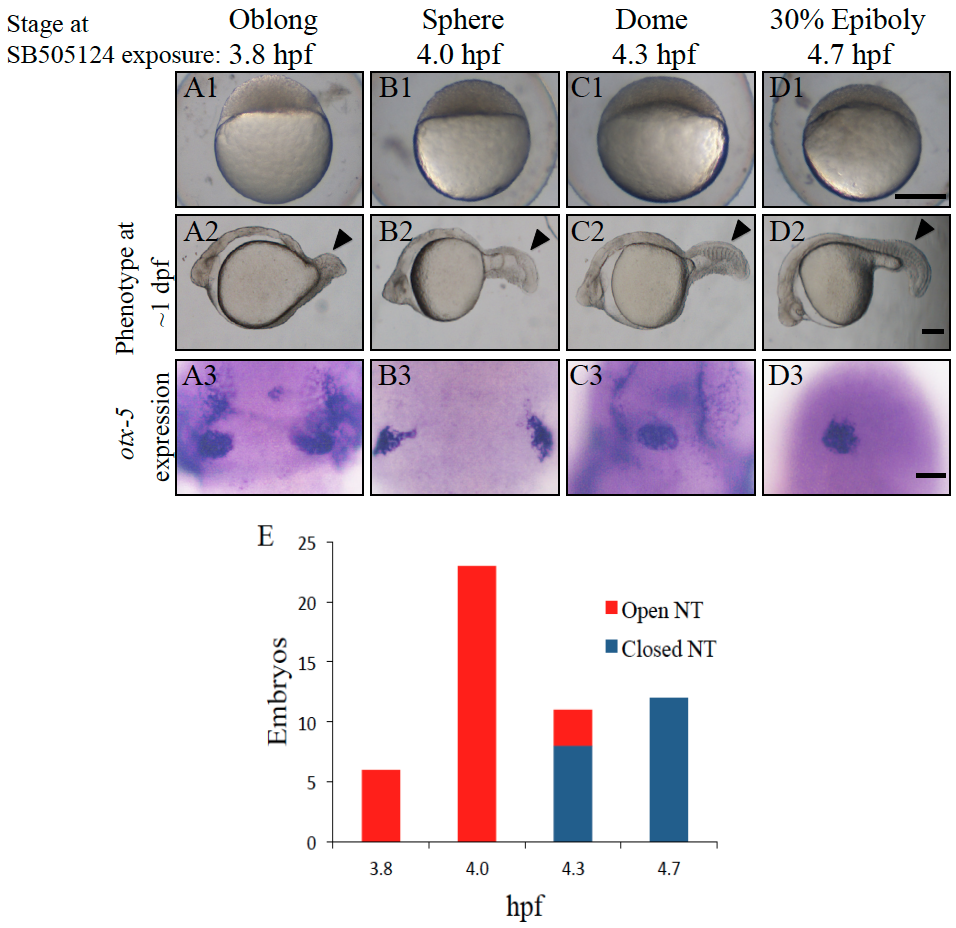


Figure 16: Neural tube closure is inhibited in embryos treated with the 75 µM Nodal inhibitor SB505124 before the late blastula stage (4.3 hpf). (A1-D1) Images of live embryos at the stage of inhibitor exposure. Lateral views, animal poles to the top. (A2-D2) Comparison of phenotypes of live 1 dpf embryos treated at different time points. Lateral views with anterior to the left. (A2) Embryo displays severe cyclopia, loss of head mesendodermal derivatives and loss of head and trunk region. One somite (arrowhead) is present in the tail region, indicating some presence of mesoderm. (B2-D2) Embryos display cyclopia, partial loss of head mesendodermal derivatives and abnormal head and trunk morphology. Reduced number of somites, totaling 3, 8 and 15 respectively (arrowheads). (A3-D3) WT embryos were assayed for *otx5* in the pineal gland. Dorsal views with anterior to the top. (A3, B3) Embryos display pineal precursors that fail to converge at the dorsal midline, resulting in a divided or elongated pineal. (C3, D3) Embryos display pineal convergence and neural tube closure, resulting in an oval pineal morphology. (E) The total number of open and closed neural tubes in WT embryos at each stage of inhibitor exposure. Scale bars: 250 µm (A1-D2), 50 µm (A3-D3).

Increased Dose Of Nodal Inhibitor Correlates With The Severity Of Neurulation Failure And Mesendodermal Loss

Studies have shown that zebrafish embryos respond in a dose-dependent manner to Nodal signaling (Schier, 2009). Consequently, a higher dosage of the Nodal inhibitor drives a greater loss of the cell autonomous induction of the mesendoderm, leading to an open neural tube. To determine if the closed neural tubes in embryos treated after 4.3 hpf were due to insufficient inhibition of Nodal signaling, we tested the effect of increased inhibitor dosage.

The temporal requirement for Nodal signaling in neural tube closure remained consistent between embryos exposed to an increased inhibitor dose (75 μM) and the embryos exposed to the lower (50 μM) dose (Fig. 16, 17). As in embryos exposed to the lower dose, embryos exposed to the higher dose had open neural tubes when treated with the inhibitor before or at 4.3 hpf (Fig. 16, 17). However, a higher percentage of embryos exposed to the increased inhibitor dose displayed an open neural tube phenotype (5.3% vs. 37.5%), suggesting the lower dose did not completely block Nodal signaling (Fig. 17). As with the lower dose, treatment with 75 μM inhibitor at 4.8 hpf, or later, did not cause open neural tubes (Fig. 17). The consistency in the temporal requirement between the two doses suggested that there is a strict time window in the requirement for Nodal signaling in anterior neurulation.

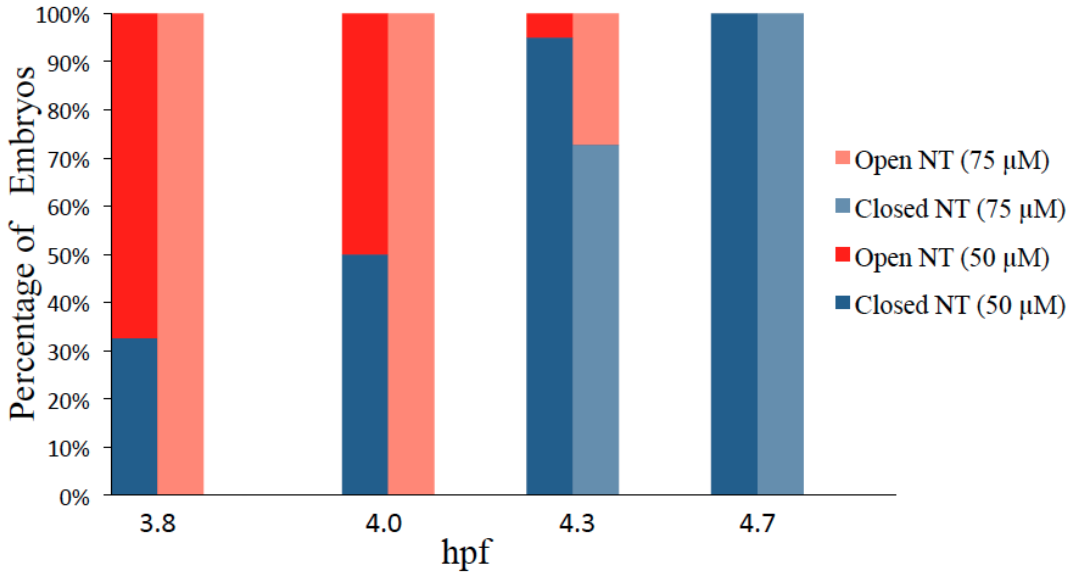


Figure 17: Temporal consistency in neural tube closure is observed between embryos exposure to high (75 µM) Nodal inhibitor and low (50 µM) Nodal inhibitor. The percent distribution of open and closed neural tubes in WT embryos exposed to different doses of Nodal inhibitor across blastula stages.

We found that the inhibitor caused severe defects in mesendoderm and mesoderm formation at the same stages where it caused open neural tube phenotypes (Fig. 16). Embryos exposed to the Nodal inhibitor at the mid-blastula stage resulted in loss of multiple mesendodermal and mesodermal derivatives, and had phenotypes similar to Nodal deficient mutants (Fig. 15, 16, 18) (Sampath *et al*, 1998). Similar to Nodal deficient mutants, treated embryos displayed complete absence of the prechordal plate, a mesendodermal derivative that requires high Nodal activity (N=34/34) (Fig. 18F) (Gritsman *et al*, 2000; Thisse and Thisse, 1999). The presumptive notochord requires intermediate Nodal activity (Gritsman *et al*, 2000; Schier and Talbot, 2005). Consistent with this, the treated embryos still had a few notochord cells, although their number was always decreased (N=24/24) (Fig. 18H). Treated embryos also displayed presence of intermediate mesodermal (IM) cells in the midbrain-hindbrain border. However, IM cells

were absent from the pronephric mesoderm, suggesting that these IM cells required a higher Nodal dose than those at the midbrain hindbrain border (N=26/36) (Fig. 18G).

Consistent that some Nodal signaling remained under the low dose of the inhibitor, embryos treated with the high dose had greater loss of mesodermal and mesendodermal tissues when compared to embryos exposed to the low dose (Fig. 18). For instance, embryos exposed during the mid-blastula stage (3.8 hpf) to the low inhibitor dose had approximately normal numbers (~30) of somites (Fig. 18E). In contrast, embryos exposed at the same stage to the high inhibitor dose generated only 1-2 somites, phenocopying embryos with a severe loss in Nodal signaling (Fig. 16A2) (Feldman *et al*, 1998; Gritsman *et al*, 1999).

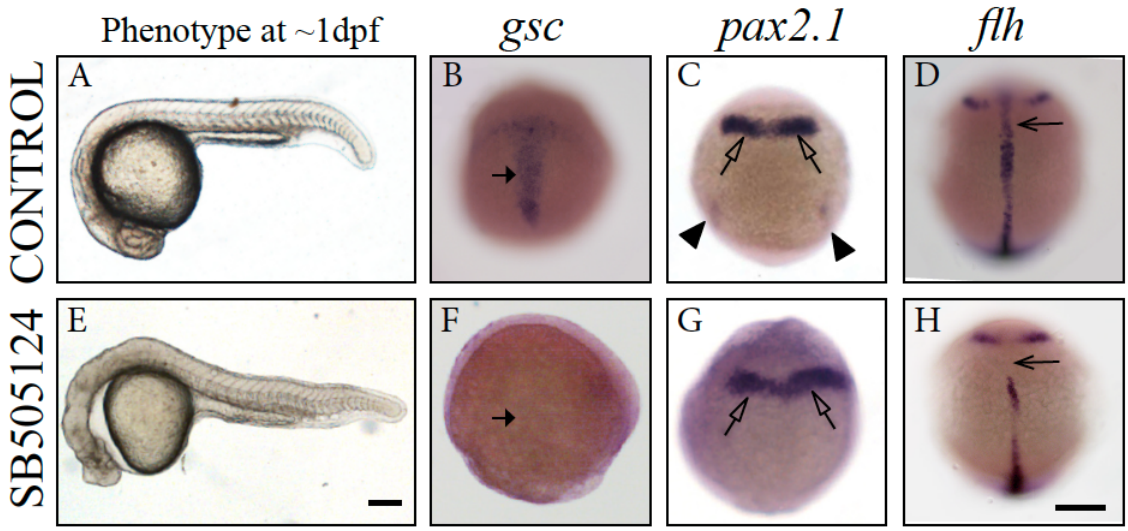


Figure 18: Exposure to Nodal inhibitor SB505124 at 3.8 hpf leads to loss of mesendodermal and mesodermal derivatives in WT embryos. (A-D) Control WT embryos exposed to DMSO and (E-H) WT embryos exposed to 50 µM Nodal inhibitor. (A) WT embryo at 1 dpf has a normal smooth and round head, a straight body axis, and complete somite formation. (B-D) Embryos were fixed and processed for expression of (B, F) *gsc*, marking the prechordal plate precursors at 9 hpf, (C, G) *pax2.1*, marking the intermediate mesoderm at 10 hpf and (D, H) *flh*, marking the notochord precursor at 12 hpf. (B) Normal *gsc* expression displays a T-shaped expression domain (arrow). (C) Normal *pax2.1* expression displays a staining in the anterior region of the embryo along the midbrain-hindbrain border (arrow) and in the posterior region in the pronephric mesoderm (arrowheads). (D) Normal *flh* expression displays staining along the dorsal midline (arrow). (E) Inhibitor treated embryo at 1 dpf displays cyclopia and abnormal morphology throughout the embryo. Lack of Nodal signaling also causes (F) reduced or absent *gsc* expression, (G) presence of *pax2.1* mRNA in the midbrain-hindbrain border (arrowheads) but none in the pronephric mesoderm, and (H) partial *flh* expression in the notochord precursors (arrow). Scale bars: 250 µm (A-H).

Differential Nodal Activity Across The Blastula Stage For Somite Induction

A difference in somite production among embryos exposed to the high dose of inhibitor also revealed a varying effect of Nodal signaling across the temporal landscape. In WT embryos, approximately 30 somites are present at 1 dpf (Van Eeden *et al*, 1996). The first somite is observed after 10 hpf followed by an additional somite formed approximately every 20-30 minutes, until a total of 30 or 31. In later development, these

somites differentiate into muscles, vertebrae, and dermis (Van Eeden *et al*, 1996). In the embryos treated with the inhibitor during the mid-blastula (3.8 hpf), 1-2 somites developed, compared to 16 somites in embryos exposed to the inhibitor during the late blastula stage (4.8 hpf) (Fig. 16). This suggests that Nodal signaling had a varying effect on mesoderm induction, mostly active right after the mid-blastula stages and milder after these stages.

Absence Of Head Mesendodermal Tissues Correlates With Open Neural Tube Phenotype

Our next goal was to map the regions of the head mesendoderm and anterior mesoderm involved in neural tube closure. Implementing a correlative approach, we used *sqt* mutants and *lefty1* overexpressing embryos, both of which have variable mesendoderm/mesoderm and neural tube phenotypes (Aquilina-Beck *et al*, 2007; Pei *et al*, 2007). The four head mesendodermal/mesodermal tissues assayed were the hatching glands, the anterior notochord, the cephalic paraxial mesoderm, and the head muscles (Fig. 19). If a head mesendodermal/mesodermal tissue was required for neurulation, then that tissue would always be present when the neural tube was closed and always absent when the neural tube was open. We found there was a strong correlation between the presence of the head mesendodermal/mesodermal tissues and a closed neural tube phenotype, but no tissue was absolutely required.

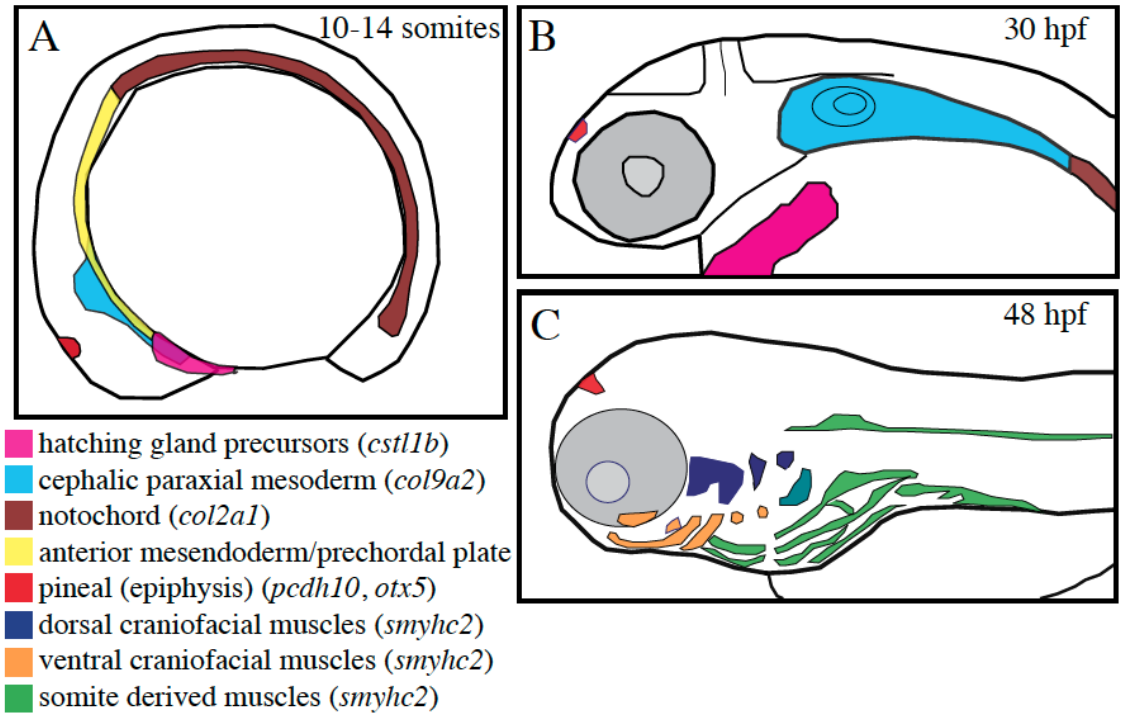
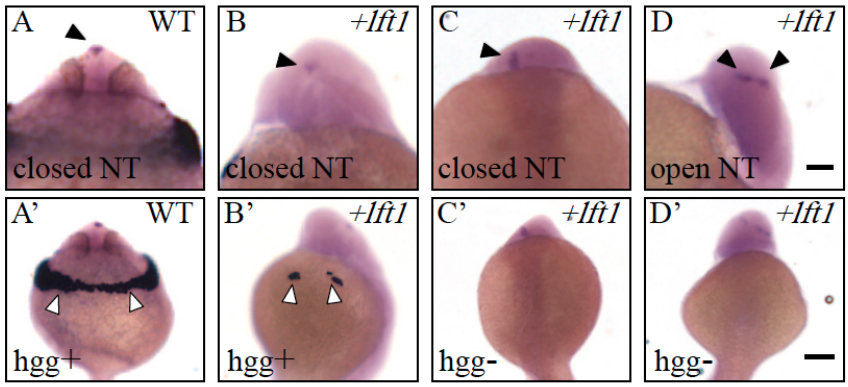


Figure 19: Regions of the head mesendoderm were assayed using a panel of markers. Schematic representation of expression patterns of mesendodermal and mesodermal tissues along the anterior-posterior axis (A) during the early somite stage, (B) the mid pharyngula period (C) and at the onset of the hatching period. Lateral views with dorsal to the top. Schematics based on gene expression patterns in digital images of developing embryos (adapted from data in Raunch *et al*, 2003; Murakami *et al*, 2006; Thisse *et al*, 2001).

The hatching glands are derived from the anterior most prechordal plate and are the first mesendodermal tissue to involute during gastrulation (Vogel and Gerster, 1997). The prechordal plate underlies the presumptive anterior neuroectoderm from the early gastrula stage, which is a stage when studies suggest ventral specification of the neuroectoderm may be initiating (Hammerschmidt *et al*, 1996). Hence, the hatching glands were strong candidates for mediating a head mesendoderm-ectoderm interaction. Embryos were categorized into four groups based on whether the hatching gland tissue was present or absent and whether the neural tube was open or closed (Fig. 20). The data reflected a strong statistical support for a relationship between neural tube closure and hatching gland

presence (*lefty1* injected: P=0.0001, *sqt*: P=0.0909; Fisher's exact test) and this correlation was positive (*lefty1* injected: φ=0.61; *sqt*: φ=0.4, Phi coefficient). Despite this correlation, the hatching gland cells were neither necessary nor sufficient for a closed neural tube (Fig. 20, 21). In both the *sqt* mutant and the *lefty1* overexpressing embryos, closed neural tube embryos were observed that had no hatching gland cells, and open neural tube embryos were observed that had hatching gland cells (Fig. 20, 21).

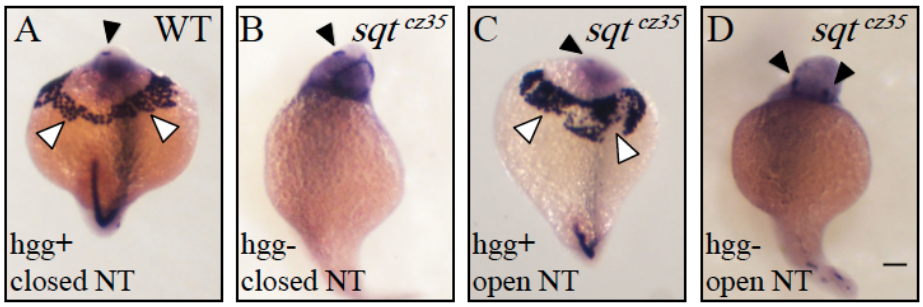
Figure 20: Hatching glands are not required for neural tube closure in *lefty1* mRNA injected embryos. Embryos were (A, A') left uninjected or (B-D') injected with *lefty1*



E

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
hgg Present	9	0	1	10
hgg Absent	7	1	28	36
Total	16	1	29	46

mRNA (+*lft1*) at the 1-2 cell stage, raised to 1 dpf and processed in tandem for expression of *cstl1b* in the hatching gland cells (hgg, white arrowheads) and *otx5* in the pineal anlage (closed arrowheads). Whether an embryo is positive (+) or negative (-) for the hgg staining is indicated. (A, A') In 1 dpf, WT, uninjected embryos, the hatching glands make a "necklace" around the anterior of the head. The pineal organ is oval indicating a closed neural tube. (B-D') *lefty1* injected embryos with an (B, B') oval pineal and the hatching glands present, (C, C') oval pineal and complete absence of the hatching glands, (D, D') elongated pineal with complete absence of the hatching glands. (E) The compilation of all combination of phenotypes identified in four independent experiments. Frontal views with dorsal to the top. Panels with the same letter are different views of the same embryo. Scale bars: 100 µm (A-D), 200 µm (A'-D').



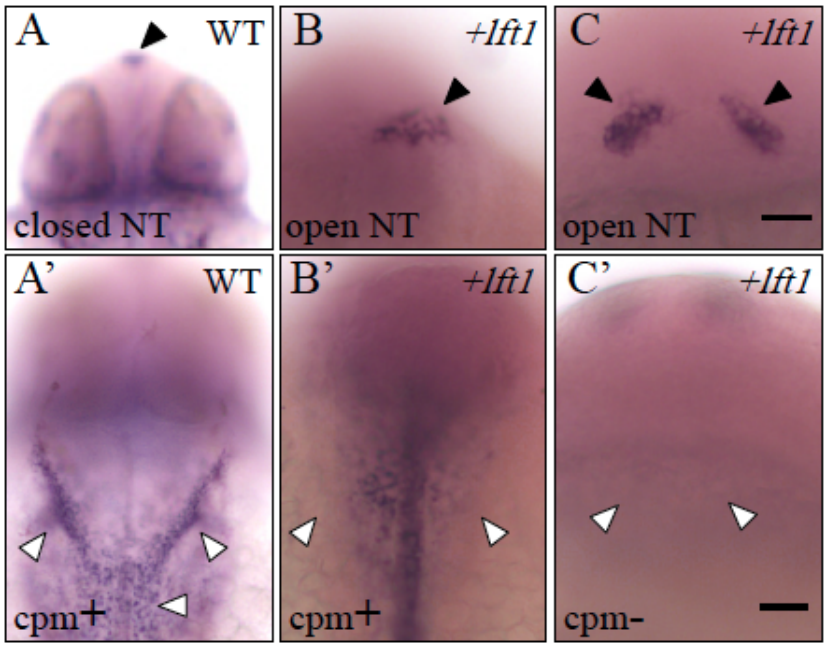
E

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
hgg Present	3	1	0	4
hgg Absent	5	5	10	20
Total	8	6	10	24

Figure 21: Hatching glands are not required for neural tube closure in *sqt* embryos. (A-D) *Sqt* embryos were raised to ~1 dpf and processed in tandem for *cstl1b* in the hatching gland cells (hgg, white arrowheads) and *otx5* in the pineal anlage (closed arrowheads). Whether an embryo is positive (+) or negative (-) for the hgg staining is indicated. (A) In 1 dpf, WT embryos, the hatching glands make a "necklace" around the anterior of the head. The pineal organ is oval indicating a closed neural tube. (B-D) *sqt* embryos with an (B) oval pineal with complete absence of the hatching glands, (C) elongated with the hatching glands present and (D) elongated with complete absence of the hatching glands. (E) The compilation of all combinations of phenotypes identified in four independent experiments. Frontal views with anterior on the top. Scale bar: 100 µm (A-D).

The Cephalic Paraxial Mesoderm (CPM) serves as the primary precursor to all cranial skeletal muscles and the pharyngeal arches. The CPM is also an organizer derived tissue key for anteroposterior patterning of the neural tube (Couly *et al*, 1993; Jessell, 2000; Stern and Foley, 1998). Studies uncovered that murine *twist* mutants with an altered CPM also displayed an open cranial neural tube, suggesting a potential role for CPM in zebrafish neurulation (Chen and Behringer, 1995). As with the hatching glands, the CPM had a strong positive correlation with neural tube closure. The *lefty1* injected

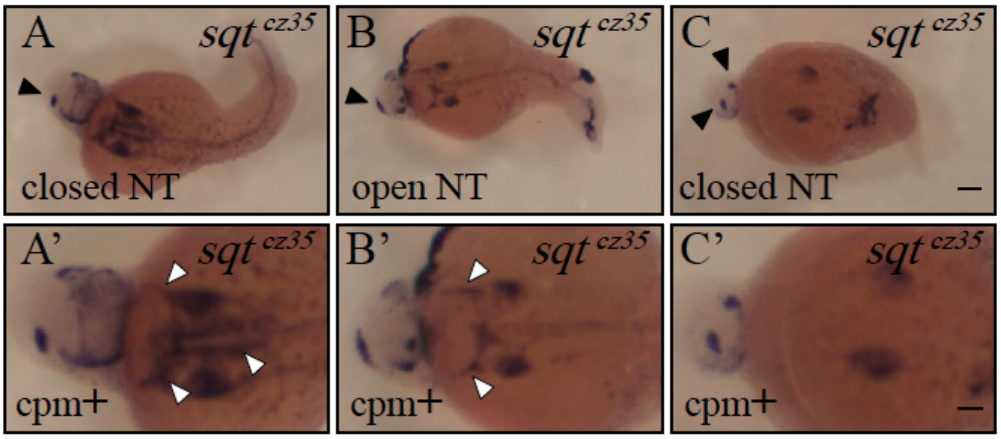
embryos generated strong statistical evidence for a relationship (P=0.0001), with a positive association between CPM and neural tube closure (φ=0.67). The *sqt* embryos displayed a weak negative correlation (φ=-0.2) and statistical evidence that there is no relationship between CPM presence and neural tube closure (P=1). However, we attributed to the lower numbers in the *sqt* correlative tests as compared to the numbers of the *lefty1* mRNA injected embryos for the variation in the correlation between the *sqt* mutants and the *lefty1* injected embryos. Nevertheless, as with the hatching glands, in both *sqt* mutants and the *lefty1* overexpressing embryos, the CPM was neither necessary nor sufficient for neural tube closure (Fig. 22, 23).



D

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
cpm Present	13	1	1	15
cpm Absent	6	5	27	38
Total	19	6	28	53

Figure 22: Cephalic Paraxial Mesoderm is not required for neural tube closure in *lefty1* mRNA injected embryos. Embryos were (A, A') left uninjected or (B-C') injected with *lefty1* mRNA (*+lft1*) at the 1-2 cell stage, raised to 1 dpf and processed in tandem for expression of *col9a2* in the cephalic paraxial mesoderm (cpm, white arrowheads) and *otx5* in the pineal anlage (closed arrowheads). Whether an embryo is positive (+) or negative (-) for the cpm staining is indicated. (A, A') In 1 dpf, WT, uninjected embryos, the cephalic paraxial mesoderm is expressed in the hindbrain region, projecting laterally from the anterior notochord. The pineal organ is oval indicating a closed neural tube. (B-C') *lefty1* mRNA injected embryos with an (B, B') elongated pineal and the cephalic paraxial mesoderm present and (C, C') divided pineal and complete absence of the cephalic paraxial mesoderm. (D) The compilation of all combinations of phenotypes identified in three independent experiments. Dorsal views with anterior on the top. Panels with the same letter are different views of the same embryo. Scale bar: 100 µm (A-C), 200 µm (A'-C').



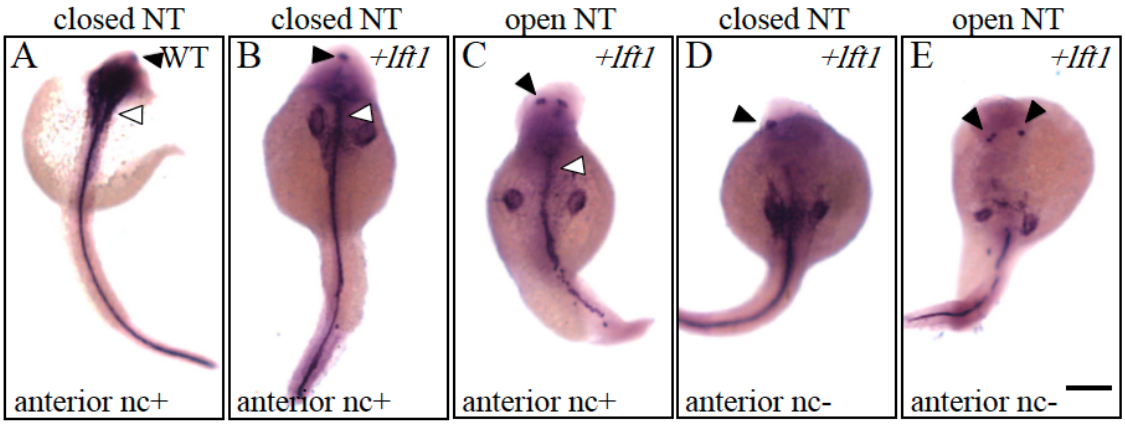
D

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
cpm Present	2	3	6	11
cpm Absent	2	1	7	10
Total	4	4	13	21

Figure 23: Cephalic Paraxial Mesoderm is not required for neural tube closure in *sqt* embryos. (A-C') *sqt* embryos were raised to 1 dpf and processed in tandem for *col9a2*, in the cephalic paraxial mesoderm (cpm, white arrowheads) and *otx5* in the pineal anlage (closed arrowheads). Whether an embryo is positive (+) or negative (-) for the cpm staining is indicated. In 1 dpf, WT embryos, the cephalic paraxial mesoderm is expressed in the hindbrain region, projecting laterally from the anterior notochord and the pineal organ is oval indicating a closed neural tube. (A-C') *sqt* embryos with an (A, A') oval pineal with the cephalic paraxial mesoderm present, (B, B') elongated with the cephalic paraxial mesoderm present and (C, C') divided with complete absence of the cephalic paraxial mesoderm. (D) The compilation of all combinations of phenotypes identified in three independent experiments. Dorsal views with anterior on the top. Panels with the same letter are different views of the same embryo. Scale bar: 100 µm (A-D), 50 µm (A'-C').

The next strongest positive correlation was observed in the analysis of the anterior notochord. In chick embryos, the notochord is attached to the median hinge point (MHP) in the anterior region of the neural plate. The attached notochord is responsible for

inductive interactions with the MHP that drive the convergence of the lateral edges of the neural plate towards each other at the dorsal midline (Johnson *et al*, 1994; Fan Tessier-Lavigne, 1994; Smith and Schoenwolf, 1997; Ybot-Gonzalez *et al*, 2002). As with the hatching glands, there was a strong statistical evidence for a relationship (*sqt* $P<0.0001$; *lefty1* injected $P<0.0001$) and a positive correlation (*sqt* $\varphi=0.31$; *lefty1* injected $\varphi=0.49$) between neural tube closure and anterior notochord presence. Despite the positive correlation and the roles in neurulation, we found the zebrafish anterior notochord was neither necessary nor sufficient for a closed neural tube (Fig. 24). Both *sqt* mutants and *lefty1* injected embryos presented a closed neural tube, while simultaneously lacking the anterior notochord. Additionally *lefty1* mRNA injected embryos possessed the anterior notochord with an open neural tube (Fig. 24).

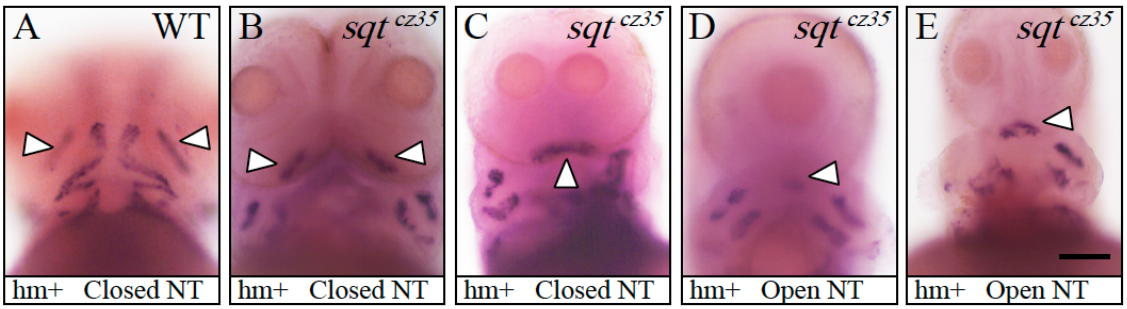


F

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
anterior nc Present	50	4	14	68
anterior nc Absent	18	9	46	73
Total	68	13	60	141

Figure 24: Anterior Notochord cells are not required for a closed neural tube closure in *lefty1* mRNA injected WT embryos. Embryos were (A) left uninjected or (B-E) injected with *lefty1* mRNA (*+lft1*) at the 1-2 cell stage, raised to 1 dpf and processed in tandem for expression of *col2ala*, in the anterior notochord (anterior nc, white arrowheads), and *otx5* in the pineal anlage (closed arrowheads). Whether an embryo is positive (+) or negative (-) for the anterior nc staining is indicated. The otic capsules, precursors to the ears were used as the division point for the anterior and posterior notochord. (A) In 1 dpf, WT, uninjected embryos, the notochord runs the length of the anterior-posterior axis. The pineal organ is oval indicating a closed neural tube. (B-D) *lefty1* mRNA injected embryos with an (B) oval pineal and the anterior notochord present, (C) divided pineal and the anterior notochord present, (D) oval pineal and the complete absence of the anterior notochord, and (E) oval pineal with the complete absence of the anterior notochord. (F) The compilation of all combinations of phenotypes identified in four independent experiments. Dorsal views with anterior on the top. Scale bar: 100 μm (A-E).

The final head mesendodermal tissues assayed were the head muscles, which had the lowest correlation with neural tube closure compared to other tissues assayed. The head muscle derivatives become apparent at approximately 50 hpf, during the hatching period, and give rise to the eyes, jaw, and the gill arch muscles in the adult (Kimmel *et al*, 1990). Studies suggest that early cellular signals to the underlying mesoderm from the migrating neural crest cells are key for spatial expressions of the head muscle derivatives (Noden, 1983a; Schilling *et al*, 1996). Furthermore, the cellular cues for the spatial organization appear to be during early development when the neural crest cells are first migrating away from the neural tube; prior to the visibility of specific head muscle precursors (Schilling and Kimmel, 1997). Subsequently, the correct inductive signaling during neurulation is key for normal head muscle formation in later development. We found *lefty1* injected embryos reflected strong evidence for a relationship (P=0.0242) (Fig. 22) and positive correlation (φ=0.4) between neural tube closure and head muscle presence. However, evidence for a relationship between neural tube closure and head muscle presence did not reach statistical significance in *sqt* embryos (P=0.5170; φ=0.1) (Fig. 25). Again, we found the head muscles were neither necessary nor sufficient for a closed neural tube (Table 3, Fig. 25).



F

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
hm Present	12	1	6	19
hm Absent	2	0	2	4
Total	14	1	8	23

Figure 25: The head muscles are not required for neural tube closure in *sqt* embryos. (A-C) *sqt* embryos were raised to 3 dpf and processed in tandem for *smyhc2*, in the head muscles (hm, white arrowheads) and *otx5*, in the pineal anlage. Whether an embryo is positive (+) or negative (-) for the hm staining is indicated. The adductor mandibulae (am) is the first muscle to develop in the head region and was used as the indicator for head muscle presence. (A) In 1 dpf, WT embryos, am precursors are in two clusters just anterior to the yolk sac and other jaw muscles. The pineal organ is oval indicating a closed neural tube. (B-E) *sqt* embryos with an (B, C) oval pineal with the am present and (D, E) divided pineal with the am present. (F) The compilation of all combinations of phenotypes identified in two independent experiments. Frontal views with anterior on the top. Scale bar: 100 µm (A-C).

Table 3. The head muscle derivatives are not necessary for neural tube closure in *lefty1* mRNA injected embryos.

	Pineal Morphology			
	Closed	Open		Total
	Oval	Elongated	Divided	
hm Present	40	2	0	42
hm Absent	5	0	3	8
Total	45	2	3	50

Discussion

In this study, we established the temporal requirement for Nodal signaling and mapped regions of mesendoderm and mesoderm important for neurulation. Nodal signaling was required up to the late blastula stage of 4.3 hpf for a closed neural tube. After 4.3 hpf, Nodal signaling remained active, but was no longer required for neural tube closure. Moreover, there was a strong correlation between the presence of head mesendodermal and anterior mesodermal tissues with neural tube closure. Yet, no single mesendodermal or mesodermal tissue was necessary for anterior neurulation. Therefore, we propose a model in which Nodal signaling acts prior to neural plate formation to induce the anterior mesendodermal and mesodermal tissues. The spatial mapping studies suggest that multiple of these Nodal-induced mesendoderm/mesoderm tissues are competent to drive closure of the overlying anterior neural tube.

Nodal Acts During Blastula Stages To Induce The Mesendodermal/Mesodermal Tissues Required For Neural Tube Closure

The first step in primary neurulation is the columnarization of the ectoderm to form the neural plate (Gammill and Bronner-Fraser, 2003). In the anterior of the zebrafish embryo, this columnarization initiates during late gastrulation and is completed by the start of the segmentation period (~10 hpf) (Blader and Strähle, 2000; Geldmacher-Voss *et al*, 2003). We found a strict temporal requirement for Nodal signaling in neurulation. This requirement occurs during the late blastula stages, which is hours before the columnarization of the neural plate. This suggests that the Nodal-induced mesendodermal and mesodermal tissues could act in the very earliest steps of

neurulation.

An early role for Nodal in anterior neurulation is supported by studies of zebrafish *oep* mutants, which lack a component of the receptor complex required for signaling by all Nodal ligands (Gritsman *et al*, 1999). Comparisons of mutants that vary in the timing of oep loss suggest that Nodal signaling is required around the mid-zygotic transition (MZT) (Aquilina-Beck *et al*, 2007). The MZT marks the developmental time point when maternal mRNA is downregulated and zygotic transcription is initiated (Tadros and Lipshitz, 2009). In zebrafish, the transition from maternal to zygotic mRNA falls within a range around 2.75 hpf, but can vary slightly from one embryo to another (Tadros and Lipshitz, 2009). Maternal Zygotic *oep* (MZ*oep*) mutants lack both maternal and zygotic *oep* mRNA, have near complete absence of mesendodermal and mesodermal derivatives, and always exhibit an open neural tube (Gritsman *et al*, 1999; Aquilina-Beck *et al*, 2007). Zygotic *oep* (*Zoep*) mutants have maternal *oep* mRNA but lack *oep* mRNA transcribed from the embryo's genome. *Zoep* mutants and can have either closed or open neural tubes (Aquilina-Beck *et al*, 2007; Lu *et al*, 2013). We hypothesize that the variation in neural tube phenotypes in *Zoep* can be attributed to the slight differences in the MZT range across embryos. The closed neural tube phenotypes observed in *Zoep* may be the result of maternal *oep* mRNA persisting long enough to compensate for the lack of zygotic mRNA. In contrast, *Zoep* mutants with open neural tubes may have a slightly earlier MZT, resulting in insufficient *oep* mRNA to mediate neurulation. The variable neurulation phenotypes in *Zoep* mutants suggest that the MZT is a critical time for Nodal signaling's role in neurulation.

An early role for Nodal-induced mesendoderm and mesoderm in neurulation is

also supported by the fact that MZ*oep* embryos have abnormal neural plate morphology (Aquilina-Beck *et al*, 2007). N-cadherin proteins are highly expressed in the neural plate of WT embryos where N-cadherin is necessary for closure of the anterior neural tube, specific cellular movements within the neuroepithelium, and convergence of the neuroepithelium to a single cell layer at the keel stage (Hatta and Takeichi, 1986; Lele *et al*, 2002; Hong and Brewster, 2006). Thus Nodal-mesendoderm interactions appear to be vital in the early steps of neurulation for regulating N-cadherin levels sufficient for neural tube closure.

Broad Region Of Mesendoderm And Mesoderm Is Important For Anterior Neural Tube Closure

Our previous studies with MZ*oep* mutants indicate that the requirement for Nodal pathway activation is not within the neural tube precursors (Aquilina-Beck *et al*, 2007). Rather, Nodal signaling promotes neurulation through induction of mesendoderm and mesoderm (Aquilina-Beck *et al*, 2007). In MZ*oep* mutants, cell autonomous activation of Nodal signaling within the mesendodermal and mesodermal precursors rescued mesendoderm and mesoderm formation and corrected the open neural tube phenotype (Aquilina-Beck *et al*, 2007). Consistent with Nodal activation being required only within the mesodermal/mesendodermal tissues, in this study we found that the Nodal signaling inhibitor SB505124 simultaneously caused loss of mesendodermal and mesodermal tissues and induced open neural tube phenotypes. Moreover, early addition of SB505124 caused loss of almost all mesendodermal and mesodermal tissues and resulted in the greatest penetrance of the open neural tube phenotype. In contrast, later addition of the inhibitor (post 4.3 hpf) caused less severe losses that were primarily localized to the

posterior mesoderm and the anterior neural tube was closed.

Our findings indicated a large region of head mesendodermal and anterior mesodermal tissues are involved in anterior neurulation. There was strong statistical support for involvement of all of the tissues assayed, which included hatching gland cells, anterior notochord, cephalic paraxial mesoderm, and head muscles. Interestingly, although there was a positive correlation with the presence of each tissue and a closed neural tube, none of the tissues were absolutely required. For example, seven of forty-six *lefty1* overexpressing embryos had complete lack of hatching gland cells but a closed neural tube.

The tissues that were analyzed were the most likely to be involved due to their spatial proximity or length of contact with the presumptive anterior neural tube. Thus, this data strongly supports a large region of anterior mesendoderm/mesoderm interacting with the overlying neuroectoderm. However, we cannot rule out the possibility that a specific tissue that we did not test is required for neural tube closure. The tissues that were analyzed may have correlated with a closed neural tube not because they are directly involved, but because they are co-induced by Nodal along with the specific tissue that is required. However, this appears unlikely as we tested tissues that span the range of the head mesendoderm/mesoderm.

We do not yet know by what mechanisms the head mesendodermal and mesodermal tissues promote anterior neurulation. One possibility is that there is a biochemical signal that is secreted by a large range of mesendodermal/mesodermal tissues. If adequate numbers of any mesendodermal/mesodermal cell type is present, then enough of this signal is produced to drive neural tube closure. Consistent with this

possibility, a large region of the head mesendoderm/mesoderm is in spatial proximity to the overlying anterior neuroectoderm (Kimmel *et al*, 1990; Solnica-Krezel and Sepich, 2012). At the start of gastrulation, the mesendodermal cells move inward through the blastopore, forming a lining beneath the future neuroectodermal cells and the yolk cell below (Solnica-Krezel and Sepich, 2012). The contact between the two tissue types persists until they undergo further specification around the end of gastrulation. Thus, the head mesendoderm/mesoderm is within a few cell diameters of the overlying neuroectoderm prior to, and during, anterior neurulation.

There is growing evidence that there are a number of signals that mediate communication between the anterior neuroectoderm and the underlying mesoderm-related tissue. Disruptions of approximately 70 genes in mice result in either spina bifida or exencephaly (Harris and Juriloff, 2006). Many of these gene encode secretory proteins that have functions including involvement in biochemical cell-cell signaling pathways, apoptosis, and cell adhesion (ex. Sonic Hedgehog, Fibroblast Growth Factors, and Wingless Integrated) (Yamanoto *et al*, 2003; Ybot-Gonzalez *et al*, 2002; Londin *et al*, 2005; Harris and Juriloff, 2006). Additionally, many of these secreted proteins are highly expressed before and during anterior neurulation in the neuroepithelium, with the high expression in the neural folds and in adjacent tissues such as the head mesenchyme (Harris and Juriloff, 2006). Thus, there are many candidates that could be mediating a biochemical interaction between the anterior mesendoderm/mesoderm and the anterior neural tube.

There is also evidence of other neural patterning events where a wide range of mesendodermal/mesodermal tissues are involved. In mice, *Engrailed* (*En*) genes have a

prominent role in the midbrain and the anterior hindbrain development (Ang and Rossant, 1993; Danielian and McMahon, 1996). Similar to our studies, the whole of the anterior lateral mesendoderm region, as opposed to a specific tissue, can induce expression of the *En* genes in the overlying neural plate (Ang and Rossant, 1993; Danielian and McMahon, 1996). Moreover, in *Xenopus*, the anterior notochord is primarily responsible for inducing *En* genes in the overlying neuroectoderm. However, in the absence of the anterior notochord, the presumptive head mesoderm anterior somites can induce *En* expression, although not as efficiently (Hemmati-Brivanlou, 1990). Consistent with our study, the data from other vertebrate systems also suggests a broad region of head mesendoderm is involved in neurulation processes.

Another model has been proposed for the role of the head mesenchyme in anterior neurulation in mice. It has been suggested that proliferation and expansion of the underlying head mesenchyme drives the lateral edges of the neural plate toward each other (Chen and Behringer, 1995; Harris and Jurilof, 2006). Although the proliferation of the mesenchyme is an established mechanism in mice, it seems unlikely that head mesendoderm proliferation drives neural tube closure in zebrafish. Unlike other vertebrates, primary neurulation in zebrafish results from the thickening and then 'sinking' of the neural plate (Lowery and Sive, 2004). Therefore, it is hard to reconcile proliferation of the underlying mesenchyme/mesendoderm causing this 'sinking' morphological event in zebrafish.

Time And Dose Model For Neural Tube Closure By Nodal Signaling

The temporal requirement of Nodal signaling for neurulation matched the time Nodal signaling is required for mesendoderm induction. Interestingly, the temporal requirement of Nodal signaling for neural tube closure was only a subset of the Nodal time requirement in mesendoderm, mesoderm, and endoderm induction (Hagos and Dougan, 2007). Nodal signaling was required up to the late blastula stage of 4.3 hpf for a closed neural tube. However, Nodal signaling actively induces mesendoderm, mesoderm, and endoderm from 2.75 hpf to 5.0 hpf (Hagos and Dougan, 2007).

Two models have been proposed for how Nodal signaling induces different endodermal, mesodermal, and mesendodermal tissue types. The Spatio-Temporal model proposes that a cumulative exposure to Nodal signaling across time is the determinant for a particular cell fate (Gritsman *et al*, 2000; Hagos and Dougan, 2007). Hence, in the Spatio-Temporal model, a longer exposure to a certain Nodal dose will result in an overall increased mesendoderm and mesoderm formation, and different durations of exposure will result in different cell types. For instance, cells with extended exposure to Nodal signals will give rise to prechordal plate whereas those with shorter exposure will give rise to somites (Hagos and Dougan, 2007). The Ratchet model suggests that cells are specified by the highest dose of Nodal to which they are exposed (Gurdon *et al*, 1995; Thisse and Thisse, 1999). Therefore in the Ratchet model, a threshold level of Nodal signaling must be met for mesendoderm and mesoderm formation. For example, exposure to consistently high Nodal dose will only result in cell fates that require a high Nodal dose.

Our findings most strongly support Nodal signaling acting according to the

Spatio-Temporal model in anterior neurulation. Consistent with the Spatio-Temporal model, exposure to Nodal inhibitor in earlier time points resulted in greater mesendodermal/mesodermal loss and subsequently to open neural tube phenotypes. This suggests that early inhibition of Nodal signaling prevented the Nodal dose critical for induction of the mesendodermal/mesodermal tissue required for neurulation. In contrast the inhibition of Nodal signaling later in development, after the critical cumulative dose had been met, did not affect neural tube closure.

Although the data most strongly fitted the Spatio-Temporal model, we cannot completely rule out the Ratchet model. Overall however, our findings are less consistent with the Ratchet model. According to the Ratchet model, early addition of the Nodal inhibitor would result in less mesendodermal/mesodermal loss than later inhibition. In this model, Nodal protein accumulates over time to reach a threshold. Early inhibition would block Nodal signaling at a time when Nodal ligand levels are predicted to be low. Thus, the effect of the inhibitor would be minimal. In earlier Nodal inhibition, there would be greater recovery time for the Nodal dose to reach the critical threshold. This would result in less mesendodermal loss and a higher percentage of closed neural tube embryos when inhibitor is added early because later in development, more Nodal ligands are predicted to be present. Thus the inhibitor would have greater effect. There would be a shorter time for Nodal signaling to recover to the high levels required for head mesendoderm/mesoderm induction and promotion of neurulation. Therefore in the Ratchet model, a later inhibition of Nodal signaling is more likely to result in an open neural tube. This is opposite to what we found, where early inhibitor addition had a greater effect than later addition.

Although our data supported the Spatio-Temporal model, some modifications to this model are required to fully explain our data. In this modified model, which we call the Time-Dose model, a cumulative dose of Nodal signaling is required for the induction of a wide region of the mesendoderm/mesoderm, which then promotes neural tube closure. However, our data suggests that the cumulative Nodal dose must be met by a strict time window by 4.3 hpf, for a closed neural tube (Fig. 26). The strict time window suggests that the Nodal signaling pathway was not equal in activity across its temporal range in mesendoderm/mesoderm induction and in neurulation. Our data suggests that time points closer to the mid-blastula stage have higher amount of the Nodal activity that is required for both neural tube closure and for mesendoderm induction (Fig. 26). The progressively decreasing effect of Nodal inhibition suggests a decrease in relevant Nodal receptor activity further from the mid-blastula (Fig. 26). After 4.3 hpf, Nodal receptors were active for mesendoderm induction and other processes of early development, but no longer required for neurulation (Fig. 26).

Figure 9

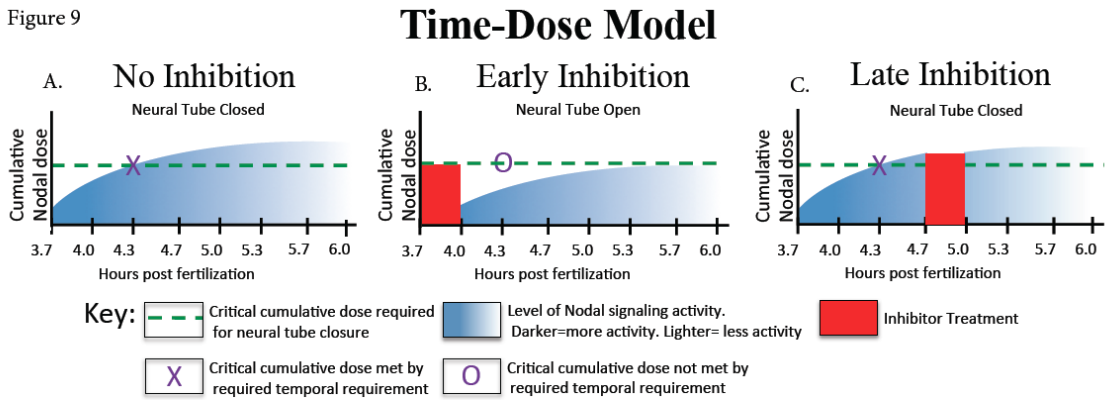


Figure 26: **The Time-Dose Model for the role of Nodal signaling in neural tube closure.** (A) Under normal conditions, WT embryos reach the critical cumulative dose of Nodal signaling by 4.3 hpf resulting in a closed neural tube. (B-C) When embryos are exposed to the Nodal inhibitor SB505124 (B) prior to 4.3 hpf, the critical cumulative dose of Nodal signaling is not met resulting in an open neural tube, but (C) after 4.3 hpf, the critical cumulative dose of Nodal signaling is met resulting in a closed neural tube.

IV. DISCUSSION

Spawning Project, Chapter 2

Summary

Chapter 2 of my thesis details new insights on fish spawning behavior and proposes a new method for zebrafish spawning with multiple benefits over the traditional spawning method (Gonsar *et al*, 2012). Our goal was to obtain daily fertilized eggs with minimal time investment for spawning tank set up. This study made three major advances. Firstly we developed a new, efficient and economic method for daily egg collection over the course of a 28-day period. Second, we found that fish spawn every 1-3 days when continuously kept together but the number of fertilized eggs varied under different density conditions. Third, we found that fish kept in single mating pairs had the highest rate of fertility compared to fish kept in higher-density tanks of 3♂×3♀ 5♂×5♀ and 7♂×7♀, where the latter had the lowest egg production.

Analysis

In Chapter 2 of my thesis, I demonstrate that zebrafish kept in our proposed method had multiple benefits over the traditional method of zebrafish breeding. The fish in the traditional spawning set up are not fed and do not get fresh water and therefore are only bred once a week. Our method allows for daily egg collection by keeping the fish on the recirculation rack with constant access to fresh water. Additionally our method allowed for egg collection everyday as opposed to the traditional method, which only allowed for obtaining eggs once a week. Therefore, within a given week, our method provided increased egg laying opportunities compared to the traditional method. Our

method was also more economical than the traditional method. Our method only required a spawning insert and a cheaply available siphon. On the contrary, the traditional method requires a bottom reservoir, a lid and the spawning insert. Finally because fish kept in our method continued to lay frequently over the course of the 28 day set-up, we propose our method is time saving requiring only a single spawning set up. Finally, research zebrafish are housed in mix gender tanks within recirculation tanks but almost never spawn (observation). However, with our modifications zebrafish continued to produce fertile eggs every 1-3 days within a recirculation system. This suggests that factors such as increased male-female interaction possible in a smaller given space of our method may drive this phenomenon.

In Chapter 2 of my thesis, I also demonstrate that zebrafish females spawn every 1-3 days, and spawn a greater number of fertilized eggs under low-density conditions. Many factors may potentially determine spawning rate and number of eggs produced. For instance, an increase in time together between males and females has been shown to increase egg production (Spence *et al*, 2008). This can potentially explain why the zebrafish continued to lay under our method but do not when they are usually housed in deep tanks on the recirculating system. The exact mechanisms that trigger spawning are not clearly understood but some evidence suggests that chasing of the female by the male zebrafish triggers the stimuli for spawning (Eaton and Farley, 1974). Chasing is a courtship behavior that begins as soon as a female-male pair is placed together (Eaton and Farley, 1974). This behavior involves the constant following of the female fish by the male zebrafish (Eaton and Farley, 1974). During the first 30 minutes of the spawning period this chasing behavior is at a rapid rate (Darrow and Harris, 2004). During this

rapid rate, males frequently nudge the female towards a potential spawning site (Spence *et al*, 2008). Males follow females around the tank and occasionally swim in tight circles around her or in figure of eight with raised fins (Spence *et al*, 2008). This triggers the egg release in female and at the same time the male releases the sperm, fertilizing the eggs.

Finally, I demonstrate that low-density tanks had higher rates of fertile egg production compared to high-density tanks. A potential explanation for the increase may be that the lower density tanks are more conducive to successful chasing behavior. Studies suggest that during courtship, females utilize the whole water column swimming to the surface and then diving down to the bottom of the spawning tank (Spence *et al*, 2008). Therefore, in lower-density tanks males have an increased area to engage in chasing with a stronger ability to avoid obstacles or to lose the female in pursuit. In lower-density tanks males also have a decreased chance of interruption by other males during the chasing behavior.

Increased density in a fish tank also increases aggression among the males, and between the female fish, which can cause a decrease in egg production (Spence *et al*, 2008). An increase in male-to-male aggression in lower-density tanks may potentially decrease the number of males available for courtship behavior per female. Additionally within a confined space a larger number of females factor as competition to other females for spawning sites. Studies also show that females exposed to the pheromones of other females for several days prior to spawning decrease or completely defer from egg production (Hurk and Lambert, 1983). We observed a dramatic drop in egg production after the first day of the 28-day course, suggesting even one day of close proximity to other females can affect egg production. Moreover, within the fish kept in high- density

tanks, we observed a negative linear rate of egg production from the three-paired fish tank to the seven-paired fish tank. Considering all the fish were kept in tanks with the same volume, it is likely that the deceased volume of water per fish in the high-density lead to faster rates of pheromone exposure. The higher rate of exposure to the pheromones may cause a stronger and faster inhibition of egg production, leading to the observed decrease in the overall egg production per female in high-density tanks.

Overall in all the different density tanks, our findings exhibited a lower egg production rate as compared to other studies (Eaton and Farley, 1974; Spence and Smith, 2006). A potential explanation is the tendency for females to avoid committing high spawning rates with closely related siblings (Gerlach and Lysiak, 2006). Olfactory cues by females result in sibling avoidance through detection of an odor (Gerlach and Lysiak, 2006). The fish used for our experiments had limited genetic diversity hence leading to a decrease in overall egg production during the 28-day period in all the tanks.

Neurulation Project, Chapter 3

Summary

The work discussed in Chapter 3 of this thesis examined the role of Nodal signaling in the development of the central nervous system. Our goal was to determine when and how Nodal signaling was required for anterior neurulation. When we began this study, it was known that the role of Nodal signaling in neurulation is early in development as demonstrated by the *oep* mutant studies (see Discussion of Chapter 3). We also knew that the necessity of Nodal signaling for neurulation is not through Nodal's activation within the neuroectodermal cell (see Discussion of Chapter 3). The studies

described in Chapter 3 made three key advances. First, we found that Nodal signaling is required up to the late blastula stage of 4.3 hpf for a closed neural tube. This is many hours prior to the initiation of neurulation when the neural plate forms. Second, we found a strong correlation between the presence of several anterior mesendoderm and mesodermal tissues and a closed neural tube. These various tissues included the hatching gland, the cephalic paraxial mesoderm, the anterior notochord and the head muscles. Third, we proposed a model for understanding the role of Nodal signaling in neural tube closure based on our findings above.

Working Model for Anterior Neurulation

In Chapter 3 of my thesis I describe that Nodal signaling induced head mesendoderm is required for neurulation up to 4.3 hpf (Fig. 27). This time window is many hours prior to formation of the neural plate and is within the time window when Nodal signaling is required for mesoderm/mesendoderm induction (Fig. 27). My findings also demonstrate that inhibition of Nodal signaling in early developmental time points correspond to greater loss of mesendoderm/mesodermal tissue and to the greatest penetrance of open neural tube phenotypes. In comparison, WT embryos with inhibition of Nodal signaling at later time points had loss of mesendodermal and mesodermal primarily limited to the posterior region with a closed anterior neural tube. These findings support a model where Nodal induced head mesendodermal and mesodermal tissues act in the early steps of neurulation during the neural plate formation (Fig. 27).

Chapter 3 of my thesis also demonstrates a prominent role for the broad region of the head mesendoderm and mesoderm in neurulation. I found that multiple head

mesendoderm/mesodermal tissues as opposed to individual tissues are competent to drive neural tube closure (Fig. 27). However we do not know how the head mesendoderm may be promoting neurulation.

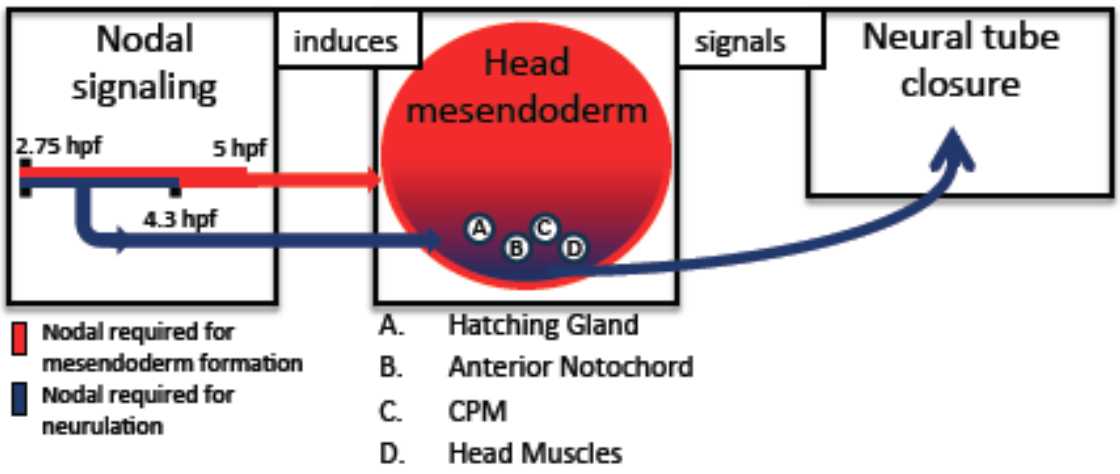


Figure 27: Schematic representation of the proposed Spatio-Temporal regulation of Nodal induced head mesendoderm in neurulation.

A growing possibility is that biochemical signaling secreted by the underling mesendoderm to the overlying ectoderm drive neurulation. We propose that the head mesendoderm could play a potential role in the expression of the N-cadherin proteins in the neural plate cells. N-cads are genes expressed by the underlying mesendoderm in the overlying neuroectoderm. N-cads have a conserved role across vertebrates as proteins important for cell adhesion and are strongly expressed in the developing neural tube of many vertebrates (Lele *et al*, 2002; Aquilina-Beck *et al*, 2007; Copp *et al*, 2003). In zebrafish N-cads are driven by Nodal signaling and promote cell polarity and convergence of the left right axis at the dorsal midline driving neural tube closure (Aquilina-Beck *et al*, 2007). Loss of *N-cad* causes disruptions in the neural rod formation and an open neural tube phenotype (Lele *et al*, 2002; Pujic *et al*, 2001; Aquilina-Beck *et al*, 2007). Likewise,

mouse *N-cad* mutants display irregularity in somite formation along with major malformations in the finals steps of neurulation resulting in NTDs but the neural tube is closed (Radice *et al*, 1997). Finally embryos that have decreased mesendoderm formation also have decreased N-cad localization in cell membranes (Aquilina-Beck *et al*, 2007). Therefore signaling of N-cad expression from the head mesendoderm/mesoderm to the overlying neuroectoderm may be a potential pathway driving the neural plate formation, the first step in neurulation.

The mice *Engrailed* genes are another set of candidate genes that have a prominent role in neurulation and driven by biochemical pathways from the underlying mesendoderm/mesoderm. Studies show that signals from the underlying anterior mesenchyme drive the expression of the *Engrailed* (*En*) genes in the mouse neuroectoderm and these genes have a vital role in the midbrain and the anterior hindbrain development (Ang and Rossant, 1993; Danielian and McMahon, 1996). Additionally, the known mouse ectoderm-mesendoderm interaction is prior to morphological changes resulting in a closed neural tube, inviting a potential role for the *En* in neural tube closure (Ang and Rossant, 1993). Similarly zebrafish *eng* genes are expressed along the midbrain hindbrain and studies suggest these genes in zebrafish may have similar roles to the genes in mice (Joyner and Martin, 1987; Davidson *et al*, 1988; Davis *et al*, 1988; Davis and Joyner, 1988). Therefore there are known signals from the head mesendoderm/mesoderm to the overlying ectoderm for neurulation in both mice and zebrafish. My findings suggest that in zebrafish, regulation of the molecular signaling is through a broad region of the underlying mesendoderm for driving neural tube closure.

My temporal and spatial findings enable me to propose a revised model for the role

of Nodal signaling and head mesendoderm in neurulation. My Time-Dose model proposes that Nodal signaling acts many hours prior to neural plate formation to induce head mesendoderm/mesodermal tissues. Therefore later in development at the onset of neurulation, signaling induced from a broad region of the mesendoderm/mesoderm tissues then drive neurulation.

Future Directions

Isolation of genes downstream of Nodal signaling promoting mesendoderm/mesoderm induction

Our overall hypothesis remains that Nodal signaling early in development regulates neural tube closure through the induction of the mesendoderm/mesoderm. The cellular mechanisms that drive neurulation are currently inadequately understood. However in the future if we are able to narrow down candidate genes that have an important role in neurulation, we can potentially assay for these in anencephalic babies and find the common alleles that cause the defect. Furthermore, it may be possible to genetically screen would be a parents to determine if they if they carry risk factors for anencephaly.

Building on my work, our next step is to first understand how the activity of Nodal signaling is regulated to produce different levels of activity over time. Chapter 3 of my thesis demonstrates that Nodal signaling had a varying effect on mesoderm and mesendoderm induction, where it was most active right after the mid-blastula transition and milder after these stages. Additionally the greater loss of mesendoderm/mesoderm tissue during early Nodal inhibitor exposure overlaps the temporal requirement of Nodal

signaling for neurulation. A potential cause for this observation could be a change in mesendodermal response to Nodal signaling over time. The change in response could be due to an overall decrease in sensitivity to Nodal signaling over time or it could be a change in the response of specific downstream Nodal gene(s). Alternately, the weaker effect of Nodal inhibition at later time points may be caused by decreasing levels of the Nodal signal over time, with early inhibition simply acting on greater absolute levels of Nodal. Identifying the potential cause of change in Nodal-mesendoderm interaction over time is crucial because it could provide valuable clues to the connection between Nodal signaling and neurulation.

To test between the potential possibilities causing a change in Nodal-mesendoderm interaction, we can quantify Nodal protein levels across specific developmental time points using Western blots (Fig. 28). The time points would range across when Nodal signal is active in mesendoderm/mesodermal, endoderm induction, spanning from the MBT though to the onset of gastrula stage (Hagos and Dougan, 2007). If the Western blot analysis demonstrates a decrease in Nodal level over time, this would suggest that the greater effect of Nodal-mesendoderm interaction at early time points is directly caused by higher levels of Nodal signaling.

However, I hypothesize that Nodal levels do not decrease over time. If true, this would suggest that that the potential cause for the varying effect of Nodal signaling is a change in mesendodermal cellular response to Nodal signaling over time, potentially through modification in regulation of specific downstream genes, causing them to activate differently even without decreasing the activation signal. In either case we expect a sequence of molecular and genetic events connecting Nodal signaling to

mesendodermal induction and eventually the generation of signals involved in neurulation. If my hypothesis is true, we can narrow our exploration to specific downstream Nodal genes, which change in response to Nodal signaling around 4.3 hpf (Fig. 28). Known downstream Nodal genes can be assessed through qRT-PCR at early and late points around the 4.3 hpf mark to isolate the change in expression levels (Fig. 28).

If qRT-PCR is unable to find Nodal-regulated genes with a high potential for inducing the head mesendoderm, an alternative option is to widen the search within unknown genes downstream of Nodal. This effort could begin with chromatin immunoprecipitation (ChIP) of the FoxH1 transcription factor in embryos before and after 4.3 hpf. FoxH1 regulates Nodal signaling therefore ChIP analysis of the transcription can identify specific genes activated directly through Nodal signaling (Fig. 28). However, we do not know how far downstream any genetic changes may be occurring, therefore using a high throughput gene sequencing technique (RNA-seq) allows for a wider net for identifying Nodal-regulated mRNA expression changes that are important for head mesendoderm/mesoderm induction (Fig. 28).

Comparing the Nodal mutants, *cyc* and MZ*oep*, we can examine all changes in the zebrafish transcriptome that lead to an open neural tube. *cyc* mutants have a closed neural tube and a functional sqt protein. On the other hand, MZ*oep* have a non-functional *sqt* and *cyc* and always have an open neural tube (Aquilina-Beck *et al*, 2007). Comparative analysis between these two Nodal mutants confines our analysis to everything downstream of the Nodal protein sqt, no matter how remotely downstream it is. The *cyc* and MZ*oep* will be compared before and after the 4.3 hpf mark, when we can evaluate

specifically for the transcriptomic changes of the Nodal-regulated genes responsible for mesendoderm induction and for neurulation. This maximizes our capability to find informative data from an abundance of data generated by RNA-seq methods.

For the two techniques mentioned, the zebrafish mesendodermal tissue can be harvested by staining with a mesendodermal marker and removed using high precision using laser capture microdissection. There are now several RNA-seq technologies appropriate for the minute quantities of tissue which can be isolated this way (Smart-seq, CEL-seq, and Quartz-seq, to name a few) (Goetz and Trimarchi, 2012; Hashimshony *et al*, 2012; Sasagawa *et al*, 2013), Finally the sequence reads of the transcripts can be identified against the current zebrafish reference genome (Zv9) administered by the Genome Reference Consortium (Collins *et al*, 2012).

Connecting the pathway

Having isolated the potential Nodal gene(s) responsible for mesendoderm induction, we can examine the neural tube morphology resulting from the decrease in the Nodal isolated genes, confirming the role of the genes in neurulation. Our next step then is to determine how this gene is acting in the head mesendoderm to promote neural tube closure. There is evidence from different vertebrates suggesting that the head mesendoderm promotes signaling to the overlying neuroectoderm that is important for neural tube formation (Chen and Behringer, 1995; Harris and Juriloff, 2006). Two potential proteins induced in the neural tube cells by the underlying mesendoderm/mesoderm are N-cad protein, responsible for neural plate formation and Engrailed proteins, responsible for midbrain and anterior hindbrain development (Ang and Rossant, 1993; Danielian and McMahan, 1996).

Our first goal in this step is to determine which signal from the mesendoderm to the overlying neuroectoderm is regulated by the isolated Nodal-regulated gene(s) from the previous section. To determine this, we can conditionally decrease the expression of the isolated Nodal gene(s) in WT embryos and determine which signal from the underlying mesendoderm to the overlying neuroectoderm is being affected. This can be done by morpholino microinjections, which will knockdown expression of the proteins encoded by the candidate genes. We can then quantify the levels of the different mesendoderm signals to the overlying neuroectoderm, using qRT-PCR or Western blots (Fig. 28). This will determine which signal(s) in the neuroectoderm are regulated by the isolated Nodal gene(s). If the isolated gene has any known downstream regulatory targets, these can be assessed in a similar fashion using targeted morpholino injections to see which of these targets in turn is connected to changes in outcome for the eventual neurulation-promoting signal to the neuroectoderm (Fig. 28).

Overall this experiment will build gaps in our current understanding to build a pathway for the regulation of neural tube closure by Nodal signaling.

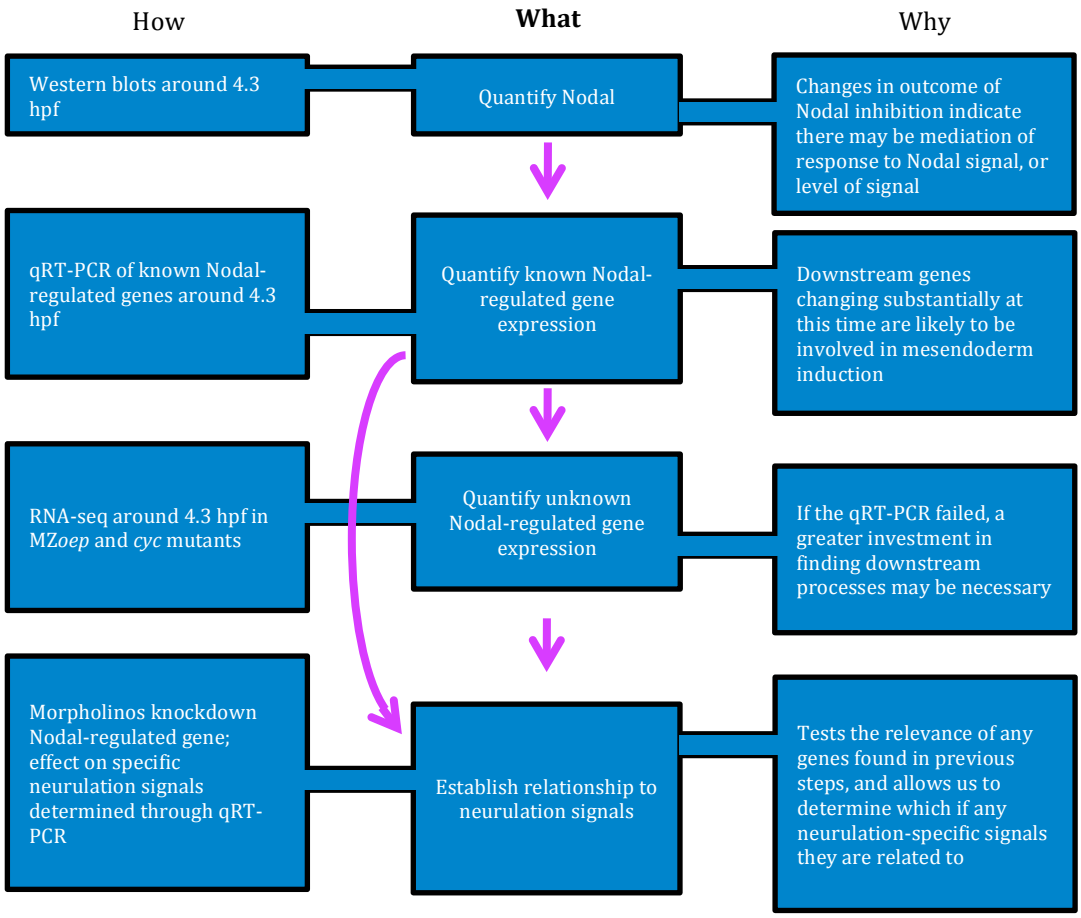


Figure 28: Outline of the future directions to build a pathway for regulation of neural tube closure by Nodal signaling

Feedback Mechanism Is An Integrating Principle

Signaling pathways play a key role in regulating an organism's development by production of feedback loops (Freeman, 2000). In Chapter 2 of my thesis, I have discussed the Nodal signaling pathway, which is regulated by positive and negative feedback mechanisms to promote development of the neural tube and many other tissues. I have also proposed a new model called the Time-Dose model, illustrating how the tightly regulated dose of Nodal signaling regulates the extent of head mesendoderm/mesoderm induction responsible for neurulation. Additionally in Chapter 3 of my thesis, I have discussed potential signals from the environment, which affect

changes in egg production of the zebrafish female. Feedback mechanisms drive the response of such signals, ultimately affecting the overall fish population density.

Within developmental biology, positive and negative feedbacks are the core producers of signaling properties for an organism's development (Freeman, 2000). The generation of the *lefty1* mRNA injected embryos in Chapter 3 of my thesis, are a result of the exploitation of the lefty1-Nodal negative feedback loop. lefty1 is a negative inhibitor in the Nodal signaling pathway, regulating all functions of Nodal signaling. One such function includes the induction of the head mesendoderm, demonstrated Chapter 3 (Green and Smith, 1990; Hamada *et al*, 2002). lefty1 functions via two mechanisms to restrict Nodal signaling. In the Nodal signaling pathway Nodal generates more Nodal through a positive feedback loop. A positive feedback occurs when a signal either induces more production of itself, less of a signal that inhibits the original signal, or more of a signal that amplifies the original signal (Robertson, 1991). lefty1 restricts Nodal from generating more Nodal, regulating the positive feedback (Hamada *et al*, 2002). Secondly lefty1 can diffuse faster than Nodal, keeping Nodal from getting out of its correct signaling range (Schier, 2003). The biochemical activity of lefty proteins is partly understood. It is thought that lefty competitively binding Nodal receptors preventing the Nodal activation (Meno *et al*, 1999).

The negative feedback loop in the BMP pathway is another example of the necessity of feedback mechanisms within developmental signaling pathways. BMP2 is expressed in high concentrations in the ventral side of the embryo, inducing embryonic cells to differentiate into ventral and lateral cells. (Gazzerro *et al*, 1998). On the dorsal side, the BMP2 inhibitor Noggin and Chordin are highly expressed, generating a BMP

concentration gradient along the dorso-ventral axis (Gazzerro *et al*, 1998). Noggin and Chordin act as an antagonist and regulates *BMP* through a negative feedback (Gazzerro *et al*, 1998). *BMP* in turn induces *Noggin*, completing a negative *Noggin-BMP* feedback loop (Gazzerro *et al*, 1998)

Within the BMP pathway, expressing the BMP binding factor cleaved cross veinless 2 (*cv2)* amplifies BMP signaling during dorso-ventral patterning of an embryo (Rentzsch *et al*, 2006). Completing the positive loop, the transcription of *clv2* is activated by the BMP signal (Rentzsch *et al*, 2006).

In developmental biology, understanding the roles of feedback mechanisms within the context of signaling pathways is fundamental. Developmental processes such as neurulation are multistep pathways, orchestrated by a host of signaling pathways functioning through different tissue types. These signaling pathways driving neurulation are in turn regulated and activated by positive and negative feedback loops. These feedback mechanisms ensure that the correct signal is sent out to the correct cells.

By exploiting our ability to disrupt the Nodal signaling feedback loop, my work demonstrates that the Nodal signaling pathway is necessary during the blastula stages of development for a closed neural tube phenotype. Additionally my work presents a model that a cumulative dose of Nodal signaling is necessary for head mesendoderm induction and consequently for neurulation. This therefore potentially provides the groundwork for understanding mechanisms involved in human NTDs, suggesting that both the developmental time and the dose of exposure are key factors for understanding cell determination. The principles of Nodal signaling within cells are similar to signaling within other biological systems where feedback mechanisms drive the homeostasis of the

system. However disruptions in the feedback loop within the signaling pathways trigger a deviation from the default feedback loops leading to adverse effects such as NTDs.

Feedback mechanisms not only produce signals for cellular fates within an organism, but they are also involved in other biological systems. For instance within an ecosystem, a negative feedback ensures a balance of participant organisms within a predator prey relationship (Robertson, 1991). An increase in prey animals allows for an increase in the number of predators. This increase in predators then decreases an excess of the prey by completing the negative loop and consequently balancing the number of prey versus predator. In a predator prey relationship, the organisms serve similar roles to the secreted proteins in a signaling pathway serving as the controlling elements by exhibiting adaptability to the given environment.

Signals are not only restricted to within and between organisms but are also derived from environmental cues. Environmental cues play a major role in triggering pathways within cells and have the capability to disrupt an organism's homeostasis triggering disease (Freeman *et al*, 2000). One example is the pancreas hormones' regulation of blood glucose level. When a high glucose diet is ingested, there is an increase in the blood sugar level (Chang *et al*, 2004). This triggers the increase in the insulin hormone in the bloodstream, reducing the blood sugar level and completing the feedback loop. However disruptions in the feedback loop for instance due to continued insensitivity to insulin causes increase in blood sugar level leading to grave health outcomes (Change *et al*, 2004). Therefore when there is a failure within the system to complete the loop in response to signal, this leads to adverse outcomes.

Chapter 2 of my thesis suggests that environmental cues could affect spawning

behavior of zebrafish. These signals from the environment drive changes in the feedback mechanisms leading to variations in the number of zebrafish eggs produced under varied density conditions. For instance signals such as female pheromones are responsible for triggering the courtship behavior in males (Hurk and Lambert, 1983). The courtship behavior in males is in turn likely to lead to female oviposition and increased egg production per female (Fig. 29). However, the female pheromones can also have an opposite effect on other females leading to a decreased in egg production (Gerlach, 2006) (Fig. 29). At the same time, other signals trigger sibling avoidance (Gerlach and Lysiak, 2006), leading to an overall decrease in egg production in both the low and the high-density tanks. Male to male aggression also results in a decreased egg production (Spence *et al*, 2008) (Fig. 29). Hence the degree of these signals emitted can imbalance the feedback loops. This imbalance deviates from the original state of the feedback loop to increase or decrease overall egg production.

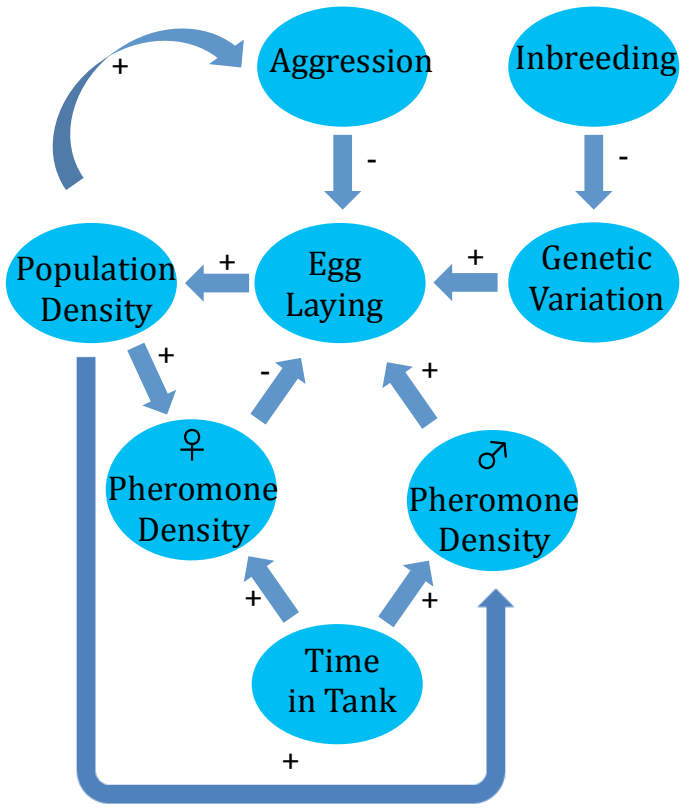


Figure 29: Environmental signals affect zebrafish egg production. Fish in high-density tanks have increased exposure to within-sex aggression. Additionally these fish are also exposed to increased female-female pheromone interaction causing a decrease in egg production. These factors contribute to an overall decrease in eggs per female in the high-density tanks, and in the wild this would complete a feedback loop to regulate the population density of a zebrafish strain. Fish on in the low-density tanks have low within-sex aggression and decreased female-female pheromone interaction leading to increase egg production. In the wild this would potentially lead to an increase in population density. Note: inbreeding was a common factor in both tanks, leading to lower egg production equally in both high and low density tanks. Also, presumably, the higher concentration of male pheromone in higher density tanks would increase egg production, although the strength of this effect does not seem to be strong enough to overcome the other, negative factors.

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