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Nodal signaling and the zebrafish organizer

ALEXANDER F. SCHIER[1] and WILLIAM S. TALBOT[2]

[1]Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, Department of Cell Biology, New York University School of Medicine, New York, U.S.A. and [2]Department of Developmental Biology, Stanford University School of Medicine, Stanford, California, U.S.A.

ABSTRACT Systematic genetic screens in zebrafish have led to the discovery of mutations that affect organizer function and development. The molecular isolation and phenotypic analysis of the affected genes have revealed that TGF-β signals of the Nodal family play a key role in organizer formation. The activity of the Nodal signals Cyclops and Squint is regulated extracellularly by the EGF-CFC cofactor One-eyed Pinhead and by antagonists belonging to the Lefty family of TGF-β molecules. In the absence of Nodal signaling, the fate of cells in the organizer is transformed from dorsal mesoderm to neural ectoderm. Differential Nodal signaling also patterns the organizer along the anterior-posterior axis, with high levels required for anterior cell fates and lower levels for posterior fates. In addition, Nodal signaling cooperates with the homeodomain transcription factor Bozozok in organizer formation and neural patterning. The combination of genetic, molecular and embryological approaches in zebrafish has thus provided a framework to understand the mechanisms underlying organizer development.

KEY WORDS: *Zebrafish, nodal, EGF-CFC, lefty, bozozok, shield.*

The zebrafish organizer corresponds to the shield, a thickening at the dorsal margin that forms at the onset of gastrulation. Transplantation of the shield can induce a complete secondary axis in host embryos, similar to the activity of the organizer as first described by Spemann and Mangold in amphibians 75 years ago (Spemann and Mangold, 1924; Oppenheimer, 1936; Shih and Fraser, 1996; Driever *et al*., 1997; Koshida *et al*., 1998; Saude *et al.,* 2000). The transplanted shield has two major roles: it signals adjacent cells, thereby dorsalizing the mesoderm and neuralizing and patterning the ectoderm, and it forms the dorsal-most mesoderm, giving rise to axial midline structures that include the prechordal plate anteriorly and notochord more posteriorly. In this review, we discuss how genetic approaches in zebrafish have led to the isolation of genes essential for organizer development and describe recent studies that have uncovered the role and regulation of the Nodal signaling pathway during organizer formation.

A genetic approach in Zebrafish

Pioneering work by Streisinger, Kimmel and their colleagues established zebrafish as a vertebrate model system that combined genetic and embryological approaches (Streisinger *et al*., 1981; Kimmel, 1989). Small-scale screens initially led to the isolation of a handful of gamma-ray induced and spontaneous mutations, including *cyclops* and *spadetail*, that cause interesting developmental phenotypes (Kimmel *et al*., 1989; Hatta *et al*., 1991). These early efforts demonstrated the potential of a forward genetic approach in zebrafish to study vertebrate development. Furthermore, the development of powerful embryological techniques such as cell transplantation and labeling allowed elegant phenotypic analyses in the transparent zebrafish embryo (Ho and Kane, 1990; Kimmel *et al*., 1990).

In order to perform large-scale screens for mutations disrupting zebrafish development, the laboratories of Nüsslein-Volhard and Driever established efficient mutagenesis and growth conditions for zebrafish (Mullins *et al*., 1994; Solnica-Krezel *et al*., 1994). The chemical N-ethyl-N-nitrosourea (ENU) induced mutations at a rate comparable to those used in the classic *Drosophila* screens performed by Nüsslein-Volhard, Wieschaus and colleagues (Nüsslein-Volhard and Wieschaus, 1980). Between 1993-1995 the two groups applied these mutagenesis conditions at a large scale and screened more than 6,000 genomes and more than a million embryos for zygotic mutations affecting zebrafish development. These studies led to the isolation of several thousand mutations causing lethal phenotypes and defined ~500 genes that when mutated lead to specific developmental defects (Driever *et al*., 1996; Haffter *et al*., 1996).

Detailed phenotypic characterization of these mutants identified two general classes of genes that affect the organizer (Schier and

Abbreviations used in this paper: cyc, cyclops; sqt, squint; oep, one-eyed pinhead; boz, bozozok; sur, schmalspur; ysl, yolk syncytial layer.

***Address correspondence to:** Alexander F. Schier. Developmental Genetics Program. Skirball Institute of Biomolecular Medicine, Department of Cell Biology, New York University School of Medicine, New York, NY 10016, U.S.A. FAX: +1-212-263-7760. e-mails: schier@saturn.med.nyu.edu; talbot@cmgm.stanford.edu

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Talbot, 1998). One class of mutants, the dorsal-ventral group, affects the function of the organizer as a dorsalizing region (Hammerschmidt *et al*., 1996b; Mullins *et al*., 1996; Solnica-Krezel *et al*., 1996). The second class, the dorsal mesoderm group, affects the development of the precursors of the prechordal plate and notochord (Hammerschmidt *et al*., 1996; Odenthal *et al*., 1996; Schier *et al*., 1996; Solnica-Krezel *et al*., 1996; Stemple *et al*., 1996).

Dorsal-ventral group: Different cell types are arranged along the dorsal-ventral axis of the blastula fate map, with dorsal fates including axial mesoderm, trunk somites and neuroectoderm and ventral fates including blood, pronephros and epidermis (Kimmel *et al*., 1995). One class of zebrafish mutants affects the overall dorsal-ventral patterning of the embryo. For instance, *dino* mutant embryos are ventralized, i.e. they have an expansion of ventral fates such as blood and epidermis (Hammerschmidt *et al*., 1996b). Conversely, mutants such as *swirl* and *snailhouse* are dorsalized, leading to an expansion of neuroectoderm and somitic mesoderm (Mullins *et al*., 1996b). These mutant phenotypes strongly resemble previously described defects induced by overactivation or inhibition of BMP signaling in *Xenopus* (De Robertis and Sasai, 1996; Harland and Gerhart, 1997). Indeed, epistatic analysis and molecular cloning has identified five genes of the dorsal-ventral group as components or regulators of the BMP signaling pathway: *swirl* corresponds to BMP2b (Kishimoto *et al*., 1997); *snailhouse* to BMP7 (Dick *et al*., 2000; Schmid *et al*., 2000); *somitabun* to smad5 (Hild *et al*., 1999); *minifin* to tolloid (Connors *et al*., 1999); and *dino* to chordin (Schulte-Merker *et al*., 1997). These mutations do not disrupt the formation of the dorsal mesoderm of the organizer, but they affect the patterning functions of the organizer. In particular, organizer derived factors such as chordin antagonize ventralizing factors such as BMP2b and BMP7. The antagonistic relationship between these factors establishes different fates along the dorsal-ventral axis.

Dorsal mesoderm group: Cells in the organizer primarily contribute to the dorsal mesoderm, giving rise to axial midline structures such as notochord and prechordal plate. The notochord is disrupted by mutations in *floating head* and *no tail*, which encode homeodomain and T-box transcription factors, respectively (Halpern *et al*., 1993; Schulte-Merker *et al*., 1994; Talbot *et al.,* 1995; reviewed in Schier and Talbot, 1998). Initial phenotypic characterization of mutants found in the large-scale screens identified additional loci important for the development of organizer derivatives. For instance, mutants in *one-eyed pinhead* lack the prechordal plate (Schier *et al*., 1997; Strähle *et al*., 1997). Likewise, *cyclops (cyc)*, *bozozok (boz), schmalspur (sur*), and *squint (sqt)* mutants display defects in axial mesoderm formation, ranging from a reduction of the prechordal plate in *cyc* mutants to loss of notochord and prechordal plate in severe *boz* mutants (Thisse *et al*., 1994; Solnica-Krezel *et al*., 1996; Schier *et al.,* 1996; Brand *et al.,* 1996; Heisenberg and Nüsslein-Volhard, 1997; Pogoda *et al.,* 2000; Sirotkin et al., 2000b). It was thus thought that these genes might provide an entry point to study the specification of axial mesoderm fates derived from the organizer. As we describe below, further molecular and genetic studies have revealed that these genes have roles that are even wider than initially anticipated.

Nodal-related signals are required for dorsal mesoderm development

Genetic analysis of *cyc* and *squint sqt* has identified these genes as key regulators of dorsal mesoderm development. The original *cyc* allele was isolated as a gamma-ray induced mutation that causes cyclopia accompanied by a loss of ventral forebrain and floor plate (Hatta *et al*., 1991). Several subsequent screens identified additional alleles induced by ENU and gamma-rays (Brand *et al*., 1996; Schier *et al*., 1996; Talbot *et al*., 1998). In addition to the ventral neural tube defects in *cyc* mutants, *goosecoid* expression in the developing prechordal plate is diminished at mid-gastrulation, and the hatching gland, a major derivative of the prechordal plate, is reduced (Thisse *et al*., 1994).

Sqt was identified as a spontaneous mutation causing cyclopia (B. Paw and L. Zon, personal communication), and phenotypic analysis showed that *sqt* mutants affect the prechordal plate at the end of gastrulation (Heisenberg and Nüsslein-Volhard, 1997). The same *sqt* allele was isolated independently from a related stock of fish as a spontaneous mutation that enhanced the phenotype of *cyc*, and further phenotypic characterization revealed that *sqt* mutants display reduced expression of dorsal mesoderm marker genes in the late blastula and a failure of shield formation at the onset of gastrulation (Feldman *et al*., 1998). At later stages, *sqt* mutants have variable defects, which can include loss of ventral diencephalon and cyclopia.

The phenotype of *sqt;cyc* double mutants is much more severe than those of the single mutants: *sqt;cyc* mutants lack not only the prechordal plate and notochord but also most other mesendodermal derivatives, including head and trunk muscle, pronephros, heart, blood, and the gut (Feldman *et al*., 1998; Figure 1). Double mutant embryos do not develop a shield and markers for dorsal mesoderm such as *goosecoid* and *floating head* are not expressed at the onset of gastrulation. Moreover, pan-mesodermal markers such as *Brachyury/no tail* are not expressed in dorsal marginal cells, which are fated to become dorsal mesoderm in wild-type. Involution of marginal cells is disrupted in *sqt;cyc* mutants, and fate mapping studies have demonstrated that dorsal marginal cells give rise to neural structures instead of dorsal mesoderm fates (Feldman *et al*., 2000). Together, these results show that *sqt* and *cyc* have overlapping functions in the development of the prechordal plate, notochord, and other mesendodermal derivatives of the head and trunk.

Using the candidate approach, the *sqt* and *cyc* genes were found to encode members of the TGF-ß superfamily related to mouse *nodal* (Feldman *et al*., 1998; Rebagliati *et al*., 1998b; Sampath *et al*., 1998; Schier and Shen, 2000). Previous analysis of mouse *nodal* mutants demonstrated that this gene is essential for formation of the primitive streak and mesoderm during gastrulation (Zhou *et al*., 1993; Conlon *et al*., 1994). Nodal-related signals were also implicated as key regulators of mesendoderm formation in *Xenopus*, since these factors can induce mesodermal and endodermal cell types in explant assays (Jones *et al*., 1995).

The expression patterns of *sqt* and *cyc* indicate that they act as spatially restricted signals that induce dorsal mesoderm development (Erter *et al*., 1998; Feldman *et al*., 1998; Rebagliati *et al*., 1998a; Sampath *et al*., 1998). Soon after the midblastula transition, *sqt* is expressed in dorsal marginal blastomeres and, slightly later, the dorsal yolk syncytial layer (YSL), a region of the embryo implicated by transplantation experiments as a source of dorsal mesoderm inducing signals (Long, 1983; Mizuno *et al*., 1996). *cyc* expression initiates in the late blastula stage, and by the end of the blastula period, both *cyc* and *sqt* are expressed around the entire circumference of the margin, consistent with their overlapping roles in the formation of the mesendoderm of the head and trunk. At the onset of gastrulation, *cyc* is strongly expressed in involuting

dorsal mesoderm, and *sqt* is expressed in a small group of dorsal marginal cells. As gastrulation progresses, *sqt* expression ceases, while *cyc* continues to be expressed in axial mesoderm throughout gastrulation. Thus the expression patterns of *sqt* and *cyc* correlate with the unique and overlapping aspects of their functions: *sqt* mutants have a disruption of dorsal mesoderm at early stages, when *cyc* expression is just beginning; *cyc* mutants have a strong phenotype at later stages, when *sqt* is not expressed; and double mutants lack endoderm and non-tail mesoderm, reflecting the overlapping marginal expression in the late blastula. Although different expression patterns account at least in part for the distinct requirements for *sqt* and *cyc* function, it remains possible that the Sqt and Cyc proteins also have different biochemical activities. Indeed, Sqt and Cyc have different activities in explant assays (Erter *et al*., 1998; Rebagliati *et al*., 1998a), and additional experiments are required to determine whether the Sqt and Cyc proteins have distinct activities in vivo.

Overexpression studies indicate that *sqt* and *cyc* are sufficient to induce dorsal mesoderm (Erter *et al*., 1998; Feldman *et al*., 1998; Rebagliati *et al*., 1998a; Sampath *et al*., 1998; Gritsman *et al*., 2000). Microinjection of synthetic mRNA encoding Sqt or Cyc can induce ectopic and expanded dorsal mesoderm. In addition, some embryos form secondary axes, much like embryos that receive ectopic organizers in shield-transplantation experiments. When overexpressed specifically in the extraembryonic YSL, *sqt* can induce dorsal mesoderm gene expression in neighboring embryonic blastomeres, demonstrating that the Sqt signal has a non-autonomous dorsal mesoderm inducing activity (Erter *et al*., 1998; Feldman *et al*., 1998). Thus the analysis of *sqt* and *cyc* has demonstrated that Nodal-related signals are necessary and sufficient for dorsal mesoderm development and that these genes are expressed appropriately to serve as endogenous signals in dorsal mesoderm formation.

One-eyed pinhead is required for Nodal signaling

The *one-eyed pinhead* (*oep*) locus was identified in the two large-scale screens in Boston and Tübingen and smaller screens in Oxford and Utah as a zygotic mutation leading to cyclopia and ventral forebrain defects (Hammerschmidt *et al*., 1996a; Schier *et al*., 1996; Schier *et al*., 1997; Strähle *et al*., 1997). Further analysis revealed that *oep* mutants also lack the anterior axial mesoderm (prechordal plate) and endoderm. The absence of *goosecoid* expression in the shield showed that normal dorsal mesoderm development is dependent on *oep* (Schier *et al*., 1997; Strähle *et al*., 1997). Double mutants for *oep* and the T-box gene *no tail* have defects in the formation of trunk mesoderm. These results demonstrated that *oep* is involved in the specification of mesendodermal cell fates.

Localizing the *oep* mutation on the zebrafish genetic map did not identify any candidate genes for this locus (Schier *et al*., 1997), necessitating a positional cloning strategy to isolate *oep* (Zhang *et al*., 1998; Talbot and Schier, 1999). This approach became feasible with the isolation of DNA markers closely linked to *oep* (Schier *et al*., 1997) and the availability of large-insert genomic libraries (Amemiya *et al*., 1999). By exploiting the large number of meioses that can be analyzed in zebrafish, genetic mapping studies limited the *oep* locus to a region of less than 100 kb (Zhang *et al*., 1998). Screening of cDNA libraries with genomic clones from this region led to the isolation of a candidate gene that rescued the *oep* mutant phenotype upon mRNA injection and was disrupted in two *oep* mutant alleles.

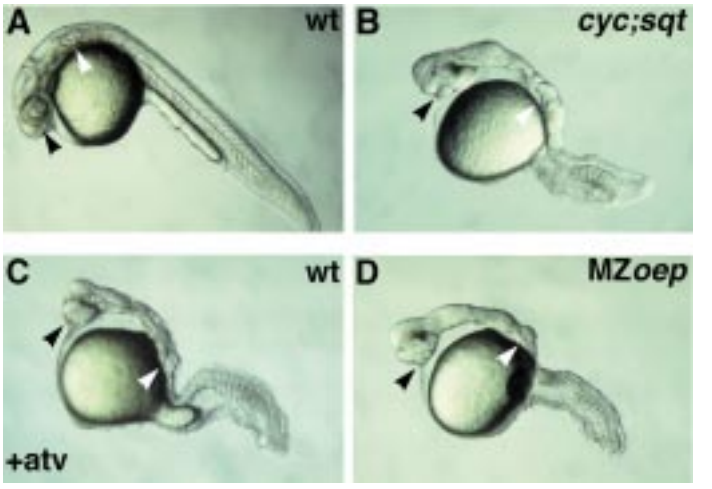

Fig. 1. Phenotypes *of* **(A)***wild-type,* **(B)** *sqt;cyc mutant,* **(C)** *wild-type embryo upon overexpression of antivin/lefty1 and* **(D)** *maternal-zygotic oep mutant at 30 hours post-fertilization. Black arrowhead points to eye, white arrowhead to ear (after Gritsman et al., 1999; Meno et al., 1999).*

Sequence analysis revealed that *oep* was a new member of the EGF-CFC family, extracellular factors previously thought to be involved in ras/MAPK signaling pathways (Shen and Schier, 2000). *Oep* mRNA is widely expressed and misexpression of *oep* does not lead to dominant phenotypes, indicating that *oep* acts as an essential and permissive factor (Zhang *et al*., 1998).

Further genetic studies demonstrated that Oep acts in the Nodal signaling pathway (Gritsman *et al*., 1999). Expression analysis showed that *oep* is not only expressed zygotically, but also maternally. The initially described mutant phenotype is caused by loss of zygotic *oep* activity. Since maternal mRNA is present in these embryos, *oep* function is not completely abolished. By injecting *oep* mRNA into zygotic *oep* mutants, it was possible to raise homozygous *oep* fish and to generate maternal-zygotic *oep* mutant progeny from homozygous adult females. Strikingly, these embryos had the same phenotype as *sqt;cyc* double mutants (Figure 1). Furthermore, maternal-zygotic *oep* mutants were found to be unresponsive to the overexpression of *cyc* and *sqt* mRNA. In contrast, activation of putative downstream components such as the Activin receptor ActRIB and the transcription factor Smad2 rescued various aspects of the mutant phenotype. In addition, overexpression of *activin* mRNA can induce dorsal mesoderm in maternal-zygotic *oep* mutants, indicating that Oep is not a general cofactor for TGF-ß signaling. These results suggested that Oep acts as an essential cofactor for Nodal proteins to activate ser/thr kinase receptors such as ActRIB (Gritsman *et al*., 1999; Schier and Shen, 2000; Figure 2). It has been proposed that Oep does not only function in Nodal signaling, but might also antagonize BMP activity (Kiecker *et al*., 2000). This suggestion is primarily based on the misexpression of a mutant (secreted) form of zebrafish Oep at non-physiological levels in *Xenopus*. It is thus unclear if these assays reflect an *in vivo* role for Oep during embryogenesis.

The molecular details of Oep's role in Nodal signaling are not yet known. Oep is not required for generating Sqt or Cyc in signaling cells. *Oep* acts cell-autonomously in the formation of prechordal plate and endoderm and thus appears to be required at the surface of responding, not signaling cells (Schier *et al*., 1997; Gritsman *et al*.,

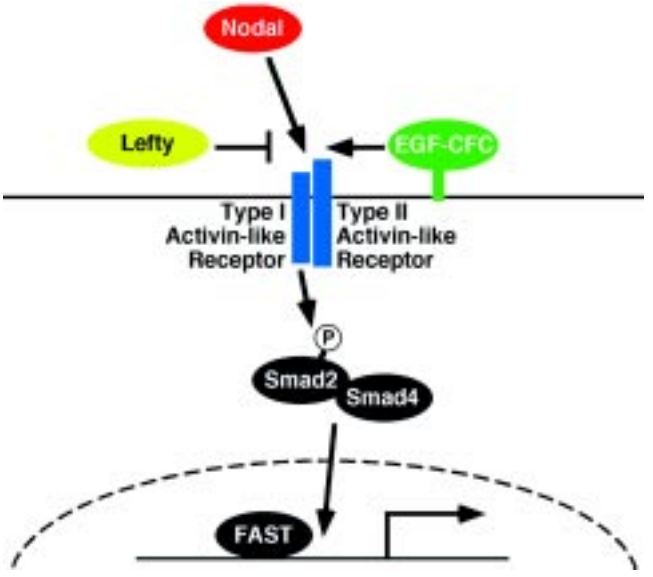

Fig. 2. The putative Nodal signaling pathway. *Loss- and gain-of-function studies indicate that Nodal signals may act with EGF-CFC cofactors to activate an Activin-like pathway involving Smad2 and FAST transcription factors. Lefty proteins act as feedback inhibitors (after Schier and Shen, 2000).*

1999). In addition, Oep is not required for generating receptor or downstream components in responding cells. The presence of a secreted mutant form of Oep between signaling and responding cells is sufficient to rescue the *oep* mutant phenotype (Gritsman *et al.*, 1999). This finding also suggests that Oep is not simply concentrating Nodal signals at the surface of responding cells. In this case, it would be expected that the secreted form of Oep could act as a dominant negative factor and block Nodal signals. It is thus most likely that Oep is part of or assembles a complex with either the ser/thr kinase receptors or Nodal signals allowing productive interaction between the ligand and its transmembrane receptors. Oep might change the conformation of the receptor or Nodal or provide an additional interaction surface for complex formation. In this model, Oep would serve as an extracellular cofactor or adaptor for Nodal signals and receptors. It is likely that the EGF-CFC factors Cripto and FRL-1 also function in Nodal signaling, because their expression in zebrafish can rescue the *oep* mutant phenotype (Gritsman *et al.*, 1999).

Lefty proteins antagonize Nodal signaling

Whereas Oep has been shown to play a role in Nodal signaling based on its loss-of-function phenotype, another class of molecules, the Lefty proteins, have been first implicated in Nodal signaling based on their gain-of-function phenotypes in zebrafish. *Lefty* genes were initially isolated in mouse as divergent TGF-ß family members (Meno *et al.*, 1996; Schier and Shen, 2000). Both *lefty1* and *lefty2* are asymmetrically expressed with respect to the L-R axis (Meno *et al.*, 1997). In addition, *lefty2* is also expressed in nascent mesoderm during gastrulation. It was initially unclear if Lefty proteins act as instructive signals similar to other TGF-ß factors or as inhibitors of TGF- ß signaling pathways. Misexpression studies in zebrafish demonstrated that Lefty proteins (initially called Antivin) act as antagonists of Nodal signaling (Bisgrove *et al.*, 1999; Meno *et al.*, 1999; Thisse and Thisse, 1999; Fig. 1). Overexpression of mouse *lefty* genes and the related zebrafish gene *antivin* (*lefty1*) induces phenotypes that strongly resemble *sqt;cyc* double mutants and maternal-zygotic *oep* mutants. The effects of *lefty* overexpression can be overcome by co-expression of *cyc* or *sqt* (Bisgrove *et al.*, 1999; Meno *et al.*, 1999). In addition, *lefty* gene expression is induced by Nodal signaling, implicating *lefty* genes as feedback inhibitors of Nodal signaling (Meno *et al.*, 1999; Fig. 2). Evidence for a requirement for *lefty2* in blocking mesoderm formation came from genetic studies in mouse. *Lefty2* mutants have an expanded primitive streak, whereas the primitive streak does not form in mouse *nodal* mutants; *nodal;lefty2* double mutants resemble *nodal* single mutants, consistent with the model that Lefty2 antagonizes the action of Nodal. In addition, heterozygosity for *nodal* also partially suppresses the *lefty2* mutant phenotype (Meno *et al.*, 1999).

How does Lefty inhibit Nodal signaling? While the molecular details are not known yet, two pieces of evidence suggest that Lefty blocks putative Nodal receptors. First, the extracellular domain of the ActRIIB Activin receptor can block the effects of both *sqt* and *lefty* overexpression (Meno *et al.*, 1999). Second, Lefty can also block signaling by Activin, suggesting that Lefty might inactivate receptors that are shared between Nodal and Activin (Thisse and Thisse, 1999).

In summary, the genetic analysis of *oep*, *cyc*, *sqt* and *lefty* has led to a model (Figure 2), wherein the interplay of these extracellular factors controls vertebrate gastrulation and organizer formation by modulating the activity of downstream receptors and transcription factors such as Smad2 and FAST1. The recent finding that the *fast1* gene is disrupted in *sur* mutants provides further genetic support for this model (Sirotkin *et al.,* 2000b; Pogoda *et al.,* 2000).

Nodal signaling patterns the dorsal mesoderm

The results described above have revealed a general role for Nodal signaling in germ-layer formation and organizer development. More recent studies have shown that Nodal signaling is also involved in patterning the organizer along the nascent anterior-posterior axis (Gritsman *et al.,* 2000). At the end of gastrulation, the dorsal mesoderm of the organizer has given rise to the axial mesoderm, consisting anteriorly of prechordal plate cells and posteriorly of notochord cells. Fate mapping and gene expression studies have revealed that this anterior-posterior pattern is already initiated in the dorsal mesoderm before gastrulation (Gritsman *et al.*, 2000). In particular, prechordal plate progenitors are located at the dorsal margin, whereas notochord precursors are present more animally, three or more cells away from the margin. These

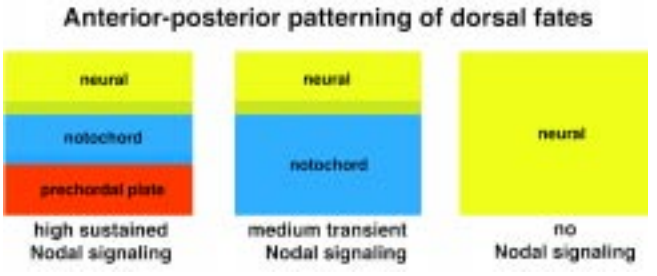

Fig. 3. Patterning of the dorsal mesoderm by Nodal signaling. *See text for details.*

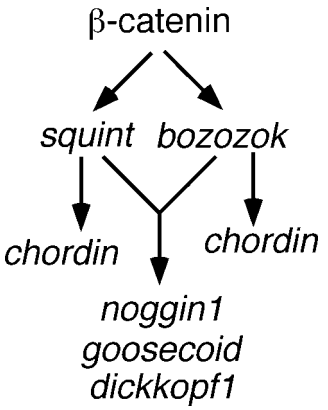

Fig. 4. Overlapping roles for Bozozok and Squint in mediating β-catenin activity to activate organizer genes. *See text for details.*

fate map territories partially overlap and roughly correspond to the expression domains of *goosecoid* (prechordal plate progenitors) and *floating head* (notochord). These observations transform the problem of anterior-posterior patterning at the end of gastrulation to the question of how cell types are specified along the animal-vegetal axis at the dorsal margin before gastrulation.

As described above, Nodal signaling is essential for both notochord and prechordal plate development. In the absence of *cyc* and *sqt* or *oep*, the shield does not form and notochord and prechordal plate fates are not specified. Instead, dorsal marginal cells acquire a neural fate (Feldman *et al.*, 2000). Interestingly, zygotic *oep* mutants have a notochord, but no prechordal plate. Moreover, *goosecoid* is not expressed at the onset of gastrulation in zygotic *oep* mutants, whereas *floating head* expression is expanded to include cells at the margin (Schier *et al.*, 1997; Gritsman *et al.*, 2000). In these embryos, maternal Oep might allow sufficient Nodal signaling to induce notochord progenitors, but not prechordal plate development. Together with the expression of *cyc* and *sqt* at the dorsal margin, these results suggested that differential Nodal signaling is involved in determining the difference between prechordal plate and notochord fates.

To further test this idea, several strategies were employed to reduce Nodal signaling and/or limit the time of its activity, including low level misexpression of Antivin and activation of Nodal signaling at different stages by addition of wild-type *oep* RNA to *oep* mutant embryos (Gritsman *et al.*, 2000). These manipulations revealed that high and sustained levels of Nodal signaling are required for the expression of *goosecoid* at the dorsal margin and the specification of prechordal plate progenitors. Transient or medium levels of Nodal signaling are sufficient for *floating head* expression and the specification of notochord fates. In addition, reduced Nodal signaling transforms cells at the margin from a prechordal plate to a notochord fate, indicating that the level of Nodal signaling can serve as a switch between the specification of different cell types (Gritsman *et al.*, 2000). In the complete absence of Nodal signaling, neither notochord nor prechordal plate cells are specified and cells at the dorsal margin give rise to neural fates (Feldman *et al.*, 2000). These results indicate that increasing levels of Nodal signaling on the dorsal side changes cell fates from neural to notochord to prechordal plate (Figure 3). This animal-vegetal pattern is then transformed into anterior-posterior patterning by the involution of dorsal mesoderm during gastrulation.

Interestingly, Nodal signaling in zebrafish not only contributes to notochord and prechordal plate formation, but is also required for the specification of the medial floor plate, an additional cell type derived from the shield region (Shih *et al.,* 1995, 1996; Gritsman *et al.,* 2000). The medial floor plate is the most ventral cell type in the spinal cord and overlies the notochord at the end of gastrulation. *Cyc* and zygotic *oep* mutants lack most of the medial floor plate (Hatta *et al.*, 1991; Schier *et al.*, 1997; Strähle *et al.,* 1997). Partial rescue of floor plate development in *cyc* mutant embryos can be achieved by expression of *cyc* mRNA in non–floor plate cells, suggesting that Cyc acts non-autonomously during floor plate induction (Sampath *et al.*, 1998). In contrast, *oep* is required cell autonomously in floor plate precursors (Strähle *et al.*, 1997; Shynia *et al.,* 1999). Since Oep is required for the reception or transmission of the Cyc signal (Gritsman *et al.*, 1999), these results establish a direct role of Nodal signaling in medial floor plate induction.

The homeobox gene *bozozok* acts in parallel to *squint* in the development of dorsal mesoderm

In addition to components of the Nodal signaling pathway, *bozozok (boz)* is another gene with a key function in organizer formation. The large-scale screen in Boston identified *boz* as a mutation reducing the development of prechordal plate, notochord, and anterior neural structures (Solnica-Krezel *et al.*, 1996; Schier *et al.*, 1996). The *boz* mutant phenotype is variable in penetrance and expressivity, but severe examples have strongly reduced *goosecoid* expression and lack a shield in the early gastrula, and develop a neural tube with deficits in ventral midline cell types and telencephalon at later stages (Fekany *et al.*, 1999; Fekany-Lee *et al.*, 2000).

Genetic mapping and molecular analysis of *boz* mutations indicated that the *boz* locus corresponds to the paired-class homeobox gene *dharma* (which we refer to as *boz* hereafter; Fekany *et al.*, 1999). Using an expression cloning approach, *boz* was isolated as a gene with potent dorsalizing activity when overexpressed in zebrafish embryos (Yamanaka *et al.*, 1998). Boz has a short sequence motif that is characteristic of transcriptional repressors (Koos and Ho, 1998), so it is possible that Boz directly represses genes with ventralizing activity, thereby indirectly promoting the expression of dorsally expressed genes such as *goosecoid*, *chordin*, and *noggin* (Yamanaka *et al.*, 1998; Fekany *et al.*, 1999; Koos and Ho, 1999). *boz* is expressed in dorsal marginal blastomeres and the dorsal YSL in the late blastula and early gastrula. Overexpression of *boz* specifically within the YSL induces dorsal mesoderm in neighboring embryonic cells, indicating

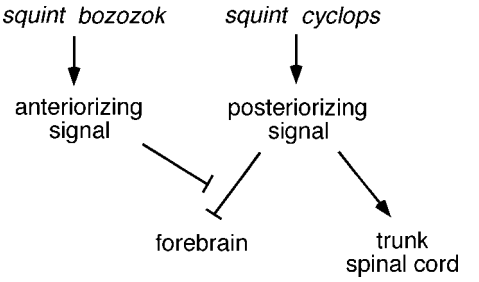

Fig. 5. Roles of Cyclops, Squint and Bozozok in anterior-posterior neural patterning. *See text for details.*

that Boz protein induces secreted factors with dorsalizing activity. This analysis of *boz* mutants and the *dharma* gene has identified another key regulator of dorsal mesoderm development.

The maternal dorsal determinant ß-catenin induces expression of *boz* and *sqt*, and several lines of evidence indicate that these genes act in parallel in the development of dorsal mesoderm (Shimizu *et al*., 2000; Sirotkin *et al*., 2000a; Dougan, Schier and Talbot, unpubl. results). First, injection of *boz* mRNA does not induce ectopic dorsal mesoderm in *sqt;cyc* mutants, suggesting that the *nodal*-related genes act downstream of or in parallel with *boz*. Second, *boz* and *sqt* are not required for proper expression of each other in the blastula, and neither gene is a strong activator of the other in overexpression assays. Third, *boz;sqt* double mutants have a fully penetrant phenotype that is more severe that that of *sqt* and *boz* single mutants. Double mutants lack notochord and prechordal plate, and also neural structures anterior to the hindbrain. Analysis of downstream gene expression also reveals parallel and overlapping roles of *sqt* and *boz*. In *boz;sqt* double mutants, *chordin* expression is strongly and consistently reduced in the blastula, whereas *chordin* is expressed at wild-type levels in *sqt* single mutants and some *boz* single mutants (*chordin* expression in *boz* mutants is variable, with some mutants showing a reduction in the early gastrula). Although it seems that the action of either *sqt* or *boz* is sufficient to activate *chordin* expression, other dorsally expressed genes, such as *goosecoid*, *noggin1*, and *dkk1* require the functions of both *boz* and *sqt* (Shimizu *et al*., 2000; Sirotkin *et al*., 2000a; Hashimoto *et al.,* 2000; Fig. 4). Taken together, these results reveal overlapping roles of the homeodomain transcription factor Boz and the Nodal-related signal Sqt.

Nodal-related signals and neural patterning

The results described above reveal essential roles for Nodal signaling and Boz in the formation of dorsal mesoderm. It is striking that the neural tube forms despite the absence of the shield and mesendodermal derivatives in these mutants. This result suggests that despite abnormal development of the organizer region and the lack of axial mesoderm, neuralizing activity is still present in the affected embryos. This apparent paradox parallels the results of embryological studies, which show that the shield and its derivatives are not required to form a neural tube (Shih and Fraser, 1996; Driever *et al*., 1997). Extirpation of the shield produces cyclopic embryos lacking notochord and prechordal plate, but the neural tube forms, although it lacks floor plate and other ventral cell types. These results indicate that organizing activities are not restricted to the shield. In molecular terms, these results imply that molecules with neural inducing activity must also be expressed outside the shield or before shield formation and in the absence of Boz and Nodal-related signals. Consistent with this, Nodal-related signals are not required for *chordin* expression (Gritsman *et al*., 1999; Sirotkin *et al*., 2000a), which is expressed in a broad dorsal domain that includes not only presumptive axial and paraxial mesoderm, but also presumptive dorsal ectoderm (Miller-Bertoglio *et al*., 1997; Schulte-Merker *et al*., 1997). Double mutants for *boz* and *sqt* have reduced *chordin* expression and are ventralized, but still form neural structures. Moreover, the neural tube of *boz;sqt;cyc* triple mutants is greatly reduced, but not absent, revealing the existence of pathways not requiring Boz and Nodal signals that can activate neural inducing molecules (Shimizu *et al*., 2000; Sirotkin *et al*., 2000a).

While neuralization is not dependent on Nodal signals, anterior-posterior neural patterning is in part controlled by Nodal signaling (Figure 5). The most striking neural phenotype in *sqt;cyc* mutants is the elimination of the trunk spinal cord (Feldman *et al*., 2000). In contrast, the tail spinal cord is present in these mutants. These results indicate that Nodal-related signals promote the development of trunk neural identity. In contrast, forebrain development is repressed by Nodal signaling. For instance, expression of forebrain markers is expanded in *sqt;cyc* and maternal-zygotic *oep* mutants and in embryos overexpressing *antivin* (Gritsman *et al*., 1999; Feldman *et al*., 2000; Shimizu *et al*., 2000; Sirotkin *et al*., 2000a; Thisse *et al*., 2000). In turn, misexpression of Nodal signals in the animal region converts cells in the forebrain fate map domain toward dorsal mesoderm or hindbrain (Erter *et al*., 1998; Feldman *et al*., 1998; Rebagliati *et al*., 1998a; Sampath *et al*., 1998; Gritsman *et al*., 1999; Gritsman *et al*., 2000; Thisse *et al*., 2000). Additional evidence for a repressive role of Nodal signals comes from the comparison of *boz;sqt* with *boz;sqt;cyc* mutants (Sirotkin *et al*., 2000a). Whereas the neural tube of *boz;sqt* mutants is greatly reduced anterior to the hindbrain, forebrain markers are expressed in *boz;sqt;cyc* triple mutants. Thus the deficit in anterior neural structures in *boz;sqt* mutants can be partly overcome by the elimination of *cyc* function (Figure 5). These results provide genetic evidence for a model of forebrain induction in *Xenopus* that postulates that Nodal signaling has to be repressed to allow forebrain formation (Piccolo *et al*., 1999). In particular, cerberus is a secreted protein that induces formation of ectopic heads when overexpressed in *Xenopus* embryos. Biochemical analysis shows that cerberus binds and antagonizes Nodal, Wnt, and BMP signals, suggesting that inhibition of these signals promotes the development of anterior neural structures.

In contrast to the results described above, genetic analysis in mouse has suggested that Nodal signaling is essential for the formation of neural structures anterior to the hindbrain (Varlet *et al*., 1997). These seemingly conflicting conclusions may reflect stage specific actions of *nodal*-related genes and their antagonists (Piccolo *et al*., 1999; Schier and Shen, 2000). For example, early Nodal signaling might be required to induce a factor involved in forebrain formation, while later Nodal signaling might induce factors that block forebrain formation (Figure 5). Consistent with this idea, Nodal-related signals induce *cer* expression, and it has been proposed that cer has a negative feedback function that later prevents Nodal-related signals from diverting prospective neural cells to mesendodermal fates (Piccolo *et al*., 1999). The absence of forebrain structures in *boz;sqt* double mutant embryos also supports an essential, but probably indirect early role for Nodal signals in forebrain formation (Shimizu *et al*., 2000; Sirotkin *et al*., 2000a). Additional work is needed to determine which neural patterning activities of Nodal signals are direct and which are mediated by secondary signals.

Conclusions

As outlined above, a combination of genetic, molecular and embryological approaches has provided a framework to describe the mechanisms underlying organizer development and function in zebrafish. The pivotal role of transcription factors such as Bozozok and of signals belonging to the Nodal and BMP families is now firmly established. Analysis of Nodal and BMP signaling has uncovered an unexpectedly complex regulation of these pathways

by extracellular cofactors (e.g. One-eyed pinhead) and inhibitors (e.g. Lefty and Chordin). Finally, differential activity of these components can specify different cell fates. Much remains to be learned. How do these and yet-to-be discovered factors interact at the molecular level? How does the organizer interact with other inductive centers to pattern the embryo? How do cells integrate extrinsic and intrinsic signals to acquire distinct developmental fates? How is cell fate linked to morphogenesis? With some key players now identified and our understanding progressing rapidly, we may have the answers to these questions within 100 years of the discovery of the organizer.

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References

AMEMIYA, C.T., ZHONG, T.P., SILVERMAN, G.A., FISHMAN, M.C. and ZON, L.I. (1999). Zebrafish YAC, BAC, and PAC genomic libraries. *Methods In Cell Biology* 60: 235-58.

BISGROVE, B.W., ESSNER, J.J. and YOST, H.J. (1999). Regulation of midline development by antagonism of lefty and nodal signaling. *Development* 126: 3253-62.

BRAND, M., HEISENBERG, C.P., WARGA, R.M., PELEGRI, F., KARLSTROM, R.O., BEUCHLE, D., PICKER, A., JIANG, Y.J., FURUTANI-SEIKI, M., VAN EEDEN, F.J., GRANATO, M., HAFFTER, P., HAMMERSCHMIDT, M., KANE, D.A., KELSH, R.N., MULLINS, M.C., ODENTHAL, J. and NÜSSLEIN-VOLHARD, C. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* 123: 129-42.

CONLON, F.L., LYONS, K.M., TAKAESU, N., BARTH, K.S., KISPERT, A., HERRMANN, B. and ROBERTSON, E.J. (1994). A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. *Development* 120: 1919-1928.

CONNORS, S.A., TROUT, J., EKKER, M. and MULLINS, M.C. (1999). The role of tolloid/mini fin in dorsoventral pattern formation of the zebrafish embryo. *Development* 126: 3119-30.

DE ROBERTIS, E.M. and SASAI, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* 380: 37-40.

DICK, A., HILD, M., BAUER, H., IMAI, Y., MAIFELD, H., SCHIER, A.F., TALBOT, W.S., BOUWMEESTER, T. and HAMMERSCHMIDT, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* 127: 343-54.

DRIEVER, W., SOLNICA-KREZEL, L., SCHIER, A.F., NEUHAUSS, S.C., MALICKI, J., STEMPLE, D.L., STAINIER, D.Y., ZWARTKRUIS, F., ABDELILAH, S., RANGINI, Z., BELAK, J. and BOGGS, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123: 37-46.

DRIEVER, W., SOLNICA-KREZEL, L., ABDELILAH, S., MEYER, D. and STEMPLE, D. (1997). Genetic analysis of pattern formation in the zebrafish neural plate. *Cold Spring Harbor Symposia on Quantitative Biology* LXII: 523-534.

ERTER, C.E., SOLNICA-KREZEL, L. and WRIGHT, C.V. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev. Biology* 204: 361-372.

FEKANY, K., YAMANAKA, Y., LEUNG, T., SIROTKIN, H.I., TOPCZEWSKI, J., GATES, M.A., HIBI, M., RENUCCI, A., STEMPLE, D., RADBILL, A., SCHIER, A.F., DRIEVER, W., HIRANO, T., TALBOT, W.S. and SOLNICA-KREZEL, L. (1999). The zebrafish bozozok locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development* 126: 1427-38.

FEKANY-LEE, K., GONZALEZ, E., MILLER-BERTOGLIO, V. and SOLNICA-KREZEL, L. (2000) The homoeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. *Development* 127: 2333-2345.

FELDMAN, B., GATES, M.A., EGAN, E.S., DOUGAN, S.T., RENNEBECK, G., SIROTKIN, H.I., SCHIER, A.F. and TALBOT, W.S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* 395: 181-5.

FELDMAN, B., DOUGAN, S.T., SCHIER, A.F. and TALBOT, W.S. (2000). Nodal-related signals establish mesendodermal and trunk neural fates in zebrafish. *Curr. Biol.* 10: 531-534.

GRITSMAN, K., ZHANG, J., CHENG, S., HECKSCHER, E., TALBOT, W.S. and SCHIER, A.F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* 97: 121-132.

GRITSMAN, K., TALBOT, W.S. and SCHIER, A.F. (2000). Nodal signaling patterns the organizer. *Development* 127: 921-932.

HAFFTER, P., GRANATO, M., BRAND, M., MULLINS, M.C., HAMMERSCHMIDT, M., KANE, D.A., ODENTHAL, J., VAN EEDEN, F.J., JIANG, Y.J., HEISENBERG, C.P., KELSH, R.N., FURUTANI-SEIKI, M., VOGELSANG, E., BEUCHLE, D., SCHACH, U., FABIAN, C. and NÜSSLEIN-VOLHARD, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* 123: 1-36.

HALPERN, M.E., HO, R.K., WALKER, C. and KIMMEL, C.B. (1993) Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell* 75: 99-111

HAMMERSCHMIDT, M., PELEGRI, F., MULLINS, M.C., KANE, D.A., BRAND, M., VAN EEDEN, F.J., FURUTANI-SEIKI, M., GRANATO, M., HAFFTER, P., HEISENBERG, C.P., JIANG, Y.J., KELSH, R.N., ODENTHAL, J., WARGA, R.M. and NÜSSLEIN-VOLHARD, C. (1996a). Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. *Development* 123: 143-51.

HAMMERSCHMIDT, M., PELEGRI, F., MULLINS, M.C., KANE, D.A., VAN EEDEN, F.J., GRANATO, M., BRAND, M., FURUTANI-SEIKI, M., HAFFTER, P., HEISENBERG, C.P., JIANG, Y.J., KELSH, R.N., ODENTHAL, J., WARGA, R.M. and NÜSSLEIN-VOLHARD, C. (1996b). dino and mercedes, two genes regulating dorsal development in the zebrafish embryo. *Development* 123: 95-102.

HARLAND, R. and GERHART, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* 13: 611-667.

HASHIMOTO, H., ITOH, M., YAMANAKA, Y., YAMASHITA, S., SHIMIZU, T., SOLNICA-KREZEL, L., HIBI, M., HIRANO, T. (2000). Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. *Dev. Biology* 217: 138-52.

HATTA, K., KIMMEL, C.B., HO, R.K. and WALKER, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* 350: 339-41.

HEISENBERG, C.P. and NÜSSLEIN-VOLHARD, C. (1997). The function of silberblick in the positioning of the eye anlage in the zebrafish embryo. *Dev. Biology* 184: 85-94.

HILD, M., DICK, A., RAUCH, G.J., MEIER, A., BOUWMEESTER, T., HAFFTER, P. and HAMMERSCHMIDT, M. (1999). The smad5 mutation somitabun blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. *Development* 126: 2149-59.

HO, R.K. and KANE, D.A. (1990). Cell-autonomous action of zebrafish spt-1 mutation in specific mesodermal precursors. *Nature* 348: 728-30.

JONES, C.M., KUEHN, M.R., HOGAN, B.L., SMITH, J.C. and WRIGHT, C.V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* 121: 3651-62.

KIECKER, C., MULLER, F., WU, W., GLINKA, A., STRÄHLE, U. and NIEHRS, C. (2000). Phenotypic effects in Xenopus and zebrafish suggest that one-eyed pinhead functions as antagonist of BMP signalling. *Mech. Dev.* 94: 37-46.

KIMMEL, C.B. (1989). Genetics and early development of zebrafish. *Trends in Genetics* 5: 283-8.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dynamics* 203: 253-310.

KIMMEL, C.B., KANE, D.A., WALKER, C., WARGA, R.M. and ROTHMAN, M.B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* 337: 358-62.

KIMMEL, C.B., WARGA, R.M. and SCHILLING, T.F. (1990). Origin and organization

of the zebrafish fate map. *Development* 108: 581-94.

KISHIMOTO, Y., LEE, K.H., ZON, L., HAMMERSCHMIDT, M. and SCHULTE-MERKER, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* 124: 4457-66.

KOOS, D.S. and HO, R.K. (1998). The nieuwkoid gene characterizes and mediates a Nieuwkoop-center-like activity in the zebrafish. *Curr. Biol.* 8: 1199-1206.

KOOS, D.S. and HO, R.K. (1999). The nieuwkoid/dharma Homeobox Gene Is Essential for bmp2b Repression in the Zebrafish Pregastrula. *Dev. Biol* 215: 190-207.

KOSHIDA, S., SHINYA, M., MIZUNO, T., KUROIWA, A. and TAKEDA, H. (1998). Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast. *Development* 125: 1957-1966.

LONG, W.L. (1983). The role of the yolk syncytial layer in determination of the plane of bilateral symmetry in rainbow trout, Salmo gairdneri Richardson. *J. Exp. Zool.* 228: 91-97.

MENO, C., GRITSMAN, K., OHISHI, S., OHFUJI, Y., HECKSCHER, E., MOCHIDA, K., SHIMONO, A., KONDOH, H., TALBOT, W.S., ROBERTSON, E.J., SCHIER, A.F. and HAMADA, H. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol. Cell* 4: 287-98.

MENO, C., ITO, Y., SAIJOH, Y., MATSUDA, Y., TASHIRO, K., KUHARA, S. and HAMADA, H. (1997). Two closely-related left-right asymmetrically expressed genes, lefty-1 and lefty-2: their distinct expression domains, chromosomal linkage and direct neuralizing activity in Xenopus embryos. *Genes to Cells* 2: 513-24.

MENO, C., SAIJOH, Y., FUJII, H., IKEDA, M., YOKOYAMA, T., YOKOYAMA, M., TOYODA, Y. and HAMADA, H. (1996). Left-right asymmetric expression of the TGF beta-family member lefty in mouse embryos. *Nature* 381: 151-5.

MILLER-BERTOGLIO, V.E., FISHER, S., SANCHEZ, A., MULLINS, M.C. and HALPERN, M.E. (1997). Differential regulation of chordin expression domains in mutant zebrafish. *Dev. Biology* 192: 537-50.

MIZUNO, T., YAMAHA, E., WAKAHARA, M., KUROIWA, A. and TAKEDA, H. (1996). Mesoderm induction in zebrafish. *Nature* 383: 131-132.

MULLINS, M.C., HAMMERSCHMIDT, M., HAFFTER, P. and NÜSSLEIN-VOLHARD, C. (1994). Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Cur. Biology* 4: 189-202.

MULLINS, M.C., HAMMERSCHMIDT, M., KANE, D.A., ODENTHAL, J., BRAND, M., VAN EEDEN, F.J., FURUTANI-SEIKI, M., GRANATO, M., HAFFTER, P., HEISENBERG, C.P., JIANG, Y.J., KELSH, R.N. and NÜSSLEIN-VOLHARD, C. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* 123: 81-93.

NÜSSLEIN-VOLHARD, C., AND WIESCHAUS, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* 287: 795-801.

ODENTHAL, J., HAFFTER, P., VOGELSANG, E., BRAND, M., VAN EEDEN, F.J.M., FURUTANI-SEIKI, M., GRANATO, M., H., M., HEISENBERG, C.-P., JIANG, Y.-J., KANE, D.A., KELSH, R.N., MULLINS, M.C., WARGA, R.M., ALLENDE, M.L., WEINBERG, E.S. and NÜSSLEIN-VOLHARD, C. (1996). Mutations affecting the formation of the notochord in the zebrafish, Danio rerio. *Development* 123: 103-115.

OPPENHEIMER, J. (1936). Transplantation experiments on developing teleosts (Fundulus and Perca). *J. Exp. Zool.* 72: 409-437.

POGODA, H., SOLNICA-KREZEL, L., DRIEVER, W. and MEYER, D. (2000). The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of nodal signaling required for organizer formation. *Curr. Biol.* 10: 1041-1049.

PICCOLO, S., AGIUS, E., LEYNS, L., BHATTACHARYYA, S., GRUNZ, H., BOUWMEESTER, T. and DE ROBERTIS, E.M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397: 707-10.

REBAGLIATI, M.R., TOYAMA, R., FRICKE, C., HAFFTER, P. and DAWID, I.B. (1998a). Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev. Biol* 199: 261-72.

REBAGLIATI, M.R., TOYAMA, R., HAFFTER, P. and DAWID, I.B. (1998b). cyclops encodes a nodal-related factor involved in midline signaling. *Proc. Natl. Aca.d Sci. U.S.A.* 95: 9932-7.

SAUDE, L., WOOLLEY, K., MARTIN, P., DRIEVER, W. and STEMPLE, D.L. (2000). Axis-inducing activities and cell fates of the zebrafish organizer. *Development* 127: 3407-3417.

SCHIER, A.F., NEUHAUSS, S.C., HARVEY, M., MALICKI, J., SOLNICA-KREZEL, L., STAINIER, D.Y., ZWARTKRUIS, F., ABDELILAH, S., STEMPLE, D.L., RANGINI, Z., YANG, H. and DRIEVER, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* 123: 165-78.

SCHIER, A.F., NEUHAUSS, S.C., HELDE, K.A., TALBOT, W.S. and DRIEVER, W. (1997). The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* 124: 327-42.

SCHIER, A.F. and SHEN, M.M. (2000). Nodal signalling in vertebrate development. *Nature* 403: 385-389.

SCHIER, A.F. and TALBOT, W.S. (1998). The zebrafish organizer. *Curr. Opin. Genet. Dev.* 8: 464-471.

SCHMID, B., FURTHAUER, M., CONNORS, S.A., TROUT, J., THISSE, B., THISSE, C. and MULLINS, M.C. (2000). Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. *Development* 127: 957-67.

SCHULTE-MERKER, S., VAN EEDEN, F.J., HALPERN, M.E., KIMMEL, C.B., NÜSSLEIN-VOLHARD, C. (1994). no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development* 120: 1009-1015

SCHULTE-MERKER, S., LEE, K.J., MCMAHON, A.P. and HAMMERSCHMIDT, M. (1997). The zebrafish organizer requires chordino. *Nature* 387: 862-863.

SHEN, M.M. and SCHIER, A.F. (2000). The EGF-CFC gene family in vertebrate development. *Trend in Genetics* 16: 303-309.

SHIH, J. and FRASER, S.E. (1995). Distribution of tissue progenitors within the shield region of the zebrafish gastrula. *Development* 121: 2755-65.

SHIH, J. and FRASER, S.E. (1996). Characterizing the zebrafish organizer: microsurgical analysis at the early-shield stage. *Development* 122: 1313-22.

SHIMIZU, T., YAMANAKA, Y., RYU, S., HASHIMOTO, H., YABE, T., HIRATA, T., BAE, Y., HIBI, M. and HIRANO, T. (2000). Cooperative roles of Bozozok/Dharma and Nodal-related proteins in the formation of the dorsal organizer in zebrafish. *Mech. Dev.* 91: 293-303.

SHINYA, M., FURUTANI-SEIKI, M., KUROIWA, A. and TAKEDA, H. (1999). Mosaic analysis with oep mutant reveals a repressive interaction between floor-plate and non-floor-plate mutant cells in the zebrafish neural tube. *Dev. Growth Differ.* 41: 135-42.

SIROTKIN, H.I., DOUGAN, S.T., SCHIER, A.F. and TALBOT, W.S. (2000a). bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm. *Development* 127: 2583-2592.

SIROTKIN, H.I., GATES, M.A., KELLY, P.D., SCHIER, A.F. and TALBOT, W.S. (2000b). fast1 is required for the development of dorsal axial structures in zebrafish. *Curr. Biol.* 10: 1051-1054.

SOLNICA-KREZEL, L., SCHIER, A.F. and DRIEVER, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* 136: 1401-1420.

SOLNICA-KREZEL, L., STEMPLE, D.L., MOUNTCASTLE-SHAH, E., RANGINI, Z., NEUHAUSS, S.C., MALICKI, J., SCHIER, A.F., STAINIER, D.Y., ZWARTKRUIS, F., ABDELILAH, S. and DRIEVER, W. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* 123: 67-80.

SPEMANN, H. and MANGOLD, H. (1924). Ueber die Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Wilhelm Roux Arch. Entw. Mech.* 100: 599-638.

STEMPLE, D.L., SOLNICA-KREZEL, L., ZWARTKRUIS, F., NEUHAUSS, S.C.F., SCHIER, A.F., MALICKI, J., STAINIER, D.Y.R., ABDELILAH, S., RANGINI, Z., MOUNTCASTLE-SHAH, E. and DRIEVER, W. (1996). Mutations affecting development of the notochord in zebrafish. *Development* 123: 117-128.

STRÄHLE, U., JESUTHASAN, S., BLADER, P., GARCIA-VILLALBA, P., HATTA, K. and INGHAM, P.W. (1997). one-eyed pinhead is required for development of the ventral midline of the zebrafish (Danio rerio) neural tube. *Genes and Function* 1: 131-148.

STREISINGER, G., WALKER, C., DOWER, N., KNAUBER, D. and SINGER, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature* 291: 293-6.

TALBOT, W.S., TREVARROW, B., HALPERN, M.E., MELBY, A.E., FARR, G., POSTLETHWAIT, J.H., JOWETT, T., KIMMEL, C.B. and KIMELMAN, D. (1995). A homeobox gene essential for zebrafish notochord development. *Nature* 378: 150-157

TALBOT, W.S., EGAN, E.S., GATES, M.A., WALKER, C., ULLMANN, B., NEUHAUSS, S.C., KIMMEL, C.B. and POSTLETHWAIT, J.H. (1998). Genetic analysis of chromosomal rearrangements in the cyclops region of the zebrafish genome. *Genetics* 148: 373-380.

TALBOT, W.S. and SCHIER, A.F. (1999). Positional cloning of mutated zebrafish genes. *Methods In Cell Biology* 60: 259-86.

THISSE, B., WRIGHT, C.V.E. and THISSE, C. (2000). Activin- and Nodal-related

factors control antero-posterior patterning of the zebrafish embryo. *Nature* 403: 425-428.

THISSE, C. and THISSE, B. (1999). Antivin, a novel and divergent member of the TGF-beta superfamily, negatively regulates mesoderm induction. *Development* 126: 229-240.

THISSE, C., THISSE, B., HALPERN, M.E. and POSTLETHWAIT, J.H. (1994). Goosecoid expression in neurectoderm and mesendoderm is disrupted in zebrafish cyclops gastrulas. *Dev. Biology* 164: 420-429.

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

YAMANAKA, Y., MIZUNO, T., SASAI, Y., KISHI, M., TAKEDA, H., KIM, C.H., HIBI, M. and HIRANO, T. (1998). A novel homeobox gene, dharma, can induce the organizer in a non-cell-autonomous manner. *Genes Dev.* 12: 2345-2353

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

ZHOU, X., SASAKI, H., LOWE, L., HOGAN, B.L.M. and KUEHN, M.R. (1993). Nodal is a novel TGF-β-like gene expressed in the mouse node during gastrulation. *Nature* 361: 543-47.