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The Function of *silberblick* in the Positioning of the Eye Anlage in the Zebrafish Embryo

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In zebrafish, as in other vertebrates, an initially singular eye field within the neural plate has to split during morphogenesis to allow the development of two separated eyes. It has been suggested that anterior progression of midline tissue within the neural plate is involved in the bilateralization of the eye field. Mutations in the recently identified *silberblick* (*slb*) gene cause an incomplete separation of the eyes. During gastrulation and early somitogenesis, the ventral midline of the central nervous system (CNS) together with the underlying axial mesendoderm is shortened and broadened in *slb* embryos. While in wild-type embryos the ventral CNS midline extends to the anterior limit of the neural plate at the end of gastrulation, there is a gap between the anterior tip of the ventral CNS midline and the anterior edge of the neural plate in *slb*. To investigate the cause for the shortening of the ventral CNS midline in *slb* we determined the fate of labeled ventral CNS midline cells in wild-type and *slb* embryos at different stages of development. In *slb*, anterior migration of ventral CNS midline cells is impaired, which indicates that migration of these cells is needed for elongation of the ventral CNS midline. The anterior shortening of the ventral CNS midline in *slb* leads to medial instead of bilateral induction of optic stalks followed by a partial fusion of the eyes at later developmental stages. The analysis of the *slb* phenotype indicates that anterior migration of midline cells within the neural plate is required for proper induction and subsequent bilateralization of an initially singular eye field. These findings may therefore provide a starting point in elucidating the role of neural plate morphogenesis in positioning of the eyes.

INTRODUCTION

In vertebrates, development of the eyes begins with the specification of eye progenitors in anterior–medial portions of the developing neural plate. Regional fate maps of the neural plate in amphibians and teleosts have shown that these progenitors are initially clustered in one coherent region of the anterior neural plate which subsequently gets subdivided into two bilateral eye fields containing the progenitors for each of the eyes (Ballard, 1973; Hirose and Jacobson, 1979; Woo and Fraser, 1995). The separation of the fields is thought to depend on signals emanating from both the underlying axial mesendoderm as well as from the ventral midline of the central nervous system (CNS) within the neural plate.

Classical experiments by Adelmann and others (Adelmann, 1930) have shown that in *Amblystoma,* partial deletions of the prechordal plate, the axial mesendoderm underlying the head, can lead to a fusion of the retinae, also known as cyclopia. This, together with other observations indicating that the neural plate initially contains a singular and coherent eye field, has lead to the hypothesis that the prechordal plate may be the source of signals responsible for the bilateralization of the eye field.

The role of the axial mesendoderm in neural patterning has been highlighted by the discovery that the axial mesendoderm can induce ventral CNS midline structures such as the floorplate within the overlying neurectoderm (for reviews see Ruiz i Altaba, 1994; Kelly and Melton, 1995). In the spinal cord, signals from the floorplate which include *shh,* a vertebrate homologue of the *Drosophila hedgehog* gene (Krauss *et al.,* 1993; Echelard *et al.,* 1993; Riddle *et al.,* 1993; Chiang *et al.,* 1996), can induce neurons with ventral character such as motoneurons. At the anterior end of the neural anlage, signals from the ventral CNS midline are thought to be involved in patterning the eye field by promoting the development of optic stalks and suppressing the differentiation of retinae (Macdonald *et al.,* 1995; Ekker *et al.,* 1995).

The separation of the eye fields not only depends on in-

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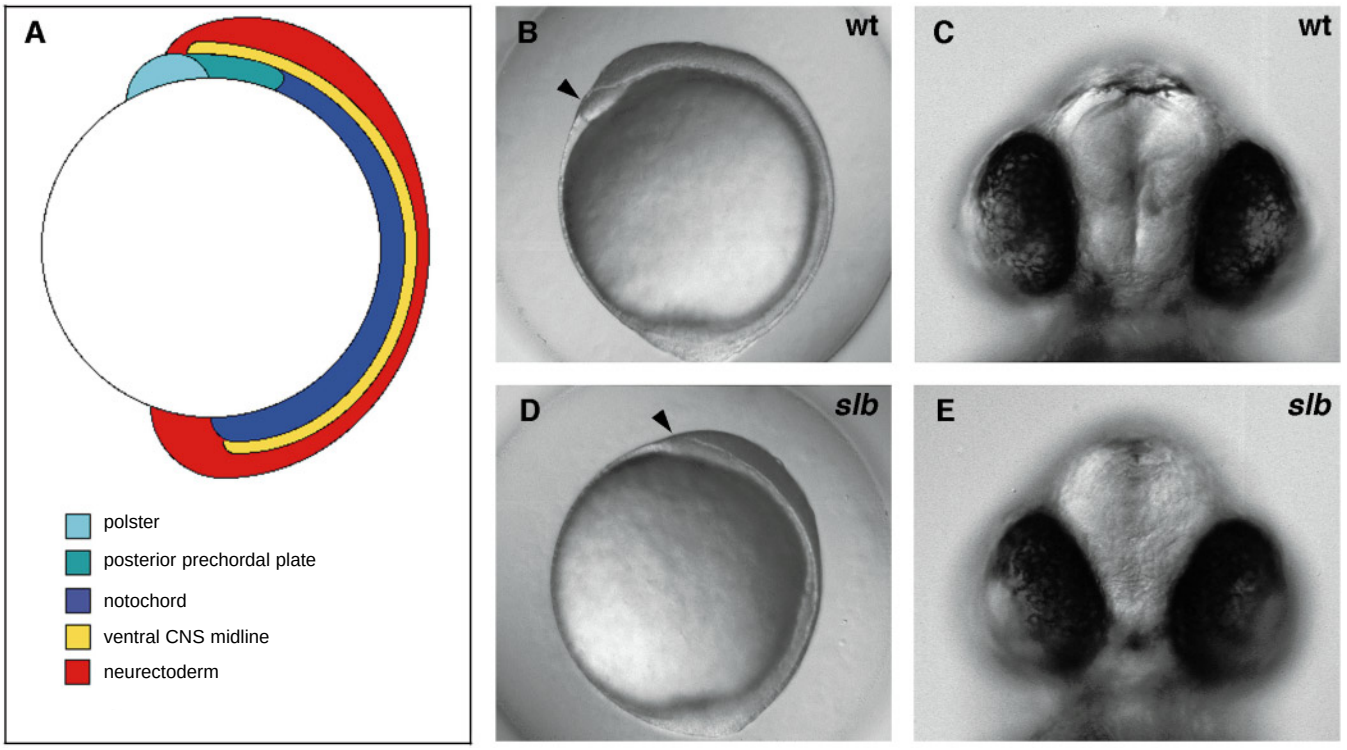

FIG. 1. *slb* embryos are shorter at bud stage. The eyes are turned inward anteriorly at later developmental stages. (A) Schematic drawing of the embryonic body axis organization at bud stage. (B, D) Side view of a wild-type (B) and *slb* (D) embryo at bud stage. (C, E) Frontal view of a wild-type (C) and *slb* (E) embryo at 48 hpf. Arrowheads in B and D indicate position of the polster. Anterior to the left.

ductive signals from adjacent tissues but also on cellular rearrangements within the neural plate. Similar to the underlying axial mesendoderm, the neural plate undergoes convergent extension during gastrulation which leads to anterior elongation of midline tissue within the neural plate (Keller *et al.*, 1992). Based on fate map studies in the zebrafish, it recently has been suggested that this elongation may result in a physical subdivision of the initially uniform eye field (Woo and Fraser, 1995).

In zebrafish, two classes of mutants have been isolated in which the position of the eyes is affected: in the first class of mutants which includes *cyclops* (*cyc*) and *one-eyed-pinhead* (*oep*) large parts of the axial mesendoderm together with the overlying ventral CNS do not form (Hatta *et al.*, 1991; Brand *et al.*, 1996; Hammerschmidt *et al.*, 1996; Schier *et al.*, 1996). The absence of the ventral CNS midline is followed by a reduction of optic stalks and a fusion of the retinae in the eyes. In the second class of mutants including *silberblick* (*slb*), *trilobite* (*tri*), and *knypek* (*kny*), the morphogenesis of the axial mesendoderm together with the overlying ventral CNS midline is disturbed, resulting in a compression rather than a reduction of midline tissue (Solnica-Krezel *et al.*, 1996; Hammerschmidt *et al.*, 1996; Heisenberg *et al.*, 1996). A shortening of the ventral CNS midline in these mutants is frequently accompanied by an incomplete separation of the eye anlagen, resulting in an anterior fusion of the optic stalks.

In this study, we show that migration of ventral CNS midline cells into the anteriormost portion of the neural plate is affected in *slb* mutants during gastrulation. The resultant anterior shortening of the ventral CNS midline in *slb* embryos causes an incomplete separation of the eye anlagen followed by a slight fusion of the eyes. These findings indicate that bilateralization of an initially singular eye field within the neural plate depends on anterior migration of ventral CNS midline cells.

MATERIALS AND METHODS

Maintenance and Breeding of Fish

Fish were maintained and bred as previously described (Mullins *et al.*, 1994).

Fish Lines and Genetics

For the analysis of the *slb* and *tri* phenotypes the slb^{tx226} and tri^{tc240} alleles were used. Similar results were obtained by using the

slb^{tz216} and *tri*^{tk50} alleles (data not shown) (Hammerschmidt *et al.*, 1996; Heisenberg *et al.*, 1996). To generate embryos homozygous for both the *slb* and *tri* mutations we identified adult fish carrying both mutations in the progeny of a cross between *slb*^{tx226}/+ and *tri*^{tc240}/+. Since *slb/tri* double mutant embryos exhibited phenotypic characteristics of both *slb* and *tri* mutations, it was possible to distinguish wild-type, *slb*, *tri*, and double mutant embryos from the bud stage on. The same procedure was used to generate *slb*^{tx226}/*ntl*^{tc41} and *slb*^{tx226}/*sqt*^{z1} double mutant embryos. Double mutants exhibited phenotypic characteristics of both of the corresponding single mutants and were therefore distinguishable from wild-type, *slb*, and *ntl* (*sqt*) embryos.

Whole-Mount in Situ Hybridization and Immunohistochemistry

Antibody and *in situ* stainings were performed as previously described (Hammerschmidt and Nüsslein-Volhard, 1993). For antibody stainings anti-Fkd2 (R. Warga, unpublished data; 1:2000) and anti-Ntl (Schulte-Merker *et al.*, 1992; 1:1000) polyclonal and anti-acetylated tubulin (Sigma; 1:1000) monoclonal antibodies were used. For *in situ* hybridization digoxygenin-labeled RNA probes were synthesized from the full-length *shh* (Krauss *et al.*, 1993), *pax2* (Krauss *et al.*, 1991a,b), *hgg1* (Thisse *et al.*, 1994), *ntl* (Schulte-Merker *et al.*, 1992), *rtk2* (Xu *et al.*, 1994), and *dlx3* (Akimenko *et al.*, 1994) cDNA clones. For double stainings (*in situ* and antibody labeling), embryos first underwent *in situ* hybridization, followed by antibody staining.

Mosaic Analysis

Transplantations of shield stage embryos were done as previously described (Hatta, 1992). Donor embryos were labeled with a mixture of biotin–dextran (10 kMW, 1.5%) and tetramethylrhodamine–dextran (10 kMW, 1.5%) (1:1). Embryos which received transplants were first observed under fluorescent light to detect rhodamine labeling. Subsequently mosaic embryos were fixed (4% PFA, 4 hr, RT), and transplanted biotin-labeled cells were visualized by using an AB–peroxidase complex (Vectastain). *In situ* hybridization on transplanted embryos was done prior to biotin detection. A homogeneous population of homozygous *slb* embryos was obtained by crossing homozygous *slb* adults, thereby allowing us the characterization of these mutants even before the appearance of the phenotype.

RESULTS

Morphological Features of silberblick (slb) Mutants

slb mutants can first be identified at bud stage by their shortened and broadened body axis. In addition, the anterior part of the prechordal plate, the ''polster,'' is flattened, elongated, and does not extend as far anteriorly as in the wild type (Figs. 1B and 1D; see also Fig. 1A for a general scheme of the embryonic body axis organization at bud stage). At 48 hr postfertilization (hpf), the eyes are slightly turned inward anteriorly, whereas no further obvious abnormalities are detectable (Figs. 1C and 1E). The gastrulation phenotype is completely penetrant, whereas the eye phenotype is only seen in about half of the mutant embryos depending on the genetic background (Heisenberg *et al.*, 1996). Homozygous embryos showing no or only a weak eye phenotype are viable and fertile in both sexes.

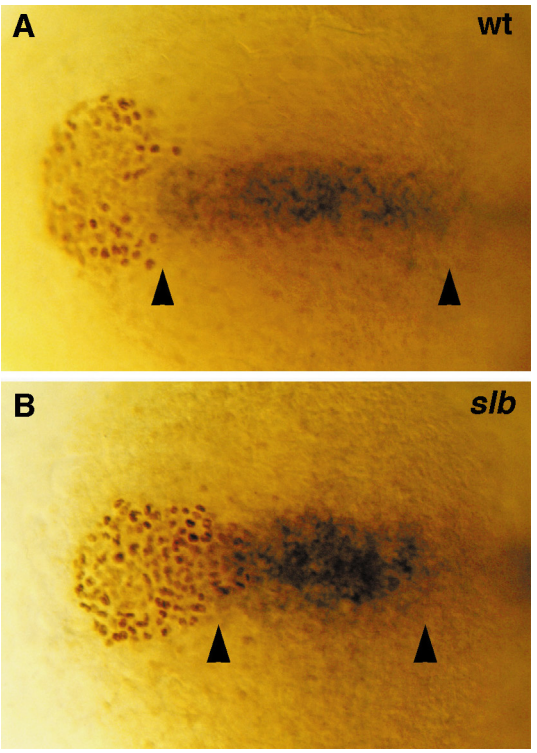

FIG. 2. The posterior prechordal plate of *slb* embryos is shortened and broadened, whereas the polster appears narrower and more elongated at bud stage. (A, B) Double labeling showing cells of the posterior prechordal plate expressing *rtk2* (blue, *in situ* staining) and polster cells stained for Fkd2 (brown, antibody staining) in a wild-type (A) and *slb* (B) embryo at bud stage. Arrowheads demarcate the length of the posterior prechordal plate along the anterior–posterior axis. Dorsal views, anterior to the left.

The Axial Mesendoderm in slb

The axial mesendoderm is composed of prechordal plate and notochord. At the anterior-most end of the prechordal plate lies the polster, a distinct domain of cells which later form the hatching gland (Kimmel *et al.*, 1990). At bud stage, the polster is narrower and more elongated in *slb* mutants compared to wild type. In contrast, the posterior prechordal plate, which can be identified by *receptor tyrosine kinase2* (*rtk2*) expression (Xu *et al.*, 1994), and the anlage of the notochord are shortened and broadened (Figs. 2A and 2B; see also Heisenberg *et al.*, 1996). These shape changes may represent a general migratory defect of axial mesendodermal cells.

We asked if *slb* gene function is required for both anterior

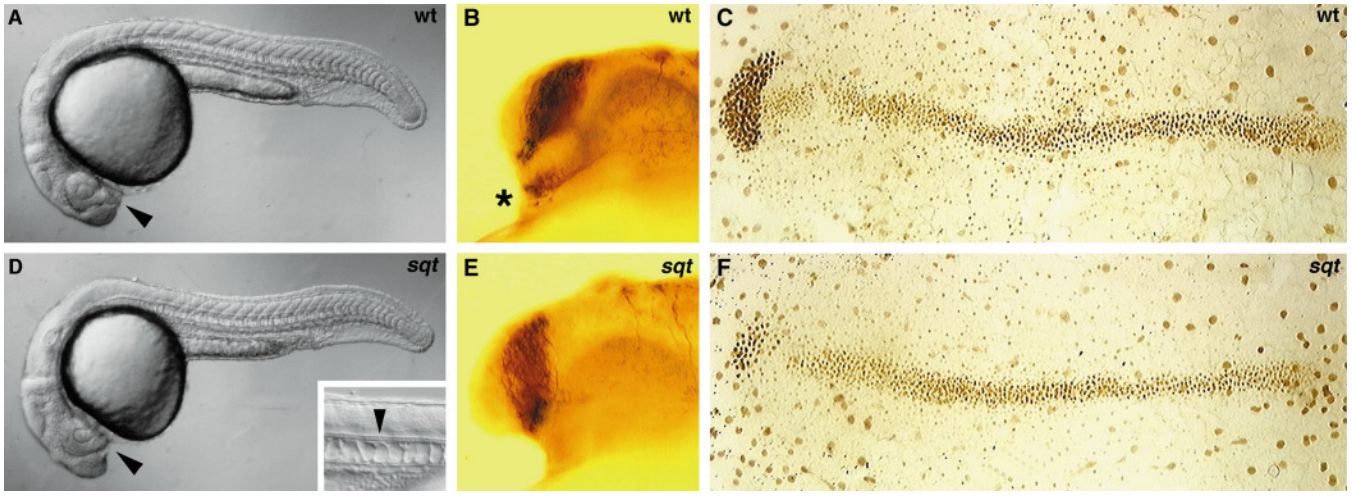

FIG. 3. In *sqt* embryos, the eyes are fused anteriorly and the ventral diencephalon as well as the underlying prechordal plate is reduced. (A, D) Side view of a wild-type (A) and *sqt* (D) embryo at 24 hpf. (B, E) The axonal scaffold stained with an anti-acetylated tubulin antibody in wild-type (B) and *sqt* (E) embryos at 24 hpf. (C, F) The axial mesendoderm stained with an anti-Fkd2 antibody in wild-type (C) and *sqt* (F) embryos at bud stage. Arrowheads in A and D point to the position of the optic stalks. Small picture in the bottom right corner of D shows the presence of a floorplate (arrowhead) in *sqt* mutants at 24 hpf. Asterisk in B demarcates the position of the nucleus of the tract of the postoptic commissur. Side (A, B, D, E) and dorsal (C, F) views, anterior to the left.

migration of polster cells and elongation of the notochord or if an initial defect in one of these structures secondarily affects the other one. We therefore generated double mutants including *slb* in which either the notochord or the polster is missing. To prevent notochord differentiation in *slb*, double mutants of *slb* and *ntl* were analyzed (*ntl* mutants lack a differentiated notochord; Halpern *et al.*, 1993), and to abolish polster formation in *slb*, double mutants for *slb* and *squint* (*sqt*) were generated.

sqt is a recessive lethal mutation. In *sqt* embryos the eyes are fused, whereas the floorplate appears normal at 24 hpf (Figs. 3A and 3D). The fusion of the eyes in *sqt* is accompanied by a reduction of the ventral diencephalon, as seen by the absence of the nucleus of the tract of the postoptic commissur stained with an anti-acetylated tubulin antibody (Figs. 3B and 3E). At bud stage, the prechordal plate is strongly reduced whereas the notochord is normal in *sqt* mutants stained with an antibody against Forkhead domain2 (Fkd2), a zebrafish homologue of the *Drosophila forkhead* gene (R. Warga and J. Odenthal, unpublished data; also described as zFkd1 in Dirksen and Jamrich, 1995; Figs. 3C and 3F).

In *slb/ntl* double mutants the polster phenotype is indistinguishable from that in *slb* alone and in *slb/sqt* double mutants the notochord phenotype is similar to that in *slb* alone (Figs. 4A and 4B and data not shown). These results are consistent with the idea that in *slb* the defects in the polster and notochord arise independently from each other.

The Ventral Midline of the Central Nervous System (CNS) in slb

The anterior extension of the ventral CNS midline within the neural plate relative to the position of the underlying axial mesendoderm was determined at bud stage. The ventral CNS midline was visualized by expression of *sonic hedgehog* (*shh*) (Krauss *et al.*, 1993), the anterior edge of the neural plate by expression of *dlx3*, a zebrafish homologue to the *Drosophila distalless* gene (Akimenko *et al.*, 1994), and finally the polster with an antibody against Fkd2. These expression patterns reveal that at bud stage the polster of wild-type embryos is positioned in front of the neural plate, whereas in *slb*, it underlies the neural plate (Figs. 5A and 5C and data not shown). In addition, the ventral CNS midline extends almost to the anterior end of the neural plate in wild type, whereas in *slb* it appears to be shortened and broadened leaving a gap between its anterior tip and the anterior edge of the neural plate (Figs. 5B and 5D). The ventral CNS midline exclusively overlies the posterior axial mesendoderm both in wild-type and *slb* embryos at bud stage, indicating that the posterior axial mesendoderm but not the polster induces ventral CNS midline structures at this stage of development.

The shortening of the ventral CNS midline in *slb* can be explained in different ways: (I) *slb* may primarily affect convergent extension/anterior migration of the axial mesendoderm, and the shortening of the ventral CNS midline results from incomplete induction by the underlying axial mesendoderm (see schematic diagram Fig. 6A I). (II) *slb* may affect convergent extension/anterior migration of both axial mesendodermal as well as ventral CNS midline cells (see schematic diagram Fig. 6A II).

In order to distinguish between these possibilities, we transplanted 10–20 cells from a labeled donor at shield stage, from a region of the shield which had previously been shown to give rise to ventral CNS midline cells at the level

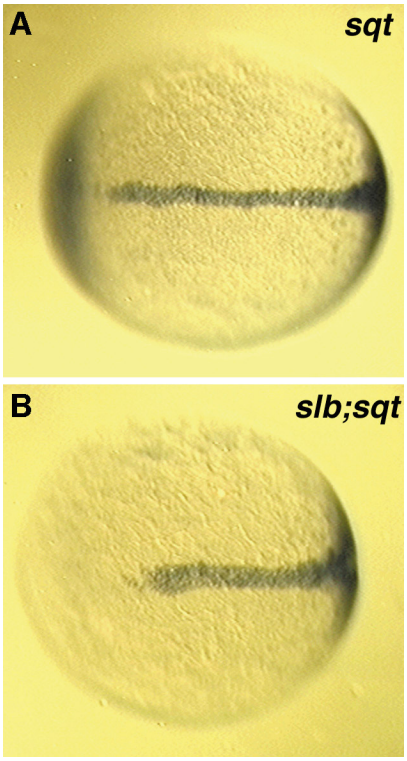

FIG. 4. The notochord is affected independently from the polster in *slb* at bud stage. (A, B) Notochord cells expressing *ntl* in a *sqt* mutant (which lacks polster cells, as revealed by probing the embryos for *hgg1* expression) (A) and a *slb/sqt* double mutant (B) at bud stage. Dorsal views, anterior to the left.

of the diencephalon (Woo and Fraser, 1995), into the same position of an unlabeled host of the same age and genotype. The position to which these ventral diencephalic midline cells had migrated in wild-type and *slb* embryos was determined at bud stage and at 15 hpf. At bud stage, the transplanted ventral diencephalic midline cells were found to be located at a similar distance from the anterior edge of the neural plate in *slb* and wild-type embryos ($n > 25$; n = number of recipients showing transplanted labeled cells) (Figs. 6B and 6E). However, at 15 hpf, transplanted cells were positioned posteriorly to the fused optic stalk anlage in *slb*, whereas in the wild type these cells were located anteriorly of the optic stalk anlage ($n > 25$) (Figs. 6C and 6F). Further in development transplanted cells formed ventral diencephalic (hypothalamic) tissue both in wild-type and in *slb* embryos (data not shown). These results show that in *slb* there is reduced anterior migration of ventral CNS midline cells from the bud stage on which supports the view that convergent extension/anterior migration is required for elongation of both the axial mesendoderm and the overlying ventral CNS midline.

We also performed transplantations of wild-type cells into mutant embryos and of mutant cells into wild-type embryos at shield stage. Twenty to 40 cells were targeted for the anlage of the ventral diencephalon and eyes (optic stalks and retinae) as well as of the prechordal plate and notochord (Hatta, 1992; Woo and Fraser, 1995). Transplantations were done into either the neurectoderm or the axial mesendoderm alone and into both of these layers together. Wild-type cells transplanted in mutant embryos were indistinguishable from mutant cells and mutant cells transplanted in wild-type embryos were indistinguishable from wild-type cells ($n > 25$) (Figs. 6D and 6G and data not shown). This indicates that *slb* function is not required cell-autonomously in both the neurectoderm and the axial mesendoderm.

Patterning of the Neural Plate in slb

Signals from the ventral CNS midline, such as *shh*, are thought to be involved in patterning of the eye anlage. It has been shown that *shh* induces the expression of *pax2*, a marker of the optic stalk anlage, in cells of the anterior neural plate adjacent to the ventral CNS midline but not within cells of the ventral CNS midline expressing *shh*

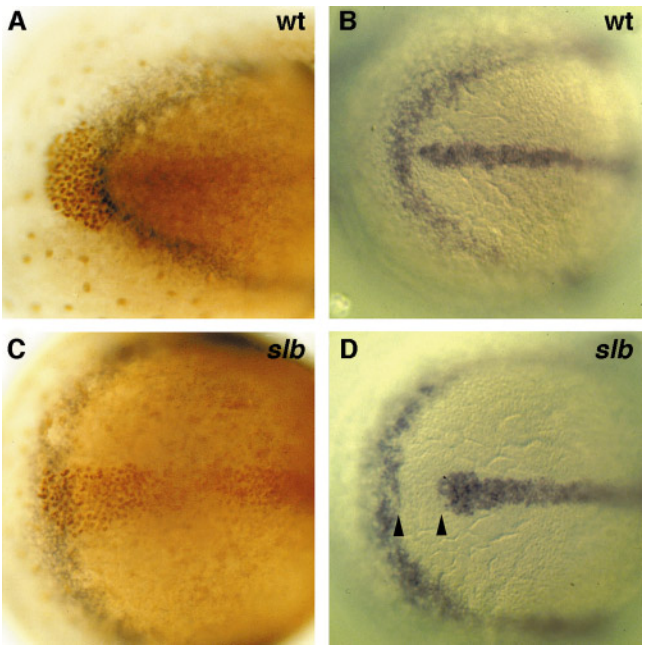

FIG. 5. Posterior displacement of the polster in *slb* is accompanied by a shortening of the *shh* expression domain at bud stage. (A, C) Double labeling staining the polster with an anti-Fkd2 antibody (brown) and the anterior edge of the neural plate by its expression of *dlx3* (blue) in a wild-type (A) and *slb* (C) embryo at bud stage. (B, D) *In situ* staining which labels the midline (*shh*) and anterior edge of the neural plate (*dlx3*) in a wild-type (B) and *slb* (D) embryo at bud stage. Arrowheads in D demarcate the gap between the anterior edge of the neural plate and the anterior end of the *shh* expression domain in *slb*. Dorsal views, anterior to the left.

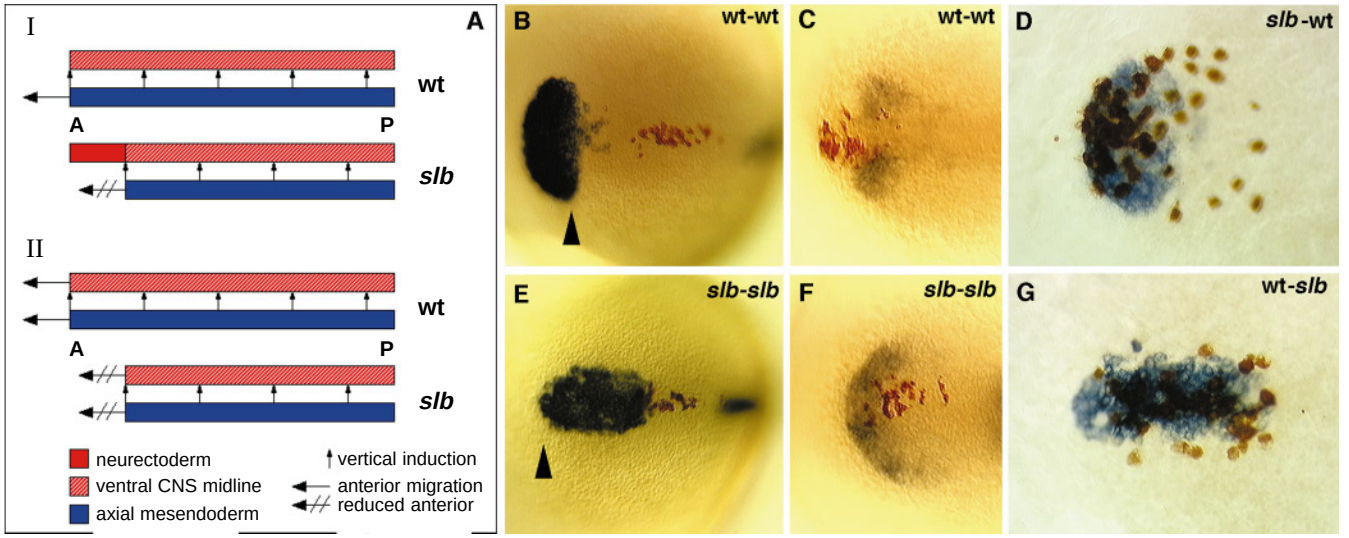

FIG. 6. Anterior migration of ventral CNS midline cells is normal at bud stage but impaired at 15 hpf in *slb*. (A) Schematic diagram showing two different models which might explain the shortening of the ventral CNS midline in *slb:* (I) reduced anterior migration of the axial mesendoderm (blue) in *slb* leads to incomplete induction of the ventral CNS midline (hatched, red) in the overlying neural plate (red). (II) Anterior migration of both the axial mesendoderm (blue) and the overlying ventral CNS midline (hatched, red) is impaired in *slb* (B, E) Position of ventral diencephalic midline cells (brown) in relation to the polster expressing *hgg1* (blue) in a wild-type (B) and *slb* (E) embryo at bud stage. Note that the anterior end of the neural keel (arrowhead) lies to the posterior of the polster in wild type whereas it lies to the anterior of the polster in *slb*. (C, F) Position of ventral diencephalic midline cells (brown) in relation to the anlage of the optic stalks expressing *pax2* (blue) in a wild-type (C) and *slb* (F) embryo at 15 hpf. (D, G) Transplantation of *slb* cells (brown) into a wild-type polster expressing *hgg1* (blue) (D) and of wild-type cells (brown) into a *slb* polster expressing *hgg1* (blue) (G) do neither phenocopy nor rescue the mutant phenotype. Dorsal views, anterior to the left.

themselves (Macdonald *et al.*, 1995; Ekker *et al.*, 1995). In *slb*, the shortening of the ventral CNS midline is followed by a fusion of the optic stalk anlage (Heisenberg *et al.*, 1996). It appears likely that the fusion of the optic stalks in *slb* is caused by the shortening of the ventral CNS midline, leading to ectopic *pax2* expression in more medial portions of the anterior neural plate, a position where the ventral CNS midline would normally be located.

Despite the optic stalk fusion, patterning of the neural plate along the anterior–posterior (a–p) axis appears to be largely normal in *slb*, as shown by the expression of markers along the a–p axis such as *pax2*, which demarcates the position of the mid–hindbrain boundary at bud stage (data not shown). Also, the general organization of the forebrain in *slb* is still intact, as shown by staining of *slb* embryos at 24 hpf with an antibody against acetylated tubulin. Acetylated tubulin allows the visualization of clusters of primary neurons and of the axonal scaffold which interconnects these clusters in the zebrafish embryonic brain (Chitnis and Kuwada, 1991). The axonal scaffold in *slb* embryos at 24 hpf appears normal with the exception of a slight deformation at anterior–ventral positions due to the fusion of the eyes (data not shown).

slb/trilobite (tri) Double Mutants

In *tri* mutants convergent extension movements are impaired during gastrulation in a manner reminiscent of the mesendodermal defect seen in *slb* (Hammerschmidt *et al.*, 1996). In contrast to *slb*, *tri* mutants form an apparently normal polster, and only a small fraction of embryos homozygous for the strong tri^{tk50} allele show a weak anterior fusion of the eyes. In addition to the convergent extension phenotype, the formation of the tailbud and subsequent outgrowth of the tail is defective in *tri* (Hammerschmidt *et al.*, 1996). We analyzed embryos homozygous for both *slb* and *tri*. In *slb/tri* double mutants the *slb* eye phenotype is strongly enhanced, whereas the *tri* tail phenotype is unchanged at 48 hpf (Figs. 7A and 7D and data not shown). The eye fusion in *slb/tri* is preceded at 15 hpf by a clearly enhanced fusion of the bilateral *pax2* expression domains demarcating the anlage of the optic stalks (Figs. 7B and 7E). Apart from the nearly complete fusion of the eyes in the double mutants, the axonal scaffold and thus the general organization of the embryonic brain appears to be intact at 24 hpf (Figs. 7C and 7F). The observation that the eye fusion is clearly enhanced in the double mutants indicates that *tri* and *slb* have partially redundant functions in the process of eye separation.

We also analyzed *slb/tri* double mutants at bud stage (Figs. 8A and 8D): the reduction in the length of the notochord in the double mutants appears to result from an addition of the single phenotypes seen in *slb* and *tri* mutants alone, indicating that both *slb* and *tri* may function in con-

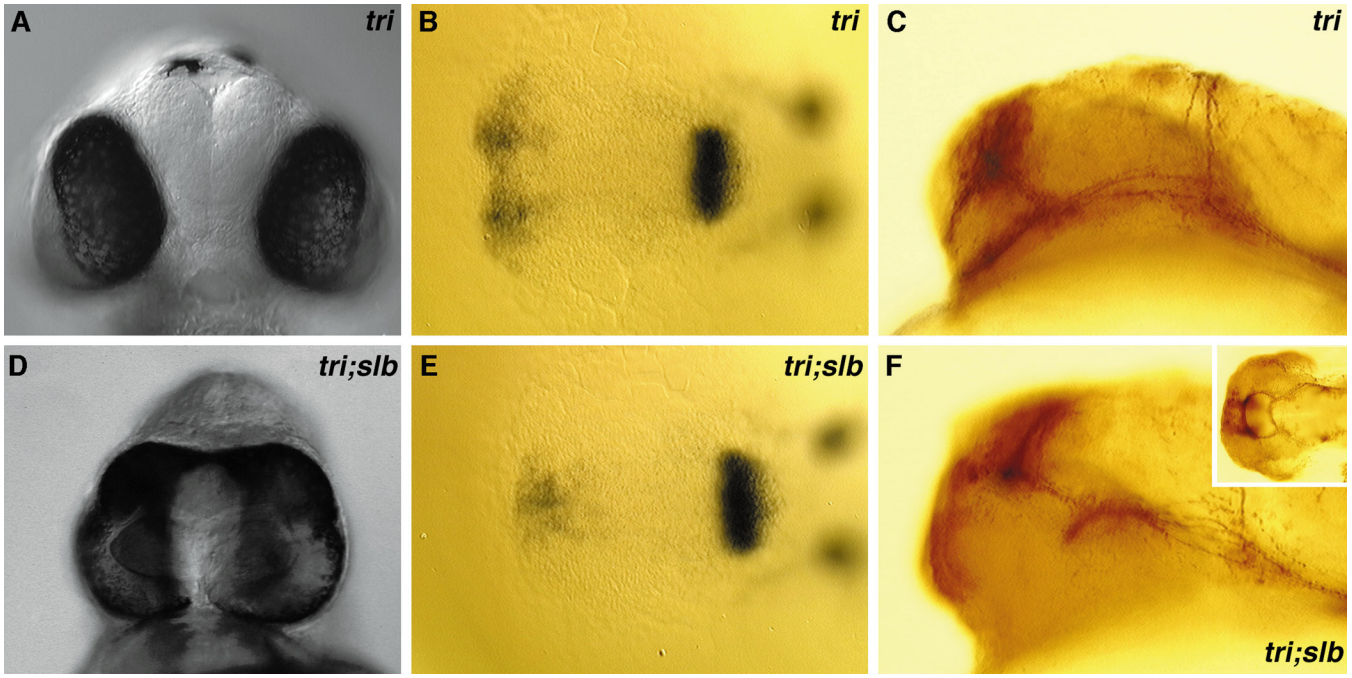

FIG. 7. In *slb/tri* double mutants the *slb* eye phenotype is strongly enhanced. (A, D) Frontal view of a *tri* (A) and *slb/tri* (D) double mutant at 48 hpf. (B, E) The anlage of the optic stalks expressing *pax2* in a *tri* (B) and *slb/tri* (E) double mutant at 15 hpf. Dorsal views, anterior to the left. (C, F) The axonal scaffold stained for acetylated tubulin appears normal in a *tri* (C) mutant and is still intact but strongly deformed in a *slb/tri* (F) double mutant at 24 hpf (the small picture in the upper right corner of F shows a dorsal view of the axonal scaffold stained for acetylated tubulin in *slb/tri* embryos at 24 hpf). Side views, anterior to the left.

vergent extension of the axial mesendoderm (Figs. 8B and 8E). In contrast, the polster phenotype and subsequently the distance between the anterior edge of the neural plate and the anterior end of the ventral CNS midline is not significantly enhanced in *slb/tri* double mutants compared to *slb* mutants alone (Figs. 8C and 8F). Therefore, *tri* function may be needed predominantly for convergent cell intercalation within the posterior axial mesendoderm whereas anterior migration of polster cells is unaffected by *tri* at bud stage. The loss of *tri* function may enhance the *slb* eye phenotype by further slowing down elongation of the posterior axial mesendoderm together with the overlying ventral CNS midline during gastrulation and early somitogenesis.

DISCUSSION

Elongation of the Axial Mesendoderm

silberblick (*slb*) gene function is required for elongation of the embryonic body axis during gastrulation. It has previously been shown that convergent cell intercalation is a common mode for elongation of the embryonic axis in gastrulating amphibians and teleosts (Keller and Danilchik, 1988; Warga and Kimmel, 1990). In *slb* mutant embryos, the notochord and the posterior prechordal plate are shortened and broadened at late gastrulation stages, suggesting that convergent cell intercalation is perturbed. It also has been reported that in *Xenopus* cells at the anterior end of the prechordal plate actively migrate toward the animal pole, thereby further contributing to axis elongation (Keller and Danilchik, 1988). The elongated and narrowed shape of the polster in *slb* could therefore also reflect a defect of cells within the prechordal plate to migrate anteriorly. We propose, based on the shape changes of the axial mesendoderm seen in *slb* embryos at late gastrulation, that *slb* is required for a mode of cell movement needed in both convergent cell intercalation and anterior migration of axial mesendodermal cells during gastrulation.

In *trilobite* (*tri*) mutants dorsal convergence of the axial and paraxial mesendoderm in trunk and tail is affected, whereas anterior migration of polster cells appears to be undisturbed (Hammerschmidt *et al.*, 1996). In contrast, *slb* is required for anterior migration of polster cells, whereas dorsal convergence of the paraxial mesoderm seems to be normal (Heisenberg *et al.*, 1996). Therefore, *tri* appears to be more generally required for dorsal convergence of posterior mesendoderm, whereas *slb* is needed for elongation of the embryonic axis through anterior migration and medial lateral intercalation of cells *within* the axial mesendoderm. The analysis of *slb/tri* double mutants shows that the notochord phenotypes of *slb* and *tri* are additive, supporting the view that both genes are required in parallel for elongation of the posterior axial mesendoderm.

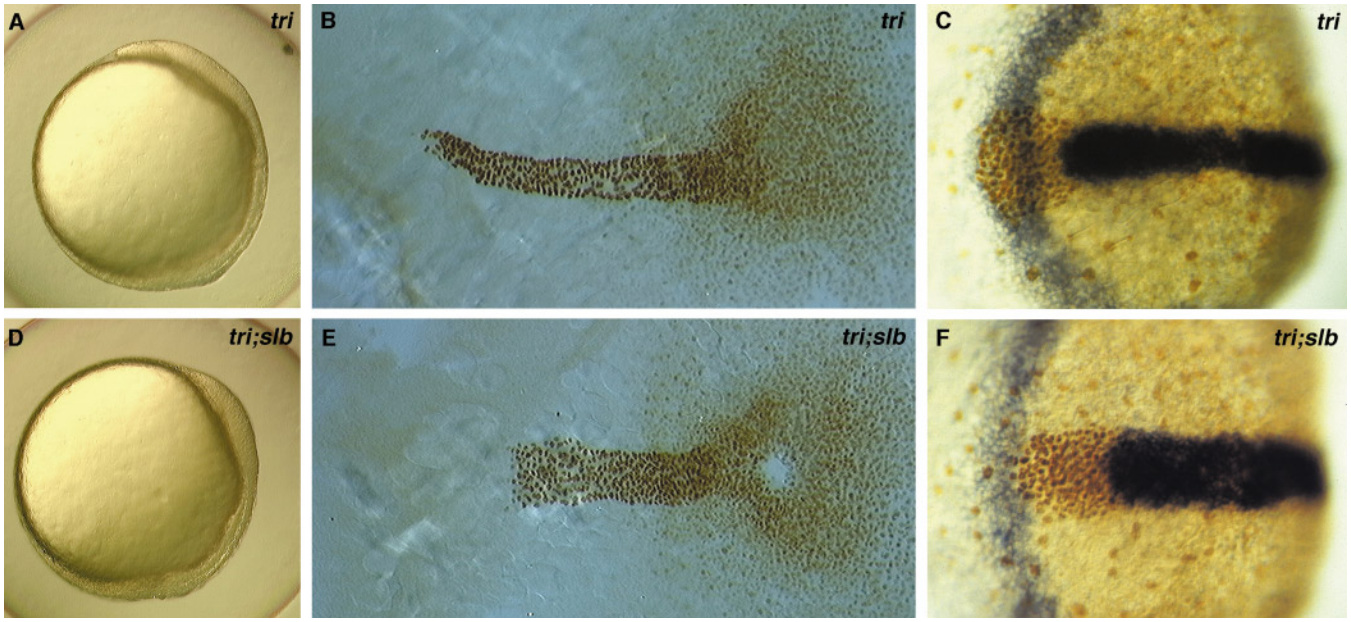

FIG. 8. In *slb/tri* double mutants the *slb* notochord defect is strongly enhanced, whereas the shape of the polster and the distance between the anterior edge of the neural plate and the anterior end of the *shh* expression domain is unchanged. (A, D) Live phenotypes of a *tri* (A) and *slb/tri* (D) double mutant at bud stage. Side views, anterior to the left. (B, E) The notochord stained for Ntl in a *tri* (B) and *slb/tri* (E) double mutant. (C, F) The polster stained for Fkd2 (brown, antibody staining), the ventral CNS midline expressing *shh* (blue, *in situ* staining), and the anterior edge of the neural plate expressing *dlx3* (blue, *in situ* staining) in a *tri* (C) and *slb/tri* (F) double mutant. Dorsal views, anterior to the left.

A third zebrafish mutant exhibiting defects in the convergent extension of the embryonic axis is *knypek* (*kny*) (Solnica-Krezel *et al.*, 1996). In *kny* mutant embryos, notochord and somites are broadened and shortened, whereas positioning of the eyes is apparently unaffected (Solnica-Krezel *et al.*, 1996). Double mutants of *tri* and *kny* exhibit a strong fusion of the eyes, revealing a partially redundant function of both *tri* and *kny* in a process that leads to the spacing of the eyes (L. Solnica-Krezel, unpublished data). It will be of interest to find out if the polster phenotype in *tri/kny* double mutants resembles that seen in *slb*. Moreover, double mutants of *slb* and *kny* will reveal if *kny*, like *tri*, can enhance the *slb* eye phenotype.

Elongation of the Ventral Central Nervous System (CNS) Midline

In *slb* embryos, the ventral CNS midline is shortened and broadened, in a similar manner to the underlying posterior axial mesendoderm. The close apposition and phenotypic similarity of these structures can be explained in different ways. It could be that the ventral CNS midline and the posterior axial mesendoderm actively elongate during gastrulation, possibly by using convergent extension/anterior migration as a common migratory mode (Keller *et al.*, 1992). This view is supported by the results of the transplantation analysis in *slb* showing that reduced anterior migration of neural plate midline cells is involved in the shortening of the ventral CNS midline. Alternatively, the shortening of the ventral CNS midline in *slb* may simply mirror the induction of ventral CNS midline tissue by the displaced underlying axial mesendoderm. This second explanation presupposes that the ventral CNS midline is only induced by posterior axial mesendoderm since no ventral CNS midline can be seen in the part of the neural plate which overlies the polster in *slb*.

Presumably both mechanisms, that is anterior migration of ventral CNS midline cells as well as ectopic induction of ventral CNS midline identity, contribute to the generation of the *slb* phenotype. Therefore inductive signals from the underlying axial mesendoderm could both determine cell identity within the ventral CNS midline and direct migration of midline cells within the neural plate.

Positioning of the Eyes

It has been shown that signals from the ventral CNS midline, such as *shh*, are able to induce the expression of *pax2*, a marker of the optic stalk anlage, within the anterior neural plate (Macdonald *et al.*, 1995; Ekker *et al.*, 1995). Notably, *pax2* expression is not induced in cells of the ventral CNS midline which endogenously express *shh*. In *slb* embryos,

the ventral CNS midline does not extend into the anterior-most portion of the neural plate at bud stage. Thus, the cells at anterior–medial positions of the neural plate which do not form a ventral CNS midline in *slb* may now be able to respond to signals from the ventral CNS midline by expressing *pax2*. This ectopic induction of *pax2* may subsequently lead to the anterior–medial fusion of the optic stalks observed in *slb* mutants. An anterior shortening of the ventral CNS midline followed by a fusion of the optic stalks has also been observed by misexpressing *wnt4* in zebrafish embryos (Ungar *et al.*, 1995).

In classical studies by Adelmann and others (Adelmann, 1930) it has been shown that in *Amblystoma* manipulation of the prechordal mesoderm can lead to deformations of the eye anlage that frequently produce cyclopic embryos. Removal of parts of the prechordal plate, as well as inhibition of prechordal mesoderm morphogenesis by treatment with lithium, has been shown to result in an incomplete separation of the eye anlagen. These studies lead to the model where an initial singular eye field within the anterior portion of the neural plate is subdivided into two bilateral fields by signals emanating from the underlying prechordal mesoderm. In more recent studies based on fate mapping of CNS progenitors in the zebrafish (Woo and Fraser, 1995), it has been shown that at early shield stages there is indeed a singular eye field and it has further been suggested that this field may be separated by the anterior migration of diencephalic progenitors. We propose that in *slb*, reduced anterior extension movements of the ventral CNS midline together with the underlying prechordal plate lead to medial instead of bilateral induction of optic stalks, thereby supporting the view that proper midline morphogenesis is essential for bilateralization of the eye anlage.

In *tri* mutants, elongation of the posterior axial mesendoderm is affected, whereas morphogenesis of the prechordal plate and spacing of the eyes appears largely normal. *tri*, however, enhances the *slb*-eye phenotype in a *tri/slb* double mutant. How could this partially redundant function of *tri* in positioning of the eyes be explained? In *tri*, elongation of the posterior axial mesendoderm is impaired. It is conceivable that extension of the posterior axial mesendoderm per se is not required for spacing of the eyes during the normal course of development. However, if prechordal plate morphogenesis is impaired, as is the case in *slb*, elongation of the remaining axial mesendoderm becomes more essential to ensure at least some degree of axis elongation necessary for extension of the ventral CNS midline and subsequent positioning of the eyes.

The only other vertebrate mutant described thus far which shows an eye phenotype reminiscent of *slb* mutants is the *talpid* mutant in the chicken (Ede and Kelly, 1964). In *talpid*, as in *slb*, aberrant morphogenesis of prechordal mesoderm is accompanied by a medial displacement of the optic stalks. However, a detailed characterization of the axial mesendodermal defect in *talpid* has not yet been carried out, which makes it difficult to judge if the defective processes are homologous to those affected in *slb*.

Patterning of the Embryonic Forebrain

It has been shown that a misspecification of ventral forebrain tissues is often associated with a reduction of anterior forebrain structures (Hatta *et al.*, 1991; Schier *et al.*, 1996; Hammerschmidt *et al.*, 1996), indicating that the formation of dorsal–ventral (d–v) and anterior–posterior (a–p) polarity within the forebrain, are linked. In *slb* mutants, however, extension of the ventral CNS midline within the forebrain anlage, an indicator for d–v axis specification, is impaired without further disturbing a–p patterning. This finding suggests that, in the developing forebrain, different and independent signals are involved in d–v versus a–p axis formation, in a way similar to what has been reported for the hindbrain and spinal cord (Ruiz i Altaba, 1994).

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REFERENCES

Adelmann, H. B. (1930). Experimental studies of the development of the eye. III. The effect of the substrate (''*Unterlagerung*'') on the heterotopic development of the median and lateral stripes of the anterior end of the neural plate of *Amblystoma*. *J. Exp. Zool.* **57,** 223–281.

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W., and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: Part of a homeobox gene code for the head. *J. Neurosci.* **14,** 3475–3486.

Ballard, W. W. (1973). A new fate map for Salmo gairdneri. *J. Exp. Zool.* **184,** 49–74.

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. M., 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,** 129–142.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. H. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383,** 407–413.

Chitnis, A. B., and Kuwada, J. Y. (1991). Elimination of a brain tract increases errors in pathfinding by follower growth cones in the zebrafish embryo. *Neuron* **7,** 277–285.

Dirksen, M. L., and Jamrich, M. (1995). Differential expression of fork head genes during early Xenopus and zebrafish development. *Dev. Genet.* **17,** 107–116.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a

member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430.

Ede, D. A., and Kelly, W. A. (1964). Developmental abnormalities in the head region of the *talpid* mutant of the fowl. *J. Embyol. Exp. Morphol.* **12,** 161–182.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T., and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5,** 944–55.

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., and Nüsslein-Volhard, C. (1993). The expression of a zebrafish gene homologous to Drosophila snail suggests a conserved function in invertebrate and vertebrate gastrulation. *Development* **119,** 1107–1118.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., Brand, M., van Eeden, F. J. M., 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. (1996). Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, *Danio rerio. Development* **123,** 143–151.

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,** 339–341.

Hatta, K. (1992). Role of the floor plate in axonal patterning in the zebrafish CNS. *Neuron* **9,** 629–642.

Heisenberg, C.-P., Brand, M., Jiang, Y.-J., Warga, R. M., Beuchle, D., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., and Nüsslein-Volhard, C. (1996). Genes involved in forebrain development in the zebrafish, *Danio rerio. Development* **123,** 191–203.

Hirose, G., and Jacobson, M. (1979). Clonal organization of the central nervous system of the frog. I. Clones stemming from individual blastomeres of the 16-cell and earlier stages. *Dev. Biol.* **71,** 191–202.

Keller, R., and Danilchik, M. (1988). Regional expression, pattern and timing of convergence and extension during gastrulation of Xenopus laevis. *Development* **103,** 193–209.

Keller, R., Shih, J., and Sater, A. (1992). The cellular basis of the convergence and extension of the Xenopus neural plate. *Dev. Dyn.* **193,** 199–217.

Kelly, O. G., and Melton, D. A. (1995). Induction and patterning of the vertebrate nervous system. *Trends Genet.* **11,** 273–278.

Kimmel, C. B., Warga, R. M., and Schilling, T. F. (1990). Origin and organization of the zebrafish fate map. *Development* **108,** 581–594.

Krauss, S., Johansen, T., Korzh, V., and Fjose, A. (1991a). Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. *Development* **113,** 1193–1206.

Krauss, S., Johansen, T., Korzh, V., and Fjose, A. (1991b). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353,** 267–270.

Krauss, S., Concordet, J. P., and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431–1444.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I., and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121,** 3267–3278.

Mullins, M. C., Hammerschmidt, M., Haffter, P., and Nüssleín-Volhard, C. (1994). Large-scale mutagenesis in the zebrafish: In search of genes controlling development in a vertebrate. *Curr. Biol.* **4,** 189–202.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Ruiz i Altaba, A. (1994). Pattern formation in the vertebrate neural plate [published erratum appears in *Trends Neurosci.* 1994 Jul;**17**(7):312]. *Trends Neurosci.* **17,** 233–243.

Schier, A. F., Neuhauss, C. C. F., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H., and Driever, W. (1996). Mutations affecting the development of the embryonic brain. *Development* **123,** 165–178.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G., and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116,** 1021–1032.

Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C. F., Malicki, J., Schier, A. F., Stanier, D. Y. R., Zwartkruis, F., Abdelilah, S., and Driever, W. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* **123,** 67–80.

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. Biol.* **164,** 420–429.

Ungar, A. R., Kelly, G. M., and Moon, R. T. (1995). Wnt4 affects morphogenesis when misexpressed in the zebrafish embryo. *Mech. Dev.* **52,** 153–164.

Warga, R. M., and Kimmel, C. B. (1990). Cell movements during epiboly and gastrulation in zebrafish. *Development* **108,** 569–580.

Woo, K., and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous system. *Development* **121,** 2595–2609.

Xu, Q., Holder, N., Patient, R., and Wilson, S. W. (1994). Spatially regulated expression of three receptor tyrosine kinase genes during gastrulation in the zebrafish. *Development* **120,** 287–299.

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