Functional Interactions of Genes Mediating Convergent Extension, knypek and trilobite, during the Partitioning of the Eye Primordium in Zebrafish

Florence Marlow,* Fried Zwartkruis,† Jarema Malicki,†‡ Stephan C. F. Neuhauss,†§ Leila Abbas,* Molly Weaver,* Wolfgang Driever,†,¶ and Lilianna Solnica-Krezel*,†,1

*Department of Molecular Biology, Vanderbilt University, Box 1820, Station B, Nashville, Tennessee 37235; †Cardiovascular Research Center, Massachusetts General Hospital, 149 13th Street, Charlestown, Massachusetts 01129; ‡Department of Ophthalmology, Harvard Medical School/MEEI, 243 Charles Street, Boston, Massachusetts 02114; §Max Plank Institute for Developmental Biology, Physical Biology, Spemannstrasse 35/I, Tuebingen MA, 72076 Germany; and *Developmental Biology, University of Freiburg, Hauptstrasse 1, Freiburg, D-79104 Germany

Vertebrate eye development in the anterior region of the neural plate involves a series of inductive interactions dependent on the underlying prechordal plate and signals from the midline of the neural plate, including Hedgehog. The mechanisms controlling the spatiotemporal expression pattern of hedgehog genes are currently not understood. Cyclopia is observed in trilobite (tri) and knypek (kny) mutants with affected convergent extension of the embryonic axis during gastrulation. Here, we demonstrate that tri mutants show a high frequency of partial or complete cyclopia, kny mutants exhibit cyclopia infrequently, while knym119 trim209 double-mutant embryos have dramatically reduced convergent extension and are completely cyclopic. We analyzed the relationships between the convergent extension defect, the expression of hedgehog and prechordal plate genes, and the formation of cyclopia in knym119 and trim209 mutants. Our results correlate the cyclopia phenotype with the abnormal location of hh-expressing cells with respect to the optic primordium. We show that cyclopia in these mutants is not due to an incompetence of tri and kny cells to respond to Hedgehog signaling. Rather, it is a consequence of exceeding a critical distance (> 40–50 μm) between hedgehog-expressing cells and the prospective eye field. We hypothesize that at this distance, midline cells are not in an appropriate position to physically separate the eye field and that HH and other signals do not reach the appropriate target cells. Furthermore, tri and kny have overlapping functions in establishing proper alignment of the anterior neural plate and midline cells expressing shh and twhh genes when the partitioning of the eye primordium takes place.

Key Words: gastrulation; Hedgehog; midline signaling; prechordal plate; cyclopia.

INTRODUCTION

During vertebrate development, a series of inductive interactions and morphogenetic movements leads to the formation of an embryo with defined polarity and three germ layers (Kessler and Melton, 1994). Further, inductive interactions within and between germ layers specify and pattern organ rudiments. Vertebrate eyes develop from the anterior region of the neural plate. During neurulation, the two optic vesicles evaginate on both sides of the forebrain while maintaining an attachment to the diencephalon through the optic stalks (reviewed in Saha et al., 1992). Subsequently, the optic vesicles invaginate to form bilayered optic cups which will give rise to the retina and the pigment epithelium. The surface ectoderm overlapping the
optic cups will form the lens placodes (Schmitt and Dowling, 1994). Early embryological experiments indicate that the entire anterior region of the neural plate has potential to give rise to retinal fates (Adelmann, 1936). However, during normal development retinal fates are restricted to more lateral positions. The retinal field is resolved by suppressing retina formation in the median of the field. The prechordal plate underlying the developing eye field has been implicated in this process. After experimental removal of the prechordal plate from amphibian and chick embryos (Adelmann, 1936; Li et al., 1997) or in zebrafish mutants in which the prechordal plate is absent or reduced, the anterior region of the neural plate has potential to give rise to retinal fates (Schier et al., 1997; Thissie et al., 1994; Strähle et al., 1997). In addition, fate-mapping experiments in zebrafish embryos indicate that the ventral diencephalic precursors might physically invade the eye field such that two retinas separated by midline cells are formed (Woo and Fraser, 1995; Varga and Westerfield, University of Oregon, Eugene, OR, personal communication).

Several recent studies indicate that the division of the eye field into two involves Hedgehog signaling from cells located in the midline of the developing neuroectoderm. Members of the hedgehog (hh) gene family, consisting of at least four members in the mouse, chick, and zebrafish, encode secreted signaling proteins that function in multiple patterning processes during animal embryogenesis (for review, see Hammerschmidt et al., 1997). Among the above family members, sonic hedgehog (shh) expression has been found in axial mesoderm and at later stages in the ventralmost cells along the developing neural tube in all vertebrates studied (Echelard, 1993; Krauss et al., 1993; Riddle, 1993; Roelink et al., 1994). In zebrafish, tiggy-winkle hedgehog (twhh) is detected in the ventral central nervous system (CNS) (Ekker et al., 1995), while yet another member of the family, echidna hedgehog (ehh), is expressed exclusively in the notochord (Currie and Ingham, 1996). The reduction or absence of shh and twhh expression in zebrafish cyclops (cyc) (Ekker et al., 1995; Krauss et al., 1993; Macdonald et al., 1995) and one-eyed pinhead (oep) (Schier et al., 1997; Strähle et al., 1997) mutants is correlated with cyclopia. Furthermore, mouse embryos homozygous for a targeted mutation in the shh gene are cyclopic (Chiang, 1996). Finally, holoprosencephaly in humans can result from mutations in the shh homologue (Belloni et al., 1996; Kelly et al., 1996; Lanoue et al., 1997; Roessler et al., 1996; Roux et al., 1997).

In the zebrafish cyc mutants, cyclopia is correlated with ectopic expression of pax6 in a bridge of tissue at the anterior pole of the neural keel in the position normally occupied by cells that form optic stalks. Concomitantly, expression of the pax2 gene in this region is dramatically reduced or absent (Ekker et al., 1995; Hammerschmidt et al., 1996a; Hatta et al., 1994; Macdonald et al., 1995). The anterior-median tissue exhibiting abnormal pax gene expression differentiates as retina, resulting in the formation of a single cyclopic eye in the mutant embryos. Ectopic expression of shh, twhh, Indian hedgehog (Ihh), or a dominant negative form of protein kinase A leads to the reduction of pax6 expression and to the suppression of retina and pigment epithelium. This is accompanied by increased pax2 expression and hypertrophy of the optic stalks in both wild-type and cyc mutant embryos (Ekker et al., 1995; Hammerschmidt et al., 1996a; Macdonald et al., 1995). A model has been proposed based on these results in which Shh or a related molecule (e.g., Twhh in zebrafish) inhibits pax6 expression and retina and pigment epithelium development in the median anterior neural tube while promoting pax2 expression and optic stalk differentiation in neighboring tissue (Macdonald et al., 1995). According to this model the spatial and temporal distribution of midline signals including HH should be critical for proper eye development.

The genes and mechanisms involved in achieving the spatial distribution of hh gene expression in vertebrate embryos are currently not understood. During gastrulation, convergent extension movements lead to a narrowing of the embryonic body in the mediolateral direction (convergence) and its extension along the anterior-posterior (AP) axis (Keller et al., 1992a; Keller, 1985, 1991; Warga and Kimmel, 1990). Convergent extension movements have been shown to be involved in the morphogenesis of the midline tissues that express hh genes, including notochord, prechordal mesendoderm, and floor plate in mouse and chick (Sausedo and Schoenwolf, 1993, 1994), as well as the neural plate in chick and frog (Keller et al., 1992b; Schoenwolf and Alvarez, 1989; Schoenwolf and Smith, 1990). Mutations in three zebrafish loci required for normal convergent extension during gastrulation exhibit synophthalmia and cyclopia. trilobite (tri) mutants (Hammerschmidt et al., 1996b; Solnica-Krezel et al., 1996) exhibit synophthalmia and cyclopia, silverblck (slb) mutants (Heisenberg et al., 1996; Heisenberg and Nüsslein-Volhard, 1997) are synophthalmic but not cyclopic, while knypek (kny) mutants (Solnica-Krezel et al., 1996) infrequently exhibit synophthalmia. slb but not tric240 mutants exhibit shortening and compression of the ventral CNS midline expressing shh which could result in the cyclopia phenotype (Heisenberg et al., 1996).

Here we analyzed the relationships between the convergent extension defect, the spatiotemporal expression pattern of hedgehog and prechordal plate genes, and the formation of cyclopia in tri and kny mutants. We present evidence that tri and kny loci functionally interact during eye development. Partial eye fusion was frequently observed in triknm209, triknm747, and triknm778 mutants and only rarely in knym119 and knym119. Double-mutant knym119triknm209 embryos exhibited a dramatic reduction of convergent extension and were invariably cyclopic. triknm209 and knym119 mutations synergistically affected the localization of shh- and twhh-expressing cells in the neural plate.
midline at the beginning of somitogenesis. At a critical distance, greater than 30–50 μm, between the cells expressing hh genes and the anterior neural plate margin, the optic primordium failed to properly divide into two separate retinal fields. The location of the posterior prechordal plate primordium failed to properly divide into two separate expressing cells nor cyclopia. These studies and recent work on the interaction between slb and tri loci reveal a genetic network required for the proper alignment of cells interacting during vertebrate head development (Heisenberg and Nüsslein-Volhard, 1997) and implicate convergent extension movements in this process.

MATERIALS AND METHODS

Fish Maintenance

Fish were maintained in 1-, 2-, and 4-liter tanks with recirculating water essentially as described in Solnica-Krezel et al. (1994).

Zebrafish Mutant Strains

tri<sup>m209</sup>, tri<sup>m747</sup>, tri<sup>m778</sup>, and knym<sup>119</sup> were identified during large-scale mutagenesis screening in the laboratory of W. Driever (Solnica-Krezel et al., 1996). The tri<sup>m778</sup> allele was identified as a low-penetrance defect. tri<sup>m778</sup> homozygous mutant embryos were observed at a frequency of 4% (n = 442) and exhibited massive degeneration during day 1 postfertilization (dpf). tri<sup>m778/m209</sup> transheterozygous embryos were observed at a frequency of 8% (n = 2119) and were viable through 5 dpf. The frequency and segregation indicate that tri<sup>m778</sup> may be a translocation allele. knym<sup>119</sup> was induced with γ-irradiation (C. Walker and C. Kimmel, Eugene, OR, personal communication). knym<sup>119</sup> tri<sup>m209</sup> double-mutant lines were generated by crossing fish heterozygous for the knym<sup>119</sup> mutation of AB genetic background with fish heterozygous for the tri<sup>m209</sup> mutation of AB genetic background.

Observation of Live Embryos

For phenotypic analysis of live mutant embryos, crosses were performed between fish of defined genotypes and embryos were collected in egg water as described in Solnica-Krezel et al. (1994). Live embryos were observed using Zeiss SV11 and STEMI 2000 dissecting microscopes, or an Axiopt microscope, and photographed using an Axioscan microscope as described in Solnica-Krezel and Driever (1994). In experiments testing the influence of temperature on mutant phenotype, crosses were performed and embryos were collected at 26–28.5°C. Embryos were transferred to either 32 or 22–23°C before the sphere stage, 4 h postfertilization (hpf). Embryos were returned to 26–28.5°C at the one-somite stage and analyzed further at 3 dpf. To quantify the penetrance and expressivity of the cyclopia phenotype, the cyclopia index (CI) index was determined as a sum of the ratio of mutants of class x (I–V) to the total number of mutants in the clutch, multiplied by the numerical value of the class (x = 1 – 5); [#class I × 1/#mutants] + [#class II × 2/#mutants] + [#class III × 3/#mutants] + [#class IV × 4/#mutants] + [#class V × 5/#mutants] (see Fig. 1 and Results for description of phenotypic classes).

Whole-Mount in Situ Hybridization

In situ hybridization was performed essentially as described in Oxtoby and Jowett (1993), except instead of washing at 60°C, washes were done at 70°C. Antisense RNA probes were synthesized from cDNAs encoding shh (Krauss et al., 1993), twhh (Ekker et al., 1995), hlx1 (Fjose et al., 1988), hgg1 (Thiess et al., 1994), pax2 (Krauss et al., 1991a), pax6 (Krauss et al., 1991b), dlx3 (Akimenko et al., 1994), and patched (ptc) (Concordet et al., 1996).

Morphometric Analysis of RNA Expression Patterns and Live Embryos

Embryos stained by whole-mount in situ hybridization were observed under a dissecting microscope to identify wild-type and mutant phenotypes. Embryos were then grouped according to phenotype and coded. Next, “blind” measurements were taken and embryos were decoded after all samples were measured. Measurements of tri<sup>m209</sup> and knym<sup>119</sup> were performed only with embryos obtained from appropriate single heterozygotes. From the progeny of knym<sup>119</sup> tri<sup>m209</sup> double heterozygotes only putative double mutants were identified and measured. Identified wild-type or mutant embryos were cleared in glycerol. Subsequently embryos were transferred to glycerol in chambers formed by bridging a coverslip across three layers of No. 2 coverslips and viewed with Nomarski optics using a Zeiss Axioplan microscope and a calibrated 10× objective. The objective was calibrated relative to an object of known dimensions using Metamorph software (Universal Imaging). Images of specific staining patterns were recorded using a Hamamatsu cooled CCD camera and viewed on a computer screen. Specific distances were measured with Metamorph image-analysis software. This was performed by drawing a straight line between two marked points (e.g., the anterior tip of the shh expression domain and the posterior edge of the dlx3 expression domain) or by tracing along a specific embryonic structure (e.g., the length of an embryo).

mRNA Injections

Wild-type and mutant embryos were collected from natural spawnings. Embryos were dechorionated with pronase and washed extensively in embryo medium as described in The Zebrafish Book (Westerfield, 1996). shh or twhh mRNAs were synthesized from DNA templates using the mMESSAGE mMACHINE kit (Ambion) and suspended in ddH<sub>2</sub>O at 100–200 ng/μl. Phenol red (0.1%) was added to the RNA solution, and embryos were injected in the yolk at the two- to eight-cell stage using a pneumatic picopump (WPI) with a known volume of RNA solution. Following injection, embryos were cultured in embryo medium. Embryos were analyzed at 30 hpf and at 48 hpf when eye and pigment epithelia are fully established.

RESULTS

Mutations in trilobite Locus Lead to Partial Cyclopia

The zebrafish tri locus has been identified by virtue of mutations that lead to decreased convergent extension of the embryonic body during gastrulation (Hammerschmidt...
function. However, intermediate phenotype rather than the complete loss of that the synopthalmia and partial cyclopia represented an trim209/m209 we conclude that synopthalmia and partial cyclopia in trim209 any clutch of trim209/m209 that identified (Table 1) (Solnica-Krezel et al., 1996b; Solnica-Krezel et al., 1996). It was possible that the trim209 and trim747 alleles were hypomorphs and that the synopthalmia and partial cyclopia represented an intermediate phenotype rather than the complete loss of function. However, trim778, which segregates like a translocation allele (Materials and Methods), led to partial cyclopia with a low penetrance in trim778 homozygous and trim209/778 transheterozygous embryos. Additionally, synopthalmia and partial cyclopia are observed in γ-irradiation-induced tri alleles (J. Topczewski and L. Solnica-Krezel, unpublished observations). Since γ-irradiation-induced and trim778 alleles most likely represent null mutations and failed to enhance cyclopia when in trans with trim209, we conclude that synopthalmia and partial cyclopia in trim209/m778 are not the consequence of a hypomorphic nature of this allele.

When trim209 heterozygotes were crossed with wild-type fish of India background, the identified AB/India hybrid heterozygotes produced tri mutant embryos with a higher incidence of cyclopia (Table 1). Five distinct degrees of cyclopia phenotypes have been distinguished when eye pigmentation is fully developed (Fig. 1). A few of cyclopia mutants with different classes of cyclopia exhibited a fully penetrant convergent extension defect and were not viable. Class I mutants exhibited eye spacing comparable to wild-type embryos (Figs. 1A and 1B); class II mutants exhibited a decreased spacing between the eyes (Fig. 1C); in mutants of class III the eyes were touching (Fig. 1D); in mutants of class IV two distinct but clearly fused eyes were observed (Fig. 1E); and class V mutants were characterized by a single cyclopic eye (Fig. 1F). In clutches of embryos obtained from two separate lines of trim209 AB/India hybrids, all five phenotypic classes were observed (Table 2). To quantify the penetrance and expressivity of the cyclopia phenotype in any clutch of tri mutants, we introduced a Cyclopia Index, CI (see Materials and Methods). In one line of trim209 AB/India hybrids the CI was determined to be 2.17 ± 0.64, while in the second line investigated the CI was 2.45 ± 0.4 (Table 2).

### The Cyclopia Phenotype in trim209 Mutants Is Cold Sensitive

Zebrafish embryos develop normally, although at different rates, at temperatures between 23 and 32°C (Kimmel et al., 1995). To test possible effects of temperature on the phenotype of tri mutants, clutches of embryos obtained from trim209 AB/India hybrids were incubated at 23, 28.5, and 32°C. Analysis of these embryos indicated that the penetrance and expressivity of the cyclopia phenotype were exacerbated at 23°C and suppressed at 32°C (Table 2). The convergent extension phenotype was fully penetrant at all temperatures examined, as indicated by measurements of the AP length and the width of the shh expression domain at the level of the otic vesicles (Table 4). Therefore, the cyclopia phenotype is cold sensitive in trim209 mutants.

kny∆m119 trilobite∆m209 Double Mutants Exhibit Complete Cyclopia and Additive Defects with Regard to Body Length

kny∆m119 mutants exhibited a very similar phenotype to tri mutants at day 1 of development (Fig. 2), except cyclopia was observed less frequently in kny∆m119 mutants in AB or AB/India hybrid background (Table 1) (Solnica-Krezel et al., 1996). Furthermore, kny∆m119 mutants exhibited a characteristic forked yolk extension and failed to develop the ventral fin (data not shown). Cyclopia was also rare in kny∆m119 tri mutants (C. Walker and C. Kimmel, University of Oregon, Eugene, OR, personal communication), and additional γ-irradiation induced alleles (J. Topczewski and L. Solnica-Krezel, unpublished observations). Therefore, the low frequency of cyclopia in kny∆m119 mutants is not likely due to a hypomorphic nature of this allele.

Considering the similar phenotypes of kny and tri, a double-mutant line, kny∆m119 tri∆m209, was generated to test for possible functional interactions between the two genes. In the progeny of kny∆m119+/− tri∆m209+/− double heterozygotes, single tri and kny mutants were identified based on the morphological characteristics described above, at expected frequencies (Fig. 2, Table 3). Furthermore, a distinct class of mutants was present at a frequency expected for double-mutant embryos. At 24 hpf these double-mutant embryos were characterized by a very short axis with a particularly shortened and dorsally kinked tail region (Fig. 2G). The AP body length of wild-type embryos was determined to be 2.3 ± 0.02 mm (n = 10) at this stage of development; tri∆m209 were ca. 0.5 mm shorter than wild type, 1.8 ± 0.03 mm (n = 10), and kny∆m119 mutants were ca. 0.6 mm shorter than wild type, 1.7 ± 0.05 mm (n = 5). The kny∆m119 tri∆m209 double mutants were half as long as wild type, 1.15 ± 0.03 mm (n = 5), indicating that the extension defect of the

### TABLE 1

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knym119 trim209 double mutant was additive. Notably, the double mutants exhibited complete cyclopia (Fig. 2G, inset). This synergistic phenotype suggested that trim209 and knym119 have overlapping functions in convergent extension as well as eye development.

Interestingly, 28% of knym119 mutants identified among the progeny of knym119+/− tri m209+/− double heterozygotes exhibited partial cyclopia. This was in contrast to the lack of cyclopia observed for knym119 mutants in AB/AB background (Table 1). It was likely that homozygous knym119 mutant embryos exhibited cyclopia when also heterozygous for the tri m209 allele. Alternatively, another enhancer of cyclopia could be present in the kny tri mutant line. To distinguish between these possibilities, crosses were performed between siblings of known genotypes at the kny and tri loci, and the cyclopia index was determined in the resulting progeny (see Materials and Methods). As expected, embryos homozygous for knym119 mutant alleles with two wild-type alleles at the tri locus rarely exhibited cyclopia (CI = 1.01 ± 0.01; n = 200). kny mutants from crosses of knym119 trim209 double heterozygotes with knym119 heterozygotes had a significantly higher cyclopia index, 1.54 ± 0.32 (n= 1009). However, among these kny mutants only half should contain one tri m209 allele. Therefore, the estimated cyclopia index for embryos of the knym119−/− tri m209−/+ genotype is 2.0 ± 0.7, considering that kny mutants with two wild-type alleles at the tri locus do not exhibit cyclopia (see above). Since less than 50% of knym119 mutants exhibited cyclopia, these observations support the notion of tri m209 being a dominant enhancer of cyclopia in knym119−/− mutants. As expected from earlier experiments, double-mutant embryos obtained from these crosses exhibited complete cyclopia (CI = 4.9 ± 0.16; n = 277).

FIG. 1. Degrees of cyclopia in tri m209 mutants. Micrographs of live embryos at 3 dpf, ventral view of head region. Five classes of the cyclopia phenotype can be distinguished in tri m209 mutants. In class I mutants (B) the eye spacing is comparable to that observed in a wild-type sibling (A). In mutants of class II (C) the eye spacing is decreased. (D) In mutants of class III eyes are marginally fused. (E) The eyes are completely fused in mutants of class IV. (F) One cyclopic eye is observed in class V mutants. Retina covered by pigment epithelium (arrow).
The Cyclopia Phenotype Is Correlated with an Abnormal Distribution of shh and twhh Transcripts in Mutant Embryos

The cyclopia phenotype is often accompanied by and most likely results from the reduction or absence of hh gene expression in the midline of the developing neural plate (Ekker et al., 1995; Krauss et al., 1993; Macdonald et al., 1995; Schier et al., 1997; Strähle et al., 1997; Chiang et al., 1996). Therefore, the degree of cyclopia could reflect the abnormal expression of shh and possibly twhh. Indeed, a shortened shh expression domain in the AP axis has been observed in slbtx226 cyclopic mutants at the onset of somitogenesis (Heisenberg and Nüsslein-Volhard, 1997). Therefore, we analyzed the expression of shh and twhh genes in trim209 mutants at permissive and restrictive temperatures, as well as in knym119 and knym119 trim209 double-mutant embryos at the beginning of somitogenesis. In situ hybridization analysis indicated that at tail bud stage the expression domain of shh and twhh genes was shorter along the AP axis and broader along the mediolateral axis for both knym119 (Figs. 3B and 3F) and trim209 (Figs. 3C and 3G) mutants (also data not shown). This phenotype was exacerbated in putative double-mutant embryos (Figs. 3D and 3H). The abnormal shape of shh and twhh expression domains was consistent with a general convergent extension defect in the mutant embryos and likely resulted from reduced convergent extension of cells specified to express hh genes. Notably, the AP shh expression domain length appeared shorter in knym119 mutants than in trim209 mutants; however, cyclopia was infrequently observed for knym119 (Figs. 3B and 3F vs Figs. 3C and 3G). This indicated that the AP shortening of the shh expression domain at the onset of somitogenesis was not sufficient for the cyclopia phenotype.

At 24 hpf, the overall AP length of the shh expression domain was shortened to a similar degree in both trim209 (Fig. 4C) and knym119 mutants (Fig. 4B). In contrast, while the rostral brain shh expression domain was normal in knym119 mutants compared to wild type (Figs. 4A and 4B), in trim209 mutants this expression domain was shortened (Solnica-Krezel et al., 1996) and was drastically compressed in knym119 trim209 double mutants (Figs. 4D and 4H). In trim209 (Fig. 4G) and knym119 trim209 (Fig. 4H) cyclopic mutant embryos, the forebrain expression domain appears to be posteriorly shifted with respect to the eyes. Therefore, the compression of the anterior portion, but not the overall AP length of the shh expression domain at 24 hpf, correlated with the cyclopia phenotype.

These observations suggested that cyclopia could be due to the abnormal location of shh-expressing cells with respect to the prospective eye field. To investigate this possibility, clutches of mutant embryos at the one-somite stage were hybridized simultaneously with shh and dlx3 probes. dlx3 transcripts are detected in a baseball-stitch band positioned around the edge of the prospective neural plate (Akimenko et al., 1994). In wild-type embryos shh and twhh midline expression domains extended anteriorly to the posterior edge of the dlx3 domain (Fig. 5A and data not shown). In contrast, a small gap between shh and dlx3 expression domains was observed for knym119 mutants (Fig. 5B), and an apparently larger gap was observed for trim209 (Fig. 5C) and knym119 trim209 mutant embryos (Fig. 5D). This observation prompted us to measure the distance between the anterior edge of shh and dlx3 domains with a digital camera and M etamorph software (see Materials and Methods). These studies revealed a correlation between the size of the gap between the shh and dlx3 expression domains in the anterior neural plate and the cyclopia phenotype (Fig. 6 and Tables 1 and 2). In wild-type embryos the average shh–dlx3 distance was smaller than 5 μm (n = 69). In knym119 mutants, in which cyclopia was only occasionally observed, this distance was significantly larger, approximately 20 ± 11 μm (n = 17). In trim209 embryos that developed at a permissive temperature, which suppressed cyclopia (CI = 1.1–1.3), the shh–dlx3 distance was greater, 30 ± 15 μm (n = 15). Notably, in trim209
embryos which developed at the restrictive temperature which leads to a high incidence of cyclopia (CI = 2.8–3.1), the average shh–dlx3 distance was markedly increased to 50 ± 22 μm (n = 9). Finally, the shh–dlx3 distance was extremely large, ca. 90 ± 27 μm (n = 12), in cyclopic knym^{m119} trilm^{m209} double-mutant embryos (CI = 5). The dimension of the shh–dlx3 gap in knym^{m119} trilm^{m209} double mutants was larger than the sum of the gaps observed for

FIG. 2. knym^{m119} trilm^{m209} double-mutant embryos exhibit additive defects in convergent extension during somitogenesis and 1 dpf, and a synergistic cyclopia defect, compared to single mutants. (A, C, E, G) Dissecting microscope images, lateral view of live embryos at 30 hpf. The length of the embryo is decreased in knym^{m119} (C) and trilm^{m209} (E) mutants and dramatically decreased in the double mutant (G) compared to wild type (A). Abnormal shape of the yolk cell and cyclopia are seen in the double mutant (G, inset). (B, D, F, H) Dorsal views of live embryos at 13 hpf using Nomarski optics. The mediolateral width and AP extension of somites (s), notochord (n), and neural keel (nk) are affected in an additive fashion in double mutants (H) compared to knym^{m119} (D) and trilm^{m209} (F) single-mutant embryos. fb, forebrain; hb, hindbrain; mb, midbrain; mb/hb, midbrain–hindbrain boundary; ye, yolk extension.
the single mutants (Fig. 6). Therefore, there was a synergistic interaction between the two mutations with respect to the size of the shh–dlx3 gap, as there was for the cyclopia phenotype. Based on these results we hypothesized that excessive distance between the hh-expressing cells and the anterior neural plate (>40–50 μm) resulted in failure to partition the optic primordium and led to synopthalmia or cyclopia.

The measurements of the AP dimension of the embryo (the length of the embryo from the anterior tip of the forming polster to the tail bud) confirmed that there was no simple relationship between the degree of the overall convergent extension defect and cyclopia (Table 4). The double mutants exhibited the shortest axis and the largest shh–dlx3 gap (Table 4). However, the AP length defect with respect to wild-type embryos was ca. 100 μm larger in knym119 than trim209 mutants (Table 4). The convergence defect was further examined by measuring the width of the

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The observed numbers of wild-type and single- and double-mutant embryos are not significantly different from those expected for two independently segregating genes. \( \chi^2 = 0.148, P > 0.975. \)

The measurements of the AP dimension of the embryo (the length of the embryo from the anterior tip of the forming polster to the tail bud) confirmed that there was no simple relationship between the degree of the overall convergent extension defect and cyclopia (Table 4). The double mutants exhibited the shortest axis and the largest shh–dlx3 gap (Table 4). However, the AP length defect with respect to wild-type embryos was ca. 100 μm larger in knym119 than trim209 mutants (Table 4). The convergence defect was further examined by measuring the width of the

**FIG. 3.** Comparative expression of shh in wild-type (A, E), trim209 (C, G), knym119 (B, F), and kny tri (D, H) embryos at bud stage (10.5 hpf). The shh expression domain is shortened in the AP direction and broadened in the mediolateral direction; this phenotype is more pronounced in knym119 (B, F) and knym119 trim209 (D, H) than in trim209 (C, G) embryos. (A–D) Lateral views, dorsal to the right and anterior at the top; (E–H) dorsal views, anterior toward the top. Tailbud (tb), prospective head region (h). Bar, 120 μm.
shh expression domain and the width of the neural plate at the level of the otic placodes marked by stronger expression of the dlx3 gene (Akimenko et al., 1994). The measurements revealed similar defects for knym119 and trim209 mutants compared to their wild-type siblings. However, the convergence defect in double mutants appeared additive.

### Prechordal Plate and hh Expression

Cyclopia is often correlated with a lack or reduction of prechordal mesendoderm (Ekker et al., 1995; MacDonald and Wilson, 1996; Schier et al., 1997; Strähle et al., 1997). Inappropriate location of the prechordal mesendoderm could result in the abnormal positioning of hh-expressing cells in the overlying neuroectoderm. During late gastrulation, expression of hlx1 homeobox gene is detected in the posterior prechordal region of the hypoblast (Fjose et al., 1994), while hhg1 is expressed in the anterior-most prechordal plate (polster) (Thisse et al., 1994). Prechordal plate is present, and the hlx1 expression domain is shorter in knym119 and trim209 mutants (Solnica-Krezel et al., 1996).

To address whether the prechordal plate determines the position of hh expression, we examined hlx1, pax2, dlx3, and hhg1 RNA expression in trim209, knym119, and knym119 trim209 double mutants by simultaneous in situ hybridization.

Examination of hhg1 and dlx3 revealed that the polster was positioned anterior to the neural plate in wild-type (Fig. 5I) and trim209 (Fig. 5K) embryos. However, in knym119 (Fig. 5J), the polster was abnormally localized beneath the neural plate, as was reported for slbBx226 mutant embryos (Heisenberg and Nüsslein-Volhard, 1997), and was partially underlying the neural plate in knym119 trim209 double-mutant embryos (Fig. 5L). There was no simple correlation between hhg1 position and cyclopia, as knym119 embryos were most affected.

Furthermore, this analysis indicated that a gap existed between the hlx1 and dlx3 expression domains for all genotypes analyzed (Figs. 5I–5L). To determine if the distance between hlx1-expressing cells and the anterior edge of the neural plate correlated with cyclopia and shh expression, measurements were taken with Metamorph software as described previously. The hlx-dlx3 gap was 6.0 ± 6.9 μm (n = 40) for wild type, 35.1 ± 13.9 μm (n = 10) for trim209, 40.9 ± 15.5 μm (n = 10) for knym119, and 45.2 ± 7.0 μm (n = 10) for knym119 trim209 embryos. Next, we compared distance changes between dlx3 and hlx1, as well as between dlx3 and shh, at temperatures that suppressed and enhanced cyclopia in trim209 mutants. The shh–dlx3 results were consistent with those described previously in this paper (Fig. 6). These studies revealed no correlation between the position of hlx1 and shh expression at this stage of development. Specifically, at the permissive temperature (32°C), the hlx1–dlx3 gap was larger compared to the normal developmental temperature. This was opposite to the hh–dlx3 gap, which was reduced at the permissive relative to normal developmental temperature. Therefore, it is not likely that the location of the hh-expressing cells at this stage of development is determined by the position of the hlx1-expressing cells within the prechordal mesendoderm.
Cyclopic knym\textsuperscript{119} trim\textsuperscript{209} Mutants Have Altered pax2 and pax6 Expression Patterns

Lack of HH signaling in cyc mutants leads to ectopic expression of the pax6 gene in the median region of the anterior neural plate and to a reduction or absence of the pax2 gene expression (Ekker et al., 1995; Hammer-schmidt et al., 1996a; Macdonald et al., 1995). Failure of HH signaling to reach the anterior region of the neural plate would be expected to produce similar changes in pax gene expression in convergent extension mutants. In wild-type embryos at 24 hpf, pax6 RNA was detected in two separate domains and was absent from the anterior-most region of neuroectoderm (Fig. 7C). However, in knym\textsuperscript{119} trim\textsuperscript{209} mutants pax6 RNA was uniformly distributed throughout the anterior neural keel including the median region normally devoid of pax6 expression (Fig. 7D). In contrast to the two distinct domains observed in wild-type siblings (Fig. 7E), pax2 RNA was expressed in a single domain centered at the midline in the knym\textsuperscript{119} trim\textsuperscript{209} double mutants (Fig. 7F). Therefore, a

![Diagram of gene expression patterns](image-url)
failure to partition the optic primordium leads to cyclopia in these mutants.

**Mutant Embryos Can Respond to Hedgehog Signaling**

Another plausible explanation for the cyclopia phenotype and the abnormalities in pax2 and pax6 gene expression described above is that tri and kny have partially redundant functions required in the neuroectoderm for HH signal reception. To test this possibility synthetic mRNAs encoding shh and twhh were injected into one- to eight-cell-stage embryos of wild-type, tri"m209, kny"m119, and kny"m119 tri"m209 genotypes. Ectopic expression of hh was confirmed by in situ hybridization. Ectopic expression of ptc in response to ectopic hh was observed, as reported by Concordet, indicating that the injected mRNA was functional (data not shown and Concordet et al., 1996). Consistent with earlier reports, the prominent phenotypes were unilateral and bilateral reduction of retina and pigment epithelium in wild-type embryos (Figs. 8A and 8B) (Ekker et al., 1995; Hammer-schmidt et al., 1996a; Macdonald et al., 1995; Ungar and Moon, 1996). Sibling tri"m209, kny"m119, and kny"m119 tri"m209 embryos injected with shh (or twhh) mRNAs exhibited a generally unchanged mutant morphology (data not shown). Therefore, at the injected doses hh mRNA did not rescue or suppress the convergent extension defect in the mutant embryos. Mutant embryos exhibited unilateral or bilateral reduction of retina and pigment epithelium as observed for wild type (Fig. 8; Table 5). Surprisingly, the frequency of

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**FIG. 6.** The shh-dlx3 gap dimensions correlate with the cyclopia phenotype in convergent extension mutants. The dimensions of the shh-dlx3 gap were measured as described under Materials and Methods. Expression was visualized by whole-mount in situ hybridization. The graph shows dimensions in micrometers of the shh-dlx3 gap (above the x axis) and their corresponding CI (below the x axis) for mutants and their wild-type siblings (see Materials and Methods for calculation of CI). Two independent samples of kny"m119 at the permissive temperature are shown, as well as one sample of tri"m209 mutant siblings incubated at permissive temperature (32°C) or restrictive temperature (23°C), and a kny"m119 tri"m209 double-mutant sample. Error bars show standard deviations for each sample.
affected mutant embryos within a clutch was greater than the frequency of affected wild-type embryos (Table 5). Furthermore, pax6 expression was suppressed in wild-type and mutant embryos injected with Shh (Twhh) RNA (data not shown). These results indicated that the anterior neuroectodermal of trim209, knym119, and knym119 trim209 mutant embryos was competent to respond to HH signaling. The eye spacing remained reduced in trim209 mutant embryos injected with hh, and knym119 trim209 mutant embryos remained cyclopic (Figs. 8D and 8E and data not shown). Therefore, ectopic HH signaling affected cell fates, but not the abnormal morphogenesis of the eye field in these mutants.

To further explore the potential of the mutants to respond to HH signaling, the expression of the ptc gene encoding a candidate receptor for Shh was analyzed (Marigo et al., 1996; Stone et al., 1996). Previous studies have reported a correlation between ptc1 mRNA accumulation and neuroectodermal and mesodermal cell response to HH signaling in zebrafish embryos (Concordet et al., 1996). At the one-somite stage, ptc1 RNA was detected in trim209 (Fig. 5G), knym119 (Fig. 5F), and knym119 trim209 (Fig. 5H) double-mutant embryos in a domain adjacent to shh-expressing cells as described previously for wild-type embryos (Fig. 5E). This provided further evidence that cells adjacent to hh-expressing cells could respond to HH signaling. When the expressions of ptc1 and dlx3 were analyzed simultaneously, a gap between the anterior edge of ptc1 and dlx3 expression domains was observed in trim209 (Fig. 5G) and knym119 trim209 (Fig. 5H) double embryos, but not in wild-type (Fig. 5E) or knym119 embryos (Fig. 5F). This indicated that hh-expressing cells could activate ptc1, a target gene, but not in the anterior-most median position of the neural keel.

Taking into account that ptc1 expression reflects the range of shh activity (Chen and Struhl, 1996; Concordet et al., 1996), this observation provides strong support for a failure of the HH signal to reach the anterior region of the neural plate in some trim209 and all knym119 trim209 mutants.

DISCUSSION

Multiple Convergent Extension Genes in Zebrafish Display Overlapping Functions in Eye Morphogenesis

Here we demonstrate that two zebrafish genes, kny and tr, required for normal convergent extension of the embryonic axis during gastrulation, functionally interact during the specification of the eye anlagen. Three alleles of the tri locus, trim209 and trim747 trim770, resulted in a low frequency of synphalmic and cyclopic mutants in the AB genetic background. The penetrance and expressivity of the cyclopia phenotype increased in AB/India genetic background. This could be explained by the presence of dominant enhancer(s) of cyclopia in India background or repressors in AB background. knym119 mutants only infrequently exhibited synphalma in AB/India hybrid background and had normal eye spacing in AB genetic background. However, knym119 trim209 double mutants exhibited cyclopia in AB background. Furthermore, knym119 homozogotes with one allele of trim209 exhibited an increased cyclopia index, indicating that trim209 is an enhancer of cyclopia in knym119 mutants.

The tri locus has also recently been shown to interact with yet another convergent extension gene, silberblick (sib) (Heisenberg and Nüsslein-Volhard, 1997). The trim240

TABLE 4
Morphometric Analysis of Convergent Extension Mutant Embryos

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>Genotype</th>
<th>AP length 1</th>
<th>AP length 2</th>
<th>wt – mΔ</th>
<th>Notochord width</th>
<th>wt – mΔ</th>
<th>dlx3 distance</th>
<th>wt – mΔ</th>
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<tr>
<td>23</td>
<td>Wild type</td>
<td>1257 ± 43</td>
<td>1295 ± 17</td>
<td>47 ± 8</td>
<td>359 ± 71</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>23</td>
<td>trim209</td>
<td>994 ± 52</td>
<td>1016 ± 41</td>
<td>271</td>
<td>60 ± 9</td>
<td>13</td>
<td>485 ± 43</td>
<td>126</td>
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<tr>
<td>32</td>
<td>Wild type</td>
<td>1355 ± 81</td>
<td>1343 ± 88</td>
<td>41 ± 8</td>
<td>344 ± 44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>trim209</td>
<td>1027 ± 57</td>
<td>1021 ± 58</td>
<td>325</td>
<td>59 ± 8</td>
<td>18</td>
<td>415 ± 69</td>
<td>71</td>
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<tr>
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<td>1390 ± 58</td>
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<td>286 ± 35</td>
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<td>knym119</td>
<td>926 ± 109</td>
<td>927 ± 107</td>
<td>459</td>
<td>51 ± 8</td>
<td>8</td>
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<td>1459 ± 69</td>
<td>43 ± 4</td>
<td>276 ± 31</td>
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<tr>
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<td>knym119</td>
<td>966 ± 100</td>
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<td>493</td>
<td>59 ± 12</td>
<td>16</td>
<td>411 ± 48</td>
<td>135</td>
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<tr>
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<td>302 ± 55</td>
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<td>880 ± 39</td>
<td>523</td>
<td>96 ± 13</td>
<td>42</td>
<td>531 ± 47</td>
<td>229</td>
</tr>
</tbody>
</table>

Note. Measurements of embryos at 1 somite stage of development are given in micrometers. Standard deviations represent variations observed in measurements of at least 10 embryos of each genotype. Cyclopia in trim209 is enhanced at low temperature (23°C) and suppressed at 32°C. (See Table 2). Anterior–posterior (AP) length of the embryo was determined by measuring from the tip of the head to the length of the otic vesicles, and the dlx3 distance was measured across the neural plate from the otic vesicle on one side to the otic vesicle on the other side of the embryo. wt – mΔ represents the difference between wild-type and mutant dimensions.
mutant investigated in this work does not manifest cyclo-
pia. However, trtc240 slbtx226 double mutants are cyclo-
pic (Heisenberg and Nüsslein-Volhard, 1997). A possible inter-
action between knym119 and slbtx226 as well as between all
three loci is currently being investigated by the construc-
tion of a knym119 slbtx226 triple mutant line (L.S.K.;
Carl-Philipp Heisenberg and Stephen Wilson, Kings Col-

**Cyclopia in tri and kny tri Double Mutants Results
from Failure to Divide a Single Eye Field**

Morphological observations as well as analysis of gene
expression patterns indicated that cyclopia in tri209 and
kny209 triple mutants was a consequence of a failure to
properly partition the single optic primordium in the ante-
orior region of the developing neural plate. While in wild-
type embryos at the 15-somite stage two distinct retinal
primordia separated in the midline by the telencephalon
anlage were visible; in knym119 tri209 mutants a single
retinal primordium was observed with a bridge of tissue
located anterior to the putative telencephalon. This bridge
type embryos. In
wild-type embryos (A) two separate eye placodes are seen (ey), and the prospec-
tive telencephalon (t) and diencephalon (d) are positioned
between the eyes at the anterior edge of the neural keel (arrow).
In knym119 tri209 mutant embryos (B) one continuous eye placode (ey) is
observed with the prospective telencephalon and diencephalon positioned posterior and dorsal to the forming eye. (C, D) pax6 and (E, F)
pax2 expression visualized by whole-mount in situ hybridization. pax6 expression in wild-type embryos (C) is detected in distal regions of
the anterior neural plate in the future diencephalon and eyes and is absent from the median region of the anterior neural plate (arrow).
knym119 tri209 double mutants have ectopic pax6 expression in the median region of the neural keel (arrow), while expression in the
prospective diencephalon is compressed and located posterior to the eye field. pax2 in wild type is expressed in two optic stalks (os)
separated by the midline; in contrast, one continuous domain is observed in knym119 tri209 double mutants with ectopic expression in the
median region of the anterior neural keel (arrow).

**FIG. 7.** Abnormal morphology of the optic primordia and ectopic expression of pax6 and pax2 genes in the proximal anterior neural plate
in knym119 tri209 embryos during the formation of a cyclopic eye. (A, B) Nomarski images of live embryos at the 16-somite stage. In
wild-type embryos (A) two separate eye placodes are seen (ey), and the prospective telencephalon (t) and diencephalon (d) are positioned
between the eyes at the anterior edge of the neural keel (arrow). In knym119 tri209 mutant embryos (B) one continuous eye placode (ey) is
observed with the prospective telencephalon and diencephalon positioned posterior and dorsal to the forming eye. (C, D) pax6 and (E, F)
pax2 expression visualized by whole-mount in situ hybridization. pax6 expression in wild-type embryos (C) is detected in distal regions of
the anterior neural plate in the future diencephalon and eyes and is absent from the median region of the anterior neural plate (arrow).
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prospective diencephalon is compressed and located posterior to the eye field. pax2 in wild type is expressed in two optic stalks (os)
separated by the midline; in contrast, one continuous domain is observed in knym119 tri209 double mutants with ectopic expression in the
median region of the anterior neural keel (arrow).
It is noteworthy that the developing eye primordium in knym119 trim209 mutants was smaller at 15 somites than in wild-type siblings. This is in contrast to cyc mutants in which the size of the optic primordia at an analogous stage of development does not appear to be significantly affected (Macdonald et al., 1995). Therefore, knym119 trim209 mutants must have patterning or morphogenetic defects in addition to the failure to divide the eye field.

Cyclopia Phenotype in knym119 and trim209 Mutants Is Correlated with the Abnormal Location of Midline Cells, Expressing hh Genes with Respect to the Anterior Neural Plate

The characteristic morphology and the abnormal expression of pax6 and pax2 genes are indicative of defects in HH signaling (Ekker et al., 1995; Macdonald et al., 1995). These could result from the absence of HH signals, their abnormal localization in the embryo, or failure of the target tissue to respond to such signals. Based on our studies we propose that in trim209 and knym119 trim209 mutants the defective partitioning of the eye field is a consequence of the abnormal location of hh-expressing cells with respect to the developing eye primordium, such that the signal cannot reach appropriate target cells. First, we provided evidence that both shh and twhh RNAs are expressed in the cyclopic mutants. Second, we demonstrated that trim209, knym119, and knym119 trim209 mutants responded to ectopic expression of twhh and shh RNAs. pax2 and ptc were upregulated in embryos ectopically expressing hh, while pax6 was downregulated, and retina and pigment epithelia were reduced. Furthermore, the mutant embryos expressed ptc1 RNA in cells neighboring the shh expression domain. These observations indicate that trim209, knym119, and knym119 trim209 mutants respond normally to HH signaling. Our studies point to abnormal localization of cells expressing hh and/or other signals as the primary defect underlying the cyclopia phenotype.

We demonstrated that in the cyclopic mutants the expression domains of the shh and twhh genes were shortened in the AP dimension at the onset of somitogenesis. Furthermore, a gap was observed between the anterior edge of shh (and twhh) in the midline and dlx3 delimiting the anterior edge of the neural plate. Notably, the dimension of the gap correlated with the degree of cyclopia in mutant embryos. We hypothesize that when the dimension of the gap exceeds 40–50 μm, the HH and/or additional signals fail to reach the target tissue resulting in cyclopia.

In imaginal discs of Drosophila larvae, HH was shown to have a limited range of action (Chen and Struhl, 1996). It has been proposed that HH limits its own ability to move through tissue by upregulating its receptor, Ptc, in responsive cells. In this model, HH secreted by signaling cells binds to Ptc or the Patched–Smoothened (Ptc Smo) receptor complex expressed at low levels in neighboring cells (Stone et al., 1996). This alleviates the inhibition of Smo signaling.
activity by Ptc and results in the upregulation of Ptc expression. High levels of Ptc in response to HH signaling would sequester the diffusing HH, effectively limiting its range of action. Thus, the regions of higher levels of Ptc adjacent to a HH source in the embryo would reveal the limits of HH signaling. It is very likely that the same mechanism limits the ability of vertebrate HH to move through tissue (Concordet and Ingham, 1994). In cyclopic convergent extension mutants, but not in wild type or convergent extension mutants that did not exhibit cyclopia, a gap was observed between the anterior border of the ptc1 and dlx3 expression domains. This supports the notion of HH signals not reaching the anterior-most region of the neural plate in cyclopic mutants.

Is the above-hypothesized failure of HH signals to reach target cells the only defect underlying the cyclopia phenotype in trilm209 and knym119 trielm209 mutants? Abnormally positioned hh-expressing cells in the mutants may also be secreting other factors involved in eye field separation. It is conceivable that these factors are also displaced in cyclopic convergent extension mutants; therefore, exposing the anterior neural plate to hh alone would not be sufficient to suppress the cyclopia phenotype. Another possibility is that the midline cells may be required to physically separate the eye field. In support of this view, recent fate-mapping experiments indicate that in zebrafish the ventral diencephalic precursors physically invade and thus partition the eye field. In support of this view, recent fate-mapping experiments indicate that in zebrafish the ventral diencephalic precursors physically invade and thus partition the eye field (Woo and Fraser, 1995; K. Woo and S. Fraser, California Institute of Technology, Pasadena, CA, and Z. Varga and M. Westerfield, University of Oregon, Eugene, OR, personal communication). Therefore, ectopic HH activity would fail to partition the eye field because the midline cells would remain abnormally located and would not separate the eye field in mutant embryos. In trilm209 and knym119 trielm209 mutants at 24 hpf the anterior compression of the shh expression domain indicates that the ventral diencephalon does not form in an appropriate position and therefore could not split the eye field either physically or through a midline-mediated signaling process, resulting in cyclopia. It remains to be determined whether the location of the diencephalon is a consequence of the abnormal location of its precursors at earlier stages of development.

Convergent Extension and Eye Patterning

What causes the abnormal location of the hh-expressing cells in cyclopic convergent extension mutants? One possibility is that the location of shh and twhh expression domains results from abnormal prechordal mesendoderm position with respect to the overlying neuroectoderm. A convergent extension defect within the prechordal plate could result in its more posterior location. As a consequence, genes activated by the prechordal plate in the overlying neuroectoderm would also occupy a more posterior position. Indeed, at the end of the gastrula stage, the posterior prechordal plate is shorter in the AP axis and broader in the mediolateral direction in trielm209 mutants as indicated by the hlx1 expression pattern (Solnica-Krezel et al., 1996). Here, we demonstrated no positional correlation between hlx1 and shh expression domains at the one-somite stage. A similarly altered shape is observed with Fkd2 expression in the prechordal plate of slb mutants (Heisenberg and Nüsslein-Volhard, 1997). Fkd2-expressing cells are not likely to be involved since their location is not correlated with shh (Heisenberg and Nüsslein-Volhard, 1997). It is possible that induction by the prechordal plate occurs at an earlier developmental stage such that the position of the prechordal plate and genes activated in the neuroectoderm may not positionally correlate following the onset of morphogenetic movements. Alternatively, a cell population within the prechordal plate other than hlx1 could be involved. BMP7 in the rat embryo has been shown to cooperate with HH in the prechordal mesoderm to induce ventral midline cell differentiation (Dale et al., 1997). In addition, cyclops, a nodal-related gene, has recently been shown to be required for normal prechordal plate development and induction of ventral fates in the CNS (Sampath et al., 1998). One would predict that, like HH, such molecules would be more posteriorly located in cyclopic trielm209, knym119, and knym119 trielm209 mutant embryos.

Another possibility is that the gap between the hh and dlx3 expression domains could result from defective convergent extension movements of ectodermal cells expressing these genes. In this view, the mediolateral narrowing
and AP extension of the hh expression domain, driven by the convergent extension movements of the entire population of midline neuroectodermal cells expressing hh, contributes to an anteriorward movement of hh-expressing cells relative to the surrounding neural plate and consequently to the partitioning of the eye field. As discussed previously, such anteriorward movement of midline cells has been observed in fate-mapping experiments (Woo and Fraser, 1995; K. Woo and S. Fraser, California Institute of Technology, Pasadena, CA, and Z. Varga and M. Westerfield, University of Oregon, Eugene, OR, personal communication). As shown in Xenopus embryos, a reduced rate of mediolateral intercalation could lead to altered expression domains for a number of genes (Shih and Keller, 1992). In knym119 and trim209 mutants the shh expression domain was not only shorter in the AP direction but also broader in the mediolateral direction. Initial analysis of gastrulation movements in trim209, knym119 and knym119 trim209 mutants demonstrated that convergent extension of mesodermal tissues and neuroectoderm was slower in trim209 and knym119 mutants relative to wild-type embryos and dramatically reduced in knym119 trim209 double mutants (L.S.K. and F.M., unpublished observations). slbtx226 cells occupy a more posterior position than their wild-type counterparts at 15 hpf. However, no difference in midline neuroectodermal cell migration during gastrulation was detected after transplantation of slbtx226 mutant and wild-type cells (Heisenberg and Nüsslein-Volhard, 1997).

It is noteworthy that we observed no simple relationship between the degree of the overall convergent extension defect and cyclopia in trim209 and knym119 mutants. knym119 mutants exhibited a shorter AP axis and shh expression domain at the onset of somitogenesis, as well as at 1 dpf, than trim209 mutants. Yet, cyclopia and the shh–dlx3 gap were more prominent in trim209 mutants. We hypothesize that the abnormal location of the hh expression domain with respect to the anterior neural plate, and consequently cyclopia, arises when morphogenetic movements of hh-expressing cells (or movements of the prechordal plate inducing them) are not coordinated with the morphogenesis of the anterior neuroectoderm. Therefore, the overall convergent extension defect would not lead to cyclopia (e.g., knym119 mutants) as long as the appropriate position of cells expressing hh with respect to the anterior edge of the neural plate is maintained. Such failure to coordinate the morphogenetic movements of cells that need to interact during eye development might reflect region-specific requirements for trim209 and knym119 function within the embryo.

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