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The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail*

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SUMMARY

The zebrafish locus *one-eyed pinhead* (*oep*) is essential for the formation of anterior axial mesoderm, endoderm and ventral neuroectoderm. At the beginning of gastrulation anterior axial mesoderm cells form the prechordal plate and express *goosecoid (gsc)* in wild-type embryos. In *oep* mutants the prechordal plate does not form and *gsc* expression is not maintained. Exposure to lithium, a dorsalizing agent, leads to the ectopic induction and maintenance of *gsc* expression in wild-type embryos. Lithium treatment of *oep* mutants still leads to ectopic *gsc* induction but not maintenance, suggesting that *oep* acts downstream of inducers of dorsal mesoderm. In genetic mosaics, wild-type cells are capable of forming anterior axial mesoderm in *oep* embryos, suggesting that *oep* is required in prospective anterior axial mesoderm cells before gastrulation.

The *oep* gene is also essential for endoderm formation and the early development of ventral neuroectoderm, including the floor plate. The loss of endoderm is already manifest during gastrulation by the absence of *axial*-expressing cells in the hypoblast of *oep* mutants. These findings suggest that *oep* is also required in lateral and ventral regions of the gastrula margin. The *sonic hedgehog (shh)*.gene is expressed in the notochord of *oep* animals. Therefore, the impaired floor plate development in *oep* mutants is not caused by the absence of the floor plate inducer *shh*. This suggests that *oep* is required downstream or in parallel to *shh* signaling. The ventral region of the forebrain is also absent in *oep* mutants, leading to severe cyclopia. In contrast, anterior-posterior brain patterning appears largely unaffected, suggesting that underlying prechordal plate is not required for anterior-posterior pattern formation but might be involved in dorsoventral brain patterning.

To test if *oep* has a wider, partially redundant role, we constructed double mutants with two other zebrafish loci essential for patterning during gastrulation. Double mutants with *floating head*, the zebrafish *Xnot* homologue, display enhanced floor plate and adaxial muscle phenotypes. Double mutants with *no tail (ntl)*, the zebrafish homologue of the mouse *Brachyury* locus, display severe defects in midline and mesoderm formation including absence of most of the somitic mesoderm. These results reveal a redundant function of *oep* and *ntl* in mesoderm formation. Our data suggest that both *oep* and *ntl* act in the blastoderm margin to specify mesendodermal cell fates.

Key words: prechordal plate, endoderm, floor plate, cyclopia, forebrain, *goosecoid, sonic hedgehog, no tail*

INTRODUCTION

A series of inductive interactions pattern the early vertebrate embryo. By the end of gastrulation the three germ layers are defined and both mesoderm and ectoderm become patterned along the anterior-posterior and dorsoventral axis. Studies in frogs have shown that this process is initiated by maternally provided signaling molecules that are located in the vegetal, presumptive endodermal region of the early embryo (Nieuwkoop, 1969; reviewed by Slack, 1994; Smith, 1995). These signals induce both mesoderm formation and an organizing center, known as the Spemann organizer, in the dorsal mesoderm (Spemann, 1938; Hamburger, 1988). The organizer has a dual role in mesoderm formation. It develops into the axial mesoderm, consisting of prechordal plate anteriorly and notochord posteriorly, and it is a source of dorsalizing signals that pattern the dorsoventral axis of the adjacent mesoderm. This process leads to the characteristic vertebrate gastrula fate map, with axial mesoderm being the most dorsal tissue type, and paraxial mesoderm (developing into somites), intermediate mesoderm (pronephros) and lateral plate mesoderm (blood) being more lateral and ventral mesodermal derivatives (Keller, 1976; Dale and Slack, 1987; Kimmel et al., 1990).

The organizer and its derivatives are also involved in the induction and patterning of the neuroectoderm (Spemann and

Mangold, 1924; Smith and Slack, 1983; reviewed by Ruiz i Altaba and Jessell, 1993; Doniach, 1995). Planar signals from the organizer and/or vertical signals from the underlying axial mesoderm are thought to neuralize the dorsal ectoderm and initiate anterior-posterior regionalization in the neuroectoderm. The notochord is subsequently involved in the induction of ventral cell types such as floor plate in the overlying neuroectoderm (van Straaten et al, 1988; van Straaten and Hekking, 1991; Placzek et al., 1990). Factors like noggin (Lamb et al., 1993), chordin (Sasai et al., 1994; Holley et al., 1995), and members of the TGFβ (Rebagliati et al., 1985; Kessler and Melton, 1994), FGF (Slack et al., 1988; Kimelman et al., 1992), wnt (Moon, 1993) or hedgehog (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ingham, 1995) families are candidate signaling molecules that mediate the inductive events in mesodermal and neural patterning.

Several genes have been identified that are expressed in the organizer region in response to mesodermal patterning signals. Most prominently, the putative transcription factors *Brachyury* (Hermann et al., 1990; Smith et al., 1991; Beddington et al., 1992; Herrmann and Kispert, 1994), *goosecoid* (Blumberg et al., 1991; Cho et al., 1991), *Pintallavis* and *HNF3β* (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992; Knoechel et al., 1992; Strähle et al., 1993; Ang et al., 1993; Ruiz i Altaba et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993), *lim1* (Taira et al., 1992) and *Xnot* (von Dassow et al., 1993) are activated as a response to mesoderm inducers. These genes are thought to execute or control the embryonic patterning initiated by inductive signals. Mutational analysis in the mouse has indicated that *lim1* (Shawlot and Behringer, 1995), *otx2* (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), *HNF3β* (Ang and Rossant, 1994; Weinstein et al., 1994) and *Brachyury* (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Herrmann and Kispert, 1994), but not *gsc* (Rivera-Perez et al., 1995; Yamada et al., 1995), are required during early development. All four mutants display serious deficits in the formation of the organizer and/or axial mesoderm. *Brachyury* mutants have a severe posterior truncation of the axis and lack a differentiated notochord. Head formation appears normal. *HNF3β* mutants do not form a distinct node (the mouse equivalent of Spemann organizer), lack a notochord and show variable head defects. Both *otx2* and *lim1* mutant mice lack structures anterior to hindbrain rhombomere 3, including the prechordal plate, midbrain and forebrain, and notochord development appears to be incomplete in some *otx2* mutant embryos.

In zebrafish, three loci have been shown to affect the formation of axial tissues, *floating head* (*flh*), *no tail* (*ntl*), and *cyclops* (*cyc*). Mutants of the *flh* gene show defects in notochord formation during midgastrulation (Talbot et al., 1995). Instead of maintaining their axial identity, *flh* mutant cells in the midline develop into paraxial muscle (Halpern et al., 1995). Molecular analysis has identified *flh* as a mutation in the zebrafish homologue of the *Xenopus Xnot* homeobox gene (Talbot et al., 1995). The *ntl* locus is the zebrafish homologue of the mouse *Brachyury* gene (Schulte-Merker et al., 1992; Halpern et al., 1993; Schulte-Merker et al., 1994). It is first expressed in the entire marginal region, and later in notochord and tail bud. Notochord precursors in *ntl* mutants appear to be present but do not differentiate properly and retain a mesenchymal appearance. Furthermore, tail formation is impaired in *ntl* mutants. Embryos mutant for the *cyc* locus display deficits in the formation of ventral neuroectoderm, leading to eye fusion and reduction of floor plate (Hatta et al., 1991, 1994). Additionally, a reduced number of hatching gland cells, and lower levels of *gsc* expression indicate that prechordal plate formation is also weakly affected in *cyc* mutants (Thisse et al., 1994).

Although less well understood than mesodermal and neuroectodermal induction and patterning, endoderm formation might rely on the same or related signals that induce mesoderm (Asashima et al., 1991; Jones et al., 1993; Cornell et al., 1995; Gamer and Wright, 1995; Henry et al., 1996). In particular, exposure of animal caps to high concentrations of mesoderm inducers like activin can also activate endoderm specific markers (Rosa, 1989; Cornell et al., 1995; Gamer and Wright, 1995). Furthermore, injection of a dominant-negative activin receptor construct inhibits the expression of some endoderm markers (Gamer and Wright, 1995; Henry et al., 1996). The zygotic downstream responses to endodermal inducers are virtually unknown. Few genes like *Mix1* (Rosa, 1989), *Xlhbox-8* (Wright et al., 1988) or members of the *HNF3* (Ang et al., 1993; Ruiz i Altaba et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993) family are expressed as an early response to endoderm formation. Genetic analysis has not yet identified any mutants that disrupt the development of the entire endoderm.

In genetic screens for additional genes that function during vertebrate pattern formation, we have discovered the *one-eyed pinhead (oep)* locus in zebrafish (Schier et al., 1996; Solnica-Krezel et al., 1996). The *oep* gene is essential for the development of several regions of the embryo, including prechordal plate, endoderm and ventral neuroectoderm. The analysis of mutant phenotypes suggest a role for the prechordal plate in dorsal-ventral but not anterior-posterior patterning of the brain. Double mutant analysis reveals a partially redundant requirement for *oep* and *ntl* in mesoderm formation.

MATERIALS AND METHODS

Strains

Fish and embryos were maintained as described by Solnica-Krezel et al. (1994) and Westerfield (1994). *oep*^{m134} (Schier et al, 1996; Solnica-Krezel et al., 1996) was isolated in the progeny of ENU-mutagenized fish from an AB background (Chakrabarti et al., 1983). *oep*^{z1} was identified in the progeny of gamma-ray mutagenized fish from an EK background (Ekk Will Waterlife Resources, Florida; Helde et al., 1994). Both alleles segregate at Mendelian ratios: 594/2318 embryos were *oep*^{m134} (25.6%); 90/354 embryos were *oep*^{z1}(25.4%); 227/883 embryos were *oep*^{m134}/*oep*^{z1} (25.7%). Both mutations are subject to modifiers in different genetic backgrounds. Mutant phenotypes are generally stronger (as judged from strong eye fusion or absence of eyes, notochord defects, absence of heart muscle) in AB and TU (Mullins et al., 1994) backgrounds, and weaker (partial eye fusion, normal notochord, formation of some hatching gland cells and heart muscle) in India (Knapik et al., 1996) or HK (Stainier et al., 1995) backgrounds. Additional *oep* alleles have been identified (Hammerschmidt et al., 1996; Strähle et al., personal communication; Kimmel et al., personal communication). Double mutants were constructed by crossing *oep*/+ heterozygous fish to *ntl*^{b160}/+ (Halpern et al., 1993) or *flh*^{n1}/+ (Talbot et al., 1995) heterozygous fish. All three loci are unlinked. *oep*/+;*flh*/+ and *oep*/+;*ntl*/+ heterozygous fish were identified by test crosses and interbred to create homozygous double mutant embryos. Mutant loci segregate in a Mendelian fashion; crosses of *oep*/+;*ntl*/+ heterozygous fish yield 699 wild-type: 251 *oep*: 220 *ntl*: 86 *oep ntl* embryos (= 8.1: 2.9: 2.6: 1.0); crosses of *oep*/+;*flh*/+ heterozygous fish yield 848 wild-type: 299 *oep*: 304 *flh*: 102 *oep flh* embryos (= 8.3: 2.9: 3.0: 1.0).

Mapping

RAPD PCR assays were performed as described by Postlethwait et al. (1994). In initial experiments, the RAPD marker 15AH.500 was found to be linked to *oep*. Since RAPD markers are strain-specific and therefore not informative in all mapping crosses, a sequence-tagged site (STS) marker for the 15AH.500 locus was generated by cloning and sequencing the RAPD fragment and synthesizing primers specific for that sequence (primer WTZ97, 5′-ACTTGCAGGAGTGGATCTGAC, and primer WTZ106, 5′-CACAAAAACACCATCTGACC). In the *oep* mapping crosses, all of the animals display an allele of the same size, but a polymorphism was evident when the amplification products were cleaved with *Taq*I (Fig. 2A). We scored the 15AH.500 STS in 85 haploid embryos in oep^{m134} mapping crosses, and found no recombinants (0±2.3 cM; Fig. 2). To determine the location of 15AH.500 on the genetic map, we scored the STS in the linkage map cross of Johnson et al. (1996). These primers detect codominant alleles, differing in size by a few nucleotides, of the 15AH.500 locus. The 15AH.500 STS, and therefore *oep*, maps to one end of LG 10 (Fig. 2B). The observation that the 20K.875 RAPD marker, previously mapped to LG 10 (Postlethwait et al., 1994; Johnson et al., 1996), is linked to *oep* (14 recombinants among 85 individuals) confirms this assignment. To test if oep^{z1} might represent a deletion of the region of the *oep* locus, PCR was performed using 12 different primers and 12 different primer pair combinations of the 15AH.500 locus. No amplification products cosegregating with *oep* were found in oep^{z1} mutant animals (data not shown). We have recently identified another marker that is deleted in oep^{z1} mutants, as judged from PCR analysis and Southern blot hybridization (J. Zhang, W. S. T. and A. F. S., unpublished results).

Phenotypic analysis

In vivo observations, in situ hybridization, and histological analysis were performed as described previously (Jowett and Lettice, 1994; Westerfield, 1994; Schier et al., 1996). Dorsalization by exposure to LiCl was performed as described by Stachel et al. (1993).

Cell transplantation

Genetically mosaic embryos were generated using cell transplantation techniques (Ho and Kane, 1990; Halpern et al., 1993). Donor embryos were injected with a mixture of lineage tracer dyes (5% rhodamine dextran (10 kDa) and 5% lysine-fixable biotin dextran (10 kDa; Molecular Probes) between the 1- and 8-cell stage. At midblastula stages 5-50 labeled cells were transplanted into isochronic host embryos. To guarantee full expressivity of the *oep* phenotype, *oep* mutant donors or hosts were derived from fish heterozygous for *oep* in a AB/TU genetic background. In the assay for hatching gland formation, host embryos were allowed to develop until 28 hpf, analyzed using fluorescence microscopy, and fixed in 4% paraformaldehyde. Hatching gland cells were identified by their size, granular morphology and their location over the yolk anterior and ventral to the head. Biotin-dextran labeled donor cells were detected using the ABC-peroxidase kit (Vector Laboratories, Inc.). Subsequently, embryos were processed for in situ hybridization with digoxigenin-labeled *hgg1* (Thisse et al., 1994) riboprobes, and alkaline phosphatase coupled anti-digoxigenin antibodies were used to assay for *hgg1* expression in hatching gland cells. When the peroxidase reaction is performed before in situ hybridization, the precipitate of the peroxidase reaction precludes the detection of *hgg1* in biotin-dextran labeled cells. Therefore, host hatching gland cells (blue) could be distinguished unambiguously from donor hatching gland cells (brown). In the assay for *gsc* expression, donor and host embryos were treated with LiCl at the 256- to 512-cell stage (Stachel et al., 1993), and fixed at 70-80% epiboly in 4% paraformaldehyde. In situ hybridization using *gsc* riboprobes was performed as described above. Embryos were then processed to detect biotin-dextran labeled donor cells using the ABC-peroxidase kit. The precipitate of the alkaline phophatase reaction does not preclude the detection of biotin-dextran labeled cells. Therefore, donor cells expressing *gsc* could be identified unambiguously as brown cells with a bluish cytoplasmic halo.

RESULTS

Isolation and mapping of the *one-eyed pinhead* locus

We have isolated two *one-eyed pinhead (oep)* alleles in genetic screens for zygotic mutations affecting zebrafish embryogenesis (Fig. 1). Allele oep^{m134} was discovered in a screen of ENU-induced mutations (Driever et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996), and oep^{z1} was isolated in a screen using gamma-rays (K. A. H. and D. Grunwald, unpublished results). Both alleles segregate as Mendelian recessive embryonic lethal mutations and display similar phenotypes (Fig. 1; see below). In addition, the gamma-ray induced oep^{z1} allele displays severe general degeneration starting at the end of the segmentation period (Fig. 1C). Transheterozygous oep^{m134}/oep^{z1} embryos show all the characteristic *oep* phenotypes but do not express the general degeneration of oep^{z1} (Fig. 1D,H).

Linkage of the RAPD marker 15AH.500 with the oep^{m134} mutation allowed mapping of the *oep* locus to one end of linkage group 10 (Fig. 2). 15AH.500 DNA is absent in oep^{z1} mutant animals (data not shown; see Materials and Methods). This result, together with the gamma-ray induced origin of oep^{z1} and the general degeneration phenotype associated with oep^{z1}, but not oep^{m134} or oep^{m134}/oep^{z1}, suggests that oep^{z1} is a deletion of the *oep* locus and one or more linked essential genes.

Essential role for *oep* in the formation of prechordal plate mesoderm

The *oep* mutant phenotype was first characterized on days 2 and 3 of embryogenesis by morphological and histological analysis, and the study of marker gene expression. Analysis of mesodermal derivatives shows that *oep* is essential for the formation of hatching gland (Figs 1L, 3B) and eye muscles (Fig. 3D) as judged from the expression of *hgg1* (Thisse et al., 1994) and *myoD* (Weinberg et al., 1996), respectively. Both structures have been proposed to derive at least in part from prechordal plate mesoderm, the anterior-most axial mesoderm (Adelmann, 1932; Jacob et al., 1984; Wachtler et al., 1984; Kimmel et al., 1990; Thisse et al., 1994; Inohaya et al., 1995; Kimmel et al., 1995). A minority of *oep* mutant embryos also show defects in the formation of notochord, the more posterior axial mesoderm (Fig. 1M-T). In particular, the notochord has a wavy, thinner appearance (Fig. 1T) or is absent most anteriorly in some *oep* mutants (Fig. 1P). Formation of somites, skeletal muscle, pronephros, and blood is not overtly affected in *oep* mutants. In a few cases the notochord is absent in the tail resulting in somite fusion ventrally.

To determine when *oep* functions, we analyzed the formation of the prechordal plate in living embryos and the expression of marker genes during gastrulation. The prechordal plate is clearly visible during midgastrulation as a knob-like structure constituting the anterior axial mesoderm (Kimmel et al., 1995). In *oep* mutant embryos this structure is not visible (Solnica-Krezel et al, 1996). During segmentation stages, hatching gland precursors form the polster, the anterior-most prechordal plate structure, in wild-type embryos, but are absent or strongly reduced in *oep* mutants (data not shown). Furthermore, expression of *islet1* (Fig. 3F; Korzh et al., 1993; Inoue et al., 1994) and *hgg1* (Fig. 3H; Thisse et al., 1994), markers for anterior prechordal plate, is absent or strongly reduced in *oep* mutant animals at the end of gastrulation. Markers that are expressed in the posterior prechordal plate region, such as *hlx1* (Fig. 3J; Fjose et al., 1994) and *gsc* (see below) are also affected in *oep* mutants. Correspondingly, the expression of *axial* (Strähle et al., 1993) and *shh* (Krauss et al., 1993), two genes expressed in both the developing prechordal plate and ventral neuroectoderm is reduced anteriorly (Fig. 3L, and data not shown). These studies demonstrate that prechordal plate development is impaired in *oep* mutants by the end of gastrulation.

Maintenance of *goosecoid* expression requires *oep*

The homeobox gene *gsc* (Blumberg et al., 1991; Cho et al., 1991; DeRobertis et al., 1992) represents the earliest specific marker for prechordal plate development during gastrulation (Fig. 4). *Gsc* is activated in dorsal mesoderm as a zygotic response to maternal dorsalizing agents like activin, Vg1 or wnt family members shortly after midblastula transition (Cho et al., 1991). In zebrafish, *gsc* is expressed in the developing shield, the region corresponding to the organizer in fish (Stachel et al., 1993; Schulte-Merker et al., 1994; Thisse et al., 1994). At the onset of gastrulation *gsc* expression is restricted to cells of the anterior-most axial mesoderm, the prechordal plate. Expression of *gsc* is correctly initiated in both *oep* and wild-type animals before gastrulation (Fig. 4A,B). At the beginning of gastrulation and thereafter, however, *gsc* expression is absent or strongly reduced in *oep* mutant embryos (Figs. 4C-H). These data suggest that *oep* is required for the development of the prechordal plate before or at the onset of gastrulation.

The finding that *gsc* expression is activated but not maintained in *oep* mutants suggests that *oep* acts downstream of mesoderm inducers as a maintenance factor for *gsc* expression or *gsc*-expressing cells. One prediction of this model is that exposure of *oep* mutants to dorsalizing agents would initiate ectopic *gsc* expression which would not be maintained because of the later requirement for *oep*. Alternatively, exposure of dorsalizing agents might induce *gsc* and other genes and bypass the requirement for *oep*. Exposure to lithium ions, a dorsalizing agent before midblastula transition, leads to the ectopic activation of *gsc* in ventral and lateral cells in wild-type

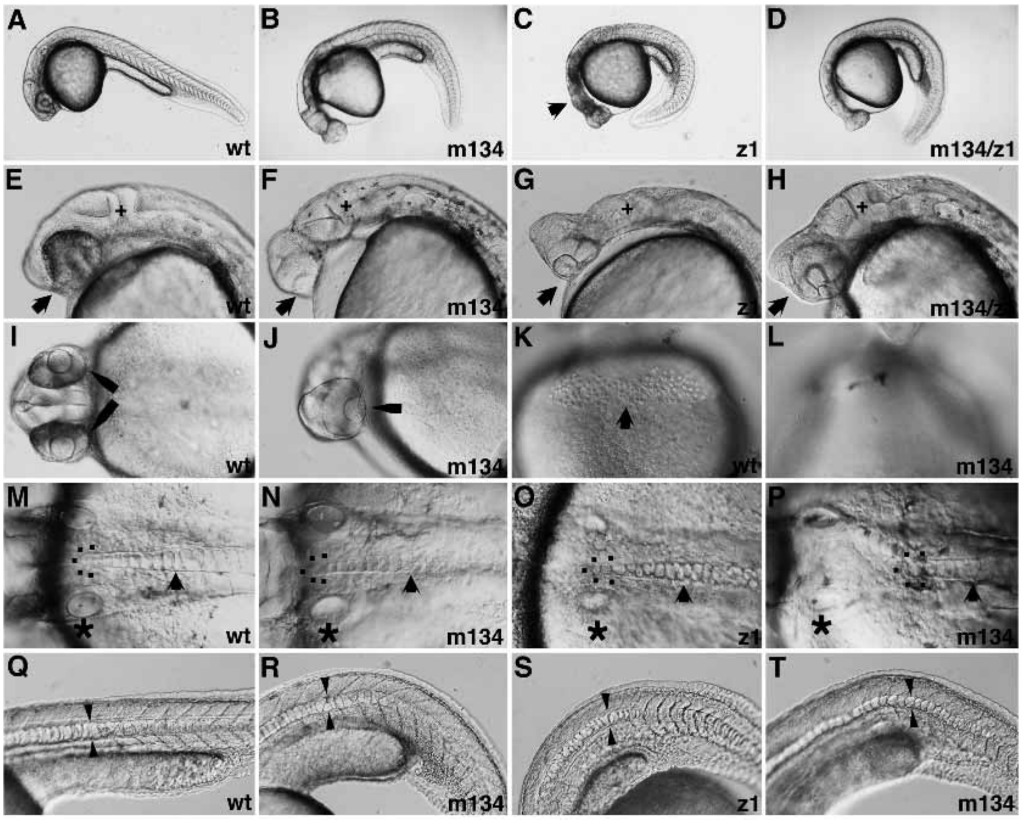

Fig. 1. Phenotype of oep^{m134} and oep^{z1} mutant embryos. (A-D) Comparison of wild-type (A), homozygous oep^{m134} (B), homozygous oep^{z1} (C) and transheterozygous oep^{m134}/oep^{z1} (D) embryos at 27-28 hpf in a lateral view. Here and in all other figures anterior is to the left and dorsal is up, except where indicated otherwise. Note the general degeneration of the head region of homozygous oep^{z1} mutant embryos (arrow in C) but not homozygous oep^{m134} or transheterozygous oep^{m134}/oep^{z1}. (E-H) Comparison of the head region of wild-type (E), homozygous oep^{m134} (F), homozygous oep^{z1} (G) and transheterozygous oep^{m134}/oep^{z1} (H) embryos at 27-30 hpf in a lateral view. Note the anterior-medial location of the single eye in *oep* mutants (arrow). + indicates the location of the midbrain-hindbrain boundary. (I,J) Ventral view of the head region of wild-type (I) and oep^{m134} mutant (J) embryos at 27-29 hpf. Arrows highlight the location of the two eyes in wild-type and the single median eye in *oep* mutant embryos. (K,L) Hatching gland formation over the yolk in wild-type (arrow in K) but not oep^{m134} mutants (L) at 38 hpf. Ventral view, anterior is up. (M-T) Notochord formation in wild-type (M,Q), oep^{m134} (N,P,R,T) and oep^{z1} (O,S) mutant embryos at 27-30 hpf. (M-P) Dorsal view of the anterior-most region of the notochord (outlined by dots). Note that the anterior extent of the notochord is normally found next to the otic vesicle (indicated by stars). In some *oep* mutants the anterior end of the notochord is located more posteriorly (P). (Q-T) Lateral view of the notochord in the trunk and tail region. Arrows outline the borders of the notochord. The floor plate and neurocoel lie directly dorsal to the notochord. Note the wavy and thinner appearance of the notochord in some *oep* mutants (T).

zebrafish (Stachel et al., 1993).We find that *gsc* expression is radially induced in lithium-treated *oep* embryos but cannot be maintained (Fig. 4I-L). This is consistent with a model in which *oep* acts downstream of dorsalizing signals and early factors that initiate *gsc* expression.

Autonomous function of *oep* during prechordal plate formation

The above analyses indicate that *oep* is required for the formation of the prechordal plate before or at the onset of gastrulation. Where does *oep* function in this process? One possibility is that *oep* acts non-autonomously, perhaps being required for the formation of a signal(s) that controls prechordal plate development. Alternatively, it could be required cell-autonomously in prechordal plate precursors. To distinguish between these possibilities, we transplanted labeled wild-type cells into mutant hosts and vice versa, and assayed for the formation of hatching gland cells (Fig. 5A-C). Wild-type cells are capable of forming hatching gland in *oep* mutants (Fig. 5C), but we never observed the formation of hatching gland cells by mutant cells in wild-type hosts (see Fig. 5 legend). Furthermore, the wild-type cells in *oep* mutants are not able to induce or recruit mutant host cells to form hatching gland (Fig. 5C). These data demonstrate that *oep* acts strictly cell-autonomously in the prechordal plate precursors of the hatching gland. We have extended these observations by transplanting cells of lithium-treated wild-type donors into lithium-treated mutant hosts and then assaying for maintenance of *gsc* expression at midgastrula stages. Consistent with the previous transplantation experiments, we find that wild-type cells are able to express and maintain *gsc* in mutant animals (Fig. 5D-F). These results demonstrate that *oep* acts cell-autonomously in prechordal plate progenitors.

Essential role for *oep* in endoderm formation

Studies in frogs suggest that the prechordal plate gives rise not only to prechordal plate mesoderm but also to pharyngeal endoderm (Keller, 1976; Shih and Keller, 1992). Since it is assumed that cells expressing *gsc* at the onset of gastrulation are fated to give rise to both prechordal plate mesoderm and pharyngeal endoderm (DeRobertis et al., 1992), the loss of *gsc* expression in *oep* mutants prompted us to determine if pharyngeal endoderm is also affected in *oep* mutants. As expected, we find that *axial* and *shh* expression (Strähle et al., 1993, Krauss et al., 1993) in the pharyngeal endoderm of *oep* mutants is absent or strongly reduced at 51 hpf (Fig. 6B,F). Surprisingly, *shh* expression, which is found throughout the entire endoderm of wild-type embryos (Fig. 6A,C), is also strongly reduced in the posterior of *oep* mutants (Fig. 6D). This demonstrates that *oep* not only affects the formation of pharyngeal endoderm, but also more posterior endodermal structures, consistent with the observation that the gut is absent or strongly reduced in *oep* mutants (Fig. 6H).

To determine when the endoderm is abnormal, we analyzed the expression of *collagen type II* (Yan et al., 1995) during the segmentation period. We find that *collagen type II* expression in the presumptive endoderm is strongly reduced in *oep* mutants at the 12-somites stage (Fig. 6J). Fate mapping studies have shown that endodermal precursor cells are located around the entire margin of the early gastrula (Kimmel et al., 1990; Warga, 1996), and after involuting acquire a flat morphology before finally reaching the dorsal midline (Warga, 1996). We have found that during gastrulation, the *axial* gene (Strähle et al., 1993) appears to be expressed in all or a subset of these endodermal precursor cells positioned in close proximity to the yolk cell (Fig. 6M-O). This is consistent with the endodermal expression of HNF3-α and -β in other species (Ang et al., 1993; Ruiz i Altaba et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993). To determine if *oep* functions early in endoderm formation, we examined the expression of *axial* during gastru-

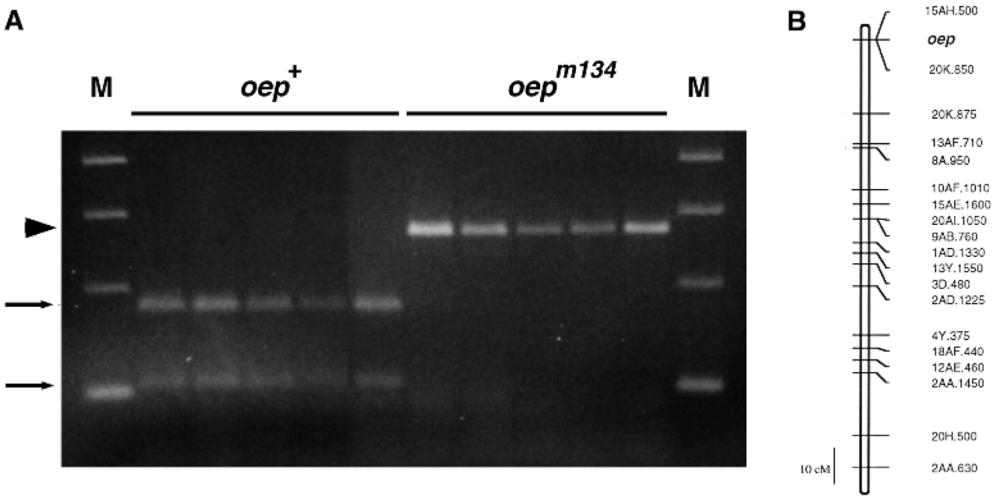

Fig. 2. Mapping of the *oep* locus. (A) Cosegregation of a polymorphism in the 15AH.500 sequence-tagged site (STS) marker with the *oep*^{m134} locus. DNA was prepared from individual haploid embryos derived from a *oep*^{m134}/+; AB/India female. The 15AH.500 STS marker was amplified and incubated with *Taq*1. *Taq*1 cuts the 15AH.500 STS marker in wild-type individuals (arrows; lanes 2-6 *oep*$^{+}$) but not in *oep*^{m134} siblings (arrowhead; lanes 7-11 *oep*^{m134}). Size standards in marker lane (M) are 400, 300, 200, and 100 bp. (B) Genetic map of linkage group 10 (LG 10; from Postlethwait et al, 1994; Johnson et al, 1996) showing the position of 15AH.500, 20K.850, and *oep*. The 15AH.500 STS was scored in 85 haploid embryos in *oep*^{m134} mapping crosses, and no recombinants were found (0±2.3 cM). The 20K.875 RAPD marker, previously mapped to LG 10 (Postlethwait et al., 1994; Johnson et al., 1996), was also found to be linked to *oep* (14 recombinants among 85 individuals). The RAPD marker 20K.850 was found to be tightly linked to *oep* (0 recombinants among 85 individuals, 0±2.3 cM) in India × *oep*^{m134} mapping crosses. This marker was not informative in the linkage map cross (Johnson et al., 1996), and the map position shown in B is inferred from its proximity to *oep*. The finding that *oep* is located at one end of LG10 is consistent with the segregation of *oep*^{m134} in early pressure (EP) crosses (17/227 EP embryos were *oep*^{m134} homozygotes, corresponding to a gene-centromere distance of 42.5 cM). None of more than 70 cloned and mapped zebrafish genes are located in this region of LG10 (J. Postlethwait, W. S. T., and M.Gates, personal communication).

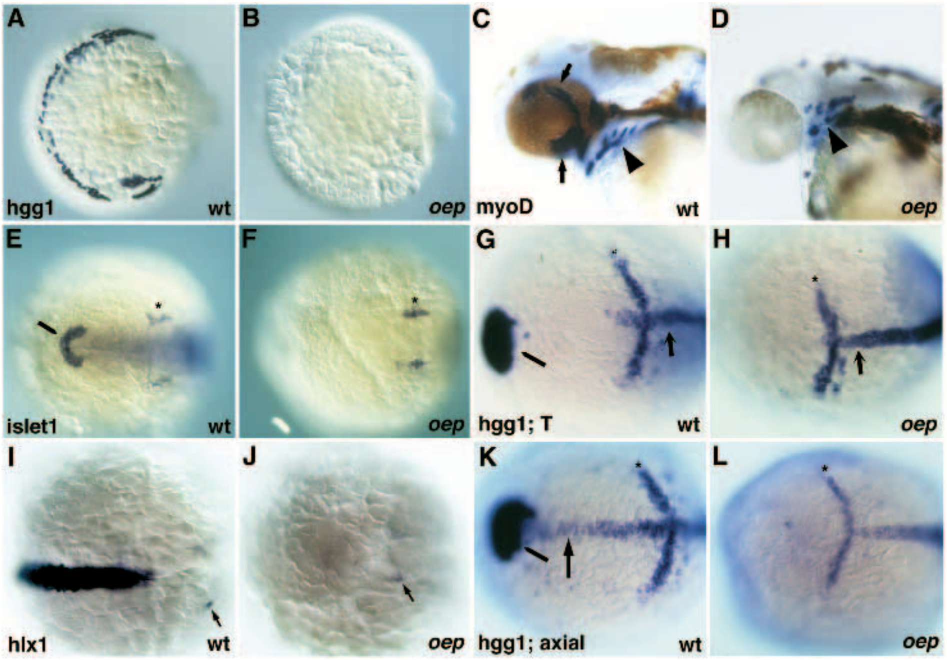

Fig. 3. Defects in the formation of the prechordal plate and its derivatives in *oep* mutants. (A,B) Formation of hatching gland cells in wild-type (A) but not *oep* mutant (B) embryos at 26 hpf as judged from the expression of the hatching gland specific gene *hgg1*. Dorsal-anterior view, ventral is to the right. (C,D) Formation of eye muscles (arrows) in wild-type (C) but not *oep* mutant (D) embryos at 42 hpf as judged from expression of *myoD*. Arrowhead indicates the location of pharyngeal muscles in wild-type and *oep* mutant embryos. (E,F) Formation of hatching gland precursors (pillow) in wild-type (E, fine arrowhead) but not in *oep* mutant (F) embryos at the 6-somites stage as judged from *islet1* expression. Stars indicate the normal formation of the trigeminal ganglion anlage. Dorsal view, anterior is to the left. (G,H) Formation of hatching gland precursors in wild-type (G, fine arrowhead) but not *oep* mutants at the end of gastrulation (bud stage) as judged from *hgg1* expression. Note the normal anterior limit of notochord precursors anterior to the krox20 stripe in rhombomere 3 (star) as judged from the expression pattern of T/*ntl* (arrow). Dorsal view, anterior is to the left. (I,J) Expression of *hlx1* in wild-type (I) and *oep* mutant embryos (J) at the tail bud stage. Arrows highlight the expression of *hlx1* in the hindbrain. Dorsal view, anterior is to the left. (K,L) Expression of *hgg1*, *axial* and krox20 in wild-type (K) and *oep* mutant embryos (L) at 90% epiboly. Note that at this stage wild-type *axial* expression in the axial mesoderm still includes the prechordal plate (arrow) with the exception of the hatching gland precursors (fine arrowhead). Star indicates the location of krox20 expression in rhombomere 3. Dorsal view, anterior is to the left.

lation. Consistent with an early requirement of *oep* in endoderm formation, *axial* expression is absent or strongly reduced in the hypoblast of *oep* mutants (Fig. 6P). Thus, *oep* is not only active in the dorsal mesoderm but is also required, directly or indirectly, in more lateral and ventral regions.

Since the endoderm has been implicated in the induction of the myocardium in frogs (Jacobson and Duncan, 1968; Sater and Jacobson, 1990; Nascone and Mercola, 1995; Schultheiss et al., 1995), we examined the heart in *oep* mutants. We find that *oep* mutants have defects in heart muscle as judged from morphological observations and the expression of α-*tropomyosin* (Fig. 6L; Thisse et al., 1993). The heart is small or absent, and sometimes cardia bifida is apparent. Pharyngeal endoderm has also been implicated in the induction of cartilage formation in pharyngeal neural crest cells (Graveson and Armstrong, 1987; Seufert and Hall, 1990), and we find that cartilage formation in the jaw region of *oep* mutants is reduced as judged from Alcian blue staining at 80 hpf (data not shown).

Role of *oep* in neural patterning

The prechordal plate has been implicated in several aspects of neural patterning (reviewed by Ruiz i Altaba, 1993). Based on embryological experiments (Spemann, 1931; Mangold, 1933; Gerhart et al., 1989; Ang and Rossant, 1993; Ang et al., 1994) and the analysis of mouse mutants in *lim1* (Shawlot and Behringer, 1995) or *otx2* (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), the prechordal plate has been proposed to be an equivalent of the head organizer, a source of signals inducing anterior neural structures including forebrain and midbrain. Additionally, embryological studies in amphibians have suggested that the prechordal plate is involved in the separation of the eye forming region (Adelmann, 1936) or the induction of the eye forming region, but not of the entire forebrain (Dixon and Kintner, 1989; Ruiz i Altaba, 1992; Papalopulu and Kintner, 1993). The absence of a prechordal plate in *oep* mutants has allowed us to test some predictions of these models in zebrafish. Morphological analysis indicates that *oep* mutants have a normal anterior-posterior patterning of the brain (Fig. 1E-H). Telencephalon, midbrain and hindbrain are distinct. Anterior-posterior patterning of the brain in *oep* mutants is also revealed by the analysis of expression patterns of *pax-a*, *pax-b*, and *krox-20* expression in forebrain, midbrain and hindbrain subregions during segmentation (Krauss et al., 1991; Püschel et al., 1992a ; Krauss et al., 1992; Püschel et al., 1992b; Oxtoby and Jowett, 1993; Fig. 7B). These data do not support models in which the prechordal plate is essential for forebrain or midbrain formation during zebrafish gastrulation.

In contrast, dorsoventral patterning in the forebrain is severely disrupted in *oep* mutant embryos, most clearly manifested as severe cyclopia (Fig. 1J). Ventral forebrain regions are not present as indicated by the loss of the hypothalamus (Fig. 1F-H) and the severe reduction or absence of *shh* (Fig. 7F; Krauss et al., 1993) and *nk2.2* (Fig. 7H; Barth and Wilson, 1995) expression in ventral neuroectoderm. These observations are in accord with classical embryological studies by Adelmann (1936), who showed that the absence of head mesoderm can lead to cyclopia, a phenotype very reminiscent of *oep*. These results provide further evidence in favor of the idea that the prechordal plate might be involved in the induction of ventral cell types in the forebrain.

Oep is not only required for the formation of ventral structures in the forebrain, but is also involved in the proper formation of the floor plate. As judged from morphological analysis and the expression of floor plate markers like *shh* (Fig. 7I-L), *typeII collagen*, *axial* or *F-spondin* (Klar et al., 1992; data not shown), the number of floor plate cells in *oep* mutants is reduced at 28 hpf. To determine when the ventral neuroectoderm phenotype becomes apparent, *shh* expression was studied at the end of gastrulation. Normal *shh* expression in the ventral neuroectoderm is not observed in *oep* mutant animals (Fig. 7D), but is found in the developing notochord. We conclude that *oep* is required for the specification of ventral neuroectoderm during gastrulation. This defect may result indirectly from the lack of proper prechordal plate formation or from a direct effect of *oep* on neuroectoderm.

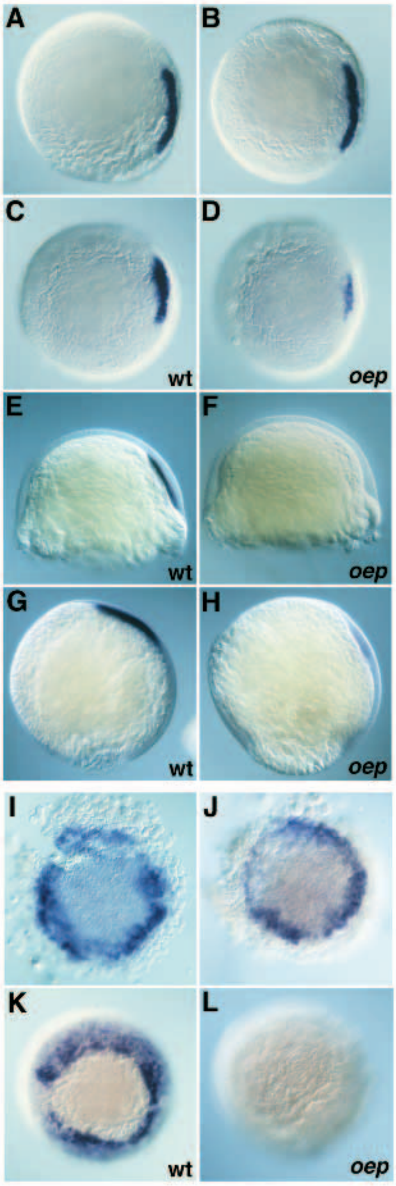

Fig. 4. Expression of *goosecoid* is initiated but not maintained in *oep* mutants. (A-H) *Gsc* expression at 45% epiboly (A,B); 50% epiboly (C,D); after shield formation (E,F) and at 90% epiboly (G,H) in wild-type (C,E,G) and *oep* mutant embryos (D,F,H). (A-D) animal pole view, dorsal is to the right; (E-H) lateral view, dorsal is to the left, animal pole is up. Embryos in A,B could be either wild-type or mutant. Note the loss of *gsc* expression in *oep* mutants at the onset of involution. (I-L) Expression of *gsc* in Li-treated embryos; animal pole view. Embryos derived from a cross of *oep*/+ heterozygous fish were incubated in 0.3 M LiCl at the 256-512 cell stage as described by Stachel et al. (1993). (I,J) 45-50% epiboly; (K,L) 9.5 hpf. 28/28 (100%) Li-treated embryos derived from a cross of *oep*/+ heterozygous fish expressed *gsc* at 45-50% epiboly in the entire margin (I,J), but 18/57 (31% compared to an expected 25%) Li-treated embryos derived from a cross of *oep*/+ heterozygous lacked *gsc* expression at 9.5 hpf (L). 81/81 (100%) Li-treated embryos derived from a cross of wild-type fish expressed *gsc* at 9.5 hpf (K).

Interaction of *oep* with *flh* and *ntl*

Two zebrafish mutants, *ntl* and *flh*, affect the formation of the notochord (Halpern et al., 1993; Schulte-Merker et al., 1994; Talbot et al., 1995). To determine the embryological effects of disrupting the development of the entire axial mesoderm and to test for possible genetic interactions, we constructed *oep flh* and *oep ntl* double mutants, and studied the expression of the *flh* and *ntl* genes in *oep* mutants.

(I) Additive defects in *oep flh* double mutants

The homeobox gene *flh*, a homologue of the Xenopus *Xnot* gene (von Dassow et al., 1993), is first expressed in the blastula margin and then becomes restricted to the shield and notochord (Talbot et al., 1995). Consistent with the studies described above, these *flh* expression domains are present in *oep* mutants (data not shown). We conclude that *oep* is not required for early *flh* expression. During somitogenesis *flh* is expressed in progenitors of the hatching gland in wild-type but not *oep* mutant embryos (data not shown).

Oep flh double mutants do not show any new dramatic defects that are not already present in the single mutants (Fig. 8E). Defects seem mainly additive as in the case of the axial mesoderm (absence or reduction of prechordal plate (*oep*) and notochord (*flh*) at the beginning of somitogenesis), or reflect the individual mutant phenotypes as in the case of cyclopia (*oep*), or somite patterning (lack of muscle pioneers as in *flh*). The correct number of somites forms, and pronephros and blood develop normally in *oep flh* double mutants.

However, more detailed inspection reveals that *oep flh* double mutants have enhanced floor plate and adaxial muscle phenotypes. A few floor plate cells are present in *oep* mutants (Figs 7K,L, 9C, 10B), and the floor plate seems normal anteriorly, but scattered posteriorly in *flh* mutant embryos (Figs 9E,F, 10C). In contrast, double mutants display a complete absence or very severe reduction of floor plate cells during segmentation (Fig. 9G,H) and at 28 hpf (Fig. 10D).

Adaxial muscle cells lie adjacent to the notochord (Thisse et al., 1993; Kimmel et al., 1995). Both *oep* and *flh* single mutants develop adaxial cells as judged from the expression of *myoD* (Weinberg et al., 1996) at the 11-somites stage (Fig. 11). Interestingly, expression of *myoD* is strongly reduced in the posterior region of *oep flh* double mutants (Fig. 11E,K). This result suggests that *oep* and *flh* or the structures that they primarily affect have a partially overlapping role in patterning structures adjacent to the axial mesoderm.

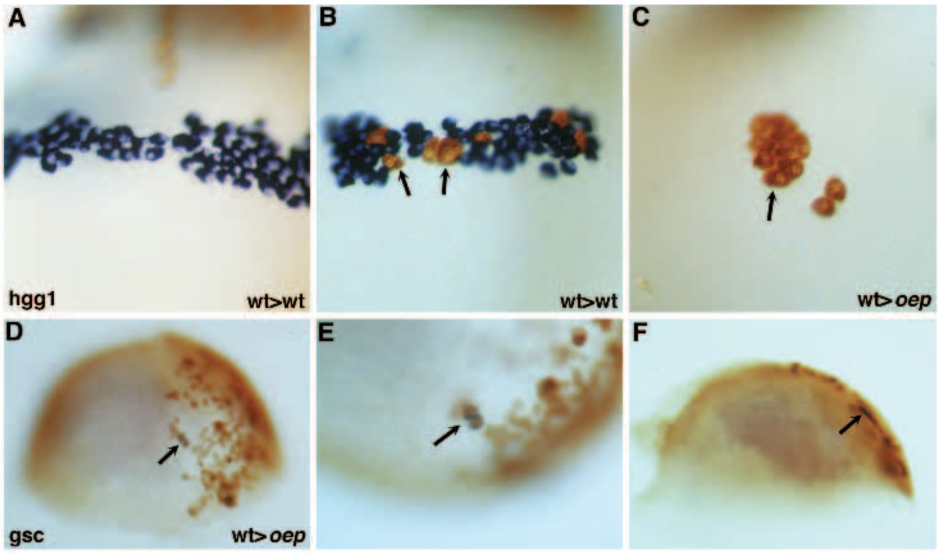

Fig. 5. Cell-autonomous role of *oep* in prechordal plate formation. (A-C) Formation of hatching gland by wild-type donor cells transplanted into wild-type (A,B) or *oep* mutant (C) hosts; ventral view, anterior is up; 28 hpf. (A) No contribution of wild-type donor cells (brown) to the hatching gland (blue) of a wild-type host. (B) Contribution of wild-type donor cells (brown, arrows) to the hatching gland (blue) of a wild-type host. (C) Formation of hatching gland by wild-type donor cells (brown, arrow) in *oep* mutant host. No host hatching gland cells (blue) form. Hatching gland cells are large, are located over the yolk anterior to the head region, and have a characteristic granular appearance. 14/99 transplants of wild-type donor cells into *oep* mutant hosts resulted in the formation of hatching gland cells by wild-type donor cells but not mutant host cells. 0/44 transplants of *oep* mutant donor cells into wild-type hosts resulted in the formation of hatching gland cells by *oep* mutant cells. Transplantations were performed as described in Materials and Methods. (D-F) Expression of *gsc* by wild-type cells transplanted into *oep* mutant hosts at 70-80% epiboly following LiCl treatment. Donor cells expressing *gsc* (arrow) are brown (biotin-dextran) and blue (*gsc* mRNA). (D) Animal pole is up; (E) higher magnification; (F) side view of embryo in D and E. Note the location of *gsc*-expressing cells in the hypoblast. 11/41 transplants of wild-type donor cells into *oep* mutant hosts led to the formation of *gsc*-expressing wild-type donor cells but not *gsc* expressing *oep* mutant host cells. Donor and host embryos were treated with Li and transplantations were performed as described in Materials and Methods.

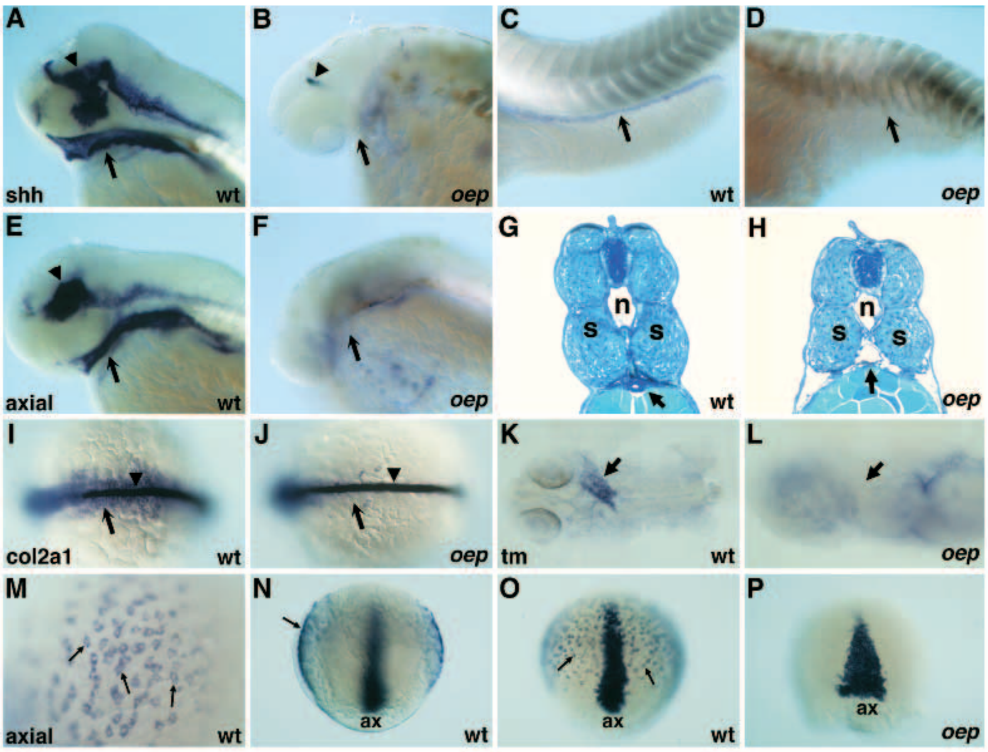

Fig. 6. Endoderm formation is impaired in *oep* mutant embryos. (A-D) Expression of *sonic hedgehog* (*shh*) in wild-type (A,C) and *oep* mutant (B,D) embryos at 51 hpf. Expression of *shh* in the pharyngeal endoderm (arrow in A,B) and gut (arrow in C,D) is normal in wild-type but not in *oep* mutant embryos. Expression in the brain region of *oep* mutants (arrowhead in B) is strongly reduced as compared to wild-type (arrowhead in A). (E,F) Expression of *axial* in wild-type (E) and *oep* mutant (F) embryos at 51 hpf. Expression of *axial* in the pharyngeal endoderm (arrow in E,F) is normal in wild-type but not in *oep* mutant embryos. Expression in the brain of *oep* mutants is also severely affected as compared to wild-type (arrowhead in E). Sagittal cross-section (5 μm) of the trunk region of wild-type (G) and *oep* mutant (H) embryo at 53 hpf. Arrow indicates the location of the gut in wild-type (G) embryo. Note the lack of tissue and the gaping hole in this region of *oep* mutants (arrow in H). n, notochord; s, somites. (I,J) Expression of the *type II collagen* gene *col2a1* in wild-type (I) and *oep* mutant (J) embryos at the 12-somites stage. Note the normal expression in the notochord (arrowhead), but severe reduction in the endoderm (arrow) of *oep* mutants (J). Dorsal view, anterior is to the left. (K,L) Expression of α-*tropomyosin* (*tm*) in the heart region (arrow) of wild-type (K) and *oep* mutant (L) embryos at 28 hpf. Note the severe reduction of *tm* in *oep* mutants. (M-P) Expression of *axial* in wild-type (M-O) and *oep* mutant embryos (P) at 80% epiboly. (M) High magnification view of *axial* expressing cells (arrows) located in the hypoblast. (N) Optical cross section reveals direct juxtaposition of *axial*-expressing cells to yolk (arrow), ax, *axial* expression in axial mesoderm. (O) Axial expression in *axial* mesoderm (ax) and presumptive endoderm (arrows) in wild-type embryos. (P) Loss of *axial* expression in the presumptive endoderm of *oep* mutants; ax, *axial* expression in the axial mesoderm of oep mutant embryos. Axial expression is laterally expanded as a result of reduced convergence and extension in *oep* mutants.

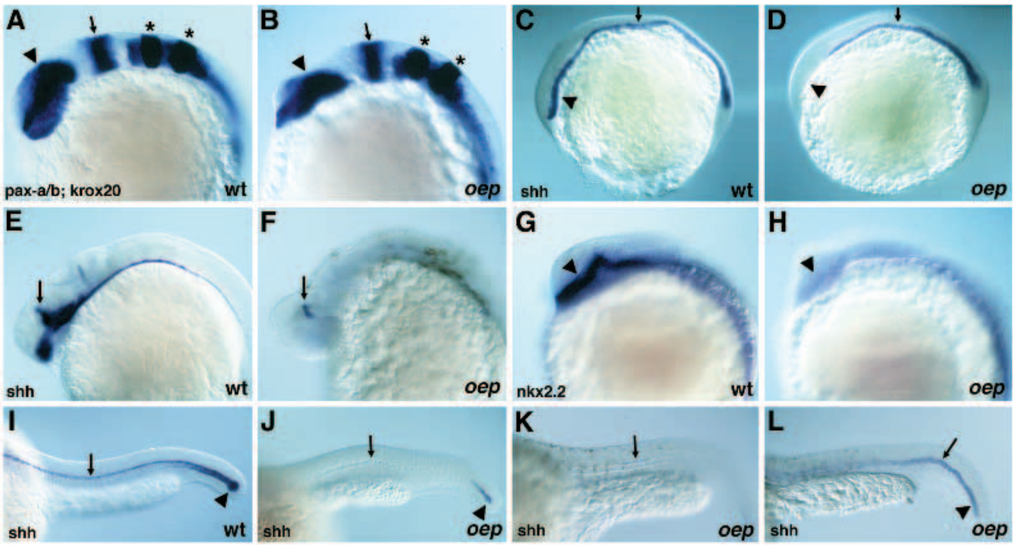

Fig. 7. Neuroectodermal phenotype of *oep* mutant embryos. (A,B) Expression of *pax-a* (arrowhead) in the forebrain, *pax-b* (arrow) at the midbrain-hindbrain boundary) and *krox-20* (stars) in rhombomeres 3 and 5 in wild-type (A) and *oep* mutant (B) embryos at the 10-somites stage. Note the apparently normal anterior-posterior organization of expression patterns. (C,D) Expression of *shh* in wild-type (C) and *oep* mutant (D) embryos at the 1-somite stage. Note the absence of *shh* expression in the head region (arrowhead) of *oep* mutants. *shh* is expressed in the notochord, but expression in the floor plate is reduced or absent (arrow) in *oep* mutant embryos. (E,F) Expression of *shh* in the head region of wild-type (E) and *oep* mutant (F) embryos at 26 hpf. *shh* is only found in the dorsal-most region next to the epiphysis in *oep* mutants (arrow). (G,H) Expression of *nk2.2* in the head region of wild-type (G) and *oep* mutant (H) embryos at the 18-somites stage. Arrowhead highlights expression in the ventral diencephalon. *shh* and *nk2.2* are expressed in adjacent domains, *shh* being the more ventral marker. (I-L) Expression of *shh* in the tail and trunk region of wild-type (I) and *oep* mutant (J-L) embryos at 26-28 hpf. Note that the floor plate (arrow) is lost (J) or reduced (K,L) in *oep* mutants. Some *oep* mutant embryos (L) show a more persistent expression of *shh* in the notochord (arrowhead).

(II) Severe deficits in mesoderm and midline formation in *oep ntl* double mutants

Ntl is first expressed in the entire marginal zone, then in the developing notochord, and finally in the tail bud (Schulte-Merker et al., 1992). Consistent with the analysis of *oep* described above, no loss of *ntl* expression was observed in *oep* mutants (Fig. 3H, and data not shown). We conclude that *oep* is not required for the regulation of *ntl* gene expression.

Oep ntl double mutants show a dramatic deficit in the formation of mesoderm (Fig. 8F). As compared to the single mutants where notochord (*ntl*), prechordal plate (*oep*), and a subset of adaxial cells (*ntl*) are affected, double mutants retain only the anterior-most somitic mesoderm as judged from the expression of α-*tropomyosin* (Fig. 12D) and *myoD* (Fig. 11F,L). Somitic defects are already apparent during gastrulation. Expression of *snail1* (Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1993) in somitic progenitors is slightly reduced in *ntl* mutant embryos (Fig. 12G), but drastically reduced or absent in *oep ntl* double mutants (Fig. 12H). The formation of ventral mesodermal cell types like blood is also reduced in *oep ntl* double mutants as judged from the expression of *gata1* (Detrich et al., 1995) during somitogenesis (Fig. 12L). Intermediate mesoderm seems to form in *oep ntl* double mutants as judged from the expression of *pax-b* (Püschel et al., 1992b; Krauss et al., 1992) and *lim1* (Toyama et al., 1995) during somitogenesis (data not shown).

Midline structures are also severely affected in *oep ntl* double mutants; *shh* is expressed in the notochord and floor plate of wild-type embryos at the 12-somite stage (Fig. 9A,B). In *oep* mutants, few floor plate cells are present, but the notochord appears normal (Figs 9C,D, 10B). In *ntl* mutants, a string of cells expresses *shh* in the trunk (Fig. 9I,J, 10E). In contrast, *oep ntl* double mutants have no *shh* expressing cells, except in the anterior trunk region (Figs

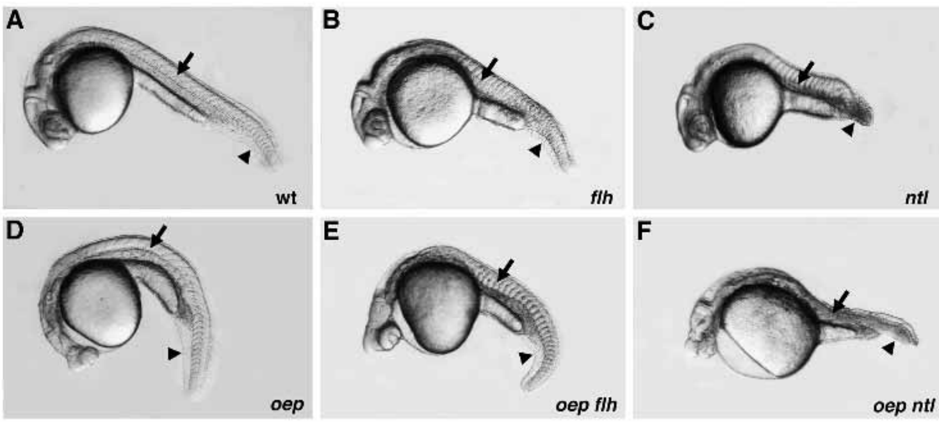

Fig. 8. Phenotype of *oep flh* and *oep ntl* double mutants. Comparison of wild-type (A), and *floating head* (*flh*; B), *no tail* (*ntl*; C), *one-eyed pinhead* (*oep*; D), *one-eyed pinhead*; *floating head* double (*oep flh*; E), and *one-eyed pinhead*; *no tail* double (*oep ntl*; F) mutant embryos at 28 hpf. Arrow indicates the notochord, arrowhead indicates posterior region. Note the mainly additive features of *oep flh* double mutants as compared to the severe defects in *oep ntl* double mutants.

9K,L, 10F). Interestingly, these cells are not located only at the midline, but are displaced laterally as patches of *shh* expressing cells (Fig. 9L). Inspection of *shh*, *axial* and *F-spondin* expression at later stages, when these genes are specifically expressed in the floor plate, suggests that the few remaining midline cells in *oep ntl* double mutants correspond to floor plate cells (data not shown). These data demonstrate that the *oep ntl* double mutants have an enhanced early midline phenotype. Consistent with this observation, expression of *axial* in the presumptive notochord is often absent in *oep ntl* double mutant embryos at the end of gastrulation (data not shown). In summary, the drastic deficits in *oep ntl* double mutants indicate that *oep* and *ntl* interact and have partially redundant roles in the formation of mesoderm and trunk midline structures.

DISCUSSION

Requirement of *oep* in prechordal plate and endoderm formation

The examination of gene expression patterns in *oep* mutants indicates that *oep* is involved in the early steps of prechordal plate and endoderm formation. Both *gsc* expression in prechordal plate progenitors and *axial* expression in presumptive endodermal cells are absent or strongly reduced in *oep* mutants. Our transplantation studies show that *oep* acts cell-autonomously in prechordal plate precursor cells. Furthermore, *oep* is also cell-autonomously required in the marginal progenitors of *axial*-expressing hypoblast cells (AS,SN&WD, unpublished results). Studies in frogs suggest that the formation of endoderm and dorsal mesoderm is initiated by maternal signaling molecules like Vg1 or activin (Rosa, 1989; Kessler and Melton, 1994; Cornell et al., 1995; Gamer and Wright, 1995; Henry et al., 1996). The initial response to these factors seems to be normal in *oep* mutants as genes like *gsc* or *Brachyury* are turned on normally after midblastula transition. This early activation has been shown to be independent of zygotic gene activity (Cho et al., 1991; Smith et al., 1991), and, as expected, is independent of the zygotic *oep* locus. As a zygotic downstream gene, *oep* is required in the execution of the programs leading to the formation of endoderm and prechordal plate.

Prechordal plate development and function

Studies in amphibian and avian embryos have suggested that pharyngeal endoderm and eye muscles are derived from the prechordal plate, the first involuting dorsal cell group (Adelmann, 1932; Keller, 1976; Jacob et al., 1984; Wachtler et al., 1984). Fate map studies suggest, but have not unequivocally demonstrated, that the prechordal plate has the same fate in zebrafish (Kimmel et al., 1990; Halpern et al., 1993; Kimmel et al., 1995). Furthermore, the anterior portion of the zebrafish prechordal plate gives rise to the pillow (polster), which later differentiates into the hatching gland (Thisse et al., 1994; Kimmel et al., 1995). The *oep* mutant phenotype and the correlation between the early absence of prechordal plate and the later deficit in eye muscles, hatching gland and pharyngeal endoderm support the view that the zebrafish prechordal plate has a fate similar to that established in other vertebrates.

The *oep* mutant phenotype is reminiscent of proposals of Adelman (1936), who suggested that the prechordal plate might be a midline signaling center. Transplanting the eye-forming region of neural plate *Amblystoma* embryos to the belly region, Adelman found that two separated eyes develop only when the underlying head mesoderm is included in the transplant. Transplanting isolated neural plate alone leads to the formation of a single, median eye. These results led to the suggestion that the prechordal plate might be involved in the formation of ventral brain structures, leading to the separation of the eye field into two units. The phenotype of *oep* mutants is consistent with this proposal. *Oep* mutants display extreme cyclopia and a loss of ventral forebrain structures. In this scenario, the primary role of *oep* is in the formation of the prechordal plate and the forebrain defects would then be a consequence of the loss of a ventralizing center underlying the

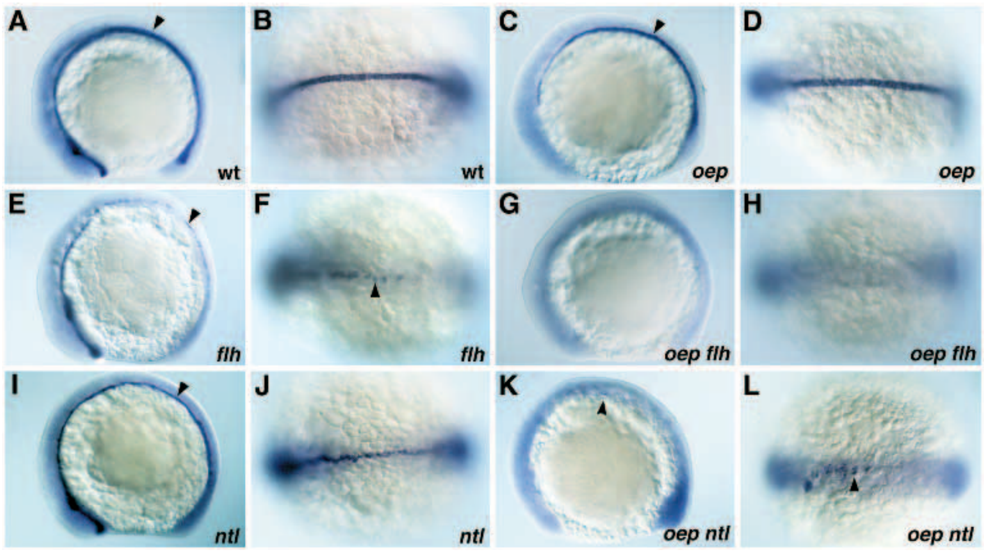

Fig. 9. Midline defects in *oep flh* and *oep ntl* double mutants. (A-L) Expression of *shh* in wild-type (A,B), *oep* mutant (C,D), *flh* mutant (E,F), *ntl* mutant (I,J), *oep flh* double mutant (G,H) and *oep ntl* double mutant (K,L) embryos at the 11-12 somites stage. (A,C,E,G,I,K) lateral view; (B,D,F,H,J,L) dorsal view of anterior trunk region. (A,B) Expression of *shh* in ventral neuroectoderm including ventral diencephalon and floor plate (arrowhead), and notochord in wild-type embryos. (C,D) Expression of *shh* in notochord and few floor plate cells (arrowhead) of *oep* mutants. (E,F) Expression of *shh* in ventral neuroectoderm, anterior floor plate and scattered posterior floor plate cells (arrowhead) in *flh* mutants. (G,H) Absence of *shh* expression in *oep flh* double mutants. (I,J) Expression of *shh* in the midline in presumptive floor plate cells (arrowhead) of *ntl* mutants. (K,L) Expression of *shh* in scattered cells in the anterior trunk (arrowhead) of *oep ntl* double mutants.

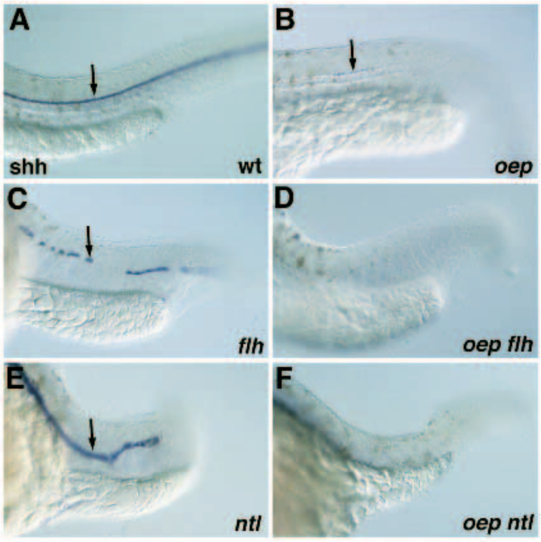

Fig. 10. Floor plate formation in *oep flh* and *oep ntl* double mutants. (A-F) Floor plate formation (arrow) detected by the expression of *shh* in the trunk/tail region of wild-type (A), *oep* (B), *flh* (C), *oep flh* (D), *ntl* (E) and *oep ntl* (F) mutant embryos at 28 hpf. Note that the floor plate appears thicker in *ntl* mutant embryos as compared to wild-type embryos.

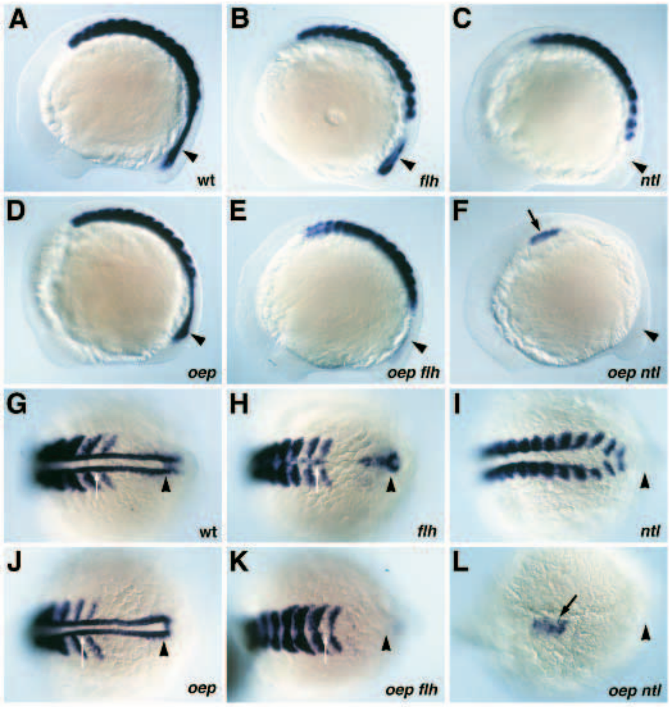

Fig. 11. Expression of *myoD* in *oep flh* and *oep ntl* double mutants. (A-F) Lateral view of *myoD* expression in wild-type (A), *flh* (B), *ntl* (C), *oep* (D), *oep flh* (E) and *oep ntl* (F) mutant embryos at the 11-12 somites stage. (G-L) Dorsal view of *myoD* expression in wild-type (G), *flh* (H), *ntl* (I), *oep* (J), *oep flh* (K), and *oep ntl* (L) mutant embryos. G-K display the posterior expression domain of *myoD*, (L) displays the anterior most and only expression domain of *myoD* in *oep ntl* double mutants. Adaxial cells located between the last two presumptive somites expressing *myoD* are indicated by a white arrow. Note that this cell population is fused in *flh* mutant embryos (H) and that *myoD* expression at this position is absent in *ntl* mutant and *oep flh* double mutant embryos. The posterior-most expression domain of *myoD* (arrowhead) is also drastically reduced in *oep flh* double mutants and absent in *ntl* mutants. Black arrow in F,L indicates the formation of a cluster of *myoD* expressing cells in the anterior trunk of *oep ntl* double mutants.

forebrain. Alternatively, *oep* might (also) have a direct autonomous function in the formation of the ventral forebrain.

The prechordal plate has also been implicated in the induction of anterior neuroectoderm (reviewed by Ruiz i Altaba, 1993; Doniach, 1995). Classical transplantation experiments have shown that presumptive head mesoderm can induce the formation of anterior neural structures in host embryos (Mangold, 1933; Spemann, 1931). In contrast, in these experiments, the presumptive posterior mesoderm induced more posterior neural structures. These and other results led to the proposal that the neural plate is patterned along the anterior-posterior axis by vertical signals from the underlying mesoderm. The head mesoderm would then correspond to the head organizer and the more posterior mesoderm to the tail organizer. The head organizer proposal has recently been revived by the finding that *lim1* mutant mice lack structures anterior to rhombomere 4 in the hindbrain (Shawlot and Behringer, 1995). As *lim1* is expressed in the organizer and prechordal plate, one possible interpretation of the *lim1* mutant phenotype is that the loss of prechordal plate and corresponding signaling function leads to the loss of the head organizer and head. Similar proposals have been put forward to explain the *otx2* mutant phenotype (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). The absence of the prechordal plate in *oep* mutants provides a test of this model in zebrafish. We find that despite the absence of prechordal plate during gastrulation, anterior-posterior patterning of the brain is largely undisturbed, allowing the formation of structures like telencephalon and midbrain. This finding supports models of planar induction (Ruiz i Altaba, 1992, Doniach et al., 1992), in which signals from the organizer (including prechordal plate precursors) directly induce anterior-posterior patterning in the neuroectoderm, without the need for underlying head mesoderm. Explant studies in frogs have also suggested that some aspects of forebrain development can be induced in a planar fashion (Papalopulu and Kintner, 1993). Thus, head organizer genes like *lim1* might exert their effects in the organizer, prior to the formation of the prechordal plate.

Endoderm formation and function

The finding that *oep* is defective in the early formation of endoderm provides the first example of such a phenotype in vertebrates. Fate map studies in zebrafish have shown that the endoderm derives from the most marginal region of the late blastula embryo, partially overlapping with mesodermal precursors (Kimmel et al., 1990, 1995; Warga, 1996). During gastrulation this cell population involutes and streams from the margin towards the animal pole. Mesoderm and endoderm are not at first distinguishable as separate germ layers but form the hypoblast, an apparently single layer of cells. Our observation that *axial* (Strähle et al., 1993) is expressed in a sub-population of hypoblast cells located in close proximity to the yolk cell, and that this cell population is specifically affected in the endoderm mutant *oep*, suggests that *axial*-expressing cells represent some or all of the endoderm precursors during gastrulation. Thus, distinct populations of mesoderm and endoderm cells are present in the hypoblast of the gastrula.

The deficit of endoderm in *oep* mutants provides a tool to study the postulated roles of endoderm in the formation of mesodermal and ectodermal tissues like heart (Jacobson and Duncan, 1968; Sater and Jacobson, 1990; Nascone and Mercola, 1995; Schultheiss et al., 1995) or pharyngeal cartilage (Graveson and Armstrong, 1987; Seufert and Hall, 1990). We find that both heart muscle and pharyngeal cartilage differentiation are compromised in *oep* mutants. Although we cannot exclude an autonomous role of *oep* in these structures, *oep* mutant defects are consistent with embryological data that suggest an inductive role for endoderm.

Requirement of *oep* in floor plate formation

The floor plate, the ventral-most cell type in the neural tube, is thought to be induced by signals from the notochord (van Straaten et al, 1988; van Straaten and Hekking, 1991; Placzek et al., 1990). The signaling molecule shh has been implicated in this process, as it can induce floor plate and is expressed in the notochord (Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994). We find that *oep* mutations disrupt early floor plate development, and that this is not caused by a failure of *oep* mutant notochord cells to express *shh*. These results suggest that *oep* might be a downstream component or response to the shh signaling cascade, or might act in a parallel pathway. Alternatively, the requirement for *oep* might be less direct, and earlier deficits in *oep* mutant animals, e.g. the absence of prechordal plate, might lead to impaired floor plate development. It is conceivable that the early contact of prechordal plate cells with the overlying neuroectoderm during gastrulation contributes to floor plate induction, either by direct induction or by priming the neuroectoderm to respond to later signals from the notochord. Analysis of genetic mosaics would determine the role of *oep* in floor plate induction, but the significant number of floor plate cells that develop in *oep* mutants has precluded this analysis. It is therefore unclear if *oep* is directly and cell-autonomously involved in the formation of floor plate, or if the ventral deficits are due to the role of *oep* in other structures like the axial mesoderm.

Interaction of *oep* with *flh*

The additive defects in *oep flh* double mutants suggest that *oep* and *flh* act primarily independently. Two structures, however, are more strongly affected in double mutants than in either of the single mutants. Loss of *flh* seems to enhance the floor plate phenotype of *oep* mutants. Whereas a variable number of floor plate cells form in *oep* or *flh* mutants, *oep flh* double mutants show a dramatic loss of floor plate cells. Furthermore, as evidenced by reduced *myoD* expression, posterior adaxial muscle cells are also affected in *oep flh* embryos. Both adaxial and floor plate cell precursors are located adjacent to the notochord, and are also contacted by the prechordal plate during gastrulation. It is conceivable that the loss of both prechordal plate and notochord in *oep flh* double mutants leads to a significant reduction of axial mesodermal signaling activity and consequently affects the induction of adjacent structures more severely that in single mutants. It is also possible that *oep* and/or *flh* play direct autonomous roles in floor plate formation and act in a partially redundant or additive manner, a function that is uncovered in double mutants.

Interaction of *oep* with *ntl*

Oep ntl double mutants show extreme defects in the formation of mesodermal cell types, revealing a partially overlapping requirement of *oep* and *ntl* in mesoderm formation. This finding offers an explanation for an enigma concerning the expression and function of *ntl*. The *ntl* gene is first expressed in the marginal zone, including all mesodermal precursor cells; however, this expression domain does not seem to be required as judged from the normal development of mesoderm like muscle, pronephros, blood or heart in *ntl* mutants (Halpern et al., 1993). Mutants for *ntl* are only defective in the proper formation of notochord, muscle pioneer cells and tail. These mutant phenotypes have been suggested to reflect the requirement and expression of *no*

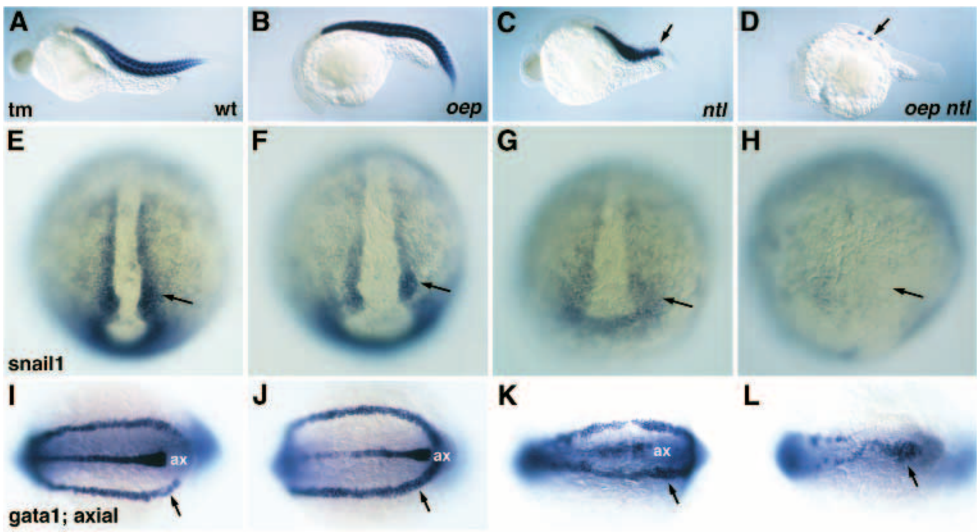

Fig. 12. Expression of mesodermal markers in *oep ntl* double mutants. (A-D) Expression of α-*tropomyosin* (*tm*) in wild-type (A), *oep* mutant (B), *ntl* mutant (C) and *oep ntl* double mutant (D) embryos at 26 hpf. Wild-type and *oep* mutant embryos have 30-32 somites at this stage, whereas *ntl* mutants have 15-17 somites (arrow). Note the dramatic deficit in *tm* expression in *oep ntl* double mutants (arrow in D). (E-H) Expression of snail 1 in wild-type (E), *oep* mutant (F), *ntl* mutant (G), and *oep ntl* double mutant (H) embryos at the bud stage. Note (arrows) the mild reduction of snail1 expression in *ntl* mutant embryos (G), and the severe deficit of snail1 expression in *oep ntl* double mutants (H). (I-L) Expression of *gata1* and *axial* in wild-type (I), *oep* mutant (J), *ntl* mutant (K), and *oep ntl* double mutant (L) embryos at the 11-somites stage. Note the severe reduction of *gata1* expressing cells (arrow) in *oep ntl* double mutant embryos (L); ax, *axial* expression in the midline. Assignment of *gata1* (arrow) and *axial* (ax) expression domains is based on the analysis of embryos stained with either probe alone (data not shown).

tail in notochord and tail bud after the onset of gastrulation (Halpern et al., 1993). Our results suggest that the early requirement for *ntl* in mesoderm formation might be partially masked by the activity of *oep*. The *oep* or *ntl* genes alone might be sufficient for normal somite and blood formation. Removing both genes simultaneously reveals their partially redundant function and leads to drastic deficits in mesoderm development.

How do *oep* and *ntl* interact ? We find that *oep* disrupts the development of *axial* expressing cells derived from the entire marginal zone of the gastrulating embryo. *Ntl* is expressed in this region during this time. It is tempting to speculate that both *oep* and *ntl* act in marginal cells to ensure proper mesoderm formation. Both *oep* and *ntl* might act to specify mesodermal structures. This suggestion is consistent with studies in frogs, showing that ectopic expression of *Brachyury* can also induce the formation of mesodermal cell types other than notochord (Cunliffe and Smith, 1992, 1994; O'Reilly et al., 1995). Alternatively, *oep* and *ntl* might also be required for the proper migration of mesendodermal cells. The latter proposal is supported by the study of wild-type and *Brachyury* mutant chimeric mouse embryos (Wilson et al., 1993, 1995). *Brachyury* mutant cells appear defective in proper mesodermal cell movements, which results in the progressive accumulation of mesoderm cells near the primitive streak. This ultimately blocks the formation of posterior mesoderm. If a similar scenario is applicable to zebrafish, *oep* and *ntl* might lead to migratory abnormalities in the marginal zone. It is interesting to note that *oep* mutants display reduced convergence-extension (Solnica-Krezel et al., 1996). Gastrulation movements could be partially blocked in *oep ntl* double mutants and lead to the observed deficits.

The idea that *ntl* has the same function in zebrafish as *Brachyury* in mouse is supported by the high degree of sequence conservation between the two genes and the resemblance of their expression patterns (Halpern et al., 1993; Schulte-Merker et al., 1994). Indeed, both *ntl* and *Brachyury* mutant embryos display defective notochord differentiation and posterior truncations. However, it is clear that several characteristics of the mouse *Brachyury* mutant phenotype are not present in the zebrafish *ntl* mutant. In particular, *Brachyury* mutants form no more than 8 somites in the anterior trunk region and lack the floor plate (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Grueneberg, 1958; Beddington et al., 1992; Dietrich et al., 1993; Conlon et al., 1995). In contrast, *ntl* mutants form all trunk somites and a floor plate. It is interesting to note that the trunk phenotype of *oep ntl* double mutants is more closely related to the *Brachyury* mutant phenotype. Namely, midline structures are severely affected and somites are found only in the anterior trunk region in *oep ntl* double mutants. We might speculate that zebrafish *oep* has some of the functions or features of the mouse *Brachyury* gene product, thereby masking a broader role of *ntl*.

Oep function in midline development

During the formation of axial mesoderm, *oep* primarily functions in the formation of the prechordal plate, whereas *flh* appears to promote notochord formation (Halpern et al., 1995; Talbot et al., 1995). The *flh* mutant phenotype has been interpreted as a cell fate specification defect. Similarly, *oep* might act as a prechordal plate cell fate specification gene. It has to be emphasized, however, that *oep* might also be involved in the formation of the notochord, a function revealed in a minority of *oep* mutants that show notochord defects, and the midline defects in *oep ntl* double mutants. Therefore, we suggest that *oep* acts in axial mesoderm precursor cells located in the organizer region where it is mostly required to allow the proper formation of the anterior-most, first involuting axial mesoderm.

The deficits in ventral neuroectodermal structures in *oep* mutants are reminiscent of the *cyclops* mutant phenotype (Hatta et al., 1991, 1994). *Cyc* embryos display partial eye fusion, absence of floor plate cells, and a slight reduction of the prechordal plate (Thisse et al., 1994). The extreme cyclopia and absence of prechordal plate demonstrate that the head phenotype of *oep* is more severe than in *cyc* mutants. Interestingly, the floor plate phenotype of *cyc* mutants appears to be more severe: only a few floor plate cells form in the tail region of *cyc* embryos. These comparisons suggest that *oep* and *cyc* are involved in the same or similar developmental pathways, but to different extents. Further, *oep* seems to be involved in additional processes as judged from the endoderm phenotype and the severe mesoderm defects in *oep ntl* double mutants.

In summary, the findings presented here establish *oep* as an essential zygotic component downstream of several inductive interactions in the vertebrate embryo, namely the formation of axial mesoderm, endoderm and ventral neuroectoderm. As a first step towards the molecular isolation of *oep*, we have mapped the *oep* locus to linkage group 10 on the zebrafish genetic map. So far, no candidate genes map to this region, and we can exclude *oep* as a mutation in a number of genes that have been invoked in organizer function or development, including *gsc*, *lim1*, *axial*, or *shh* (J. Postlethwait, W. S. T., and M. Gates, personal communication). Phenotypic analysis and the study of genetic mosaics suggest that *oep* candidate genes should be expressed in the dorsal mesoderm and the marginal zone at the onset of gastrulation. The molecular isolation of the *oep* locus should offer further insights into how *oep* functions in the patterning of all three germ-layers, and into the nature of its interaction with *ntl*.

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