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To my parents

Preface

A number of people have contributed importantly to my scientific career at UCSF: Didier Stainier, my thesis advisor, whose guidance and support have been invaluable; Marc Tessier-Lavigne, my first scientific advisor at UCSF and a member of both my orals and thesis committees; Tris Parslow, who has been tremendously supportive as the MSTP director and a member of my thesis committee; Gail Martin, who chaired my thesis committee; Raul Andino, who taught me most of what I know about molecular biology; and Kathleen Raneses, Jana Toutolmin, and Sue Adams, who together have helped me in innumerable ways.

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The text of chapter 2 of this dissertation is a reprint of the material as it appears in Developmental Biology. Michael Rothenberg performed some of the initial gene expression studies on the *casanova* mutant as a rotation student under my supervision. Gilbert L. Henry provided the sequence of the *Xenopus Mixer* cDNA prior to publication. Didier Y. R. Stainier supervised the research that forms the basis for this and the other chapters of this dissertation.

The text of chapter 3 has been submitted for publication in Current Biology.

The text of chapter 4 has been submitted for publication in Development.

Abstract

Gut feelings: Molecular and genetic analysis of endoderm development in zebrafish

Jonathan Alexander

The mechanisms that underlie the formation of the endoderm in vertebrates have only recently begun to be explored. Using the molecular and genetic methods available in zebrafish I have investigated several aspects of this problem. I first demonstrate an essential role for the zebrafish locus *casanova* in the initial formation of the endoderm. *casanova* mutants lack a gut tube, show no molecular evidence of regional endodermal differentiation, and appear to lack endoderm entirely from the onset of gastrulation. Mosaic analysis indicates that *casanova* functions cell-autonomously within the endodermal progenitors. I have then used overexpression studies in wild-type and different mutant zebrafish embryos to assemble a genetic pathway that underlies formation of the zebrafish endoderm. My data suggest that nodal-related TGF-β growth factors together with the One-eyed pinhead protein act through type I TGF-β receptors such as TARAM-A to induce and maintain the expression of the gene *mixer*. Mixer, in a manner that depends upon *casanova*, then promotes expression of the *sox17* gene, initiating the process of endoderm differentiation. I also describe molecular and genetic evidence that indicate an important distinction between the gut endoderm and the pharyngeal endoderm in zebrafish, and suggest that the pharyngeal endoderm may in fact be mesodermal in origin. Lastly, I have begun to investigate the role of bone morphogenetic proteins in endoderm development. My results suggest that bone morphogenetic proteins may play a role in both the formation and anterior-posterior patterning of the endoderm in zebrafish, providing the first evidence of an endogenous role for these factors in vertebrate endoderm development.

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Chapter 1: Introduction

General considerations

The vertebrate embryo is composed of cells derived from three fundamental germ layers: the ectoderm, which forms the nervous system and the epidermis; the mesoderm, which forms muscle, blood, and other connective tissue; and the endoderm, which forms the lining of the gut tube, gut-associated organs such as the liver and the pancreas, as well as the lining of the respiratory tract. These germ layers, which are thought to represent the earliest division of the embryo, form and receive their initial patterning through a series of inductive interactions that occur during gastrulation (Slack, 1993; Harland and Gerhart, 1997). Identifying and understanding the functions of the factors that induce and pattern these germ layers, and the molecules that act within the germ layers to receive and interpret these signals, represent fundamental goals of vertebrate developmental biologists.

The induction and patterning of both the mesoderm and the ectoderm, and in particular the neuroectoderm, have been the subjects of intensive investigations during the past several years. This work has resulted in a detailed, although still incomplete, delineation of the molecular networks that underlie the early development of these tissues. Members of the Transforming Growth Factor-β (TGF-β) , Fibroblast Growth Factor (FGF), and Wnt families of secreted growth factors, as well as secreted antagonists of many of these molecules, have been shown to function as important inducers of numerous early events in the development of the ectoderm and mesoderm (Harland and Gerhart, 1997; Kessler and Melton, 1994; Lemaire and Kodjabachian, 1996). Within the cells of the ectoderm and mesoderm, receptors and downstream signal transduction components corrresponding to each of the above growth factor families have been demonstrated to receive and transmit these signals to the cell nucleus, where they affect the expression of a wide variety of genes. Amongst the many downstream targets of these signalling pathways, particular attention has been paid to a variety of transcription factors (Lemaire

and Kodjabachian, 1996). These transcription factors likely function in combination to direct the expression of other genes whose actions together define the character and behavior of the ectodermal and mesodermal cell populations in which they are expressed.

The early development of the endoderm, in contrast, has until recently remained largely unexplored. Indeed, many studies involving the endoderm have focused principally upon its role as a source of signals that affect development of the ectoderm and mesoderm, and only incidentally upon development of the endoderm itself. Thus, much less is known about the molecules that control the early development of the endoderm, as compared to the ectoderm or mesoderm, and only very recently have investigators begun to turn their attention to such questions.

Origin of the endoderm in the early vertebrate embryo

The pregastrula location of the endodermal progenitors has been determined by fate mapping studies in each of the principal vertebrate model systems. In *Xenopus* the gut endoderm derives primarily from the yolk-rich cells of the vegetal hemisphere (Dale and Slack, 1987; Keller, 1975; Keller, 1976). These cells lie below where the blastopore will form and therefore do not actually involute during gastrulation. Additionally, a group of cells known as the suprablastoporal endoderm forms the endoderm that lines the floor of the pharyngeal cavity (Keller, 1975). As with the mesoderm and ectoderm, the embryonic dorsal-ventral axis in the endoderm appears to correspond to the mature anterior-posterior axis (Gamer and Wright, 1995; Henry et al., 1996). Thus, dorsal vegetal cells express genes characteristic of, and contribute to, more anterior endodermal derivatives, while ventral vegetal cells form more posterior endodermal derivatives.

Prior to gastrulation in both chick (Hatada and Stern, 1994) and mouse (Lawson et al., 1991) embryos the endodermal progenitors are clustered in a region of the epiblast near the future site of node formation. These cells are therefore amongst the first to ingress

through the primitive streak as gastrulation begins. In the chick it also appears that more medially situated endodermal progenitors contribute to dorsal aspects of the gut tube, while the lateral and ventral aspects of the gut tube derive from endodermal progenitors that originate more laterally in the pregastrula embryo (Hatada and Stern, 1994).

In the zebrafish late blastula the endoderm arises entirely from the marginal-most four blastomere tiers, so that zebrafish endodermal progenitors also involute very early in gastrulation (Warga and Nusslein-Volhard, 1999). Endodermal progenitors form throughout the entire marginal zone of the zebrafish embryo, although the likelihood that the progeny of a given marginal blastomere will later contribute to the endoderm is higher in dorsal regions of the embryo (Warga and Nusslein-Volhard, 1999). As in *Xenopus*, in zebrafish dorsally located endodermal progenitors in general give rise to more anterior endodermal derivatives, while endoderm that derives from ventral marginal cells contributes to posterior endodermal structures (Warga and Nusslein-Volhard, 1999).

In mouse, chick, and zebrafish embryos the pregastrula endodermal and mesodermal fate maps overlap substantially (Hatada and Stern, 1994; Lawson et al., 1991; Warga and Nusslein-Volhard, 1999). This contrasts with the situation in *Xenopus*, in which the future endoderm is spatially segregated from progenitors of the other germ layers prior to gastrulation (Keller, 1975; Keller, 1976). This difference may relate to the essential role played by the maternally deposited vegetally localised transcription factor VegT in *Xenopus* endoderm formation (Zhang et al., 1998). Whether VegT homologues are similarly required for endoderm development in other vertebrates is not known.

The molecular basis of endoderm formation

Much of our knowledge regarding the molecular control of endoderm development comes from studies of the amphibian *Xenopus laevis*. The endodermal progenitors in *Xenopus* commit to an endodermal fate by early in gastrulation (Heasman et al., 1984;

Wylie et al., 1987), but prior to this stage they can be redirected to other fates by various experimental manipulations (Gamer and Wright, 1995; Henry et al., 1996; Wylie et al., 1987). Importantly, substantial endodermal differentiation occurs in isolated *Xenopus* vegetal pole explants (Gamer and Wright, 1995; Henry et al., 1996), suggesting that the endoderm forms through a process that is largely cell- and/or tissue-autonomous.

Many of the same growth factors that influence early mesodermal and ectodermal development may also induce formation of the endoderm. FGFs and the secreted bone morphogenetic protein (BMP) antagonists chordin and noggin have been suggested to play a role in *Xenopus* endoderm induction. Overexpression of chordin and noggin is able to promote the expression of at least some endodermal markers in *Xenopus* animal cap explants (Sasai et al., 1996), while FGFs have been suggested to influence endoderm formation both positively and negatively (Gamer and Wright, 1995; Henry et al., 1996; Jones et al., 1993). The physiological importance of such molecules in the development of the endoderm remains unclear.

Substantial evidence implicates Activin-related growth factors as important for endoderm induction. High levels of Activin and related TGF-β molecules induce endodermal gene expression in *Xenopus* animal caps (Gamer and Wright, 1995; Henry et al., 1996; Jones et al., 1993), and an endogenous role in *Xenopus* dorsal endoderm formation has been demonstrated for a Vg1-like activity (Joseph and Melton, 1998). Recent studies in zebrafish support the idea that Activin-related signals play an important role in endoderm formation in vivo. First, overexpression of low levels of the putative secreted Activin inhibitor Antivin deletes the endoderm with little or no effect on mesoderm development (Thisse and Thisse, 1999). Second, zebrafish *one-eyed pinhead* (*oep*) mutants lack endoderm, in addition to prechordal plate and ventral neuroectoderm (Schier et al., 1997). Oep is required for signalling by nodal-related molecules (Gritsman et al., 1999), and these results therefore suggest that sustained or high levels of nodal signalling are required for endoderm formation. Interestingly, a constitutively active form of the type

I TGF-β receptor TARAM-A, designated TARAM-A*, has been shown to direct cells cell-autonomously to an endodermal fate in both wild-type and *oep* mutant embryos (Peyrieras et al., 1998). Finally, embryos mutant for both *cyclops* and *squint*, two genes that encode nodal-related growth factors, as well as embryos that lack both maternal and zygotic One-eyed pinhead protein, form essentially no endoderm or mesoderm (Feldman et al., 1998; Gritsman et al., 1999). In these cases involution of the marginal zone does not occur and therefore no endoderm or mesoderm forms. Considered together, the various data suggest that high levels of Activin-related molecules such as Vg1 and nodals play an important role in endoderm induction, although what their precise functions are remains unknown.

Substantial progress has been made recently in the identification of genes that may act within the endodermal progenitors to initiate or promote their differentiation in response to inducing signals. Several *Mix* homeobox genes have been shown to be expressed in the *Xenopus* endodermal precursors early in gastrulation (Ecochard et al., 1998; Henry and Melton, 1998; Rosa, 1989). Functional studies suggest that these genes, and two in particular--*Mixer* and *milk*--, are important for endoderm formation in *Xenopus* (Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998). The precise roles played by the *Mix* genes, individually and collectively, in endoderm development are not yet clear, but one critical function appears to be the maintenance of *Xsox17* expression in the prospective endoderm (Henry and Melton, 1998).

The HMG-box transcriptional activators Xsox17α and -β (here referred to collectively as Xsox17) appear to function as important intrinsic regulators of endodermal formation (Hudson et al., 1997). Originally identified in a screen for *Xenopus* genes differentially expressed in the vegetal pole during gastrulation, *Xsox17* shows an endodermally restricted expression pattern from the onset of gastrulation (Hudson et al., 1997). Overexpression of Xsox17 promotes endodermal gene expression in animal cap explants, while expression of a fusion between Xsox17 and the repressor domain of *Drosophila* Engrailed (EnR) inhibits endoderm differentiation in both isolated vegetal pole

explants and the intact frog embryo (Hudson et al., 1997). Importantly, co-expression of the Xsox17-EnR fusion together with Mixer blocks the endoderm-inducing ability of Mixer, suggesting that Mixer promotes endoderm formation principally through Xsox17 (Henry and Melton, 1998).

Overview

The work described in this dissertation addresses several aspects of the early development of the endoderm in zebrafish. In chapter 2 I demonstrate an essential cell-autonomous role for the zebrafish locus *casanova* in the initial formation of the endoderm. *casanova* represents the first genetically defined locus that is specifically required for endoderm formation in a vertebrate. I then use overexpression studies in wild-type and different mutant zebrafish embryos, in work detailed in chapter 3, to assemble several different endodermal regulators into a molecular pathway that underlies endoderm formation. This pathway links many of the currently known regulators of endoderm formation into a single coherent framework that should be useful in assessing the roles of newly identified potential regulators of vertebrate endoderm development. In chapter 4 I present molecular and genetic evidence that indicate an important distinction between the gut endoderm and the pharyngeal endoderm in zebrafish. These results suggest that the pharyngeal endoderm has many characteristics in common with the mesoderm, and argue more generally that the use of molecular genetic criteria to describe the developmental relationships between cells may prove more useful than current germ layer assingments. In chapter 5 I describe studies on the roles of bone morphogenetic proteins in the formation and early anterior-posterior patterning of the endoderm, which provide the first evidence that these molecules may play an endogenous role in early endoderm development.

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Chapter 2: *casanova* plays an early and essential role in endoderm formation in zebrafish

SUMMARY

The cellular and molecular mechanisms that regulate endoderm development in vertebrates have only recently begun to be explored. Here we show that the zebrafish locus *casanova* plays an early and essential role in this process. *casanova* mutants lack a gut tube and do not express any molecular markers of endoderm differentiation. The early endodermal expression of genes such as *axial*, *gata5*, and *fkd2* does not initiate in *casanova* mutants, indicating that the endoderm is defective from the onset of gastrulation. Mosaic analysis demonstrates that *casanova* functions cell-autonomously within the endodermal progenitors. We also report the isolation of a zebrafish homologue of *Mixer*, a gene important for early endoderm formation in *Xenopus*. *casanova* does not encode zebrafish Mixer, and *mixer* expression is normal in *casanova* mutants, indicating that *casanova* acts downstream of, or in parallel to, *mixer* to promote endoderm formation. We further find that the forerunner cells, a specialised group of non-involuting dorsal mesendodermal cells, do not form in *casanova* mutants. Studies of *casanova* mutants do not support an important role for the forerunner cells in either dorsal axis or tail development, as has been previously proposed. In addition, although different populations of mesodermal precursors are generated normally in *casanova* mutants, morphogenetic defects in the heart, vasculature, blood, and kidney are apparent, suggesting a possible role for the endoderm in the morphogenesis of these organs.

INTRODUCTION

The three fundamental germ layers of the vertebrate embryo--ectoderm, mesoderm, and endoderm--form during gastrulation. The induction and patterning of the ectoderm and mesoderm have been studied extensively, resulting in a detailed though still incomplete understanding of how these tissues arise (Kessler and Melton, 1994; Slack, 1993). In contrast, development of the endoderm, which forms the gut tube, its associated organs such as the liver and pancreas, and the lining of the respiratory tract, has until recently been relatively unexplored.

Most of our knowledge about endoderm development comes from studies of the amphibian *Xenopus laevis*. The endoderm in *Xenopus* arises from the yolk-rich cells of the vegetal hemisphere (Dale and Slack, 1987). These cells commit to an endodermal fate by early in gastrulation, but prior to this stage they can be redirected to other fates by various experimental manipulations (Heasman *et al.*, 1984; Henry *et al.*, 1996; Wylie *et al.*, 1987). Importantly, substantial endodermal differentiation occurs in isolated *Xenopus* vegetal pole explants (Gamer and Wright, 1995; Henry *et al.*, 1996; Jones *et al.*, 1993), suggesting that the endoderm forms through a process that is largely cell- and/or tissue-autonomous.

Certain growth factors that induce mesoderm can also induce endoderm. For example, the related transforming growth factor TGF-β superfamily members Activin and Vg1 are capable of inducing the expression of several endodermal markers in isolated *Xenopus* animal caps (Gamer and Wright, 1995; Henry *et al.*, 1996; Jones *et al.*, 1993); experiments using an inhibitory Vg1 ligand confirm an endogenous role for a Vg1-like activity in dorsal endoderm formation in *Xenopus* (Joseph and Melton, 1998). Fibroblast growth factors and the secreted bone morphogenetic protein antagonists chordin and noggin may also function in *Xenopus* endoderm induction (Henry *et al.*, 1996; Jones *et al.*, 1993; Sasai *et al.*, 1996). Together these observations suggest a general model in which

the high levels of mesoderm inducers produced by vegetal cells create a local signalling environment that directs these vegetal cells themselves to an endodermal fate.

A critical player in *Xenopus* endoderm induction is the T-box transcription factor VegT. Ectopic expression of *VegT* in animal caps causes expression of several endodermal markers (Horb and Thomsen, 1997). Conversely and significantly, depletion of the vegetally localised maternal deposit of *VegT* using anti-sense oligonucleotides blocks endoderm formation entirely (Zhang *et al.*, 1998). These results further support the idea that *Xenopus* endoderm induction occurs at least tissue-autonomously. How the VegT-regulated zygotic genes interact with the signalling pathways described above to induce endoderm formation is not known.

Several recently identified zygotically expressed genes may act within the presumptive endoderm in response to inducers such as Activin. Two *Xenopus* homologues of the mouse *Sox17* gene, *Xsox17α* and *Xsox17β*, are capable of directing presumptive ectodermal tissue to an endodermal fate (Hudson *et al.*, 1997). Expression of *Xsox17* becomes restricted to the endoderm at the onset of gastrulation and is induced in animal caps by treatment with activin (Hudson *et al.*, 1997). Overexpression of *Xsox17* results in high levels of endodermal marker expression in isolated *Xenopus* animal caps, while overexpression of a fusion of Xsox17 and the repressor domain of *Drosophila* Engrailed (EnR) inhibits the expression of such markers in both vegetal pole explants and activin-treated animal caps (Hudson *et al.*, 1997). *Mix* homeobox genes also appear to play an important role in endoderm formation. Several such genes have been isolated in *Xenopus*, all of which show endodermal expression and are induced in animal caps by activin treatment (Ecochard *et al.*, 1998; Henry and Melton, 1998; Rosa, 1989; Tada *et al.*, 1998). Expression of at least some *Mix* genes is also induced by VegT . Ectopic overexpression of *Mix* genes results in different degrees of endodermal gene expression in isolated *Xenopus* animal caps; two in particular, *Mixer* and *milk*, induce high levels of endodermal marker expression (Ecochard *et al.*, 1998; Henry and Melton, 1998).

Experiments using Mixer-EnR and Xsox17-EnR fusions strongly suggest that Mixer acts upstream of Xsox17 and likely promotes endoderm development principally or perhaps entirely through Xsox17 (Henry and Melton, 1998). The restriction of *Xsox17* expression to the presumptive endoderm by Mixer (and perhaps other Mix proteins) therefore likely represents a critical early event in endoderm formation.

Mutational analyses have identified few genes essential for vertebrate endoderm formation. Tetraploid embryo-ES cell aggregation experiments in mouse demonstrate an essential role for the transcription factor HNF3β in fore- and midgut development (Dufort *et al.*, 1998). Zebrafish zygotic *one-eyed pinhead* (*oep*) mutants lack endoderm as well as prechordal plate and ventral neuroectoderm (Schier *et al.*, 1997). *oep* encodes a member of the EGF-CFC protein family that appears to act as an essential cofactor in signalling by Nodal-related growth factors (Gritsman *et al.*, 1999). Also, zebrafish embryos mutant for both *squint* and *cyclops*, two genes that encode Nodal-related growth factors, form essentially no mesendoderm (Feldman *et al.*, 1998). Zebrafish embryos lacking both maternal and zygotic One-eyed pinhead protein display an identical phenotype (Gritsman *et al.*, 1999). In these cases involution does not occur, however, leaving it unclear whether these factors directly induce mesendodermal fates or promote the cell movements necessary for mesendoderm formation during gastrulation.

In this report we demonstrate an essential role for the zebrafish locus *casanova* (*cas*) in endoderm development. *cas* mutants appear to lack endoderm entirely from the onset of gastrulation. *cas* functions cell-autonomously within the endodermal progenitors, and acts either downstream of, or in parallel to, a zebrafish Mixer homologue. *cas* mutants also appear to lack forerunner cells, and display morphogenetic defects in several mesodermal derivatives. *cas* thus represents the first locus specifically required for endoderm formation in a vertebrate, and provides a unique opportunity to analyse the role of the endoderm in patterning the embryo.

MATERIALS AND METHODS

Strains

Adult zebrafish and embryos were maintained and staged as decribed (Westerfield, 1995). The *cas*^{ta56} and *knypek*^{m119} (*kny*) mutations were identified in screens for ENU-induced embryonic-lethal mutations (Chen *et al.*, 1996; Solnica-Krezel *et al.*, 1996).

Phenotypic analysis

In situ hybridisations were performed as described (Alexander *et al.*, 1998). For sectioning, embryos were embedded in JB4 (Polysciences) and counterstained with neutral red. Labelling of forerunner cells with syto-11 was perfomed as described (Cooper and D'Amico, 1996). Photographs were taken on either a Leica MZ12 stereo microscope or a Zeiss Axioplan using Kodak Ektachrome 160T or Fujichrome 1600 ASA film, and processed using Adobe Photoshop 4.0.

Cell transplantation

Cell transplantations were performed essentially as described (Ho and Kimmel, 1993). Cells were transplanted from labelled donor to unlabelled host embryos at mid- to late-blastula stages. Host embryos were fixed at approximately 80% epiboly (mid-gastrula stage), and donor embryos were raised to determine their genotype. Host embryos were examined for expression of the *axial* gene, and biotin-labelled donor cells were subsequently detected using the ABC-peroxidase kit (Vector Laboratories, Inc.).

Isolation of *mixer*

A fragment of *mixer* was isolated from mid-gastrula stage cDNA using degenerate PCR primers 5'-CCCGAGTGCAGGTGTGGTTYCARAA-3' and 5'-GGTGTTCATGTCGGGTGTGATNDTYTTRTT-3' and standard touchdown PCR

protocols. Gene-specific primers were then used to screen a gastrula stage cDNA library by PCR for a full-length *mixer* clone. The GenBank accession number for *mixer* is AF121771.

Linkage Analysis

We identified a single-strand conformational polymorphism in the *mixer* 3' untranslated region (UTR) in a line containing the *cas*^{ta56} allele. *cas*^{ta56} does not segregate with *mixer* (data not shown) and therefore *cas* does not encode Mixer.

RESULTS

cas mutants lack a gut tube

The *cas* locus is defined by a single recessive allele, cas^{ta56}, identified in a large-scale screen for mutations that affect zebrafish embryonic development (Chen *et al.*, 1996). *cas* mutants are first identifiable at approximately 24 hours post fertilisation (hpf) by the presence of cardia bifida--bilateral hearts resulting from a failure of cardiac fusion to occur. *cas* mutants exhibit pericardial edema and collapsed brain ventricles, common to all zebrafish cardiac mutants, as well as a thickened yolk extension (Fig. 2.1A,B). The *cas* mutation was originally classified as affecting heart formation (Chen *et al.*, 1996). Light microscopic examination at 36 hpf and later stages, however, reveals that *cas* mutants entirely lack a gut tube. The absence of a gut tube is most easily seen just behind the yolk extension where the intestine exits the body at the anal opening, immediately anterior to the pronephric ducts (Fig. 2.1C). *cas* mutants have neither an anal opening nor an intestine, nor is a well-formed pronephric duct visible (Fig. 2.1D). Small cysts form posterior to the yolk extension in *cas* mutants (Fig. 2.1D). We hypothesise that these cysts result from the failure of the pronephric ducts to form normally in the absence of a gut tube (see below).

Histological sections of 48 hpf embryos confirm the absence of a gut tube in *cas* mutants. While the *sonic hedgehog* (*shh*)-expressing gut tube is clearly visible between the notochord and the yolk in wild-type embryos (Fig. 2.1E), in *cas* mutants the notochord is positioned almost directly atop the yolk (Fig. 2.1F). Floor plate cells in *cas* mutants express *shh* normally (Fig. 2.1E,F), but no other *shh*-expressing cells are seen, suggesting that the endoderm is absent in *cas* mutants.

cas mutants do not express molecular markers of endoderm differentiation

In order to test further whether any endoderm is present in *cas* mutants we examined the expression of various endoderm markers. Several genes that encode

transcription factors related to *Drosophila* Forkhead are expressed in the endoderm during zebrafish development (Odenthal and Nusslein-Volhard, 1998). *axial*, a zebrafish homologue of mouse *HNF3β* (Strahle *et al.*, 1993), is expressed in the anterior endoderm and the ventral neuroectoderm during somitogenesis (Fig. 2.2A). No endodermal *axial* expression is detectable in *cas* mutants, although neural expression of *axial* appears normal (Fig. 2.2B). Two other *forkhead*-related genes, *fkd7* and *fkd2*, are expressed in the endoderm as well as in subpopulations of the neural crest and axial mesoderm (Fig. 2.2C) (Odenthal and Nusslein-Volhard, 1998). Again *cas* mutants specifically lack endodermal expression of *fkd7* and *fkd2* (Fig. 2.2D and data not shown). Additionally, *fkd7* expression reveals that the hypochord forms but is shortened posteriorly in *cas* mutants (Fig 2.2D).

The *gata* genes encode zinc finger-containing transcription factors, several of which are expressed in the developing gut and heart (Laverriere *et al.*, 1994). We examined the expression of *gata4* in wild-type and *cas* mutant embryos during late somitogenesis. At these stages wild-type embryos express *gata4* in the myocardium as well as a region of the endoderm from which the liver will later develop (Fig. 2.2E). Cardiac expression of *gata4* is evident in *cas* mutants, demonstrating cardia bifida, but no endodermal *gata4* expression is seen (Fig. 2.2F). Examination of *gata6* expression in wild-type and *cas* mutant embryos yielded similar results (data not shown). Thus, we see no molecular evidence for the presence of endoderm in *cas* mutants during somitogenesis stages.

The endoderm is defective in *cas* mutants from the onset of gastrulation

Endodermal expression of *axial* initiates soon after the onset of gastrulation (Fig. 2.3A), and is maintained throughout gastrulation (Fig. 2.3C). These *axial*-expressing cells are identifiable as endodermal precursors by their close apposition to the yolk and their large flattened morphology (Warga and Nusslein-Volhard, 1999). *cas* mutants specifically lack endodermal *axial* expression, while *axial* expression in the prechordal plate and

notochord appears normal (Fig. 2.3B,D). These data indicate that the endoderm in *cas* mutants is defective when the hypoblast first forms.

The zebrafish *gata5* homologue has recently been isolated and shown to be expressed in endodermal precursors as the hypoblast forms in the early gastrula (Rodaway *et al.*, 1999). We therefore examined *gata5* expression in wild-type and *cas* mutant embryos. By mid-gastrulation *gata5* expression appears in endodermal precursors distributed throughout the forming hypoblast (Fig. 2.3E). This endodermal *gata5* expression is strikingly absent in *cas* mutants (Fig. 2.3F). Endodermal expression of *fkd2* (Odenthal and Nusslein-Volhard, 1998) also initiates during gastrulation (Fig. 2.3G), but again is specifically absent in *cas* mutants (Fig. 2.3H). These data reinforce the conclusion that the endoderm in *cas* mutants is defective, and perhaps entirely absent, from the onset of gastrulation.

cas functions cells-autonomously in the endoderm

The above results demonstrate that the endoderm is abnormal in *cas* mutants from a very early stage of development. This defect could result from a failure by *cas* mutants to generate endoderm-inducing signals. Alternatively, the presumptive endodermal progenitors in *cas* mutants may fail to receive or to respond to such signals. In order to test directly where *cas* functions in endoderm development we used cell transplantation to create genetic mosaics (Ho and Kimmel, 1993). We then assessed the behavior of wild-type cells transplanted into *cas* mutant hosts and vice versa by analysingthe expression of *axial*. Wild-type cells transplanted into *cas* mutant hosts can form endoderm, as assayed by their expression of *axial*, their endodermal morphology (Warga and Nusslein-Volhard, 1999), and their lateral location in the embryo (Fig. 2.4). In contrast, *cas* mutant cells were never observed to form endoderm (Fig. 2.4 and data not shown). These experiments demonstrate that *cas* functions cell-autonomously within the endoderm to allow its proper development.

A zebrafish *Mixer* homologue is expressed normally in the prospective mesendoderm in *cas* mutants

The homeobox gene *Mixer* has recently been shown to play a key early role in *Xenopus* endoderm formation (Henry and Melton, 1998). The early occurrence of the *cas* endoderm defect and the cell-autonomy of *cas* function in the endoderm led us to examine the relationship between *cas* and *Mixer*. We used degenerate PCR to isolate a zebrafish *mix* gene (Fig. 2.5A). BLAST searches (Altschul *et al.*, 1990) suggest that this gene is most closely related to *Xenopus Mixer*, and we therefore refer to it provisionally as *mixer*. The zebrafish Mixer homeodomain shows 69% identity with that of chick CMIX (Peale *et al.*, 1998; Stein *et al.*, 1998), and resembles about equally those of *Xenopus* Mixer (58% identity) and Milk (56% identity) (Fig. 2.5B). We used a SSCP polymorphism in the *mixer* 3' UTR to analyse linkage with *cas*. We found that they are not linked, demonstrating that *cas* does not encode zebrafish Mixer (data not shown).

We next examined *mixer* expression. In wild-type embryos we first detect *mixer* expression at the sphere stage in a small group of dorsal cells (Fig. 2.5C). By dome stage *mixer* expression has spread circumferentially throughout the marginal zone, and also appears in the dorsal YSL (Fig. 2.5D). At subsequent stages we do not detect *mixer* expression in the YSL. Expression in the marginal zone persists through the onset of gastrulation (Fig. 2.5E,F). Soon afterwards *mixer* expression is downregulated, and by 60% epiboly is undetectable (data not shown). *mixer* expression in *cas* mutants is indistinguishable from that seen in wild-type embryos at all stages (data not shown).

We also compared *mixer* expression to two other genes expressed in the marginal zone of the pregastrula zebrafish embryo, *no tail* (*ntl*) and *gata5*. *ntl* encodes the zebrafish homologue of mouse Brachyury (Schulte-Merker *et al.*, 1994) and is expressed in all cells that will involute to form the hypoblast (i.e. both endoderm and mesoderm) (Fig. 2.5G) (Schulte-Merker *et al.*, 1992). Prior to the onset of gastrulation *gata5* is expressed in a subset of the marginal zone from which all of the endoderm as well as some mesoderm will

emerge (Fig. 2.5H) (Rodaway *et al.*, 1999; Warga and Nusslein-Volhard, 1999). The *mixer* expression domain appears quite similar to the that of *ntl*, and includes substantially more of the marginal zone than the *gata5* expression domain (compare Figs 2.5F-H). Thus, expression of *mixer*, unlike that of its *Xenopus* homologue, is not restricted to the prospective endoderm.

cas mutants lack forerunner cells

The forerunner cells (FRs) first appear as a group of highly endocytic cells located at the dorsal margin of the late blastula zebrafish embryo (Cooper and D'Amico, 1996). From there they migrate along the YSL in front of the advancing blastoderm margin, and upon completion of epiboly come to occupy a position deep within the tailbud at the chordoneural hinge (Cooper and D'Amico, 1996; Melby *et al.*, 1996). Shortly thereafter the FR cluster expands to form Kupffer's vesicle, a fluid-filled sac unique to the teleost tailbud (Cooper and D'Amico, 1996). Late in somitogenesis Kupffer's vesicle disappears and the progeny of the FRs contribute to the notochord, muscle, and mesenchyme of the tail (Melby *et al.*, 1996).

The FRs have been proposed to represent the endodermal aspect of the neurenteric canal, a transiently existent space that connects the ependymal canal (the lumen of the spinal cord) to the anus at the end of gastrulation in numerous chordates (Cooper and D'Amico, 1996; Gont *et al.*, 1993). This hypothesis implies that the FRs are endodermal in origin. Given the absence of other endodermal derivatives in *cas* mutants, we examined Kupffer's vesicle, which the FRs normally form (Fig. 2.6A), in embryos derived from a *cas/+* heterozygote intercross. Light microscopic observation of embryos at the 6-somite stage revealed that Kupffer's vesicle did not form in approximately one quarter (17/77) of these embryos (Fig. 2.6B); when raised these embryos were all *cas* mutants. This observation demonstrates a defect in the FRs in *cas* mutants.

In order to assess the FRs earlier in development we examined expression of the *ntl* gene in wild-type and *cas* mutant embryos. At 80% epiboly *ntl* is expressed throughout the involuting cells of the germ ring and in the developing notochord; *ntl* is also expressed in the FRs (Melby *et al.*, 1996), visible as an area of *ntl* expression that extends posteriorly from the notochord below the level of the margin (Fig. 2.6C). FR *ntl* expression was absent in one quarter (16/64) of the embryos derived from a *cas*/+ heterozygote intercross (Fig. 2.6D), while *ntl* expression in the germ ring and notochord was normal. Thus *cas* mutants appear to lack forerunner cell expression of *ntl*.

Functional defects in the FRs could cause the lack of FR *ntl* expression and the failure of Kupffer's vesicle to form in *cas* mutants. Alternatively, *cas* mutants may not form FRs at all. In order to test the latter hypothesis we treated embryos from a *cas*/+ heterozygote intercross at the dome stage with syto-11, a fluorescent dye that labels the highly endocytic FRs (Cooper and D'Amico, 1996). This procedure permits visualisation under fluorescence microscopy of the forerunner cell cluster late in gastrulation (Fig. 2.6E). Approximately one quarter of the embryos (6/22) lacked a fluorescent forerunner cell cluster (Fig. 2.6F); when raised these embryos proved to be *cas* mutants. We also used Nomarski optics to examine the dorsal margin of shield stage embryos derived from a *cas*/+ heterozygote intercross (Fig. 2.6G); this technique allows direct visualisation of the forerunner cell cluster (Melby *et al.*, 1996). Again, in approximately one quarter of the embryos (10/36) no FRs were seen (Fig. 2.6H), and when raised these embryos were indeed *cas* mutants. Considered together these data demonstrate that the FRs do not form in *cas* mutants.

Defective morphogenesis of mesodermal derivatives in *cas* mutants

The *cas* mutation was originally identified because it causes cardia bifida, as shown by expression of the cardiac-specific homeobox gene *nkx2.5* (Fig. 2.7A,B) (Chen and Fishman, 1996; Lee *et al.*, 1996). In order to determine whether other mesodermal

derivatives develop abnormally in *cas* mutants we examined the expression of various mesodermal markers. The bilaterally positioned endothelial precursors express the receptor tyrosine kinase gene *tie2* (Lyons *et al.*, 1998), and normally assemble smoothly in the midline to form the trunk vasculature during somitogenesis (Fig. 2.7C) (Liao *et al.*, 1997). These endothelial precursors are disorganised in *cas* mutants (Fig. 2.7D); more anterior populations of endothelium, including the endocardium, are similarly abnormal (data not shown). The *gata1* gene labels differentiating erythroblasts arranged in bilateral stripes within the posterior lateral plate mesoderm (Detrich *et al.*, 1995). During somitogenesis these cells move towards each other and join in the midline (Fig. 2.7E). This medial movement is also perturbed in *cas* mutants (Fig. 2.7F). Lastly, the *pax2.1* gene (Mikkola *et al.*, 1992) is expressed in the nephrogenic mesoderm, which shows a similar arrangement to the differentiating erythroblasts (Fig. 2.7G). Again, as development proceeds the pronephric ducts move medially in wild-type embryos, but fail to do so normally in *cas* mutants (Fig. 2.7H); the kidneys also appear positioned more laterally in *cas* mutants. Thus, precursors of at least four different mesodermal organs--heart, vasculature, blood, and kidney--exhibit morphogenetic defects in *cas* mutants.

We considered the possibility that these mesodermal defects could result from decreased dorsal convergence during and after gastrulation. However, several lines of evidence argue against this. First, the notochord in *cas* mutants is not abnormally broad (Fig. 2.3G,H). Second, the *pax2.1*-expressing spinal commissural interneurons (Mikkola *et al.*, 1992) are not wider apart in *cas* mutants as compared to wild-type (Fig. 2.7G,H). Finally, we examined both the endoderm and these same mesodermal derivatives in *knypek* (*kny*) mutant embryos, which exhibit dramatically diminished convergence and extension (Solnica-Krezel *et al.*, 1996). *kny* mutants form endoderm that appears essentially normal although more broadly spread across the embryo (Figs 2.7I,J). The trunk endothelium in *kny* mutants is also spread more broadly across the midline (Fig. 2.7L). *kny* mutants do manifest morphogenetic abnormalities in the blood and kidney precursors by the end of

gastrulation that appear to be similar to *cas* mutants, but in *kny* mutants the *pax2.1*-expressing spinal commissural interneurons are more widely spaced than normal (data not shown). These data suggest that the convergence and extension defect in *kny* mutants affects all three germ layers. Importantly, however, *kny* mutants do not exhibit cardia bifida and their endothelium does not appear disorganised (Fig. 2.7K,L). Also, the morphogenetic defects in the kidney and blood progenitors of *cas* mutants do not appear until after the 10-somite stage, more than three hours later than in *kny* mutants, further suggesting that the underlying problem in the two mutants is different. Considering the above results, we conclude that the *cas* mesodermal defects likely do not result from a general defect in dorsal convergence, but rather from a more specific failure of lateral mesodermal cells to undergo the appropriate medial migration.

DISCUSSION

An essential role for *cas* in endoderm formation

Little is known about the genetic networks that control development of the vertebrate endoderm. Recently, however, several genes have been identified that appear to play key roles in the early events of endoderm formation. Important functions for these genes--in particular *Xsox17*α and -β and *Mix* homeobox genes such as *Mixer* and *milk*--are suggested by their endodermally-restricted expression patterns, their ability to promote endodermal gene expression when ectopically overexpressed, and, conversely, their ability to inhibit endodermal gene expression in presumptive endodermal tissue when fused to the *Drosophila* EnR domain (Ecochard *et al.*, 1998; Henry and Melton, 1998; Hudson *et al.*, 1997; Lemaire *et al.*, 1998; Tada *et al.*, 1998). It will be important to test whether mutations in these genes confirm their presumed roles in this process. In addition, genetic analyses have demonstrated required roles for mouse *HNF3*β and zebrafish *oep*, *cyclops* and *squint* in formation of part or all of the endoderm (Dufort *et al.*, 1998; Feldman *et al.*, 1998; Gritsman *et al.*, 1999; Schier *et al.*, 1997).

Our data demonstrate an early and essential requirement for the zebrafish *cas* locus in formation of the endoderm. *cas* mutants exhibit no evidence of endodermal differentiation; they lack a gut tube and show no endodermal gene expression during somitogenesis and pharyngula stages. Most interestingly, *cas* mutants lack endodermal expression of *axial*, *gata5*, and *fkd2* from the onset of gastrulation. These data place *cas* upstream of these early endodermal markers and suggest that the endoderm in *cas* mutants is not merely defective but in fact may not form. What becomes of the endodermal progenitors in *cas* mutants is not known. These cells may die, although we have not observed increased apoptosis in *cas* mutants during gastrulation (M.R. and D.Y.R.S., unpublished data). Alternatively these cells may be respecified, for example to mesodermal

fates. Testing this possibility will require the isolation of markers specific for the involuted mesoderm.

Mosaic analysis demonstrates that *cas* functions cell-autonomously within the endodermal progenitors, presumably either to receive or respond to endoderm-inducing signals. Directed misexpression of a constitutively active type I TGF-β receptor (TARAM-A*) in a single blastomere of 16-cells stage zebrafish embryos cell-autonomously directs the progeny of that blastomere to an endodermal fate (Peyrieras *et al.*, 1998). Interestingly, TARAM-A* misexpression also restores both endoderm and prechordal plate formation in *oep* mutants (Peyrieras *et al.*, 1998). The fact that the prechordal plate forms normally in *cas* mutants suggests that *cas* is required specifically in the endoderm, either downstream of or in parallel to *oep* and TARAM-A*, but experiments to test this hypothesis directly are needed.

Many studies have focused upon the the role(s) of the endoderm in the induction or patterning of the mesoderm and ectoderm (see for example Nieuwkoop, 1969; Bouwmeester *et al.*, 1996). Given the apparent complete lack of endoderm in *cas* mutants, the mutant embryos' relatively normal appearance (Fig. 2.1) is quite striking. Endodermal cells may be transiently present and able to fulfill their normal signalling functions in *cas* mutants. Alternatively, the relatively normal appearance of *cas* mutants may result from the fact that in non-amphibian embryos these signalling functions appear to be performed at least in part by extraembryonic tissues, for example, in zebrafish the YSL and in mouse the visceral endoderm (Beddington and Robertson, 1998; Fekany *et al.*, 1999; Koos and Ho, 1998; Yamanaka *et al.*, 1998). At the same time, the mesodermal defects in *cas* mutants suggest potentially important roles for the endoderm in later morphogenetic events (see below). Further analyses of *cas* mutants will provide a unique opportunity to address the various roles played by the endoderm during vertebrate development.

Mesendodermal expression of a zebrafish *Mixer* homologue

The early cell-autonomous role of *cas* in endoderm development led us to examine the relationship between *cas* and *Mixer*. We have isolated a zebrafish *Mix* gene that we call *mixer*. Determining whether this gene represents an authentic zebrafish *Mixer* equivalent will require both functional analyses and the isolation of additional zebrafish *mix* genes. *mixer* is not linked to *cas*, and *mixer* expression is normal in *cas* mutants, suggesting that *cas* acts either downstream of, or in parallel to, *mixer* to regulate endoderm formation.

The *mixer* expression pattern raises intriguing questions regarding how endoderm and mesoderm are segregated before and during gastrulation. Unlike in *Xenopus*, where *Mixer* expression is restricted to the presumptive endoderm, the *mixer* expression domain at the onset of gastrulation appears to encompass most if not all of the marginal cells that express *ntl*; both presumptive endoderm and mesoderm therefore express *mixer*. These results suggest that zebrafish embryos may utilise a different mechanism to segregate mesoderm from endoderm than does *Xenopus*. In *Xenopus* vegetally localised maternal VegT appears to play a critical role in this process (Zhang *et al.*, 1998). A zebrafish homologue of VegT, encoded by the *spadetail* locus, is not maternally expressed (Griffin *et al.*, 1998), perhaps providing further evidence of a difference between zebrafish and *Xenopus*. Comparison of the expression domains of *Brachyury* homologues reveals yet another distinction between zebrafish and *Xenopus*; while expression of the *Xenopus Brachyury* homologue *Xbra* is restricted to the mesoderm (Smith *et al.*, 1991), in the zebrafish all involuting cells (i.e. both endoderm and mesoderm) express *ntl* (Schulte-Merker *et al.*, 1992). Interestingly, all ingressing cells in both mouse and chick gastrulae also express *Brachyury* (Beddington *et al.*, 1992; Kispert *et al.*, 1995). This observation may suggest that these organisms use a mechanism to segregate endoderm from mesoderm that is more similar to that used in zebrafish than that in *Xenopus*, although how this segregation is achieved remains unknown. Understanding how the pregastrula expression of zebrafish *gata5* is restricted to the portion of the marginal zone from which the endoderm

will emerge may provide some insight into this question, as likely will the molecular identification of *cas* and subsequent analysis of its expression.

Forerunner cell development requires *cas*

Our data illuminate several aspects of FR cell biology. First, it has not been clear to which germ layer the FRs belong. Kupffer's vesicle has been proposed to represent the endodermal aspect of the neurenteric canal, implying that the FRs are endodermal (Cooper and D'Amico, 1996). On the other hand, the various fates to which the FRs' progeny ultimately contribute--notochord, muscle, and mesenchyme of the tail--are generally considered mesodermal (Melby *et al.*, 1996). We believe the fact that *cas* is required for the formation of the FRs (Fig. 2.6), similar to the role of *cas* in endoderm development, provides genetic evidence supporting the endodermal assigment of the FRs. However, we have not directly tested whether *cas* acts cell-autonomously in the FRs. The β-catenin signalling pathway that determines the embryonic dorsal axis also appears essential for formation of the FRs (Fekany *et al.*, 1999). We therefore propose that the FRs represent a specialised dorsal subset of the endoderm.

A related point concerns the germ layer assignment of the hypochord. Studies in amphibia have concluded that this structure derives from the endoderm (Lofberg and Collazo, 1997), and the same has therefore been assumed to be true in zebrafish (Appel *et al.*, 1999). However, while truncated posteriorly (Fig. 2.2), the hypochord clearly forms in *cas* mutants, which suggests that the hypochord may not be endodermal. It has recently been proposed that in zebrafish Notch-Delta signalling plays a role in the allocation of dorsal midline cells to the ectoderm (floorplate), mesoderm (notchord), and endoderm (hypochord) (Appel *et al.*, 1999). Considering that the hypochord is present in *cas* mutants, we would suggest an alternative interpretation of these results; that Notch-Delta signalling acts to subdivide a common progenitor population into three different derivatives; the floorplate, the notochord, and the hypochord. We would propose that this progenitor

population is most likely mesodermal: its formation clearly does not require *cas*, as floor plate, notochord, and hypochord are all present in *cas* mutants, arguing against an endodermal assignment; and studies in chick and zebrafish have demonstrated a close embryologic and molecular genetic relationship between the notochord and floor plate (Halpern *et al.*, 1997; Teillet *et al.*, 1998).

The functions of the FRs or their derivative, Kupffer's vesicle, in zebrafish development remain mysterious. The appearance of the FRs at approximately 30% epiboly provides the earliest morphological landmark of the embryo's dorsal aspect (Cooper and D'Amico, 1996). LiCl treatment results in the appearance of ectopic FRs, while strongly affected zebrafish *bozozok* mutants lack FRs, indicating that FR formation lies downstream of the same β-catenin signalling pathway that specifies the dorsal axis (Cooper and D'Amico, 1996; Fekany *et al.*, 1999). These observations have led to the idea that the FRs may play a role in the induction or maintenance of dorsal mesoderm (Cooper and D'Amico, 1996; Fekany *et al.*, 1999). While our studies were not exhaustive, we see no evidence for this hypothesis; *cas* mutants express prechordal plate and notochord markers normally and are not cyclopic (Figs. 2.1 and 2.3; J.A. and D.Y.R.S., unpublished data). It is also formally possible that FR precursors are transiently present in *cas* mutants and provide these functions. The fact that Kupffer's vesicle does not form in *ntl* mutants has suggested a possible role for this structure in tail development (Melby *et al.*, 1996). Again, our results provide no clear evidence for such a function; the tailbud extends in *cas* mutants and contains a normal number of somites (Fig. 2.1). As noted above, the hypochord in *cas* mutants does appear shortened posteriorly (Fig. 2.2). The hypochord and floor plate connect in the tail of the late somitogenesis and pharyngula stage embryo, a position defined as the chordoneural hinge (Gont *et al.*, 1993). As the FRs and their derivative, Kupffer's vesicle, sit at this exact place during tail extension, the hypochord defect in *cas* mutants may relate to the absence of the FRs. Other than this possibility, however, we have not identified any specific essential function for the FRs in zebrafish development.

A possible role for the endoderm in mesodermal morphogenesis

While the mesoderm appears to differentiate normally in *cas* mutants, at least initially, several mesodermal organs--the heart, vasculature, blood, and kidneys--display morphogenetic defects (Fig. 2.7). These defects are unlikely to result from reduced dorsal convergence, as *kny* mutants do not show similar abnormalities despite being strongly defective in convergence and extension (Fig. 2.7). Also, the normal width of the notochord and normal spacing of the spinal commissural interneurons in *cas* mutants argue against a general defect in dorsal convergence.

These morphogenetic defects may be due to the cell-autonomous action of *cas* in each of these mesodermal cell populations, or may instead result non-autonomously from the absence of the endoderm. For example, the endoderm may provide signals that guide mesodermal morphogenesis, may serve as a substrate for the migration of mesodermal cells, and/or may move adherent mesodermal cells in the course of its own morphogenesis. The cardia bifida in *gata4*-mutant mouse embryos provides one example of an endodermal defect that apparently underlies abnormal mesodermal morphogenesis (Narita *et al.*, 1997). Studies of *oep* mutants also support a role for the endoderm in the morphogenesis of the heart (Schier *et al.*, 1997; Peyrieras *et al.*, 1998), vasculature (Fouquet *et al.*, 1997), and kidneys and blood (J.A. and D.Y.R.S., unpublished data). Resolution of this issue in *cas* mutants awaits direct testing of the cell-autonomy of these morphogenetic defects.

It is also notable that together the two *cas* 'hearts' together appear to contain as much myocardial tissue as do wild-type embryos (compare Figs. 2.7A and 2.7B), and that the *cas* 'hearts' beat and express all myocardial markers thus far tested (M.R., J.A., and D.Y.R.S., unpublished data; Yelon et al., in press). Numerous studies have suggested important roles for the endoderm in the induction, differentiation, and/or maturation of the myocardium (see for example Jacobson and Sater, 1988; Gannon and Bader, 1995; Schultheiss *et al.*, 1995). Endodermal precursors may be transiently present in *cas* mutants and provide sufficient signals to induce and promote the differentiation of the myocardium.

Alternatively, some other tissue may provide these signals. Clearly further studies are needed to resolve the potential roles of the endoderm in zebrafish heart development.

Conclusion

The results presented in this report establish that the *cas* locus is required for endoderm and FR formation in zebrafish. Our data also suggest that *cas* plays a direct or indirect role in the morphogenesis of numerous mesodermal derivatives. We have initiated efforts to isolate *cas* by positional cloning. We expect that the molecular identification of *cas*, and the elucidation of its relationship to other genes that act in the formation of the endoderm, will represent fundamental steps towards achieving a detailed understanding of vertebrate endoderm development.

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Figure 2.1. *cas* mutants lack a gut tube. Nomarski optical images (A-D) and histological sections (E,F) of embryos at 24 hpf (A,B), 36 hpf (C,D), and 48 hpf (E,F). Compared to a wild-type sibling (A), the *cas* mutant (B) shows pericardial edema (arrowhead), collapsed brain ventricles (asterixes), and a thicker yolk extension (arrow). In wild-type embryos (C) the still-forming intestine (arrow) and anal opening (black arrowhead) are visible, immediately anterior to the pronephric ducts (white arrowhead). Neither the intestine nor the anal opening is evident in *cas* mutants (D); arrowhead indicates the cyst present in *cas* mutants. (E) The *shh*-expressing gut tube (arrow) sits between the notochord (arrowhead) and the yolk in wild-type embryos. In *cas* mutants (F) no gut tube or *shh*-expressing endodermal cells are evident and the notochord (arrowhead) rests nearly upon the yolk. Expression of *shh* in the floor plate is evident in both wild-type and *cas* mutant embryos. (A-D) Lateral views, anterior to the left and dorsal to the top; (E,F) transverse sections through the upper trunk and yolk ball.

Figure 2.2. *cas* mutants do not express molecular markers of endoderm differentiation. Wild-type (A,C,E) and *cas* mutant (B,D,F) embryos were examined at the 25-somite stage (21.5 hpf) for expression of *axial* (A,B), *fkd7* (C,D), and *gata4* (E,F). (A) *axial* is expressed in the ventral neuroectoderm and anterior endoderm (arrowhead); endodermal *axial* expression is absent from *cas* mutants (B). Similarly, *fkd7* and *gata4* are expressed in the endoderm of wild-type (arrowheads in C,E) but not *cas* mutant (D,F) embryos. *fkd7* is also expressed in the floor plate and hypochord (C), which is posteriorly shortened in *cas* mutants (arrow in D). Endodermal *gata4* expression defines a region of the gut tube that will form the liver. Myocardial *gata4* expression (arrow in E) illustrates the cardia bifida present in *cas* mutants (arrows in F). (A-D) Lateral views, anterior to the left, dorsal to the top; (E,F) dorsal views, anterior to the left.

Figure 2.1

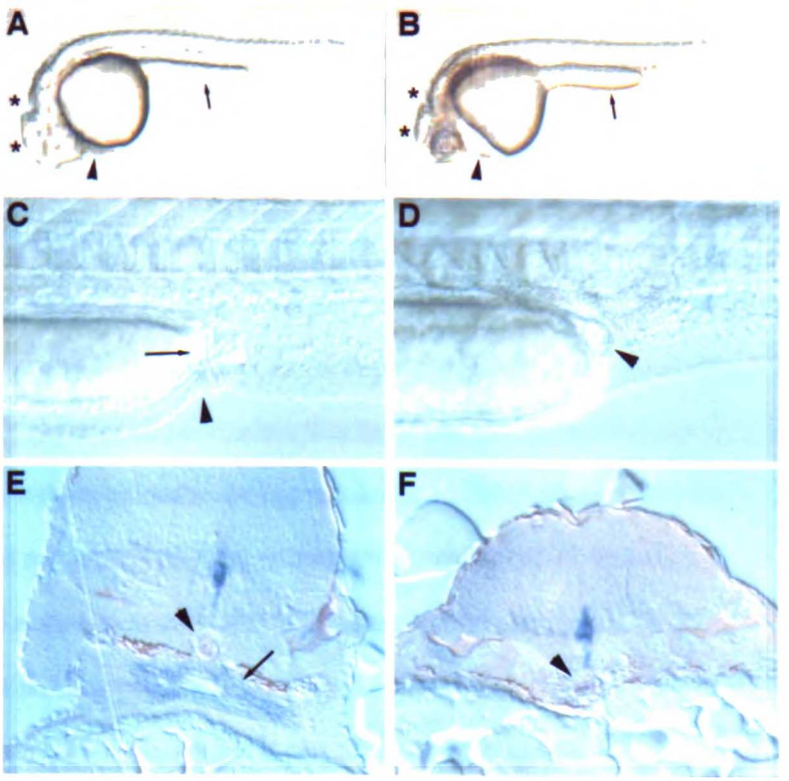

Figure 2.2

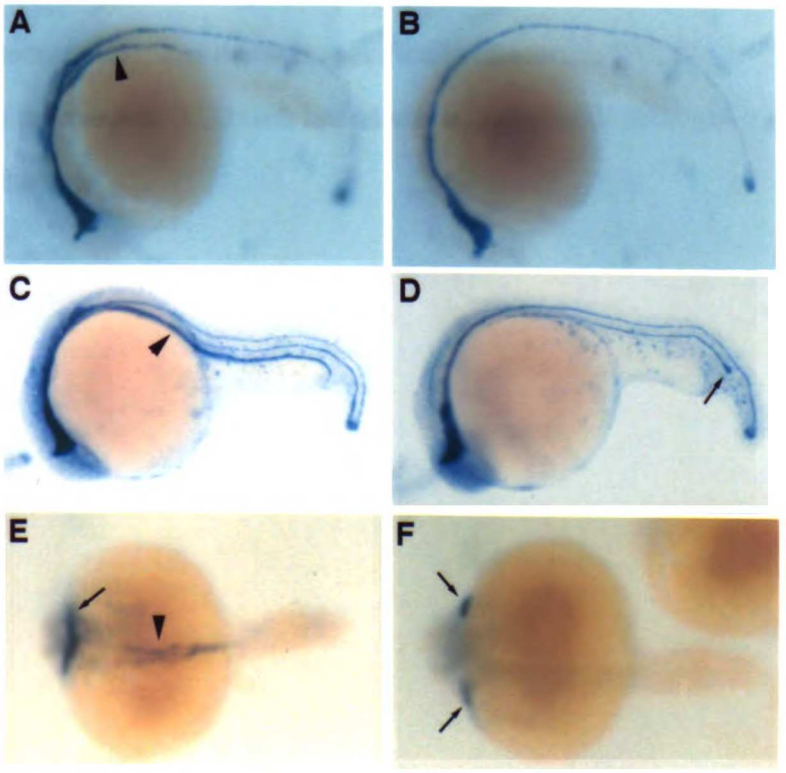

Figure 2.3. The endoderm is defective in *cas* mutants from the onset of gastrulation. *axial* (A-D), *gata5* (E,F), and *fkd2* (G,H) expression in wild-type (A,C,E,G) and *cas* (B,D,F,H) mutant embryos at shield (A,B), 80% epiboly (C-F), and 90% epiboly (G,H) stages. Soon after the onset of gastrulation (A) *axial* is expressed in the embryonic shield and endodermal precursors located throughout the hypoblast. Endodermal *axial* expression is absent in *cas*^{ta56} mutants, while expression in the embryonic shield is normal (B); the few *axial*-expressing cells just ouside the shield in the *cas* mutant are likely notochord precursors that have not yet completed dorsal convergence. At mid-gastrulation wild-type embryos express *axial* in the endodermal precursors and the prechordal plate and notochord (C); no endodermal expression of *axial* is seen in *cas* mutants (D). *gata5* expression also identifies endodermal precursors within the hypoblast of wild-type (E) but not *cas* mutant (F) embryos. *gata5* expression in the anterior lateral mesoderm precursors and YSL, which is out of focus (E), appears normal in *cas* mutants (F). *fkd2* is expressed in endodermal precursors in wild-type embryos, as well as in the YSL and axial mesoderm (G). *cas* mutants specifically lack endodermal *fkd2* expression (H). (A,B) Animal pole views; (C,D) left lateral views, anterior to the top; (E-H) dorsal views, anterior to the top.

Figure 2.4. *cas* acts cell-autonomously in the endodermal progenitors. A *cas* mutant host at 80% epiboly into which wild-type cells were transplanted contains several *axial*-expressing endodermal precursors in the lateral hyboblast (A). These cells all derive from the wild-type donor as they also contain the biotin dextran lineage tracer (brown stain in B); no mutant host cell was ever observed to form endoderm, as judged by *axial* expression. Under higher magnification (C) the presence of biotin dextran in the *axial*-expressing endodermal precursors is clearly seen (arrowheads indicate brown cells with purple cytoplasm); several cells not expressing *axial* also contain biotin dextran (arrows indicate brown cells). In 53 wild-type to wild-type control transplantations, we observed four cases in which transplanted cells formed axial-expressing endoderm (data not shown). Wild-type cells transplanted into *cas* mutant hosts formed endoderm in five of 77 cases ($p = 0.5\text{-}0.9$). *cas* mutant cells were never observed to form endoderm when transplanted into wild-type hosts (33 events; $p < 0.1$). (A,B) Right lateral views, anterior to the top; (C) high magnification view of B.

Figure 2.3

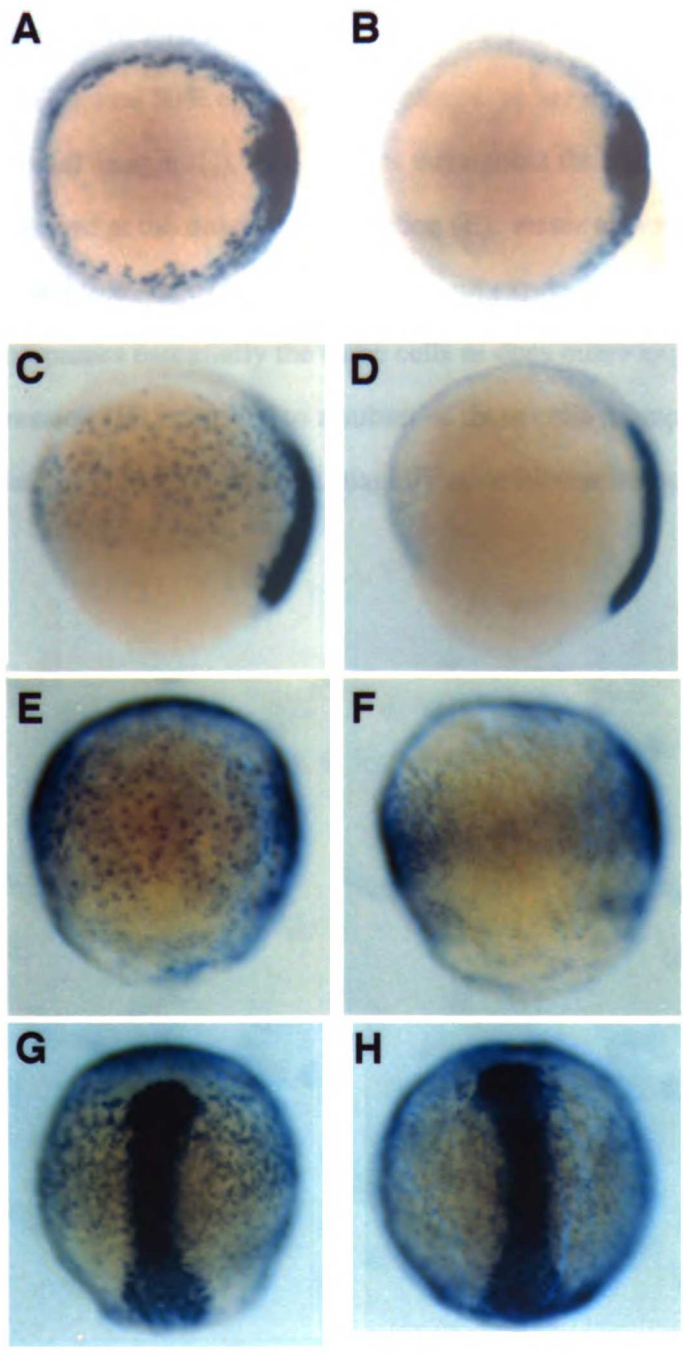

Figure 2.4

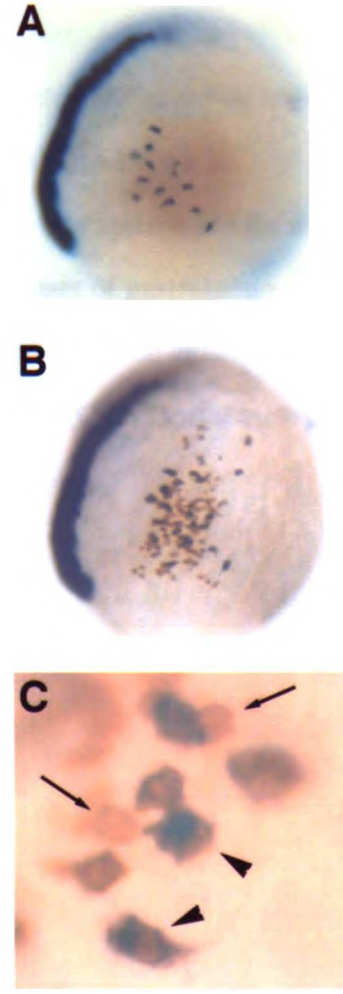

Figure 2.5. Sequence and expression of a zebrafish *Mixer* homologue. (A) Predicted amino acid sequence of zebrafish Mixer; the homeodomain and a C-terminal acidic domain are underlined. (B) Comparison of the homeodomains of zebrafish Mixer, CMIX, *Xenopus* Mixer (XMixer), and Milk; dashes indicate conserved residues. Expression of *mixer* (C-F), *ntl* (G) and *gata5* (H) in wild-type embryos at sphere (C), dome (D), and 50% epiboly (E-H) stages. *mixer* expression initiates in a group of cells at the dorsal margin (C), then spreads throughout the marginal zone (D) where it is maintained at the onset of gastrulation (E). *mixer* also appears to be expressed in the dorsal YSL at dome stage (arrowhead in D). *ntl* expression (G) at the onset of gastrulation encompasses essentially the same cells as does *mixer* expression (F), while *gata5* expression (H) is limited to a subset of these cells (compare F-H). (C,D) Dorsal views; (E-H) lateral views. (F-H) High magnification Nomarski optics images.

Figure 2.5

A

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1    MAVVHGNLGNGHLKQFQLFPNADMERRTGMHTSDYALQDRMKFLDGASRA  50
51   DNVSLFTHRRKRTNFTQQQIDVLEKVYLDTKYPDIYLREKLEALTGLPES 100
101  RIQVWFQNRRAKSRRQVGISIPHKTSGNILTPNNLLMHQFTTHQNHSGLE 150
151  NLQRTSTFTADSFHQQLINSSEEKISTIKGDIYDPTTIPCMFSRTQENPI 200
201  KTDHLSVVVPRSYTQQYPKEHEQFTHSANHAKSTMKQFLVEYDNFPPNKT 250
251  IGPEMKVVIPPLPSQSNFMMSSSSPKHIACSVQNMSVQTQPELFATFSPI 300
301  RASEAVEFSDSDSDWERDAMSGFNGFI                         327
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B

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Mixer  HRRKRTNFTQQQIDVLEKVYLDTKYPDIYLREKLEALTGLPESRIQVWFQNRRAKSRRQ
CMIX   Q-----S--AA--ET--L-FQ--M--------R-ADATQI-----------------R
XMixer Q-----FYS-NKL----QFFQTNM----HH--E-AKRIYI---------------E--D
Milk   N-----YYSPSDLAL--QYFQTNM----HQ--E-ARQM---------------S-A---
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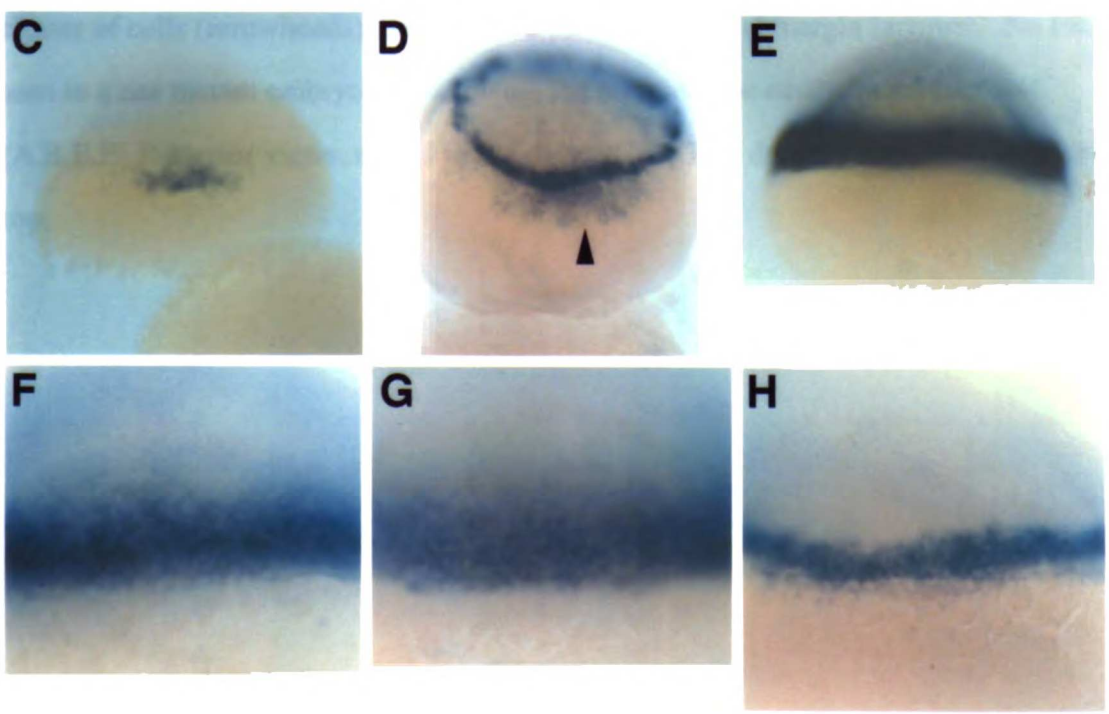


Figure 2.6. The forerunner cells do not form in *cas* mutants. Wild-type and *cas* mutant embryos were examined by light microscopy (A,B); in situ hybridisation for expression of *ntl* (C,D); syto-11 fluorescence (E,F); and Nomarski optics (G,H). Embryos are at the following stages: (A,B) 6-somite; (C,D) 70% epiboly; (E,F) 90% epiboly; and (G,H) shield. Kupffer's vesicle, formed by the forerunner cells (FRs), is easily seen in the tail of wild-type embryos (arrowhead in A) but not in *cas* mutants (B). *ntl* is normally expressed in the FRs (arrowhead in C) but this expression is lacking in *cas* mutants (D). The FR cluster (arrowhead in E) can be visualised by labelling with the fluorescent dye syto-11; cells of the enveloping layer (EVL) also take up syto-11 and thus fluoresce. *cas* mutants appear to lack a FR cluster (F); several syto-11-labelled EVL cells are seen. Using Nomarski optics the FRs can be directly visualised at the shield stage as a cluster of cells (arrowheads) that obscures part of the dorsal margin (arrows). No FRs are seen in a *cas* mutant embryo (H), allowing the margin to be easily traced (arrows). (A,B,E,F) Posterior views, dorsal to the right; (C,D,G,H) dorsal views, anterior to the top.

Figure 2.6

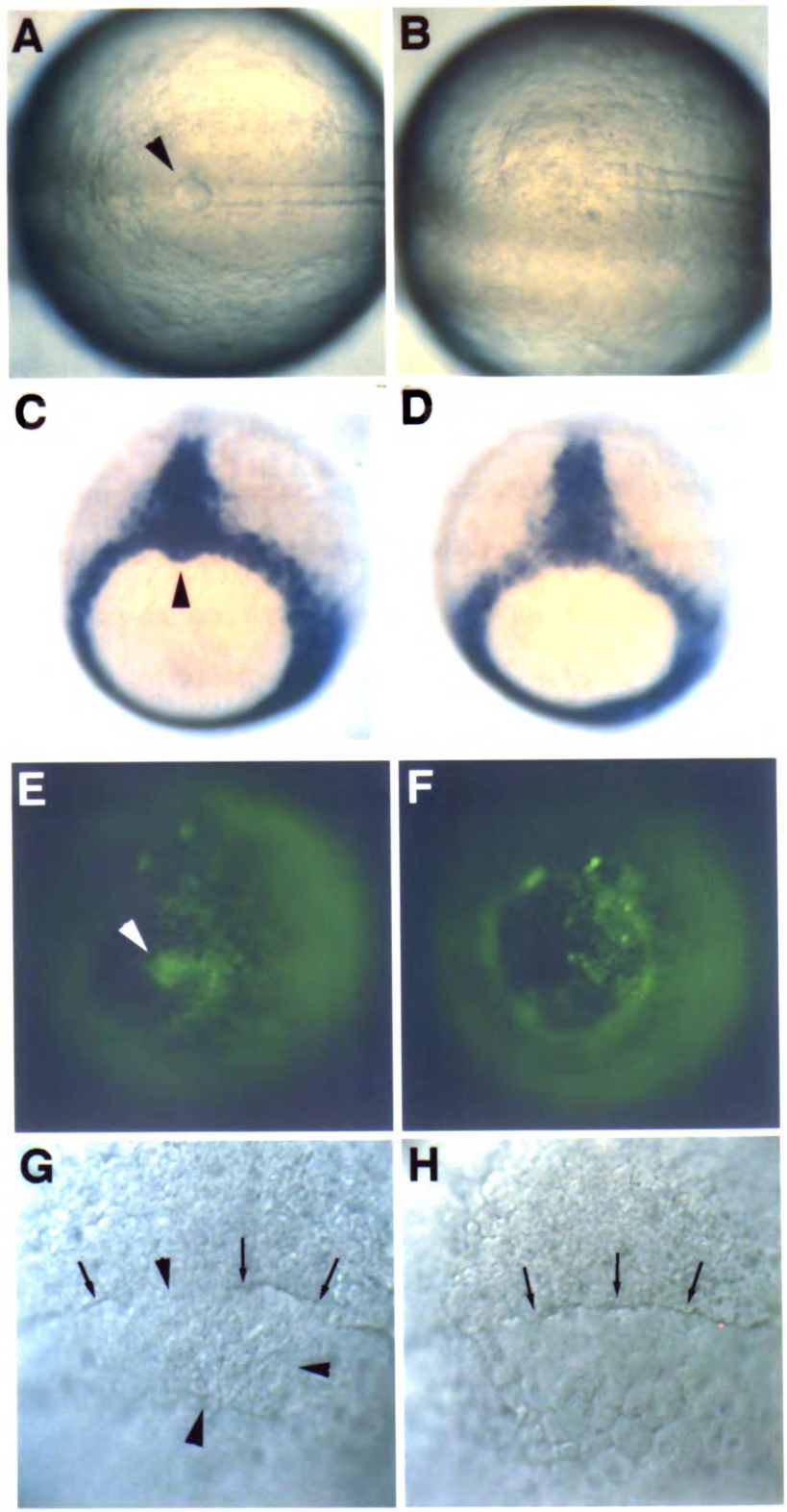
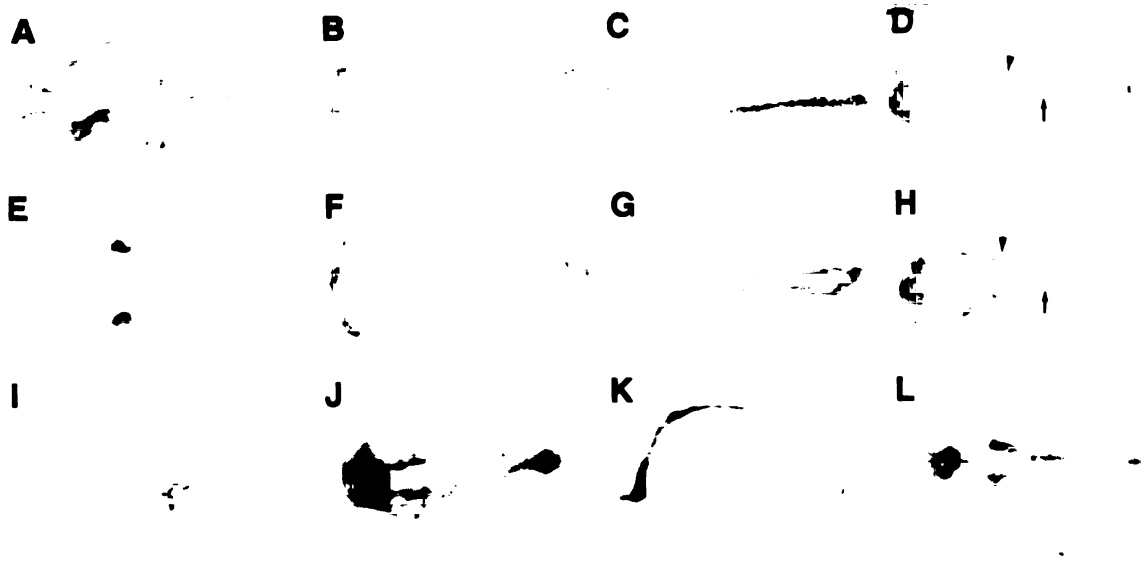


Figure 2.7. Morphogenesis of several mesodermal derivatives is defective in *cas* mutants. Wild-type (A,B,C,D), *cas* (E,F,G,H), and *kny* (I,J,K,L) embryos showing expression of *nkx2.5* (A,E,I), *tie2* (B,F,J), *gata1* (C,G), *pax2.1* (D,H), and *axial* (K,L). Embryos are at 24 hpf (A,E,I-L) and 21.5 hpf (B-D,F-H). By 24 hpf the definitive heart tube has formed in wild-type embryos (A) while two 'hearts' are evident in *cas* mutants (E). Pharyngeal endodermal expression of *nkx2.5* is also missing in *cas* mutants (compare A to E). Endothelial precursors assemble smoothly in the midline of wild-type (B) but *cas* mutant (F) embryos. In wild-type embryos the bilateral red blood cell precursors meet at the midline (C), but are positioned more laterally in *cas* mutants (G). The bilateral pronephroi (arrowheads) and pronephric ducts are also positioned more laterally in *cas* mutants (compare D to H); the arrows indicate the *pax2.1*-expressing spinal commisural interneurons. *kny* mutants form endoderm normally, although it is more broadly distributed (K,L), do not exhibit cardia bifida (I), and their trunk endothelium appears smoothly although again more broadly arranged in the midline (J). All are dorsal views, anterior to the left, except (K), which is a lateral view, anterior to the left.

Figure 2.7



Chapter 3: A molecular pathway underlying endoderm formation in zebrafish

SUMMARY

Recent studies have identified several potentially important regulators of vertebrate endoderm development. These include Activin-related growth factors and their receptors; transcriptional regulators such as Mixer, Xsox17, and HNF3β; the zebrafish EGF-CFC protein One-eyed pinhead; and the zebrafish locus *casanova*, which plays an essential cell-autonomous role in endoderm formation. We have used overexpression studies and the analysis of different zebrafish mutants to assemble a molecular pathway that underlies endoderm formation. We demonstrate that a zebrafish *Sox17* homologue is expressed during gastrulation exclusively in the endoderm. *casanova* mutants lack all *sox17* expression. Overexpression of *mixer* induces ectopic *sox17*-expressing cells in wild-type embryos, but does not rescue *sox17* expression or endoderm formation in *casanova* mutants. *mixer* overexpression does promote endoderm formation in *one-eyed pinhead* mutants. Overexpression of a constitutively active form of the type I TGF-β receptor TARAM-A also promotes *sox17* expression in wild-type and *one-eyed pinhead* mutant embryos, but not in *casanova* mutants. Finally, we demonstrate that the nodal-related molecules Cyclops and Squint and the One-eyed pinhead protein are essential for normal *mixer* expression. These data indicate that the following pathway underlies zebrafish endoderm formation: the nodal-related growth factors Cyclops and Squint activate receptors such as TARAM-A; Oep also appears to act upstream of such receptors; signals transduced by these receptors lead to the expression of the homeobox gene *mixer*; Mixer then acts through *casanova* to promote the expression of *sox17* and the differentiation of the endoderm.

INTRODUCTION

Unravelling the genetic networks that in vertebrates guide formation of the three germ layers--ectoderm, mesoderm, and endoderm--represents a fundamental goal of developmental biologists. Substantial progress has been made towards achieving a detailed understanding of the early development of the ectoderm and mesoderm. Investigations of the genes that control the initial formation of the endoderm, on the other hand, have only re recently begun.

The pregastrula location of the endodermal progenitors has been mapped in each of the principal vertebrate model systems. The yolk-rich cells of the vegetal hemisphere give rise to most of the gut endoderm in *Xenopus* (Dale and Slack, 1987; Keller, 1975; Keller, 1976). In both mouse and chick embryos the endodermal progenitors are gathered near the future site of node formation, and early in gastrulation ingress through the anterior primitive streak (Hatada and Stern, 1994; Lawson et al., 1991). In the zebrafish late blastula the endoderm arises entirely from the marginal-most four blastomere tiers, and hence, similar to the mouse and chick, endodermal progenitors involute very early during gastrulation (Warga and Nusslein-Volhard, 1999). While in mouse, chick, and zebrafish embryos the pregastrula endodermal and mesodermal fate map domains overlap substantially (Hatada and Stern, 1994; Lawson et al., 1991; Warga and Nusslein-Volhard, 1999), in *Xenopus* most of the prospective gut endoderm is spatially segregated from progenitors of the other germ layers prior to gastrulation (Dale and Slack, 1987; Keller, 1975; Keller, 1976) deposited vegetally localised transcription factor VegT in *Xenopus* endoderm formation (Zhang et al., 1998a). Whether VegT homologues are similarly required for endoderm development in other vertebrates is not known.

Many of the same growth factors that influence early mesodermal and ectodermal development may also induce formation of the endoderm. BMP and FGF signalling pathw ays have been suggested to affect endoderm formation in various ways (Gamer and

Wright, 1995; Henry et al., 1996; Jones et al., 1993; Sasai et al., 1996), although their physiologic importance for this process remains unclear. Substantial evidence implicates Activin-related growth factors as important for endoderm induction. High levels of Activin and related TGF-β molecules induce endodermal gene expression in *Xenopus* animal caps (Gamer and Wright, 1995; Henry et al., 1996; Henry and Melton, 1998; Hudson et al., 1997; Jones et al., 1993), and an endogenous role in *Xenopus* dorsal endoderm formation has been demonstrated for a Vg1-related activity (Joseph and Melton, 1998). Recent studies in zebrafish support the idea that Activin-related signals play an important role in endoderm formation. Overexpression of low levels of the putative TGF-β inhibitor Antivin appears to delete the endoderm with little or no effect on mesoderm development (Thisse and Thisse, 1999). Also, zebrafish *one-eyed pinhead* (*oep*) mutants appear not to form endoderm, in addition to lacking prechordal plate and ventral neuroectoderm (Schier et al., 1997). *oep* encodes an EGF-CFC protein required for signalling by nodal-related growth factors (Gritsman et al., 1999; Zhang et al., 1998b). Finally, in embryos mutant for both *cyclops* (*cyc*) and *squint* (*sqt*), two genes that encode nodal-related growth factors, or embryos that lack both maternal and zygotic One-eyed pinhead protein, involution of the marginal zone does not occur and therefore these mutants form essentially no endoderm or mesoderm (Feldman et al., 1998; Gritsman et al., 1999). Together these data suggest that high levels of Activin-related molecules such as Vg1 and nodals play an important role in endoderm induction, although it remains unclear what their precise functions in this process are.

Substantial progress has been made recently in the identification of genes that likely act within the endodermal progenitors to initiate or promote their differentiation in response to inducing signals. Several *Mix* homeobox genes have been shown to be expressed in the *Xenopus* endodermal precursors early in gastrulation (Ecochard et al., 1998; Henry and Melton, 1998; Rosa, 1989; Tada et al., 1998). Functional studies suggest that these genes, and two in particular--*Mixer* and *milk*--, are important for endoderm formation in *Xenopus*

(Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998; Tada et al., 1998). The precise roles played by the *Mix* genes, individually and collectively, in endoderm development are not yet clear, but one critical function appears to be the maintenance of *Xsox17* expression in the prospective endoderm (Henry and Melton, 1998).

The HMG-box transcription factors Xsox17α and -β (here referred to collectively as Xsox17) appear to function as important intrinsic regulators of endoderm formation. Originally identified in a screen for *Xenopus* genes differentially expressed in the vegetal pole during gastrulation, *Xsox17* shows an endodermally restricted expression pattern from the onset of gastrulation (Hudson et al., 1997). Overexpression of *Xsox17* promotes endodermal gene expression in animal cap explants, while expression of a fusion between Xsox17 and the repressor domain of *Drosophila* Engrailed (EnR) inhibits endoderm differentiation in both isolated vegetal pole explants and the intact frog embryo (Hudson et al., 1997). Importantly, co-expression of the Xsox17-EnR fusion together with Mixer blocks the endoderm-inducing ability of Mixer, indicating that Mixer likely acts to promote endoderm formation principally through Xsox17 (Henry and Melton, 1998).

Most recently the zebrafish *casanova* (*cas*) locus has been demonstrated to play an early and essential role in endoderm formation. *cas* mutants do not form a gut tube, never express any regional markers of endodermal differentiation, and appear to lack endoderm entirely from the onset of gastrulation [Alexander et al., submitted]. Mosaic analysis indicates that *cas* acts cell-autonomously within the endodermal progenitors, where it appears to be required for endoderm development downstream of, or in parallel to, a zebrafish *Mixer* homologue [Alexander et al., submitted].

In this report we use overexpression studies and the analysis of different zebrafish mutants to assemble the above described endodermal regulators into a molecular pathway that underlies zebrafish endoderm formation. The data suggest that the nodal-related growth factors Cyclops and Squint act through their receptors to induce the expression of *mixer*. Oep also appears to be required upstream of such receptors for *mixer* expression.

Mixer promotes the expression of *sox17* in a manner that depends absolutely upon *cas*, leading to formation of the endoderm. Together these results demonstrate important endogenous roles for several of these genes in endoderm development, and begin to elucidate in detail the molecular origins of this germ layer.

MATERIALS AND METHODS

Zebrafish strains

Adult zebrafish and embryos were maintained as described (Westerfield, 1995). Embryos were derived from natural matings of identified heterozygotes. The following mutant alleles were used: *cas*^{ta56} (Haffter et al., 1996), *oep*^{z1} (Schier et al., 1997), *cyc*^{b16} (Hatta et al., 1991), *cyc*^{m294} (Sampath et al., 1998), *sqt*^{cz35} (Feldman et al., 1998), and *oep*^{tz257} (Haffter et al., 1996). In all cases where embryos were fixed at stages prior to the time when homozygous mutants were clearly identifiable, the presence of homozygous mutants amongst sibling embryos was confirmed.

Isolation of zebrafish *sox17*

A fragment of zebrafish *sox17* was isolated from mid-gastrula stage cDNA using degenerate PCR primers 5'-ATGGTNTGGGCNAARGA-3' and 5'-GCYTCYTCVACRAADGG-3' and standard touchdown PCR protocols. Gene-specific primers were then used to screen a gastrula cDNA library by PCR for a full-length *sox17* clone. The GenBank accession number for *sox17* is AF168614.

In situ hybridisation

Wholemount in situ hybridisations were performed as previously described (Alexander et al., 1998). The *sox17* riboprobe was synthesised from EcoRI-digested plasmid pS-1, which contains the first 964 nucleotides of *sox17* subcloned into pBluescript-SK+, using T7 RNA polymerase. Other riboprobes were prepared according to published instructions.

RNA injections

pCS2+mixer, which contains the coding region and portions of the 5' and 3' untranslated regions of zebrafish *mixer* subcloned into the plasmid pCS2+, was linearised using NotI. *Xenopus* Mixer and Mixer-EnR, TARAM-A, TARAM-A*, and Antivin templates were prepared as described (Henry and Melton, 1998; Peyrieras et al., 1998; Thisse and Thisse, 1999). Capped transcripts for injection were synthesised using the SP6 mMessage Machine kit (Ambion). RNA concentrations were determined by comparison to an RNA standard using agarose gel electrophoresis. RNA was diluted to the appropriate concentration in 0.2 M KCl containing 0.5% phenol red, and embryos were injected at the 1- to 4-cell stage.

RESULTS

Expression of a zebrafish *Sox17* homologue in the endoderm and forerunner cells

We used degenerate PCR to isolate a zebrafish homologue of *Sox17* (Figure 3.1a). The predicted zebrafish Sox17 protein is highly related to mouse Sox17 (Kanai et al., 1996) and *Xenopus* Xsox17α and -β (Hudson et al., 1997) within its N-terminal region and putative HMG box (Figure 3.1b), but otherwise shows only limited sequence conservation. We first detect expression of *sox17* prior to the onset of gastrulation in a dorsally located group of marginal cells (Figure 3.1c,d). These cells correspond to the non-involuting endocytic marginal zone cells that will later form the forerunner cells (Cooper and D'Amico, 1996). We and others have previously proposed that the forerunner cells represent a specialised dorsal subset of the endoderm (Cooper and D'Amico, 1996) [Alexander et al., submitted]. *sox17* expression is maintained in the forerunner cells during gastrulation (Figure 3.1e-j), and illuminates their migration towards the vegetal pole, in front of the advancing blastoderm margin, as epiboly proceeds. Early in somitogenesis the forerunner cells form the lining of Kupffer's vesicle (Figure 3.1o) (Cooper and D'Amico, 1996) and soon thereafter cease to express *sox17* (Figure 3.1p).

Endodermal expression of *sox17* begins soon after the onset of gastrulation. As cells of the marginal zone involute to form the hypoblast, a subpopulation initiates *sox17* expression circumferentially throughout the hypoblast (Figure 3.1e,f). Their large size, morphology, and close apposition to the yolk identify these *sox17*-expressing cells as endodermal precursors (Warga and Nusslein-Volhard, 1999). Endodermal *sox17* expression continues throughout gastrulation, and at midgastrula stages resembles endodermal expression of the *axial* gene, a zebrafish homologue of mouse *HNF3β* (Strahle et al., 1993)(compare Figure 3.1g,h and 3.1k,l). At the end of gastrulation, however, a group of endodermal cells expressing *sox17* but not *axial* is evident in the area around the

tail bud (compare Figure 3.1i,j and 3.1m,n). These data show that zebrafish *sox17* is expressed in the endodermal precursors and forerunner cells during early development, and also reveal the existence of a subset of endodermal precursors that express *sox17* but not *axial* in the posterior of the zebrafish embryo.

Endodermal *sox17* expression disappears early in somitogenesis (Figure 3.1o), and is not detected again until 48 hours post-fertilisation (hpf) in a ventrally positioned group of cells in the left upper trunk (Figure 3.1q,r). We do not know the precise identity of these cells, but believe they contribute to all or part of the endodermally derived swim bladder.

cas mutants do not express *sox17*

We have previously demonstrated that *cas* mutants lack endodermal gene expression entirely from the onset of gastrulation [Alexander et al., submitted]. Given the endodermally restricted pattern of *sox17* expression, and its postulated importance for endoderm formation, we wished to determine whether *sox17* was expressed in *cas* mutants. We found that one quarter of the embryos derived from *cas/+* heterozygote intercrosses showed no *sox17* expression at any stage examined (30% epiboly to 48 hpf) (Figure 3.2a-d), indicating that *cas* mutants do not express *sox17*. The absence of the *sox17*-expressing cells in *cas* mutants at 48 hpf further indicates that these cells are endodermal. *cas* thus acts upstream of *sox17* in development of both the endoderm and the forerunner cells, which we have previously suggested represent a specialised dorsal subset of the endoderm [Alexander et al., submitted].

Zebrafish *oep* mutants also appear not to form endoderm (Schier et al., 1997). We therefore examined *sox17* expression in embryos derived from intercrosses between *oep/+* heterozygotes. This analysis revealed a dramatic decrease or complete absence of *sox17*-expressing endodermal and forerunner cells in *oep* mutants throughout gastrulation (Figure 3.2e-h). Other *oep* mutant phenotypes have been previously reported to display similar

variability (Schier et al., 1997; Strahle et al., 1997). These results confirm previous data suggesting that little if any endoderm forms in *oep* mutants (Schier et al., 1997).

mixer overexpression rescues endoderm formation in *one-eyed pinhead* but not *cas* mutants

The homeodomain protein Mixer has been suggested to play an important role in the maintenance of *Xsox17* expression in the presumptive endoderm in *Xenopus* (Henry and Melton, 1998). As shown above, *cas* is required for *sox17* expression in zebrafish. In order to explore the relationship between *mixer*, *cas*, and *sox17* in zebrafish, we examined the effects of the overexpression of zebrafish *mixer* [Alexander et al., submitted] on *sox17* expression. Control embryos injected with *GST-GFP* RNA showed normal *sox17* expression (Figure 3.3a), while overexpression of *mixer* in wild-type embryos resulted in the formation of ectopic *sox17*-expressing cells (Figure 3.3d). These ectopic *sox17*-expressing cells were primarily located more vegetally than the advancing blastoderm margin and resembled forerunner cells morphologically. Within the hypoblast of wild-type embryos we saw no obvious expansion of the *sox17*-expressing endoderm in response to *mixer* overexpression. Experiments using *Xenopus Mixer* RNA (Henry and Melton, 1998) yielded essentially identical results (data not shown), while overexpression of a *Xenopus Mixer-EnR* fusion (Henry and Melton, 1998) resulted in the loss of *sox17*-expressing endodermal precursors from regions of the hypoblast (data not shown). Together these data suggest that *mixer* acts upstream of *sox17* in zebrafish.

We also examined the effects of *mixer* overexpression on the expression of *axial*, which is expressed in the endoderm and the dorsal mesoderm during gastrulation (Schier et al., 1997; Strahle et al., 1993). These analyses revealed that convergence and extension of the dorsal mesoderm was strongly inhibited in *mixer*-injected embryos (Figure 3.3g,j,m), an effect that was also evident morphologically at 28 hpf (data not shown). Overexpression of *Xenopus Mixer* produced similar effects (data not shown). As seen for

sox17 expression above, *mixer* overexpression did not increase the number of *axial*-expressing endodermal precursors in wild-type embryos (Figure 3.3j,m).

When we examined the effects of *mixer* overexpression in embryos derived from *cas/+* heterozygote intercrosses we obtained quite different results. Similar to uninjected or control *GST-GFP*-injected *cas* mutants (Figure 3.3b), one quarter of these embryos showed absolutely no *sox17* expression (Figure 3.3e and Table 3.1). These data indicate that overexpression of *mixer* cannot rescue *sox17* expression in *cas* mutants. Examination of *axial* expression similarly demonstrated the inability of *mixer* to rescue endoderm formation in *cas* mutants (Figure 3.3h,k,n and Table 3.1). *mixer* overexpression did inhibit convergence and extension in *cas* mutants (Figure 3.3k,n), suggesting that Mixer was active in these embryos. *Xenopus Mixer* overexpression also failed to rescue *sox17* or endodermal *axial* expression in *cas* mutants (data not shown). Thus, overexpression of *mixer* cannot overcome the block to endoderm formation that results from the *cas* mutation. These data place *cas* genetically downstream of Mixer.

In order to test whether *mixer* overexpression could rescue endoderm formation in *oep* mutants we performed injections as described above into embryos derived from *oep/+* heterozygote intercrosses. Control-injected *oep* mutants contained few if any *sox17*- or *axial*-expressing endodermal precursors at mid-gastrula stages (Figure 3.3c,i and Table 3.1). In contrast, *mixer*-injected *oep* mutants, while still clearly distinguishable from their wild-type siblings, contained a significantly increased number of *sox17*- and *axial*-expressing endodermal precursors (Figure 3.3f,l,o and Table 3.1). *Xenopus Mixer* was similarly able to rescue *sox17*- and *axial*-expressing endoderm in *oep* mutants (data not shown). These results demonstrate that, at least in certain contexts, Mixer can promote endoderm formation. Furthermore, these data indicate that Mixer functions downstream of *oep*, and that high levels of Mixer are sufficient to promote endoderm formation in the absence of zygotic Oep function.

A constitutively active form of the type I TGF-β receptor TARAM-A cannot promote *sox17* expression in *cas* mutants

The previous results show that even high levels of Mixer activity cannot overcome the block to endoderm formation imposed by the *cas* mutation. Another potent inducer of endoderm is a constitutively active form of the zebrafish type I TGF-β receptor TARAM-A, designated TARAM-A* (Peyrieras et al., 1998). Injection of RNA encoding TARAM-A* into a single blastomere of a 16-cell stage zebrafish embryo cell-autonomously directs the progeny of that blastomere to endodermal fates (Peyrieras et al., 1998). Widespread high-level *TARAM-A** overexpression inhibits epiboly and converts the entire blastoderm into mesendoderm (see below). In the absence of molecular markers specific for particular lineages, it is difficult to interpret gene expression in such morphologically abnormal embryos. The endodermal specificity of *sox17* expression, however, allowed us to analyse the effects of *TARAM-A** misexpression without directing the RNA to single blastomeres.

Injection of *TARAM-A** RNA into wild-type zebrafish embryos at the 1-2-cell stage strongly inhibited epiboly, and resulted in the expression throughout the entire blastoderm of numerous mesendodermal marker genes, including *no tail* (*ntl*) (Schulte-Merker et al., 1994), the zebrafish homologue of mouse *T/Brachyury*, *goosecoid* (*gsc*) (Stachel et al., 1993; Thisse et al., 1994), *mixer* [Alexander et al., submitted], and *axial*, as well as the endoderm-specific gene *sox17* (Figure 3.4a-d and Table 3.2). Injection of similar amounts of RNA encoding wild-type TARAM-A had negligible effects upon morphology or gene expression (data not shown), as previously reported (Peyrieras et al., 1998). These results demonstrate that widespread overexpression of *TARAM-A** directs most or all cells in the embryo to a mesendodermal fate.

*TARAM-A** misexpression in single blastomeres at the 16-cell stage has also been reported to rescue prechordal plate and endoderm formation in *oep* mutants (Peyrieras et al., 1998). We found that overexpression of *TARAM-A** in embryos derived from *oep/+*

heterozygote intercrosses resulted in expression of the prechordal plate marker gene *gsc* (Figure 3.4e), the endoderm marker gene *sox17* (Figure 3.4f), and the mesendodermal marker genes *mixer* and *axial* (data not shown) in all injected embryos (Table 3.2). These results suggest that assaying gene expression in embryos injected with *TARAM-A** at the 1-cell stage yields results consistent with those obtained from the single-blastomere injection experiments (Peyrieras et al., 1998).

Injection of *TARAM-A** RNA into embryos derived from *cas/+* heterozygote intercrosses yielded dramatically different results from injections into wild-type or *oep* mutant embryos. While TARAM-A* overexpression promoted the expression of mesendodermal markers such as *gsc*, *mixer*, and *axial* in all embryos (Figure 3.4g and data not shown; Table 3.2), *cas* mutants did not express *sox17* (Figure 3.4h and Table 3.2). Thus, *cas* is required downstream of TARAM-A* for the expression of *sox17*.

TGF-β signals induce *mixer* expression

The results presented above and the work of others (Henry and Melton, 1998) implicate the activation of *Mixer* expression as an important step in the pathway leading to endoderm formation. We therefore sought to understand how *mixer* expression is regulated in the embryo. Previous studies in *Xenopus* demonstrate that high levels of Activin-related signals are able to induce the expression of various *Mix* genes (Ecochard et al., 1998; Henry and Melton, 1998; Rosa, 1989). Consistent with these studies, *TARAM-A** overexpression causes pan-embryonic *mixer* expression in wild-type and *cas* and *oep* mutant embryos (Figure 3.4b,g and data not shown; Table 3.2), demonstrating that TGF-β signalling is able to promote *mixer* expression in zebrafish.

We next tested whether TGF-β molecules are necessary for *mixer* expression in vivo. To do so, we overexpressed RNA encoding the putative TGF-β inhibitor Antivin (Thisse and Thisse, 1999) in wild-type embryos. Overexpression of 40 pg of *antivin* RNA results in embryos that form essentially no endoderm or mesoderm except for a small

amount of tail muscle (Figure 3.5a). Prior to the onset of gastrulation these embryos show a gap in the normally continuous marginal zone expression of *ntl* (Figure 3.5b)(Thisse and Thisse, 1999). *mixer* expression, in contrast, is entirely absent in *antivin*-injected embryos (Figure 3.5c). These results suggest that a TGF-β signal(s) is required endogenously for *mixer* expression in zebrafish.

nodal-related growth factors are required for normal *mixer* expression

Recently the phenotype of embryos mutant for both of the *nodal*-related genes *cyc* and *sqt* has been described. *cyc; sqt* double mutants form essentially no endoderm or mesoderm, and display a dorsal gap in *ntl* expression (Feldman et al., 1998). These phenotypes closely resemble those that result from the overexpression of high levels of *antivin*. We therefore examined *mixer* expression in *cyc* and *sqt* single mutants and *cyc; sqt* double mutants. *mixer* expression is normal in *cyc* mutants (Figure 3.6a,b), while in *sqt* mutants the mixer expression domain is thinner along the animal-vegetal axis and exhibits a dorsal gap (Figure 3.6c,d). Strikingly, *cyc; sqt* double mutants exhibit a barely detectable level of *mixer* expression (Figure 3.6e,f). These data demonstrate that the nodal-related growth factors Cyc and Sqt are required for normal *mixer* expression.

As mentioned above, Oep is a membrane-associated cofactor required for signalling by nodal-related growth factors (Gritsman et al., 1999). Given the importance of nodal-related signals for *mixer* expression, we examined *mixer* expression in zygotic *oep* mutants. *mixer* expression in *oep* mutants initiates normally (data not shown). As epiboly proceeds, however, *oep* mutants show a region of variably decreased or absent *mixer* expression in the dorsal margin (Figure 3.6g), the size of which increases over time (Figure 3.6h). These data suggest that zygotic Oep is required for the maintenance of *mixer* expression, and that this maintenance function is most important dorsally.

We hypothesised that maternally supplied Oep (Gritsman et al., 1999) may permit the substantial amount of *mixer* expression seen in zygotic *oep* mutants. In order to test

this idea, we examined *mixer* expression in embryos that lack both maternal and zygotic Oep (*MZoep* mutants) (Gritsman et al., 1999). We found that, similar to *cyc; sqt* double mutants, *MZoep* mutants exhibit only barely detectable *mixer* expression (figure 3.6i,j). *mixer* expression in embryos that lack maternal Oep only is indistinguishable from wild-type (data not shown), indicating that zygotic Oep is sufficient for normal *mixer* expression.

DISCUSSION

The results described in this report advance our understanding of the mechanisms that control vertebrate endoderm formation in several important ways. In particular, the ability to examine the expression of different putative endodermal regulators, and to overexpress some of these same molecules, in wild-type and mutant zebrafish embryos has allowed us to assess the endogenous importance of several of these genes, and to begin to assemble a molecular pathway that underlies endoderm formation (Figure 7). Although it is difficult to predict exactly how much of this pathway will be conserved in other organisms, we believe that the framework our studies provide will greatly facilitate analyses of endoderm development in all vertebrates.

Sox17-related genes and vertebrate endoderm formation

Recent studies have demonstrated an important role for *Xenopus* Xsox17 in the formation of endoderm (Hudson et al., 1997). We have isolated a zebrafish *sox17* gene and shown that it is expressed in the endoderm and forerunner cells, which we and others have previously suggested represent a specialised dorsal subset of the endoderm (Cooper and D'Amico, 1996). These results suggest that *Sox17*-related genes may play a role in endoderm formation in other vertebrates. The expression pattern of mouse *Sox17* during gastrulation has not been reported, nor has a chick *Sox17* homologue been described, but these may be fruitful avenues for further studies concerning endoderm formation in these organisms.

Comparison of *sox17* and *axial* expression at the tailbud stage reveals a posterior population of endodermal cells that expresses *sox17* but not *axial*. In the mouse *HNF3β* is expressed in, and required for the development of, the foregut and midgut endoderm (Dufort et al., 1998). *axial*, a homologue of *HNF3β*, is similarly expressed in approximately the anterior two-thirds of the endoderm throughout embryonic development

[this report and (Odenthal and Nusslein-Volhard, 1998)], suggesting that these genes may represent true orthologues. Whether *axial* is similarly required for the development of this subset of the zebrafish endoderm awaits the isolation of an *axial* mutant. More importantly, the difference between the endodermal expression domains of *sox17* and *axial* indicates that by the end of gastrulation the zebrafish endoderm already possesses some anterior-posterior patterning.

cas acts upstream of *sox17*

We have previously demonstrated an essential cell-autonomous role for *cas* in endoderm formation in zebrafish [Alexander et al., submitted]. Here we show that *cas* mutants never express *sox17*, implicating *cas* as an essential upstream regulator of *sox17* expression. The absolute requirement for functional *cas* activity to achieve *sox17* expression, and the severity and specificity of the *cas* mutant endodermal phenotype [Alexander et al., submitted], together highlight the importance of *cas* for vertebrate endoderm formation. We are currently pursuing the molecular identification of the *cas* locus and expect that this information will inform numerous aspects of the work discussed in this and other reports.

The role of *mixer* in zebrafish endoderm formation

Both zebrafish and *Xenopus* Mixer promote the formation of ectopic *sox17*-expressing cells in zebrafish embryos, while overexpression of a *Xenopus* Mixer-EnR fusion blocks endoderm formation. Together with the fact that *mixer* overexpression is able to rescue endoderm formation in *oep* mutants, these results demonstrate a role for zebrafish Mixer in endoderm formation. During normal *Xenopus* development expression of *Xsox17* actually precedes that of *Mixer*, suggesting that Mixer likely acts to maintain *Xsox17* expression in the presumptive endoderm during gastrulation (Henry and Melton, 1998; Hudson et al., 1997). Zebrafish *mixer* expression precedes *sox17* expression in the

endoderm and the forerunner cells, however, which, together with our functional studies, demonstrates unequivocally that *mixer* acts upstream of *sox17* in zebrafish. Whether Mixer is in fact necessary for *sox17* expression and endoderm formation awaits the identification and analysis of a *mixer* mutant.

Importantly, *mixer* overexpression does not rescue *sox17* expression or endoderm formation in *cas* mutants, placing *cas* between *mixer* and *sox17* in a genetic pathway underlying endoderm formation. Our experiments do not distinguish whether the biochemical activities of Mixer and *cas* in fact act sequentially, or instead function in parallel, to activate *sox17* expression.

The ability of Mixer overexpression to rescue endoderm formation in zygotic *oep* mutants is striking, especially given that, at least initially, these embryos are able to express *mixer* relatively normally due to maternally supplied Oep (Gritsman et al., 1999; Zhang et al., 1998b). These results imply that zygotic *oep* mutants lack endoderm not because they fail to express *mixer*, but because they lack some other function dependent upon Oep. It may be that one of these additional functions is somehow to activate Mixer post-transcriptionally, either directly or indirectly, a step which may be unnecessary in the presence of high levels of Mixer activity. Alternatively, high levels of Mixer activity may simply allow the embryo to bypass another Oep-regulated event(s) that is normally required for endoderm formation.

Interestingly, only a subset of the earliest-involuting cells in the zebrafish margin later express *sox17* and form endoderm (Warga and Nusslein-Volhard, 1999), despite the fact that all such cells express *mixer* [Alexander et al., submitted]. Additionally, *mixer* overexpression in wild-type zebrafish embryos causes the formation of ectopic *sox17*-expressing cells that resemble forerunner cells in their morphology and location, but does not appear to cause any obvious increase in the amount of endoderm that forms. Together these data imply that in zebrafish additional factors besides Mixer, for example perhaps *cas*

and/or *gata5* (Rodaway et al., 1999), may regulate the initiation of endodermal *sox17* expression and limit endoderm formation in the presence of high levels of Mixer activity.

cas acts downstream of TGF-β signalling in endoderm formation

Previous work has shown that the constitutively active type I TGF-β receptor TARAM-A* can cell-autonomously direct cells to an endodermal fate in both wild-type and *oep* mutant embryos (Peyrieras et al., 1998). These data place *oep* genetically upstream of TARAM-A signalling. Consistent with these results, we demonstrate that TARAM-A* overexpression also promotes *sox17* expression in both wild-type and *oep* mutant embryos. The inability of TARAM-A* to induce *sox17* expression in *cas* mutants places *cas* downstream of such signalling. This is not entirely surprising, as *cas* is also genetically downstream of Mixer, and TARAM-A* promotes *mixer* expression. Nonetheless, the complete block to *sox17* expression in *cas* mutants is striking, and emphasises the critical importance of *cas* for *sox17* expression and endoderm formation.

nodal-related growth factors induce *mixer* expression

Numerous studies have suggested a role for Activin-related growth factors in the induction of endoderm (Gamer and Wright, 1995; Henry et al., 1996; Hudson et al., 1997; Jones et al., 1993; Joseph and Melton, 1998; Rodaway et al., 1999). However, these studies have not conclusively demonstrated the physiologic importance of Activin signalling for endoderm induction, nor have they succeeded in identifying the endogenous endoderm inducer(s). Our results address both of these issues. Overexpression of the putative TGF-β inhibitor Antivin (Thisse and Thisse, 1999) demonstrates a critical in vivo role for TGF-β molecules in the normal expression of *mixer*. *cyc; sqt* double mutants express only barely detectable levels of *mixer*, identifying these nodal-related molecules as the principal endogenous inducers of *mixer* expression. *MZoep* mutants similarly exhibit only barely detectable *mixer* expression, consistent with this critical role for nodal-related

signals in normal *mixer* expression. Additionally, these data provide genetic evidence for the ability of Antivin to antagonise the effects of nodal-related molecules, as others have recently suggested (Bisgrove et al., 1999).

Comparison of the expression patterns of *mixer* and *sqt* and *cyc* supports the idea that nodal-related signals regulate *mixer* expression. *mixer* expression initiates in the dorsal blastoderm soon after the onset of *sqt* expression and then spreads circumferentially throughout the marginal zone [Alexander et al., submitted], as does *sqt* (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998). Expression of *cyc* throughout the marginal zone initiates slightly later than does *sqt* expression (Rebagliati et al., 1998; Sampath et al., 1998). *mixer* is also is expressed in the dorsal yolk syncytial layer [Alexander et al., submitted], another site of *sqt* expression (Erter et al., 1998; Feldman et al., 1998). Finally, marginal zone expression of both *sqt* and *cyc* disappears soon after the onset of gastrulation (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998), as does *mixer* expression [Alexander et al., submitted].

The *mixer* expression defects in *sqt* mutants are intriguing, as *cyc* is also expressed throughout the marginal zone. The fact that the *mixer* expression domain is thinner along the animal-vegetal axis seems unlikely to result simply from a decreased dose of nodal-related signals, as a similar defect is not apparent in *cyc* mutants. Rather, this fact along with the dorsal gap in *mixer* expression seen in *sqt* mutants, suggests that cells within the marginal zone respond differently to Sqt and Cyc. In the dorsal margin this difference may result from β-catenin signalling within the dorsal region of the embryo that directly or indirectly modifies the response of the dorsal blastomeres to nodal-related signals. For example, the dorsal marginal cells may only be sensitive to nodal-related signals during a particular time window that precedes the onset of *cyc* expression (Rebagliati et al., 1998; Sampath et al., 1998). Alternatively, Sqt may itself be a more potent inducer of dorsal mesendoderm than is Cyc, as recent results suggest (Erter et al., 1998).

While Antivin overexpression eliminates *mixer* expression entirely, a very low level of *mixer* expression is detectable in both *cyc; sqt* double mutants and *MZoep* mutants. These results suggest that another TGF-β related molecule(s) may also induce some *mixer* expression. Zebrafish embryos do contain maternally supplied Vg1 (Dohrmann et al., 1996) and Activin (Rodaway et al., 1999), which could be responsible for the slight amount of *mixer* expression present in *cyc; sqt* and *MZoep* mutants. In *Xenopus* it has been suggested that zygotically expressed nodal-related molecules relay earlier endoderm- and/or mesoderm-inducing signals from Activin or related ligands (Osada and Wright, 1999). Our results hint at a similar mechanism operating in zebrafish.

Recent experiments have demonstrated that the maternally deposited vegetally localised transcription factor VegT is required for endoderm formation in *Xenopus* (Zhang et al., 1998a). How VegT directs cells to an endodermal fate is not known, but, given the importance of zygotically expressed nodal-related factors for endoderm induction in zebrafish, it may be that VegT promotes the expression of nodal-related molecules by cells of the vegetal hemisphere (Jones et al., 1995), which then induce endoderm in an autocrine or paracrine manner. Recent results in *Xenopus* do suggest that *Xsox17β* expression depends upon cell-cell interactions (Zorn et al., 1999), and that nodal-related molecules may be important for the expression of at least dorsal endodermal marker genes (Osada and Wright, 1999; Zorn et al., 1999). On the other hand, a shortened form of Cerberus that specifically binds and inhibits nodal-related molecules does not prevent *Xsox17β* expression in the *Xenopus* embryo (Piccolo et al., 1999). Thus, it may be that both VegT and nodal-related signals are important for endoderm induction, but that their relative importance varies in different vertebrates.

Conclusion

Considered together our results advance the current understanding of zebrafish endoderm formation provided by previous studies (Feldman et al., 1998; Gritsman et al.,

1999; Peyrieras et al., 1998; Schier et al., 1997) [Alexander et al., submitted], and suggest that the following molecular pathway underlies endoderm formation in zebrafish (Figure 7): the nodal-related signals Cyc and Sqt activate appropriate receptors, for example perhaps TARAM-A; Oep also appears to act upstream of such receptors; signalling through these receptors and downstream signal transduction pathways leads to the induction of normal levels of *mixer* expression; Mixer, in a manner that requires *cas* function, activates *sox17* expression, thus initiating endodermal differentiation. As discussed above, it seems that an additional TGF-β molecule(s) likely induces some *mixer* expression (X in Figure 7), and that some other factor(s) may also regulate *sox17* expression (Y in Figure 7).

We wish to emphasise that the relationships described in the above pathway are not necessarily direct. Determining which if any are indeed direct will require biochemical analyses. Further experiments will also be necessary to determine the relevance of this pathway to endoderm formation in other vertebrates. Importantly, this pathway potentially links many of the currently known regulators of vertebrate endoderm formation into a single coherent framework, and should therefore be useful in assessing the roles of newly identified players in this process.

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FIGURE LEGENDS

Figure 3.1. Sequence and expression of zebrafish *sox17*. **(a)** The predicted amino acid sequence of zebrafish Sox17. **(b)** Comparison of the HMG boxes of zebrafish Sox17, mouse Sox17 (MoSox17) (Kanai et al., 1996), and *Xenopus* Xsox17α and -β (Hudson et al., 1997). The blue boxes highlight residues conserved in all four proteins. The zebrafish Sox17 HMG box is 80% and 78% identical to those of the mouse and *Xenopus* proteins, respectively. **(c-j)** Dorsal (c) and animal pole (d) views of an embryo at 50% epiboly showing *sox17* expression in the forerunner cells (arrowhead). Dorsal (e) and animal pole (f) views of an embryo at shield stage, when endodermal *sox17* expression initiates. *sox17* is also expressed in the forerunner cells at this stage (arrowhead). Dorsal (g) and lateral (h) views of an embryo at 80% epiboly demonstrate *sox17* expression in both the endodermal progenitors and forerunner cells (arrowhead). Lateral (i) and posterior (j) views of a tailbud stage embryo reveal that endodermal *sox17*-expressing cells extend into the tailbud. **(k-n)** Dorsal and lateral views of an embryo at 80% epiboly, showing expression of the *axial* gene. *axial* is expressed in both the dorsal mesoderm (arrow) and the endodermal progenitors. Lateral (m) and posterior (n) views of a tailbud stage embryo showing *axial* expression. *axial*-expressing endodermal cells do not extend as far posteriorly as do *sox17*-expressing endodermal cells (compare i and j to m and n). **(o)** A posterior view of an embryo at the 5-somite stage shows *sox17* expression in the forerunner cells as they form Kupffer's vesicle (arrowhead). No endodermal *sox17* expression remains at this stage. **(p)** No *sox17* expression is detectable at the 10-somite stage; lateral view. **(q,r)** *sox17* expression is apparent at 48 hpf in a ventrally located group of cells (arrowhead), seen in dorsal (q) and lateral (r) views. The riboprobe used is indicated in the lower left corner of each panel.

Figure 3.1

(a)

(b)

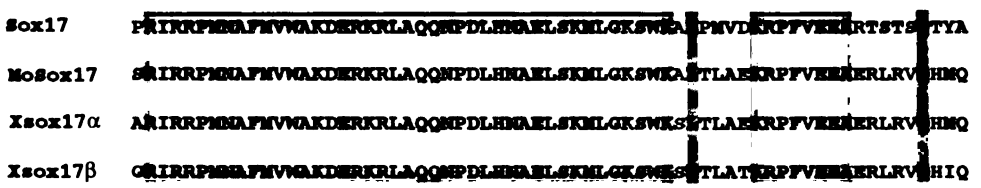

Figure 3.1

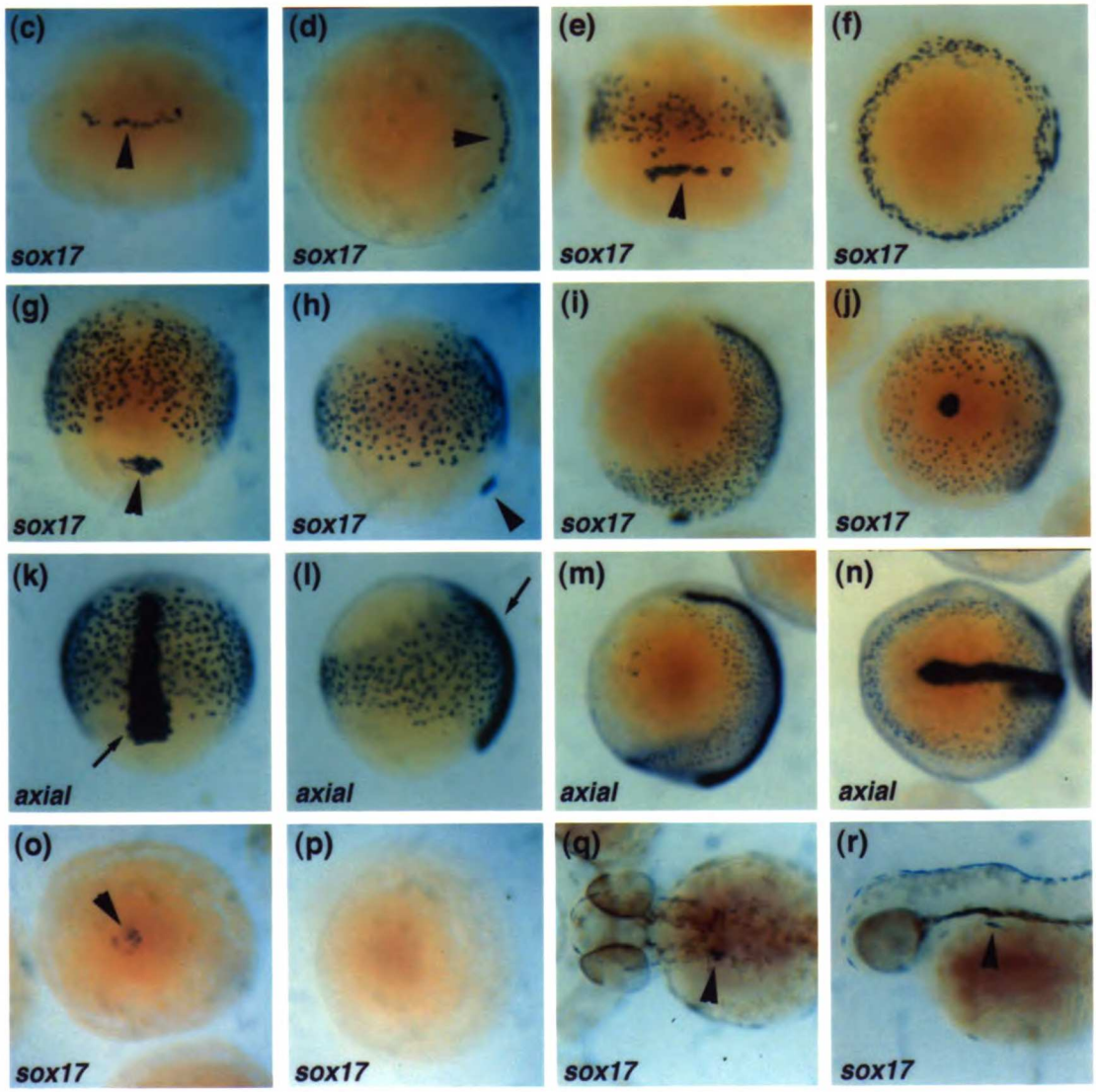
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Figure 3.2. *sox17* expression in *cas* and *oep* mutants. (a-d) *cas* mutants do not express *sox17* at any stage examined: (a) 50% epiboly (animal pole view), (b) shield stage (lateral view), (c) 80% epiboly (lateral view), and (d) 48 hpf (dorsal view). **(e-h)** A few or no *sox17*-expressing endodermal precursors (arrows) and forerunner cells (arrowheads) are seen in zygotic *oep* mutants at late shield (e,f) and 80% epiboly (g,h) stages. (e) and (g) are lateral views, (f) and (h) are dorsal views. The genotype of the embryo is indicated in the lower right corner of each panel.

Figure 3.2

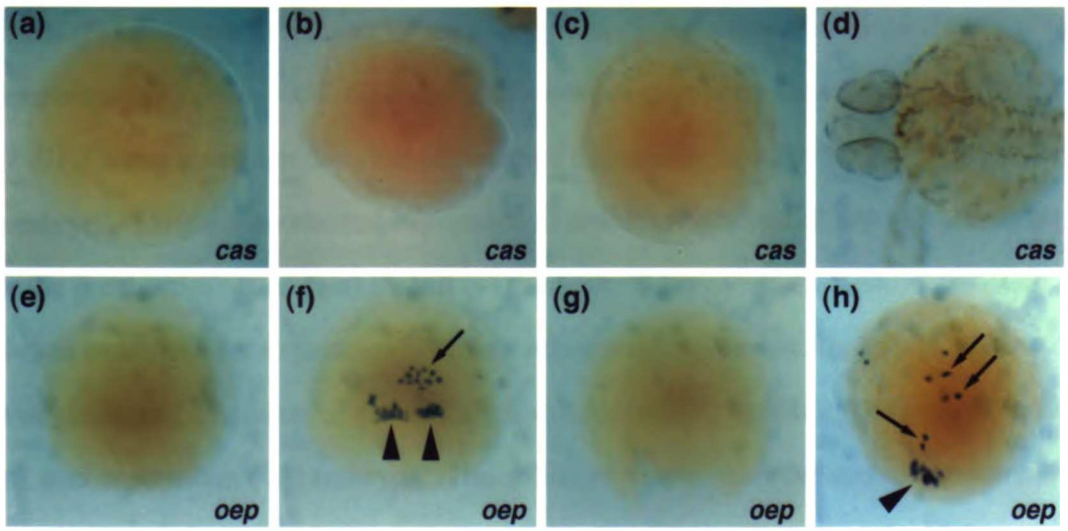
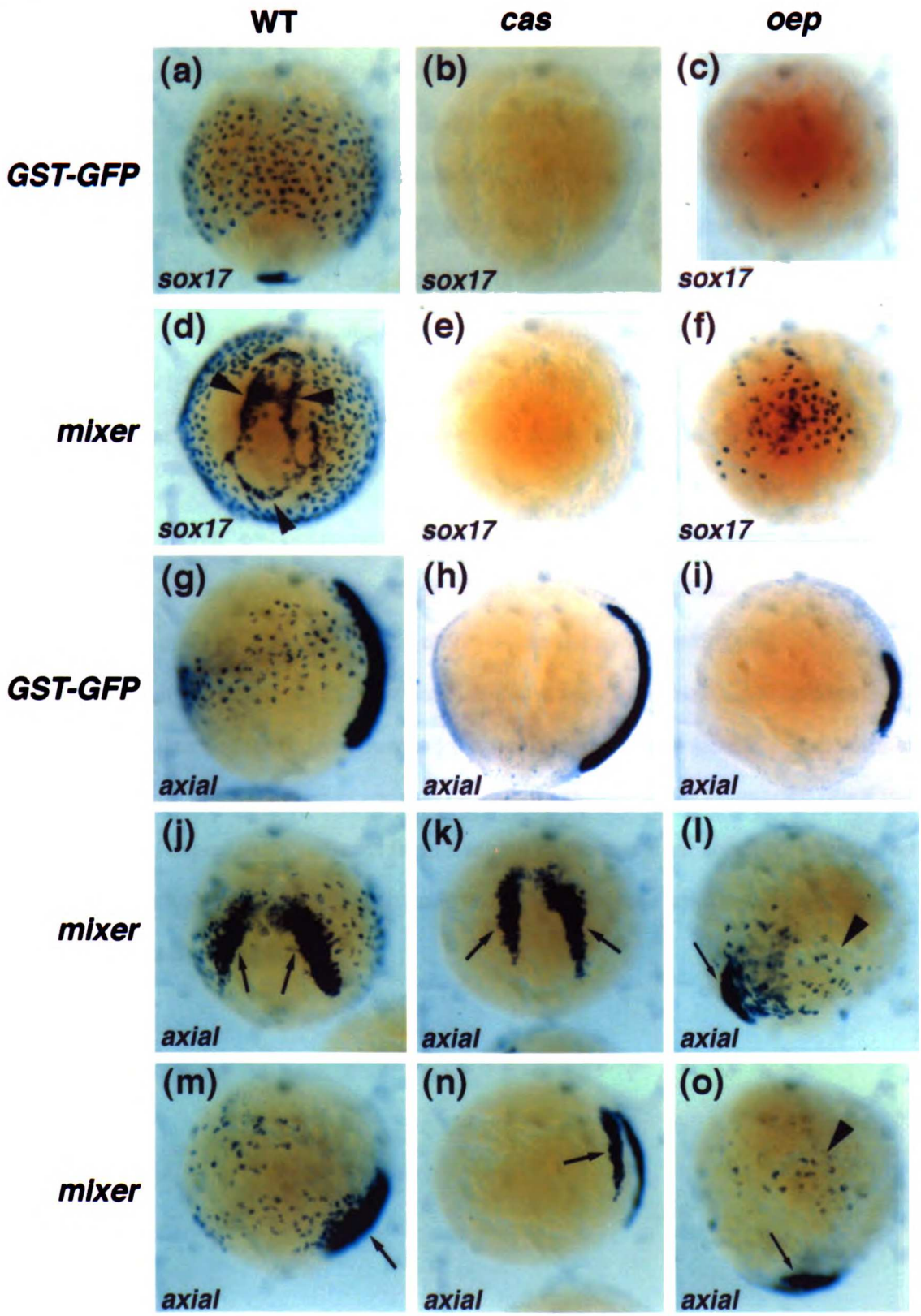


Figure 3.3. *mixer* overexpression rescues endoderm formation in *oep* but not *cas* mutants. **(a-c)** Dorsal (a) and lateral (b,c) views of control *GST-GFP*-injected embryos examined for *sox17* expression . *GST-GFP* overexpression has no effect upon *sox17* expression in wild-type (a), *cas* (b), or *oep* (c) embryos. **(d-f)** Posterior (d) and lateral (e,f) views of embryos following overexpression of 200 pg of *mixer* RNA and detection of *sox17* expression. Additional *sox17*-expressing cells that resemble forerunner cells in their location and morphology are present in wild-type embryos (arrowheads in d). *mixer* overexpression does not rescue *sox17* expression in *cas* mutants (e), but in *oep* mutants (f) a substantial number of *sox17*-expressing endodermal cells are evident. **(g-i)** Lateral views of control *GST-GFP*-injected embryos examined for expression of *axial*. *GST-GFP* overexpression does not affect *axial* expression in wild-type (g), *cas* (h), or *oep* (i) embryos. Dorsal mesodermal expression of *axial* is shortened in *oep* mutants as they lack the prechordal plate. **(j-o)** Dorsal (j-l) and lateral (m-o) views of *mixer*-injected embryos examined for expression of *axial*. *mixer* overexpression strongly inhibits convergence and extension of the dorsal mesoderm (arrow) in wild-type (j,m), *cas* mutant (k,n), and *oep* mutant (l,o) embryos. The ability of Mixer to rescue endoderm formation in *oep* (arrowheads in l,o) but not *cas* (k,n) mutants is evident. The genotype of the injected embryo is indicated along the top of the figure, and the RNA injected is indicated on the left. The riboprobe used is indicated in the lower left corner of each panel.

Figure 3.3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oep
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(m)
(n)
(o)
sox17
axial

Figure 3.4. TARAM-A* cannot promote endodermal gene expression in *cas* mutants. Lateral views of embryos injected with 90 pg of *TARAM-A** RNA and then examined for the expression of various mesendodermal and endodermal marker genes after approximately 8 hours of development. **(a-d)** *TARAM-A** overexpression in wild-type embryos converts essentially the entire blastoderm into mesendoderm that expresses *gsc* (a), *mixer* (b), *axial* (c), and *sox17* (d). **(e,f)** *TARAM-A** overexpression promotes widespread expression of *gsc* (e) and *sox17* (f) in *oep* mutants. **(g,h)** *TARAM-A** overexpression in *cas* mutants results in the widespread expression of *mixer* (g) but not *sox17* (h). In each panel the genotype of the embryo is indicated in the lower right corner and the riboprobe used is indicated in the lower left corner; WT, wild-type.

Figure 3.5. *antivin* overexpression eliminates *mixer* expression. Wild-type zebrafish embryos were injected with 40 pg of *antivin* RNA. **(a)** Lateral view of an *antivin*-injected embryo at 28 hpf. These embryos consist almost entirely of neuroectoderm, including an eye (arrowhead) and otic vesicles (arrow), and epidermis, and contain no endoderm or mesoderm except for a small amout of tail muscle. **(b,c)** Animal pole views of *antivin*-injected embryos at 50% epiboly examined for expression of *ntl* (b) or *mixer* (c). *ntl* expression is deleted from a region of the marginal zone (b), while *mixer* expression is entirely absent (c) in such embryos. The riboprobe used is indicated in the lower left corner of each panel.

Figure 3.4

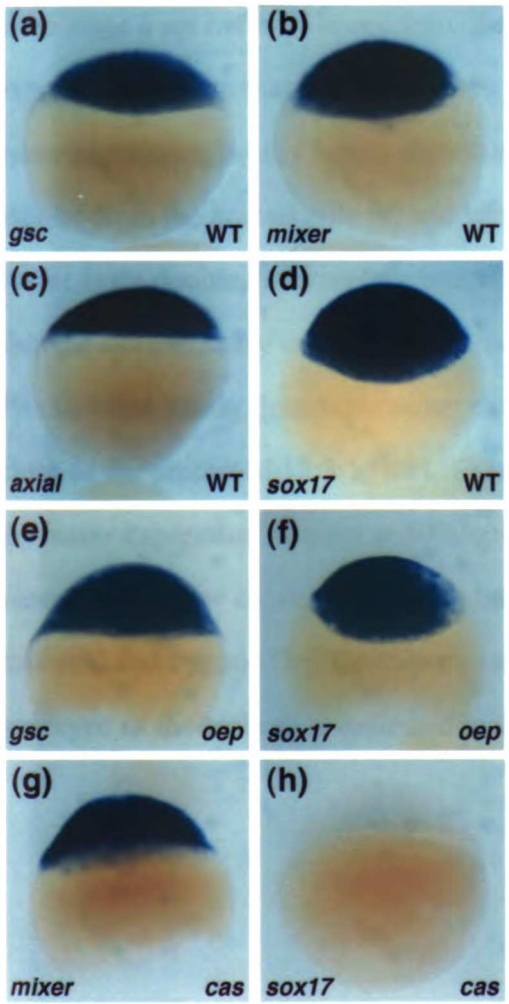


Figure 3.5

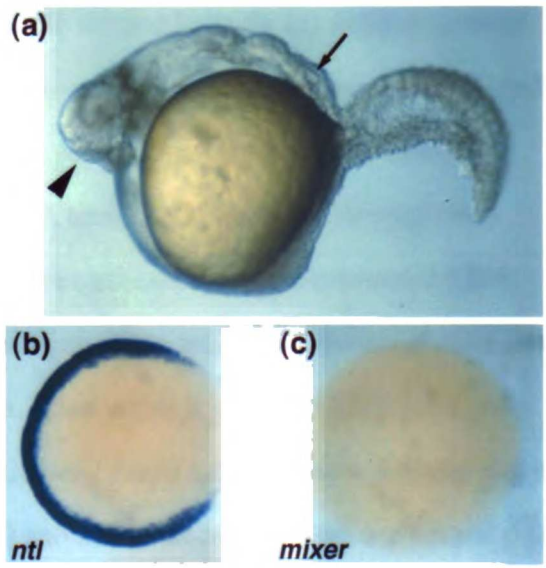
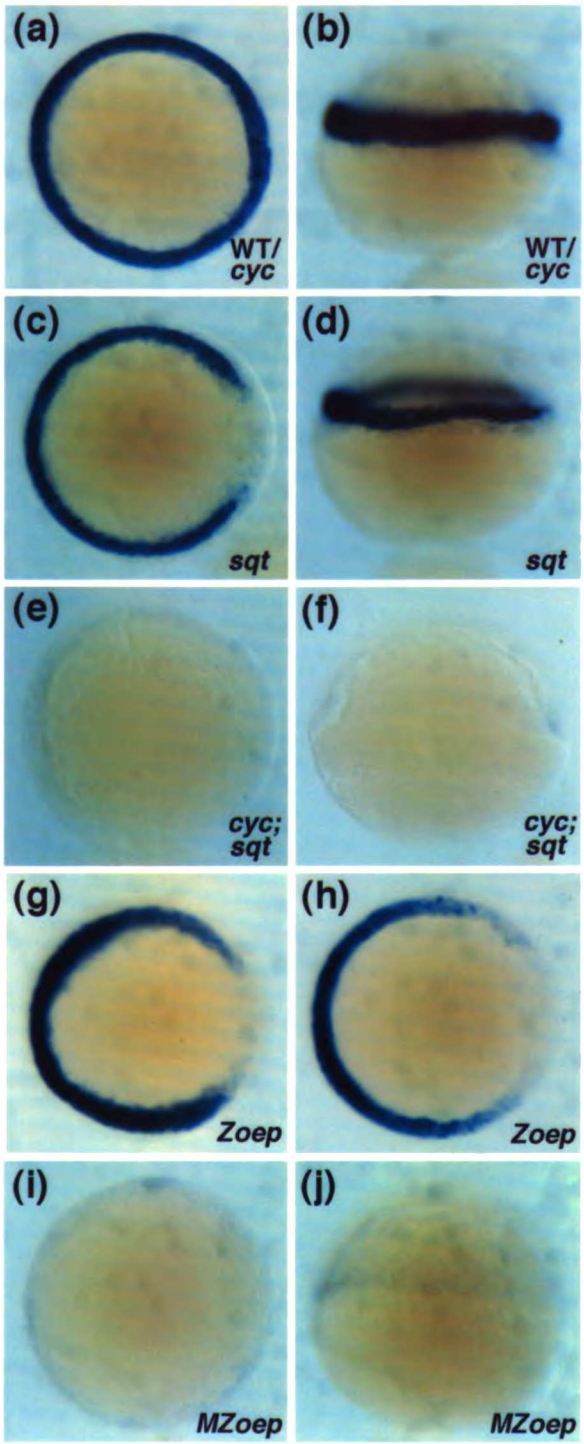


Figure 3.6. nodal-related signals are required for normal *mixer* expression. **(a,b)** Animal pole (a) and lateral (b) views of a wild-type or *cyc* mutant embryo at 50% epiboly, showing normal *mixer* expression throughout the marginal zone. **(c,d)** At the same stage a *sqt* mutant viewed from the animal pole (c) and laterally (d) exhibits a *mixer* expression domain that is thinner along the animal-vegetal axis and has a dorsal gap. **(e,f)** *mixer* expression is only barely detectable in a *cyc*; *sqt* double mutant at 50% epiboly, viewed from the animal pole (e) and laterally (f). A total of 281 embryos derived from *cyc*/+; *sqt*/+ double heterozygote intercrosses were examined for *mixer* expression; 208 showed normal *mixer* expression, 54 had a dorsal gap in the *mixer* expression domain, and 19 exhibited barely detectable *mixer* expression. This yields a ratio of 11.8:3.1:1.1, very close to the predicted 12:3:1 ratio. **(g,h)** Zygotic *oep* (*Zoep*) mutants show a dorsal gap in the *mixer* expression domain at 50% epiboly (g) and shield stage (h). Both are animal pole views. **(i,j)** *mixer* expression is only barely detectable in an embryo that lacks both maternal and zygotic Oep (*MZoep* mutant), viewed from the animal pole and laterally. The genotype of the embryo is indicated in the lower right of corner each panel; WT, wild-type.

Figure 3.6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sqt
(g)
Zoep
(h)
Zoep
(i)
MZoep
(j)
MZoep

Figure 3.7. A molecular pathway underlying endoderm formation in the zebrafish. The model integrates the relationships between various zebrafish endodermal regulators demonstrated in this and other reports (Feldman et al., 1998; Gritsman et al., 1999; Peyrieras et al., 1998) [Alexander et al., submitted]. The arrows are not meant to imply direct interactions. X represents a TGF-β molecule(s) other than Cyc or Sqt that induces a small amount of *mixer* expression (see discussion). Y represents a hypothetical additional factor(s) regulating *cas* or *sox17* in parallel to Mixer (see discussion).

Figure 3.7

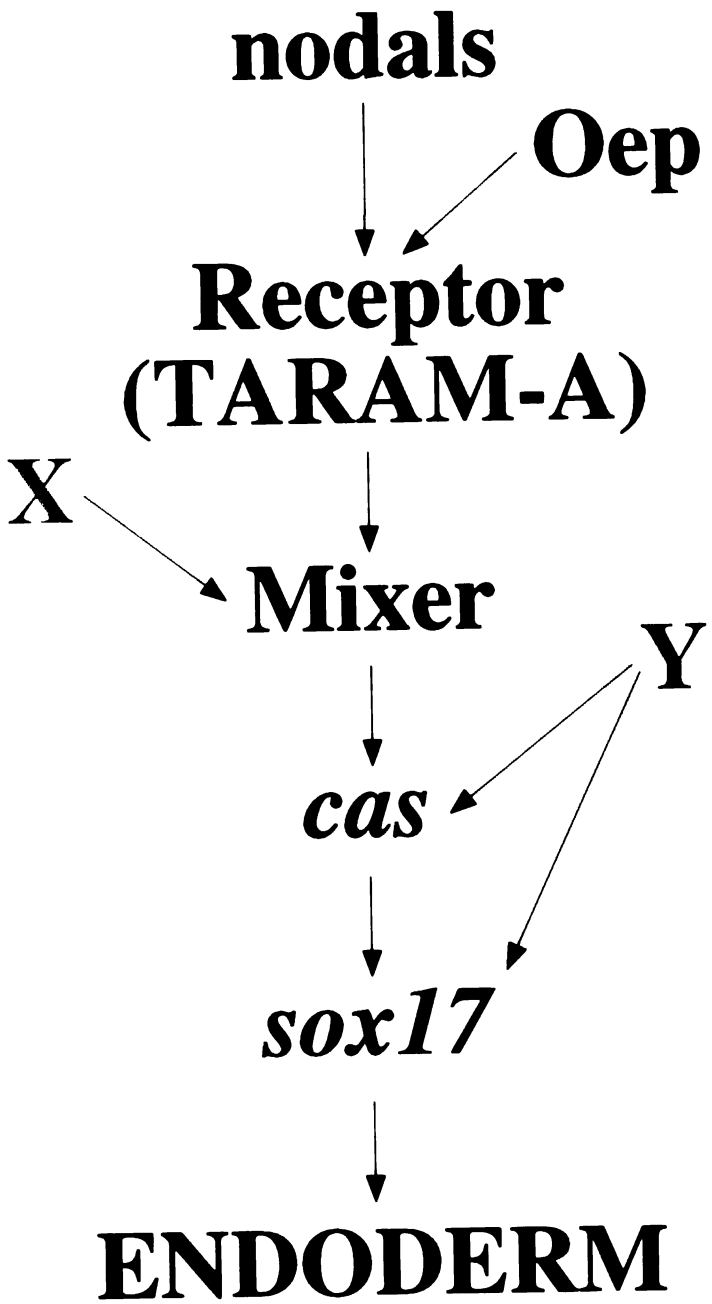

Table 3.1

***mixer* overexpression rescues endoderm formation in *oep* but not *cas* mutants**

		sox17-expressing cells			
Injected RNA	Embryos	normal/increased	25-100	1-25	0
mixer	*cas*/wild-type	53/73	0/73	0/73	20/73
	oep^{z1}/wild-type*	62/84	18/84	3/84	1/84
GST-GFP	*cas*/wild-type	26/40	0/40	0/40	14/40
	oep^{z1}/wild-type*	26/38	1/38	11/38	0/38

		axial-expressing endodermal cells			
Injected RNA	Embryos	normal/increased	25-100	1-25	0
mixer	*cas*/wild-type	64/84	0/84	0/84	20/84
	oep^{z1}/wild-type*	60/76	8/76	6/76	2/76
GST-GFP	*cas*/wild-type	35/41	0/41	0/41	6/41
	oep^{z1}/wild-type*	25/39	0/39	3/39	11/39

**mixer*-injected oep^{z1} mutants contained an average of 45.8 ± 23.6 *sox17*-expressing cells (n = 22); *GST-GFP*-injected oep^{z1} mutants contained an average of 12.2 ± 8.2 *sox17*-expressing cells (n = 12). Comparison of these values using a two-sided t-test yields a test statistic of 4.7 ($p < 0.0003$). *mixer*-injected oep^{z1} mutants contained an average of 28.8 ± 24 *axial*-expressing cells (n = 16); *GST-GFP*-injected oep^{z1} mutants contained an average of 1 ± 2.2 *axial*-expressing cells (n = 14). Comparison of these values using a two-sided t-test yields a test statistic of 4.2 ($p < 0.0003$).

Embryos were injected with 200 pg of RNA at the 1-4-cell stage, and analysed at 90-100% epiboly.

Table 3.2

TARAM-A* **overexpression promotes endodermal gene expression in *oep* but not *cas* mutants**

	Gene expression				
Embryos	*ntl*	*gsc*	*mixer*	*axial*	*sox17*
–					
wild-type	21/21	20/20	20/20	20/21	27/27
cas/wild-type	NA	30/30	31/32	31/33	111/151 (74%)
oep^{z1}/wild-type	NA	24/24	20/20	NA	19/19

Embryos were injected with 90 pg of RNA at the 1-4-cell stage, and analysed after approximately 8 hours of development. NA, not assessed.

Chapter 4: Gut and pharyngeal endoderm originate from distinct cell populations in zebrafish

SUMMARY

In vertebrate embryos the anterior-most endoderm, the so-called pharyngeal endoderm, lines the gill arches, where it forms the branchial pouches, and regions of the pharyngeal cavity. Here we show that, in the zebrafish pharynx, endodermal cells expressing the NK homeobox gene *nkx2.3* lie adjacent to, but do not overlap with, endodermal cells that express genes characteristic of the gut endoderm such as *sonic hedgehog*. By the end of gastrulation, precursors of the gut endoderm and the pharyngeal endoderm exhibit distinct molecular, morphological, and spatial characteristics. Importantly, the *casanova* and *one-eyed pinhead* loci are required for formation of the gut endoderm but not for formation of the *nkx2.3*-expressing pharyngeal endoderm. Together these results demonstrate important molecular and genetic distinctions between the gut endoderm and the pharyngeal endoderm, and indicate that these two cell types may arise from distinct progenitor populations within the zebrafish embryo. Based upon molecular, genetic, and morphological characteristics, we suggest that the pharyngeal endoderm is in fact more like the mesoderm. More generally, we suggest that using molecular and genetic criteria to describe the developmental relationships between different cell populations may prove more informative than current germ layer assignments which rely largely upon the location and/or fate of the particular cells in question.

INTRODUCTION

All cells that contribute to the mature vertebrate organism derive from one of the three fundamental germ layers--ectoderm, mesoderm, and endoderm--which are thought to represent the earliest divisions within the embryo. These germ layer assignments are based primarily upon the ultimate fate and function of the particular cells in question, as well as to some extent upon their location within the early embryo.

In vertebrates the endoderm forms the lining of the digestive and respiratory tracts, as well as gut-associated organs such as the liver and the pancreas. The anterior-most endoderm, which lines regions of the mouth and pharyngeal cavity in higher vertebrates, and forms the pharyngeal pouches that line the gill arches in fish, is termed the pharyngeal endoderm. The potential role of this subset of the endoderm in heart development has been studied intensively (Jacobson and Sater, 1988), but less is known about the development of the pharyngeal endoderm itself.

Substantial progress has been made recently in elucidating the molecules and genetic networks that regulate the formation of the endoderm, in particular through studies conducted in *Xenopus* and zebrafish. These studies implicate nodal-related signals and their receptors, the One-eyed pinhead (Oep) protein, Mixer and other Mix-related homedomain proteins, the HMG-box transcription factor Sox17, and the zebrafish *casanova* (*cas*) locus as important regulators of endoderm development (Feldman et al., 1998; Osada and Wright, 1999; Peyrieras et al., 1998; Schier et al., 1997; Gritsman et al., 1999; Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998; Hudson et al., 1997; Alexander et al., submitted; Alexander and Stainier, submitted) Whether these same molecules are similarly important for the formation of the pharyngeal endoderm has not been tested directly.

Here we present data indicating that in zebrafish the pharyngeal endoderm and the gut endoderm derive from distinct precursor populations. We first demonstrate that cells

expressing markers of the pharyngeal endoderm or the gut endoderm occupy adjacent but non-overlapping regions of the pharynx. Furthermore, as early as the end of gastrulation the precursors of the pharyngeal endoderm and the gut endoderm exhibit distinct molecular, morphological, and spatial characteristics. Finally, we show that precursors of the pharyngeal endoderm are present in both *cas* and *oep* mutants, although pharyngeal pouch morphogenesis is disrupted. The *cas* and *oep* loci have previously been shown to be essential for the formation of all the gut endoderm. These results demonstrate important molecular and genetic distinctions between the gut endoderm and the pharyngeal endoderm. Based upon molecular, genetic, and morphological characteristics, we suggest that the pharyngeal endoderm may in fact be more similar to the anterior lateral plate mesoderm than to the gut endoderm. More generally, we suggest that using molecular genetic criteria to describe the developmental relationships between different cell populations may provide a more useful classification than current germ layer assignments which are based largely upon the ultimate fate of the particular cells in question.

MATERIALS AND METHODS

Strains

Adult zebrafish and embryos were maintained and staged as decribed. The *cas*^{ta56} and *oep*^{m134} alleles were identified in screens for ENU-induced embryonic-lethal mutations (Driever et al., 1996; Haffter et al., 1996).

Phenotypic analysis

In situ hybridisations were performed as described (Alexander et al., 1998). All riboprobes were prepared as described in the original publications. For sectioning, embryos were embedded in JB4 (Polysciences) and counterstained with neutral red. Photographs were taken on either a Leica MZ12 stereo microscope or a Zeiss Axioplan using Kodak Ektachrome 160T, and processed using Adobe Photoshop 4.0.

RESULTS

Non-overlapping distribution of the pharyngeal endoderm and the gut endoderm in zebrafish larvae

During larval stages the zebrafish gene *sonic hedgehog* (*shh*) is expressed throughout the entire gut endoderm (Krauss et al., 1993; Schier et al., 1997). At the same stages the NK homeobox gene *nkx2.3* is expressed in the pharyngeal endoderm (Lee et al., 1996). Comparison of *shh* expression (Fig. 4.1A) and *nkx2.3* expression (Fig. 4.1B) in wild-type embryos at 48 hours post fertilisation (hpf) reveals distinct locations of the gut and pharyngeal endoderm. While the endoderm that lines the pharyngeal cavity expresses *shh* (arrowhead in Fig. 4.1A), the the cells that form the pharyngeal pouches do not appear to express this gene but do express *nkx2.3* (arrowheads in Fig. 4.1B). Indeed, the anterior endodermal expression domain of *shh* appears to fit almost exactly in between the bilaterally situated *nkx2.3*-expressing pharyngeal pouches (compare Fig. 4.1A to 4.1B). *nkx2.3* expression is also apparent in a more anterior crescent-shaped group of cells (arrow Fig. 4.1B).

We compared the expression domains of *shh* and *nkx2.3* in more detail by examining histological sections of embryos stained as above. At the level of the eyes *shh*-expressing endodermal cells line the pharyngeal cavity (Fig. 4.1C). *shh*-expressing cells are also found in the ventral forebrain (arrow in Fig. 4.1C). Sections at similar levels reveal that *nkx2.3*-expressing cells lie more laterally and superficially in the embryo (Fig. 4.1D). Slightly more posteriorly, at the level of the anterior tip of the notochord (arrowhead in Fig. 4.1E), *shh*-expressing endodermal cells line the lumen of the gut tube (Fig. 4.1E). In contrast, at the equivalent level the *nkx2.3*-expressing cells form the pharyngeal pouches (Fig. 4.1F). Importantly, endodermal cells lining the gut tube do not express *nkx2.3*, and conversely no *shh* expression is evident in the pharyngeal endodermal cells lining the gill slits (compare Fig. 4.1E to 4.1F). Altogether these results indicate that

the *shh*-expressing gut endoderm and the *nkx2.3*-expressing pharyngeal endoderm occupy distinct regions of the zebrafish pharynx at 48 hpf.

Molecular, morphological, and spatial distinctions between the precursors of the gut endoderm and the pharyngeal endoderm at the end of gastrulation

The non-overlapping arrangement of the *nkx2.3*-expressing pharyngeal endoderm and the *shh*-expressing gut endoderm within the larval pharynx suggested that these cells might derive from different precursor populations earlier in development. We therefore compared the precursors of the pharyngeal endoderm to those of the gut endoderm in wild-type embryos at tailbud stage (near the end of gastrulation). At this stage expression of *nkx2.7*, a second NK homeobox gene, initiates in the precursors of the pharyngeal endoderm, which will later express *nkx2.3*, and the heart, which will later express *nkx2.5* (Lee et al., 1996). We also included a probe for *shh*, which at this stage reveals the location of the prechordal plate and notochord (Krauss et al., 1993), in order to facilitate comparisons. The *nkx2.7*-expressing cells form a 'V' whose vertex sits anterior to, and is substantially separated from, the rostral tip of the prechordal plate, and whose arms extend postero-laterally within the embryo (Fig. 4.2A,B). The heart arises from the postero-medial portion of each arm, while the remainder of the cells are thought to contribute to the pharyngeal endoderm (Lee et al., 1996); at this stage these different precursor populations are not morphologically distinguishable.

In order to visualise the precursors of the gut endoderm we examined the expression of the HMG-box transcription factor gene *sox17*, which is expressed in these cells along the entire length of the embryo until early somitogenesis stages (Alexander and Stainier, submitted). As above, we also included a *shh* probe. *sox17*-expressing gut endodermal precursors are present as a discontinuous layer within the hypoblast, the outline of which roughly traces a triangle laid upon the embryo: the anterior vertex of this

triangle lies just rostral to the anterior tip of the prechordal plate, where two or three *sox17*-expressing cells are present (Fig. 2C,D).

Comparison of the expression patterns of *nkx2.7* and *sox17* at this stage reveals two important points. First, the expression domains of the two genes appear to overlap only minimally if at all (compare Fig. 4.2A,B to 4.2C,D); that is, the *sox17*-expressing cells do not express *nkx2.7*, and conversely the *nkx2.7*-expressing cells do not express *sox17*. Second, the cellular morphologies and arrangements of the *nkx2.7*- and *sox17*-expressing populations are quite distinct. The *nkx2.7*-expressing cells appear round and closely packed together (Fig. 4.2A,B). In contrast, the *sox17*-expressing cells exhibit the large, flattened morphology previously described for gut endoderm precursors (Warga and Nusslein-Volhard, 1999), and are spread discontinuously throughout the hypoblast (Fig. 4.2C,D). Considered together, these data indicate that by the end of gastrulation precursors of the pharyngeal endoderm and the gut endoderm express different, mutually exclusive marker genes, occupy distinct spatial domains within the embryo, and exhibit quite different cellular morphologies and arrangements.

cas and *oep* mutants form pharyngeal endoderm precursors

Both *cas* and *oep* mutants have previously been demonstrated to lack gut endoderm entirely from the onset of gastrulation, and to be required cell-autonomously for endoderm formation (Gritsman et al., 1999; Schier et al., 1997; Alexander et al., submitted; Alexander and Stainier, submitted). *oep* encodes an EGF-CFC protein that is required for signalling by nodal-related growth factors (Gritsman et al., 1999; Zhang et al., 1998), while the molecular identity of the *cas* locus is not yet known. In order to determine whether these two loci are similarly required for the formation of the pharyngeal endoderm, we examined *nkx2.3* expression at the 20-somite stage in wild-type, *cas*, and *oep* mutants. In wild-type embryos at this stage, the *nkx2.3*-expressing cells have initiated morphogenesis of the pharyngeal pouches, the outlines of which are just beginning to

emerge (Fig. 4.3B). Interestingly, in both *cas* and *oep* mutants a significant number of *nkx2.3*-expressing cells are present, arranged in a horseshoe-shaped collar around the head of the embryo (Fig. 4.3C,D). The position and morphology of these cells strongly resemble those of the pharyngeal endoderm in wild-type embryos at slightly earlier stages, prior to the initiation of pharyngeal pouch morphogenesis (Fig. 4.3A). Although it is difficult to assess precisely, given their abnormal morphogenetic arrangement, it appears that in *cas* and *oep* mutants there are comparable numbers of *nkx2.3*-expressing cells as in wild-type. Also, the morphogenetic movements of these *nkx2.3*-expressings cells are not merely delayed in *cas* and *oep* mutants, as these cells remain positioned around the head of the embryo as late as 48 hpf (data not shown). These results suggest that pharyngeal endoderm precursors form relatively normally in these two mutants but that their morphogenesis is disrupted, and stand in stark contrast to the complete absence of the gut endoderm in *cas* and *oep* mutants (Schier et al., 1997; Alexander et al., submitted; Alexander and Stainier, submitted).

DISCUSSION

Distinct precursor populations form the gut endoderm and pharyngeal endoderm in zebrafish

In vertebrates the cells that line the gut tube and the respiratory tract and form the gut-associated organs, are considered to be of endodermal origin. We would argue, based upon the results presented in this report, that two distinct populations of endoderm, pharyngeal endoderm and gut endoderm, exist within the zebrafish embryo. First, in the 48 hpf zebrafish larva the *nkx2.3*-expressing cells of the pharyngeal pouches do not overlap with the *shh*-expressing cells lining the mouth and pharynx, indicating that these two cell populations occupy different, adjacent domains within the pharyngeal region. The precursors of these two endodermal populations display a similar non-overlapping spatial relationship near the end of gastrulation. Comparison of the *nkx2.7*- and *sox17*-expressing cells at this early stage also emphasises the distinct cellular morphologies and arrangements characteristic of these different endodermal precursor populations; the *nkx2.7*-expressing pharyngeal endoderm precursors are round and closely packed, while the *sox17*-expressing gut endoderm precursors display a large, flattened shape and are discontinuously distributed within the hypoblast. Finally, and most importantly, despite lacking gut endoderm entirely, both *cas* and *oep* mutants form *nkx2.3*-expressing pharyngeal endoderm. These results demonstrate different genetic requirements for the formation of these two endodermal populations, strongly suggesting that they derive from distinct precursors within the zebrafish embryo.

Without direct lineage analysis it is not possible to know for certain that the endodermal cells which express *nkx2.3* or *shh* at 48 hpf do in fact derive from the *nkx2.7*- or *sox17*-expressing cells seen at the tailbud stage, respectively. *nkx2.7* expression persists in the pharyngeal endoderm until at least 24 hpf (Lee et al., 1996), and during these later stages is indistinguishable from *nkx2.3* in the pharyngeal endoderm. *sox17*

expression at the tailbud stage in the anterior gut endoderm is indistinguishable from endodermal *axial* expression (Alexander and Stainier, submitted), which is maintained until at least 48 hpf in the anterior gut endoderm where it is indistinguishable from *shh* expression (Odenthal and Nusslein-Volhard, 1998; Schier et al., 1997; J.A. and D.Y.R.S., unpublished data). Therefore, although detailed lineage analysis will be required to address this issue conclusively, it seems likely that the *nkx2.7*- and *sox17*-expressing cell populations present at tailbud stage contribute directly to the *nkx2.3*- and *shh*-expressing endoderm seen at 48 hpf, respectively.

Fate mapping studies of the zebrafish late blastula have revealed that the endodermal progenitors lie within the most marginal four blastomere tiers (Warga and Nusslein-Volhard, 1999). These studies also indicated that within the endodermal lineage the embryonic dorsal-ventral axis correlates with the mature anterior-posterior axis; more dorsally located marginal cells form anterior structures such as the pharyngeal endoderm and esophagus, while the liver, pancreas, and intestine derive from progressively more lateral and ventral endodermal progenitors (Warga and Nusslein-Volhard, 1999). These studies did not, however, reveal a distinct domain of the pregastrula embryo from which the pharyngeal endoderm, as defined by expression of *nkx2.3*, arises. There are two reasons that likely explain this. First, in these studies the definition of pharyngeal endoderm did not distinguish between the cells that form the branchial pouches and the cells that line the pharyngeal cavity. Rather, any cell contributing to the anterior-most endoderm in the embryo was classified as pharyngeal endoderm. Second, these same studies demonstrate that the fate map domains of various tissues overlap quite significantly, both within and across the germ layers (Warga and Nusslein-Volhard, 1999). Thus, even applying a revised and more specific definition of pharyngeal endoderm, it is not clear that distinct domains of cells which contribute to the *nkx2.3*-expressing pharyngeal endoderm and the *shh*-expressing anterior gut endoderm would emerge from fate mapping studies.

It is unclear whether in other vertebrates the pharyngeal endoderm and the gut endoderm may similarly arise from distinct precursor populations. The superficial pharyngeal endoderm in *Xenopus* represents the only endoderm, as defined classically, that originates suprablastoporally (Keller, 1975; Keller, 1976). Explant and extirpation studies have demonstrated that these cells are necessary for normal formation of the dorsal mesoderm and indeed possess inductive properties characteristic of the dorsal mesoderm (Shih and Keller, 1992a; Shih and Keller, 1992b). Thus, the *Xenopus* suprablastoporal endoderm exhibits traits that resemble in many ways the dorsal mesoderm (Purcell and Keller, 1993), suggesting that these cells may similarly represent a distinct population of endodermal progenitors. Whether the *Xenopus* suprablastoporal endoderm is in any way related to the *nkx2.3*-expressing pharyngeal endoderm in zebrafish merits further study.

A possible role for the gut endoderm in pharyngeal pouch morphogenesis

Although the pharyngeal endoderm is present in both *cas* and *oep* mutants, its morphogenesis does not occur normally. In wild-type embryos the *nkx2.3*-expressing pharyngeal endoderm migrates medially from its initial arrangement in a collar about the head of the embryo to form the pharyngeal pouches. This migration does not occur in *cas* or *oep* mutants, so that the pharyngeal endoderm remains in an arrangement similar to that of the pharyngeal endoderm in wild-type embryos at earlier stages. It is possible that both the *cas* and *oep* gene products are required within the pharyngeal endoderm in order to execute this morphogenetic program. However, the failure of the pharyngeal endoderm to migrate medially is strongly reminiscent of morphogenetic defects in different mesodermal organs present in both *cas* and *oep* mutants (Fouquet et al., 1997; Schier et al., 1997; Alexander et al., submitted). These mutants exhibit cardia bifida (bilateral hearts that result from a failure of the bilateral precardiac mesoderm to fuse medially), a broadened arrangement of the blood and kidney precursors, and lateral displacement and disorganisation of the trunk endothelium. We have suggested that these phenotypes may

result cell non-autonomously from a failure of these different mesodermal cell populations to undergo normal medial migration in the absence of the gut endoderm (Alexander et al., submitted), a hypothesis which has been shown to be true for the cardia bifida seen in *oep* mutants (Peyrieras et al., 1998). We therefore suggest that the abnormal pharyngeal endoderm morphogenesis in *cas* and *oep* mutants also occurs secondarily to the absence of the gut endoderm, and hypothesise that the defects in pharyngeal endoderm morphogenesis in *cas* and *oep* mutants result cell non-autonomously.

Towards a molecular genetic classification of cell populations during development

Classical assignments of particular cells in the vertebrate embryo to the three fundamental germ layers are based largely upon the ultimate fate and function of the derivatives of the particular cells in question, as well as upon the cells' position within the early embryo. Thus, the cells that line the gut tube are considered endodermal, the cells that form connective tissue are considered mesodermal, and the cells of the nervous system and skin are considered ectodermal. Consistent with these assignments, the progenitors of these different cells types are generally grouped near each other and to some extent separate from other cells prior to and during gastrulation (Hatada and Stern, 1994; Keller, 1975; Keller, 1976; Lawson et al., 1991; Warga and Nusslein-Volhard, 1999).

There are, however, exceptions to these generalisations. For example, cells of the neural crest contribute to a variety of fates in the mature organism. The cells of the peripheral nervous system, obviously neural fates, derive from this population, but so do the pharyngeal arches, which form non-neural connective tissue, including much of the bone and cartilage in the head of the organism (Schilling et al., 1996). Thus, cells from one germ layer can clearly contribute to tissues classically viewed as being derived from a different germ layer.

The results presented in this report suggest that the pharyngeal endoderm in zebrafish may represent another such exception. While these cells do line the pharyngeal pouches and regions of the pharyngeal cavity in the larva, and thus serve 'endodermal' functions, they exhibit several characteristics that are strikingly different from the gut endoderm. First, the pharyngeal endoderm does not express genes characteristic of the gut endoderm, such as *sox17* or *shh*. Second, the closely packed arrangement and round morphology of the pharyngeal endoderm precursors differ markedly from the widely spaced, large flattened cells of the gut endoderm (Warga and Nusslein-Volhard, 1999). Third, and most importantly, formation of the pharyngeal endoderm does not appear to require either the *cas* or *oep* loci, both of which are essential for gut endoderm formation (Schier et al., 1997; Alexander et al., submitted; Alexander and Stainier, submitted). In fact, consideration of these same characteristics--expression of NK homeobox genes (Lee et al., 1996), a closely packed arrangement of round cells (Warga and Nusslein-Volhard, 1999), and morphogenetic defects in *cas* (Alexander et al., submitted) and *oep* mutants (Fouquet et al., 1997; Schier et al., 1997)--suggests that the pharyngeal endoderm in zebrafish is quite similar to the anterior lateral plate mesoderm, and thus may be more properly assigned to this germ layer.

More generally, we would suggest that as our knowledge of the molecular and genetic controls that underlie development increases, the classification of cells as ectodermal, mesodermal, or endodermal will become less important. Rather, it may now be possible to begin to describe groups of cells based upon the combination of regulators that are known to function within them, as determined by either molecular or genetic means. We believe that such an approach will better illuminate the developmental relationships between different cell populations, and thus facilitate future efforts to elucidate the molecular pathways that control development.

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Figure 4.1. Comparison of the anterior gut endoderm and the pharyngeal endoderm in zebrafish larvae. Wild-type embryos were examined at 48 hpf for expression of *shh* (A, C, E) or *nkx2.3* (B, D, F), and then viewed either in wholemount (A, B) or transverse histological section (C-F). In wholemounts, gut endodermal expression of *shh* (A) marks the pharyngeal cavity (arrowhead) and the anterior gut tube (asterisk). *shh* is also expressed by midline ventral neuroectodermal cells (arrow). *nkx2.3* expression (B) labels the pharyngeal pouches (arrowheads) and a more anterior crescent of pharyngeal endoderm cells (arrow), as well as smooth muscle cells of the posterior gut tube (asterisk). The vertical lines indicate the approximate level of the section shown in the corresponding panel (C-F). At the level of the eyes, *shh*-expressing gut endoderm lines the pharyngeal cavity (C), while the *nkx2.3*-expressing pharyngeal endoderm lies more laterally (D). At the level of the anterior tip of the notochord, expression of *shh* (E) labels the endoderm of the gut tube, while the *nkx2.3*-expressing cells form the pharyngeal pouches (F). The arrows in C and E indicate neuroectodermal *shh* expression, and the arrowheads in E and F indicate the notochord. The probe used is indicated in the lower left of each panel. A and B are dorsal views with anterior to the left; C-F are transverse sections with dorsal to the top.

Figure 4.1

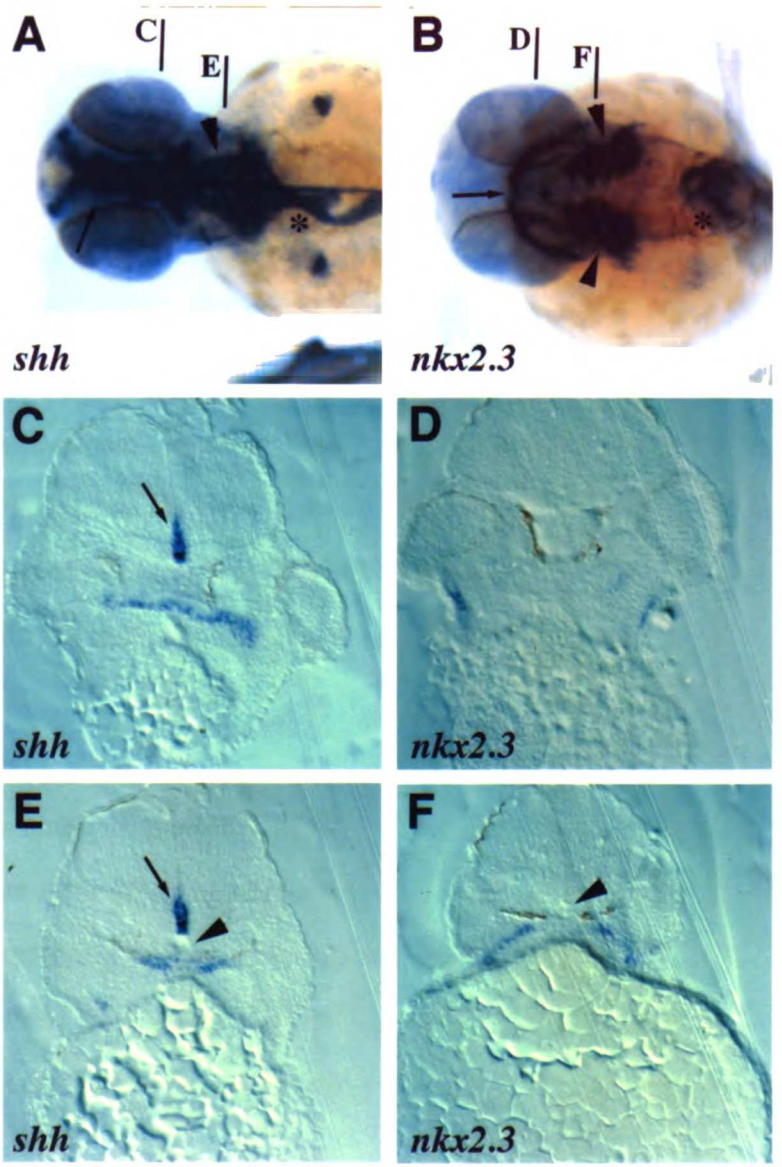


Figure 4.2. The pharyngeal endoderm and the gut endoderm exhibit distinct spatial, molecular, and morphological characteristics at the end of gastrulation. Expression of *nkx2.7* and *shh* (A,B) or *sox17* and *shh* (C,D) in wild-type embryos at the tailbud stage. The *nkx2.7*-expressing pharyngeal endoderm and precardiac mesoderm form a 'V' in the anterior of the embryo (A), whose vertex is positioned well in front of the anterior tip of the *shh*-expressing prechordal plate (arrowhead). The arrows indicate the presumed location of the precardiac mesoderm within the *nkx2.7* expression domain. Under higher power the *nkx2.7*-expressing pharyngeal endodermal cells appear round and relatively closely packed (B). The *sox17*-expressing gut endoderm precursors form a discontinuous sheet of cells (C) that extends caudally from just anterior of the *shh*-expressing prechordal plate (arrowhead). As seen at higher power, these *sox17*-expressing cells exhibit a large, flattened morphology and are well-separated from neighboring endodermal cells (D). Arrowheads in all panels indicate the *shh*-expressing prechordal plate. The probes used are indicated in the lower left of each panel. All are dorso-anterior views. B and D are high power views of the embryos shown in A and C, respectively.

Figure 4.3. Pharyngeal endoderm precursors form in *cas* and *oep* mutants. *nkx2.3* expression in wild-type embryos at the 12-somite (A) and 20-somite (B) stages, and in *cas* (C) and *oep* (D) mutants at the 20-somite stage. In wild-type embryos at the 12-somite stage (A) the *nkx2.3*-expressing cells are initially arranged in a collar around the front of the head. By the 20-somite stage (B) the *nkx2.3*-expressing cells have begun to migrate into the anterior of the embryo and initiate morphogenesis of the pharyngeal pouches (arrowheads in B). In *cas* (C) and *oep* (D) mutants the *nkx2.3*-expressing cells are present in approximately normal numbers, but these morphogenetic movements do not occur and the pharyngeal endoderm remains positioned around the the head. The abnormal shape of the cyclopic *oep* mutant head is evident (D). The genotype of the embryo is indicated in lower right corner of each panel. All views are dorso-anterior.

Figure 4.2

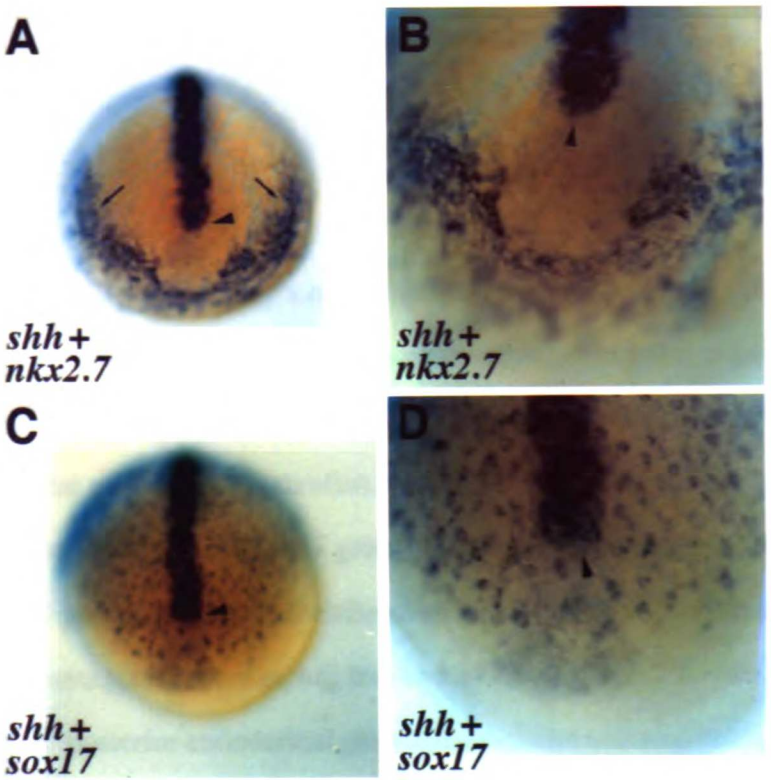
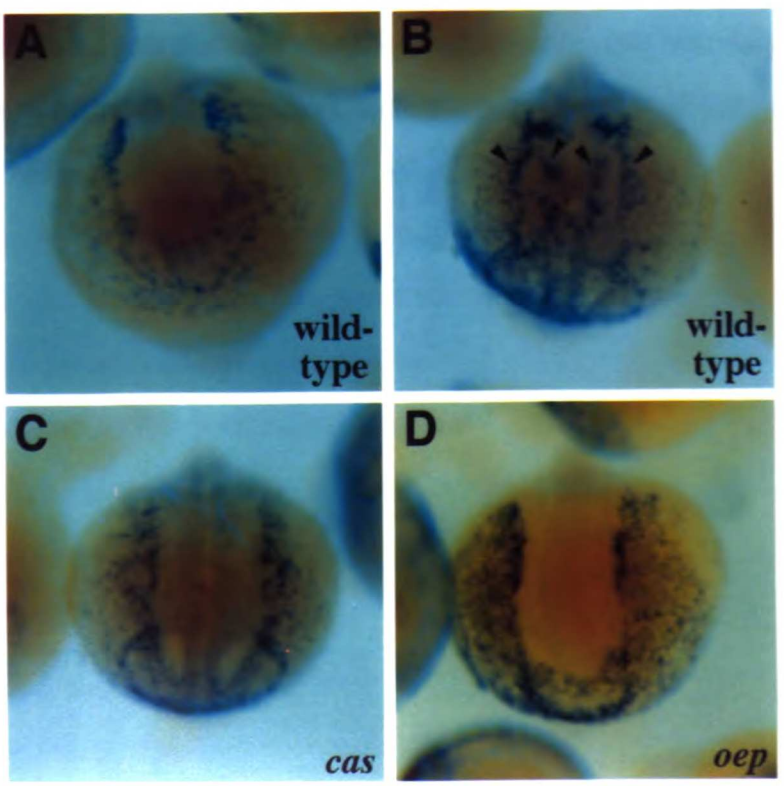


Figure 4.3



Chapter 5: A possible role for bone morphogenetic proteins in the formation and patterning of the zebrafish endoderm

SUMMARY

Bone morphogenetic proteins play a critical role in generating the early dorsal-ventral patterning of the vertebrate embryo. Here we provide evidence that bone morphogenetic proteins may also be important for both the formation and the early anterior-posterior patterning of the endoderm in zebrafish. The zebrafish mutants *swirl* and *snailhouse*, which define the *bmp2* and *bmp7* genes, respectively, exhibit increased numbers of endodermal precursors, while the *chordino* mutant, which lacks the bone morphogenetic protein antagonist chordin, has less endoderm than normal. Additionally, there appear to be fewer posterior endodermal precursors in *swirl* and *snailhouse* mutants as compared to wild-type, while the number of these cells in *chordino* mutants may be increased. These results suggest that bone morphogenetic proteins may both negatively affect the generation of endodermal precursors, and contribute to the initial anterior-posterior patterning of the endoderm.

INTRODUCTION

The mechanisms that impart dorsal-ventral patterning to the early vertebrate embryo have been the subject of intense study in recent years (reviewed in Harland and Gerhart, 1997). The model that has emerged, primarily from studies in *Xenopus*, suggests that a ventralising signal, consisting of bone morphogenetic protein (BMP) -2, -4, and/or -7, is produced by cells throughout the embryo. Chordin and noggin, which act as antagonists of these ventralising factors (Piccolo et al., 1996; Zimmerman et al., 1996), are secreted by the dorsal organiser and limit the degree and range of their effects. An additional level of regulation is provided by the Xolloid protease, which functions as an inhibitor of the secreted inhibitor chordin (Piccolo et al., 1997). The combined actions of these various players result in a gradient of BMP activity, highest ventrally and lowest dorsally, that translates into the assumption of different fates by the prospective mesoderm and ectoderm. Ventral marginal cells receive the highest levels of BMP signalling and form primarily blood and intermediate mesodermal derivatives such as kidney, while ventral ectoderm forms epidermis; more lateral and dorsal marginal cells experience lower BMP activity and form somites, heart, and notochord, while dorsal ectoderm forms neural tissue.

The identification and molecular analysis of dorsalised and ventralised zebrafish mutants have largely confirmed the roles suggested for many of the above described factors. The strongly dorsalised *swirl* (*swi*) and snailhouse (*snh*) mutants (Mullins et al., 1996) have been demonstrated to result from mutations in zebrafish *bmp2* (Kishimoto et al., 1997) and *bmp7* (B. Schmid, personal communication), respectively. Conversely, the ventralised mutant *dino* (*din*) (Hammerschmidt et al., 1996) has been shown to carry a mutation in the zebrafish *chordin* gene, and has therefore been renamed *chordino* (Schulte-Merker et al., 1997). Finally, mutations in zebrafish *zolloid* (Blader et al., 1997) have been shown to cause the mildly dorsalised *minifin* mutant (Connors et al., 1999). Detailed gene expression studies in these mutants have also demonstrated an important role for BMP

signals in maintaining their own expression before and during gastrulation (Kishimoto et al., 1997; B. Schmid, personal communication).

Whether BMP signals affect development of the vertebrate endoderm has for the most part not been examined. Overexpression of chordin or noggin has been shown to promote endodermal marker gene expression in *Xenopus* animal cap explants (Sasai et al., 1996), suggesting that high levels of BMP activity may inhibit endodermal differentiation. Similar results have not been universally reported (G. L. Henry, personal communication), however, and the endogenous importance of BMPs in *Xenopus* endoderm formation remains unclear. Fate mapping studies of the zebrafish late blastula margin demonstrate that while endoderm progenitors can be found throughout the entire marginal zone, more dorsally located cells give rise to a higher proportion of endoderm only clones (Warga and Nusslein-Volhard, 1999). These results are consistent with the suggestion from the *Xenopus* studies that low levels of BMP activity are more compatible with endoderm formation. The zebrafish fate mapping studies further demonstrate a correlation between the dorso-ventral position of a presumptive endodermal cell within the pregastrula margin and its progeny's anterior-posterior position along the larval gut tube (Warga and Nusslein-Volhard, 1999): dorsally located marginal cells contribute to the pharynx; the stomach, pancreas and liver emerge from more lateral cells; and the intestine and anus derive from the most ventral endodermal progenitors.

The isolation of genes expressed by the endodermal precursors early in zebrafish development (Alexander and Stainier, submitted), together with the dorsalised and ventralised zebrafish mutants described above, present an opportunity to examine the roles played by BMPs during early endoderm development. Here we show that dorsalised zebrafish mutants contain more endoderm, while the ventralised mutant *chordino* contains less endoderm than normal. Additionally, comparison of the expression of the early endodermal markers *sox17* and *axial* suggests that BMP signals may influence the anterior-posterior patterning of the endoderm during gastrulation.

MATERIALS AND METHODS

Strains

Adult zebrafish and embryos were maintained and staged as decribed. The *swi*, *snh*, and *din* mutants were identified in a screen for ENU-induced embryonic-lethal mutations (Mullins et al., 1996).

Phenotypic analysis

In situ hybridisations were performed as described (Alexander et al., 1998). The *sox17* and *axial* riboprobes were prepared as described (Alexander and Stainier, submitted; Strahle et al., 1993).

RESULTS

Abnormal numbers of endodermal precursors in dorsalised and ventralised zebrafish mutants

We have previously demonstrated that a zebrafish homologue of the mouse *Sox17* gene is expressed in endodermal precursors from shield stage until the end of gastrulation (Alexander and Stainier, submitted). In order to determine whether the endoderm is affected by dorsalising or ventralising zebrafish mutations, we examined *sox17* expression at the tailbud stage in *swi*, *snh*, and *din* mutants (Hammerschmidt et al., 1996; Mullins et al., 1996). In wild-type embryos at this stage, *sox17* expression reveals that the endodermal progenitors are found along the length of the embryonic axis, from the prechordal plate anteriorly into the tailbud posteriorly, and extend laterally to the lateral boundaries of the embryo itself (Figure 5.1A,B). The anterior-posterior extent of the endoderm at the tailbud stage in the dorsalised mutants *swi* and *snh* is not changed (Figures 5.1C,D). However, *sox17*-expressing endodermal precursors encircle the entire dorsal-vental extent of these mutant embryos, including the normally endoderm-free ventral region (Figures 5.1C,D). In contrast, in *din* mutants at the tailbud stage the lateral boundaries of the endoderm are narrowed (Figure 5.1E,F). More mildly affected dorsalised or ventralised mutants, such as *lost-a-fin* or *ogon*, showed normal *sox17* expression (data not shown). These data suggest that at the tailbud stage mutations that decrease BMP activity increase the number and distribution of endodermal precursors, while mutations that increase BMP activity decrease the number and distribution of these cells.

In order to determine at what point BMP activity affects endoderm development, we examined *sox17* expression in *swi*, *snh*, and *din* mutants earlier in gastrulation. At shield, 60% epiboly, and 80% epiboly stages, from early to mid-gastrulation, we were unable to distinguish mutant and wild-type embryos based upon *sox17* expression (data not shown). These results suggest that initial endoderm formation occurs normally in the dorsalised and

ventralised mutants, and that only later in gastrulation does BMP activity affect the generation of endodermal precursors.

Dorsalising and ventralising mutations affect posterior endoderm formation in zebrafish

sox17 appears to be expressed in all endodermal precursors during gastrulation (Alexander et al., submitted). During these same stages *axial*, a zebrafish homologue of *HNF3β* (Strahle et al., 1993), is also expressed in the endoderm (Schier et al., 1997), but is excluded from a posterior population of endodermal precursors (Figure 5.2A,B) (Alexander et al., submitted). Thus, comparison of endodermal *sox17* and *axial* expression reveals that some anterior-posterior patterning of the endoderm is already present by the tailbud stage.

In order to determine whether BMP activity may affect this anterior-posterior patterning of the endoderm, we compared *sox17* and *axial* expression in *swi*, *snh*, and *din* mutants at the tailbud stage. The presence of *sox17*-positive, *axial*-negative endodermal precursors can clearly be seen in the posterior of wild-type embryos (Figure 5.2A,B). In *swi* and *snh* mutants, however, *axial*-expressing endodermal precursors extend nearly, if not equally, as far posteriorly as do *sox17*-expressing cells (Figure 5.2C-F). *din* mutants, meanwhile, appear to show a relative increase in the number of *sox17*-positive, *axial*-negative endodermal precursors (Figure 5.2G,H). Together, these data suggest that decreased BMP activity, as in *swi* and *snh* mutants, expands the *sox17*-positive, *axial*-positive (anterior) endodermal domain, while increased BMP activity, as in *din* mutants, expands the *sox17*-positive, *axial*-negative (posterior) endodermal domain.

DISCUSSION

The role of BMPs in the formation and patterning of the ectodermal and mesodermal germ layers has been extensively investigated during the past several years (Harland and Gerhart, 1997). These studies have revealed that dorsally produced BMP antagonists modify the function of broadly produced BMPs to generate a gradient of BMP activity. This gradient reads out in the assumption of different fates by cells of the prospective mesoderm and ectoderm: high levels of BMP activity promote the development of ventral fates from each of these germ layers (e.g. blood from the mesoderm and epidermis from the ectoderm), while low levels of BMP activity translate into dorsal fates (e.g. muscle and notochord from the mesoderm and neural tissue from the ectoderm).

Whether BMPs are similarly important for endoderm development has not been extensively explored. The secreted BMP antagonists chordin and noggin have been reported to direct *Xenopus* animal cap explants to an endodermal fate, as assayed by the expression of the *endodermin* gene (Sasai et al., 1996). However, others have obtained conflicting results (G. L. Henry, personal communication). Given the uncertain relation of animal cap studies to normal development, these data leave unclear whether BMPs play an endogenous role in vertebrate endoderm formation.

Our results suggest that the zebrafish mutations *swi*, *snh*, and *din* (Hammerschmidt et al., 1996; Mullins et al., 1996), all of which perturb levels of BMP activity (Kishimoto et al., 1997; Schulte-Merker et al., 1997; B. Schmid, personal communication), do affect both the formation and patterning of the endoderm. The dorsalised mutants *swi* and *snh* show an expanded number and distribution of the endodermal precursors at tailbud stage, while ventralised *din* mutant contain fewer endodermal precursors than normal. Additionally, *swi* and *snh* mutants appear to contain relatively more *sox17*-positive, *axial*-positive (anterior) endoderm, while the *sox17*-positive, *axial*-negative (posterior) endoderm

seems to be expanded in *din* mutants. These results demonstrate an endogenous role for BMPs in endoderm development in zebrafish.

The expanded distribution of the endoderm in *swi* and *snh* mutants may partially result from aberrant cell movements. During gastrulation cells of all three germ layers normally converge towards the dorsal midline. This dorsal convergence is reduced in *swi* and *snh* mutants (Mullins et al., 1996), which may explain why the endodermal precursors cover the entire circumference of these mutant embryos. At the same time, there are clearly more endodermal precursors in *swi* and *snh* mutants than in wild-type embryos, indicating that BMP signals must also affect the generation of the endoderm.

How and when BMPs affect the generation of the endodermal precursors is not clear. At early and mid-gastrula stages *swi*, *snh*, and *din* mutants cannot be distinguished by the expression of the endodermal marker gene *sox17*, suggesting that the initial formation of the endoderm is not affected by BMP signalling. Fate mapping studies have shown that the endodermal progenitors are located near the margin in the pregastrula zebrafish embryo, and involute early in gastrulation (Warga and Nusslein-Volhard, 1999). These facts suggest that BMPs must affect the generation of endodermal precursors after these cells have already involuted. The same fate mapping studies have also revealed that relatively more of the endoderm emerges from dorsally located marginal progenitor cells than from ventral progenitors (Warga and Nusslein-Volhard, 1999). Thus, it may be that following their involution and determination as endodermal precursors, ventral endodermal precursors are normally inhibited from dividing by high levels of BMP activity, while lower levels of BMPs allow more dorsal endodermal precursors to divide extensively. In combination with the dorsal convergence movements described above, this effect of BMPs would create the distribution of endodermal precursors seen at tailbud stage and the different relative contributions of ventral and dorsal endodermal precursors to the mature endoderm. In *swi* and *snh* mutants ventral endodermal precursors would experience lower

levels of BMP activity than normal, and thereby would continue to divide, resulting in both an increased number and an abnormal distribution of the endodermal precursors.

At the tailbud stage anterior endoderm appears to be expanded in the dorsalised mutants *swi* and *snh*, while posterior endoderm may be expanded in ventralised *din* mutants. Thus, BMPs also seem to play a role in the patterning of the endoderm. These results are consistent with the observed relationship between early dorsal and later anterior fates in vertebrate development, and suggest that BMPs may affect cell fate in all three germ layers in a concerted fashion. Whether BMPs play an important role in the later patterning of the endoderm is unclear, as other aspects of development are grossly perturbed in *swi*, *snh*, and *din* mutants (Mullins et al., 1996), making the endoderm difficult to analyse. Additionally, more subtle aspects of early endodermal patterning may be affected by BMP levels, but in the absence of markers that reveal such patterning this possiblity cannot be addressed.

Clearly, the data presented here represent only a preliminary delineation of the importance of BMP signalling in endoderm development. Nonetheless, our results provide the first evidence that BMPs play an endogenous role in endoderm formation in vertebrates, and should encourage further efforts to understand in greater detail the various effects of these molecules on endoderm development.

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Figure 5.1. Abnormal endoderm formation in *swi*, *snh*, and *din* mutants. *sox17* expression in wild-type (A,B), *swi* (C), *snh* (D), and *din* (E,F) mutant embryos at the tailbud stage. A, C, D, and E are lateral views; B and F are dorsal views. The genotype of the embryo is indicated in the lower left corner of each panel; WT, wild-type. In wild-type embryos *sox17*-expressing endodermal precursors extend rostro-caudally from the prechordal plate into the tailbud (A), and laterally to the edges of the embryo (B). The rostro-caudal extent of the endoderm is unchanged in *swi* (C) and *snh* (D) mutants, but endodermal precursors extend abnormally to cover the ventral region of the embryo in these mutants. In *din* mutants both the rostro-caudal (E) and lateral (F) extent of the endoderm is diminished.

Figure 5.2. Dorsalising and ventralising zebrafish mutations affect anterior-posterior endodermal patterning. *sox17* (A,C,E,G) and *axial* (B,D,F,H) expression in wild-type (A,B), *swi* (C,D), *snh* (E,F), and *din* (G,H) mutant embryos at the tailbud stage. All are posterior views. The genotype of the embryo is indicated in the lower left corner of each panel; WT, wild-type. Comparison of endodermal *sox17* (A) and *axial* expression (B) in wild-type embryos reveals the existence of a posterior endodermal population that expresses *sox17* but not *axial*. In *swi* (C,D) and *snh* (E,F) mutants *sox17*- and *axial*-expressing endodermal cells appear to extend almost equally into the posterior of the embryo. In *din* mutants (G,H) *sox17*-expressing endodermal precursors populate the tailbud region, while *axial* -expressing endodermal cells appear not to reach as far posteriorly as in wild-type.

Figure 5.1

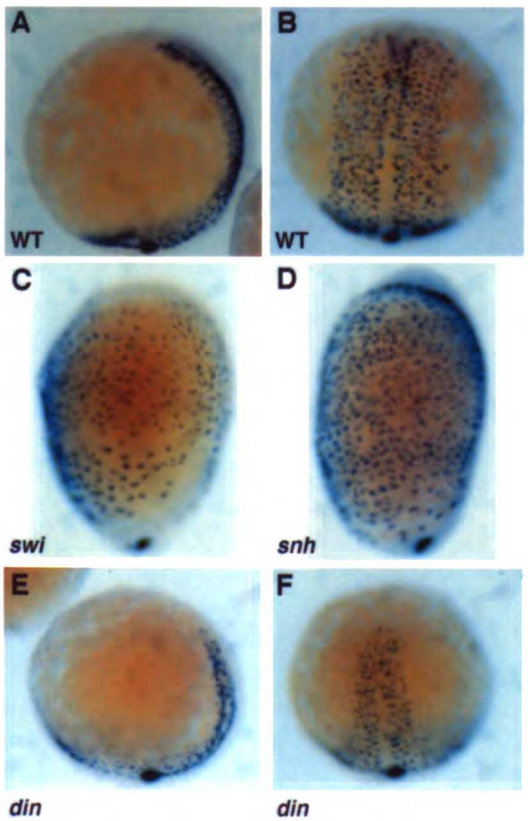

Figure 5.2