casanova Plays an Early and Essential Role in Endoderm Formation in Zebrafish

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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 parallel to, mixer to promote endoderm formation. We further find that the forerunner cells, a specialized group of noninvoluting 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 morphogenesis of these organs. © 1999 Academic Press

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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 signaling 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 localized maternal deposit of VegT using antisense 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 signaling 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 likely promotes endoderm development principally or perhaps entirely through Xsox17 (Henry and Melton, 1998). The maintenance of Xsox17 expression in 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-brain 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 signaling 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 parallel to, a zebrafish Mixer homologue. cas mutants also appear to lack forerunner cells and display morphogenetic defects in several mesodermal derivatives.

MATERIALS AND METHODS

Strains

Adult zebrafish and embryos were maintained and staged as described (Westerfield, 1995). The cas<sup>−/−</sup> and knypek<sup>−/−</sup> (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 hybridizations were performed as described (Alexander et al., 1998). For sectioning, embryos were embedded in JB4 (Polysciences) and counterstained with neutral red. Labeling of forerunner cells with syto-11 was performed as described (Cooper and D'Amico, 1996). Photographs were taken on either a Leica MZ12 stereomicroscope or a Zeiss Axioplan using Kodak Ektachrome 160T or Fujichrome 1600 ASA film and processed using Adobe Photoshop 4.0.

Cell Transplantation

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

Isolation of mixer

A fragment of mixer was isolated from midgastrula-stage cDNA using degenerate PCR primers 5'-CCGAGTCGAGGTTG-
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GTYCARAA-3' and 5'-GGTGTTCATGTCGGGTGTG-ATNDTTYTTRTT-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<sup>166</sup> allele. cas<sup>166</sup> 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<sup>166</sup>, 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 h postfertilization (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 (Figs. 1A and 1B). 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. 1C). cas mutants have neither an anal opening nor an intestine, nor is a well-formed pronephric duct visible (Fig. 1D). Small cysts form posterior to the yolk extension in cas mutants (Fig. 1D). We hypothesize 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. 1E), in cas mutants the notochord is positioned almost directly atop the yolk (Fig. 1F). Floor-plate cells in cas mutants express shh normally (Figs. 1E and 1F), 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. 2A). No endodermal axial expression is detectable in cas mutants, although neural expression of axi is normal (Fig. 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. 2C) (Odenthal and Nusslein-Volhard, 1998). A gain cas mutants specifically lack endodermal expression of fkd7 and fkd2 (Fig. 2D and data not shown). Additionally, fkd7 expression reveals that the hypochord forms but is shortened posteriorly in cas mutants (Fig. 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. 2E). Cardiac expression of gata4 is evident in cas mutants, demonstrating cardia bifida, but no endodermal gata4 expression is seen (Fig. 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 axi initiates soon after the onset of gastrulation (Fig. 3A) and is maintained throughout gastrulation (Fig. 3C). These axi-expressing cells are identifiable as endodermal precursors by their close apposition to the yolk and their large flattened morphology (Warga and Nusslein-Volhard, 1993). cas mutants specifically lack endodermal axi expression, while axi expression in the prechordal plate and notochord appears normal (Figs. 3B and 3D). 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 midgastrulation gata5 expression appears in endodermal precursors distributed throughout the forming hypoblast (Fig. 3E). This endodermal gata5 expression is strikingly absent in cas mutants (Fig. 3F). Endodermal expression of fkd2 (Odenthal and Nusslein-Volhard, 1998) also initiates during gastrulation (Fig. 3G), but again is specifically absent in cas mutants (Fig. 3H).

These data reinforce the conclusion that the endoderm in cas mutants is defective, and perhaps entirely absent, from the onset of gastrulation.
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 analyzing the expression of axial. Wild-type cells transplanted into cas

**FIG. 1.** cas mutants lack a gut tube. Nomarski optical images (A–D) and histological sections (E, F) of embryos at 24 (A, B), 36 (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 (asterisks), 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.
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. 4). In contrast, cas mutant cells were never observed to form endoderm (Fig. 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 Mesoderm 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 ze-
brafish mix gene (Fig. 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. 5B). We
used a SSCP polymorphism in the mixer 3’ UTR to analyze
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 em-
byros we first detect mixer expression at the sphere stage in
a small group of dorsal cells (Fig. 5C). By dome stage mixer
expression has spread circumferentially throughout the
marginal zone and also appears in the dorsal YSL (Fig. 5D).
At subsequent stages we do not detect mixer expression in
the YSL. Expression in the marginal zone persists through
the onset of gastrulation (Figs. 5E and 5F). Soon afterward
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. 5G)
(Schulte-Merker et al., 1992). Prior to the onset of gestra-
lation gata5 is expressed in a subset of the marginal zone
from which all of the endoderm as well as some mesoderm
will emerge (Fig. 5H) (Rodaway et al., 1999; Warga and
Nusslein-Volhard, 1999). This pregastrula phase of gata5
expression is normal in cas mutants (data not shown). The
mixer expression domain appears quite similar to that of ntl
and includes substantially more of the marginal zone than
the gata5 expression domain (compare Figs. 5F–5H). Thus,
expression of zebrafish 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
depictive 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 epi-
boly 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 somi-
togenesis Kupffer’s vesicle disappears and the progeny of
the FRs contribute to the notochord, muscle, and mesen-
chyme of the tail (Melby et al., 1996).

The FRs have been proposed to represent the endodermal
aspect of the neurureteric 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. 6A), in embryos derived from a
cas+/ heterozygote intercross. Light microscopic observa-
tion of embryos at the six-somite stage revealed that

FIG. 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 mutants, while expression in the embryonic shield is normal (B); the few axial-expressing cells
just outside the shield in the cas mutant are likely notochord precursors that have not yet completed dorsal convergence. At midgastrulation 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.

FIG. 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 hypoblast (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
(arrow indicate brown cells). In 53 wild-type to wild-type control transplants, we observed four cases in which transplanted cells
formed axial-expressing endoderm (data not shown). Wild-type cells transplanted into cas mutant hosts formed axial-expressing endoderm
in 5 of 77 cases (P = 0.5–0.9). cas mutant cells were never observed to form axial-expressing 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.
FIG. 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 (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.
Kupffer's vesicle did not form in approximately one-quarter (17/77) of these embryos (Fig. 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. 6C). FR ntl expression was absent in one-quarter (16/64) of the embryos derived from a cas+/+ heterozygote intercross (Fig. 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+/ cas/1 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 visualization under fluorescence microscopy of the forerunner cell cluster in wild-type embryos (Fig. 6E). Approximately one-quarter of the embryos (6/22) lacked a fluorescent forerunner cell cluster (Fig. 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. 6G); this technique allows direct visualization of the forerunner cell cluster (Melby et al., 1996). Again, in approximately one-quarter of the embryos (10/36) no FRs were seen (Fig. 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 (Figs. 7A and 7B) (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 endodermal 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. 7C) (Liao et al., 1997). These endodermal precursors are disorganized in cas mutants (Fig. 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 toward each other and join in the midline (Fig. 7E). This medial movement is also perturbed in cas mutants (Fig. 7F). Last, the pax2.1 gene (Mikkola et al., 1992) is expressed in the nephrogenic mesoderm, which shows an arrangement similar to that of the differentiating erythroblasts (Fig. 7G). Again, as development proceeds the nephric ducts move medially in wild-type embryos, but fail to do so normally in cas mutants (Fig. 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 (Figs. 3G and 3H). Second, the pax2.1-expressing spinal commissural interneurons (Mikkola et al., 1992) are not wider apart in cas mutants compared to wild-type (Figs. 7G and 7H). 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. 7K and 7L). The trunk endothelium in kny mutants is also spread more broadly across the midline (Fig. 7J), 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 disorganized (Figs. 7J and 7L). Also, the morphogenetic defects in the kidney and blood progenitors of cas mutants do not appear until after the 10-somite stage, more than 3 h 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 appropriate medial migration.

DISCUSSION

An Essential Role for cas in Endoderm Formation

The genetic networks that control development of the vertebrate endoderm have only recently begun to be explored. Several genes that appear to play key roles in the early events of endoderm formation have now been identified. Important functions for these genes—in particular Xsox17α and Xsox17β 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 HNF3β 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 (Figs. 1 and 2). Most interestingly, cas mutants lack endodermal expression of axial, gata5, and fkd2 from the onset of gastrulation (Fig. 3). 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 (Fig. 4), presumably either to receive or to respond to endoderm-inducing signals. Directed misexpression of a constitutively active type I TGF-β receptor (TARAM-A*) in a single blastomere of 16-cell-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 parallel to, oep and TARAM-A*, but experiments to test this hypothesis directly are needed.

Many studies have focused upon the role(s) of the endoderm in the induction or patterning of the mesoderm.

**FIG. 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 hybridization 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 visualized by labeling 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-labeled EVL cells are seen. Using Nomarski optics the FRs can be directly visualized 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.
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. 1) is quite striking. Endodermal cells may be transiently present and able to fulfill their normal signaling functions in cas mutants. Alternatively, the relatively normal appearance of cas mutants may result from the fact that in nonamphibian embryos these signaling 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; Yamana et al., 1998). At the same time, the mesodermal defects seen in cas mutants (Fig. 7) may suggest a role(s) 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, based upon its overall homology to Xenopus Mixer, we call mixer. Whether this gene represents an authentic zebrafish Mixer equivalent awaits further studies. mixer is not linked to cas, and mixer expression is normal in cas mutants, suggesting that cas acts either downstream of, or parallel to, mixer to regulate endoderm formation.

The mixer expression pattern (Fig. 5) raises intriguing questions regarding how endoderm and mesoderm are segregated before and during gastrulation. Unlike in Xenopus, in which 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 utilize a mechanism to segregate mesoderm from endoderm that is different from that of Xenopus. In Xenopus vegetally localized 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 expres-
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. 6), similar to the role of cas in endoderm development, provides genetic evidence supporting the endodermal assignment of the FRs. However, we have not directly tested whether cas acts cell autonomously in the FRs. The β-catenin signaling 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 specialized dorsal subset of the endoderm.

A related point concerns the germ-layer assignment of the hypochord. Studies in amphibians have concluded that this structure derives from the endoderm (Lobberg 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), 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 signaling plays a role in the allocation of dorsal midline cells to the ectoderm (floor plate), mesoderm (notochord), 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 signaling acts to subdivide a common progenitor population into three different derivatives: the floor plate, 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 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 the 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 bozok mutants lack FRs, indicating that FR formation lies downstream of the same β-catenin signaling 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. 1 and 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. 1). As noted above, the hypochord in cas mutants does appear shortened posteriorly (Fig. 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. 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. 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 nonautonomously 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 biphid in gata4-mutant mouse embryos provides one example of an endodermal defect that apparently underlies abnormal mesodermal morphogenesis (Narita et al., 1997). Studies of oe mutants also support a role for the endoderm in the morphogenesis of the heart (Schier et al., 1997; Peyreras 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" appear to contain as much myocardial tissue as do wild-type embryos (compare Figs. 7A and 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., 1999). 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; Schulteis 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 gata4 will represent fundamental steps toward achieving a detailed understanding of vertebrate endoderm development.

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References


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homologue of the mouse T (Brachyury) gene. Development 120, 1009–1015.

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