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Early events in the developments of the vertebrate myocardium and endoderm

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To my parents

Preface

This text represents the thought and effort of a small community, without which I would have accomplished little. Certainly, this work would have been impossible without the advice, insight and advocacy of Didier Stainier, an unflaggingly supportive and evenhanded thesis advisor. I hope someday to serve my own students as well. Much of my progress as a scientist stemmed not from classes, textbooks or even my research, but from provocative discussions with others in the lab. While all of my labmates have been terrific friends and coworkers, I am especially grateful to four who have been particularly supportive: Wayne Liao has demonstrated daily how to be an outstanding scientist and, despite close quarters, has never griped about my taste in music; Alex Navarro has done an outstanding job at keeping the fish (and consequently, me) happy; Emily Walsh has been a great friend with whom I have shared both lab and class; and Jon Alexander has been both a wonderful friend and the scientific trailblazer for most of my work. Without Jon, I would have lacked both the resources and inspiration to explore endoderm development.

I also owe a debt of gratitude to those who, by enticing me out of the lab, have maintained my sanity over these last four years. The perpetually looming scientific crisis unfailingly appeared surmountable after retreating to be with my family. I am lucky enough to share my life with Christine Ma, who has ceaselessly challenged me to maintain perspective on what is truly important.

And lastly, I am very thankful for the guidance of my thesis committee -- Gail Martin, Jane Gitscher and Cori Bargmann -- which made graduate school both edifying and fun.

The text of chapter 2 is a reprint of the material as it appears in Genes and Development. Adam Rodaway, a postdoctoral fellow in the laboratories of Nigel Holder and Roger Patient, provided the zebrafish *gata5* cDNA prior to publication. Jonathan Alexander helped characterize the zebrafish *gata5* expression pattern and provided reagents that allowed *gata5* to be mapped to the *faust* locus. Deborah Yelon provided the *cmlc1* riboprobe prior to publication.

Chapter 5 is the product of a collaboration with Cheol-Hee Kim and Ajay Chitnis. Cheol-Hee Kim synthesized the Notch3(ICD) construct.

Abstract

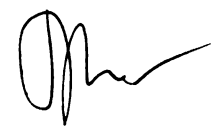
Early events in the development of the vertebrate myocardium and endoderm

--an investigation into the heartless and gutless

Jeremy F. Reiter

In this dissertation, I describe some mechanisms by which pattern is translated into the formation of different tissues within the early vertebrate embryo. Using the molecular and genetic tools available in the study of zebrafish development, I have investigated the early development of the myocardium, the muscular layer of the heart, and the endoderm, the germ layer which gives rise to the gut. I have demonstrated that the *faust* locus encodes the zebrafish homolog of Gata5, an evolutionarily conserved zinc finger transcription factor. *faust/gata5* mutants do not form functional hearts or guts. Further analysis of *faust/gata5* mutants revealed that Gata5 regulates the expression of several myocardial genes, including *nkx2.5*, a homolog of the *Drosophila tinman* gene. In the endoderm, Gata5 promotes endodermal differentiation and the formation of a normal complement of endodermal progenitors. When overexpressed *gata5* can increases endodermal and myocardial gene expression and produce ectopic, rhythmically contracting myocardial tissue. These results indicate that Gata5 performs a unique and central role in the development of both myocardium and endoderm. Furthermore, through overexpression studies and mutant analysis, I have demonstrated that *gata5* expression is induced by Nodal signaling, and that Gata5 acts in parallel with another transcription factor, Bon, to promote the differentiation of both the myocardium and endoderm.

In addition to Nodal signals, Notch and Wnt signals are present in the germ ring during the formation of the endodermal progenitors. Overexpression experiments suggest that Wnt signaling promotes and Notch signaling antagonizes endoderm formation. Cumulatively, these results suggest that blastomeres form different tissues depending on the differential integration and interpretation of Nodal, Wnt and Notch signals.



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Chapter 1: Introduction

At the beginning of vertebrate development, the single cell that will produce the entire animal undergoes a series of rapid divisions, generating a mass of morphologically indistinguishable cells. One of the first morphogenetic manifestations of differentiation among these cells is the division of the embryo into the three primary germ layers. The most superficial layer, the ectoderm, gives rise to the skin and the nervous system. The deep layer, the endoderm, gives rise to the epithelial lining of the gut and lungs as well as the pancreas and the liver. Between the ectoderm and the endoderm lies the mesoderm. A large number of tissues, including blood, connective tissue, kidney, endothelium, muscle and skeleton arise from the mesoderm. The heart is formed from paired primordia found in a discrete region of mesoderm anterior and lateral to the somites called the anterior lateral plate mesoderm (ALPM). The myocardial primordia migrate to, and fuse at, the embryonic midline where they will form a functional heart. This migration is the first, critical step in a complex series of morphogenetic movements required for heart function and viability.

Myocardial development

The heart has long been of special interest to developmental biologists, as the first functioning organ and the source of many clinically-relevant human birth defects. The incidence of heart defects among live births is about one percent (Hoffman 1995), making congenital cardiac defects a source of grave human privation. Study of the zebrafish has allowed vertebrate heart development to be dissected genetically, and may provide insight into the molecular defects underlying human congenital defects.

Understanding of the molecular control of heart development has progressed rapidly over the last few years. One essential insight has been the appreciation of the role of Nk homeobox transcription factors in heart development. Genetic analysis of the

development of the dorsal vessel, the *Drosophila* heart equivalent, first suggested a role for Nk homeobox transcription factors in heart development. *tinman*, an Nk-domain and homeobox-containing (or *nkx*) gene, is expressed in the dorsal mesoderm and dorsal vessel during *Drosophila* development (Bodmer et al. 1990). Loss-of-function *tinman* mutants completely lack dorsal vessels (Bodmer 1993). *nkx* genes are also expressed in the developing hearts of vertebrates including mouse, chick, *Xenopus laevis* and zebrafish (reviewed in Evans 1999), implying that some functions of Nk transcription factors have been conserved over large phylogenetic distances. Although *Drosophila* appear to express only one *nkx* gene in the dorsal vessel, several are expressed in vertebrates, one of which, *nkx2.5*, is the earliest known marker of the vertebrate myocardial precursors (Komuro and Izumo 1993). Murine *nkx2.5* mutants exhibit severely disrupted heart development, but do express many myocardial genes and do form a beating heart tube (Lyons et al. 1995). Perhaps embryos carrying mutations in more than one *nkx* gene will show earlier and more profound deficits in myocardial development, deficits more akin to those of *Drosophila tinman* mutants.

Gata factors comprise another family of transcription factors implicated in myocardial development. Gata factors are zinc finger transcriptional activators that bind to the consensus sequence WGATAR (reviewed in Evans 1997). Gata1, 2 and 3 are required for hematopoietic development (reviewed in Orkin and Zon 1997). Gata4, 5 and 6 are expressed in the ALPM, the endoderm and extraembryonic tissues (Laverriere et al. 1994; Jiang and Evans 1996; Morrisey et al. 1997; Reiter et al. 1999; Rodaway et al. 1999). Gata factors physically interact with each other (Charron et al. 1999) and with other transcription factors such as NF-AT3 (Molkentin et al. 1998) to synergistically activate transcription. Regulatory elements containing Gata motifs are found in a wide variety of myocardial and endodermal genes, including *B-type natriuretic peptide promoter* (*BNP*) (Grepin et al. 1994), *atrial natriuretic factor* (*ANF*) (Durocher et al. 1996), *cardiac troponin C* (Ip et al. 1994), *cardiac troponin I* (Murphy et al. 1997; Di Lisi et al. 1998),

m2 muscarinic acetylcholine receptor (Rosoff and Nathanson 1998), *slow myosin heavy chain 3* (Wang et al. 1998), *IFABP* (Gao et al. 1998), *gastric H+/K+-ATPase* (Maeda et al. 1996) and *HNF4* (Morrisey et al. 1998). As with *nkx* genes, the requirement for Gata factors in heart development has been conserved through evolution; *Drosophila pannier* encodes a Gata factor required for formation of the dorsal vessel (Garcia-Garcia et al. 1999).

Gata factors and Nkx2.5 regulate each other's expression and activity in complicated ways. Recently, two separate *cis*-regulatory elements upstream of mouse *nkx2.5* have been shown to depend upon Gata-binding motifs, suggesting that Gata factors directly control *nkx2.5* expression (Searcy et al. 1998; Lien et al. 1999). Conversely, expression of *gata6* may depend upon an Nkx2.5-responsive regulatory element (Schwartz and Olson 1999). Post-translationally, Gata4 and Gata5 can cooperate with Nkx2.5 to synergistically activate target gene expression (Durocher et al. 1997; Lee et al. 1998; Sepulveda et al. 1998). Structure/function studies have suggested that Gata factors potentiate transactivation by Nkx2.5 by binding to and inactivating the autorepressive C-terminus of Nkx2.5. Together, these results give measure of the complex manner in which Nkx2.5 and Gata factors regulate each other both transcriptionally and post-translationally.

Heart development is also regulated by surrounding tissues. The endoderm is in close physical association with the ALPM from gastrulation stages in mammals, birds, amphibia and fish (reviewed in Nascone and Mercola 1996). The endoderm apposed to the ALPM can promote myocardial differentiation in mesodermal explants, even in explants from normally non-cardiogenic regions (Nascone and Mercola 1995; Schultheiss et al. 1995). The endoderm underlying the ALPM produces the bone morphogenetic proteins Bmp2 and Bmp4, both of which are members of the TGFβ family of signaling proteins (Schultheiss et al. 1997). Bmp2 and Bmp4 are able to mimic the inductive effects of the endoderm and induce the expression of a wide range of cardiac genes

including Nkx2.5 (Schultheiss et al. 1997). Therefore, the endoderm appears to be the source of important signals that regulate the differentiation of the myocardial primordia.

In several zebrafish heart mutants, the myocardial primordia fail to migrate to the embryonic midline, producing a developmental defect known as cardia bifida. The cardia bifida mutations include *faust* (*fau*), *casanova* (*cas*), *one-eyed pinhead* (*oep*), *bonnie and clyde (bon)*, *hands off*, *miles apart* and *natter* (Chen et al. 1996; Stainier et al. 1996). The *hands off* mutation profoundly reduces the amount of myocardial tissue formed, but does not appear to affect the development of the endoderm (D. Yelon, D.Y.R. Stainier, unpublished results). However, either endoderm formation, differentiation or morphogenesis are affected in each of the other cardia bifida mutants. It remains to be determined whether this correlation between myocardial and endodermal defects reflects a regulatory relationships between these two tissues or shared genetic requirements.

cas mutants fail to form a gut or any of the gut-associated organs (Alexander et al. 1999). Moreover, the absence of endodermal gene expression during gastrulation stages in *cas* mutants indicates that the endoderm is defective from a very early stage (Alexander et al. 1999). Despite the absence of gut endoderm, *cas* mutants form a normal number of myocardial precursors which express many myocardial genes such as *nkx2.5*, *gata4*, *gata5* and *gata6* at normal levels (Alexander et al. 1999). However, as noted above, the myocardial primordia do not migrate to the embryonic midline. These results suggest that in zebrafish, the gut endoderm is not required for myocardial differentiation or growth, but does direct myocardial morphogenesis. However, pharyngeal endoderm does form in *cas* mutants (Alexander et al. 1999), and so it remains possible that the zebrafish pharyngeal endoderm induces or sustains myocardial gene expression. Indeed, the pharyngeal endoderm is closely associated with the myocardial primordia from the end of gastrulation in both zebrafish and mouse development (Kaufman 1992; Lee et al. 1996).

oep mutants, like *cas* mutants, exhibit both cardia bifida and an absence of gut endoderm (Schier et al. 1997). *oep* encodes an EGF-CFC protein related to mouse *Cripto*

and *Cryptic* that is essential for Nodal signaling (Zhang et al. 1998b; Gritsman et al. 1999). Blastomeres expressing a constitutively-active type I TGFβ receptor, TARAM-A*, can become endoderm, even in *oep* mutants (Peyrieras et al. 1998). Restoration of endoderm through *TARAM-A** overexpression non-autonomously restores fusion of the myocardial primordia in *oep* mutants (Peyrieras et al. 1998), further indicating that endoderm is essential for fusion of the myocardial primordia in zebrafish.

Endoderm development

As endoderm development is intimately associated with heart development, it is unsurprising that many of the zebrafish mutations initially recognized as affecting heart development also affect endodermal development. Although less is known about endoderm development than heart development, our understanding of the molecular control of endoderm formation has recently benefited from the identification of several essential genes. Much of this progress has come from studies of one organism, the amphibian *Xenopus laevis*.

Xenopus endoderm develops from the large yolky cells of the vegetal hemisphere (Keller 1976; Dale and Slack 1987). *Xenopus* vegetal explants express endodermal genes such as *endodermin* and *intestinal fatty acid binding protein* (*IFABP*), suggesting that endoderm is autonomously specified (Gamer and Wright 1995; Henry et al. 1996). One possible mechanism for autonomous specification of endoderm is the vegetal deposition of an endodermal determinant. The restricted vegetal expression of VegT, a maternally-supplied T-box transcription factor, raises the possibility that VegT could be such an endodermal determinant (reviewed in Stennard 1998). In animal cap assays, VegT is able to induce the expression of both mesodermal and endodermal genes (Horb and Thomsen 1997). Conversely, depletion of VegT message using an antisense oligonucleotide abolishes endoderm formation and endodermal gene expression (Zhang et al. 1998a).

The only identified transcriptional target of VegT is *Bix1*, a homeobox-containing gene expressed in the vegetal hemisphere and able to induce endodermal genes when misexpressed (Tada et al. 1998). Other potential targets of VegT include genes encoding secreted proteins such as Xwnt8 and the TGFβ signaling molecules Activin B, Derrière and the Nodal-related factors Xnr1, 2 and 4 (but not Xnr3), and endodermal regulators such as Sox17α and β, Mix.1, Milk and Bix1-3 (Clements et al. 1999; Yasuo and Lemaire 1999). Sox17 (α and β) are HMG-domain transcription factors expressed in the vegetal pole from blastula stages. Overexpression of Sox17 in *Xenopus* animal caps induces the expression of late endodermal genes and overexpression of a dominant interfering engrailed-repressor Sox17 fusion (Sox17-EnR) prevents endoderm development both in vegetal pole explants and the intact embryo (Hudson et al. 1997). Mix.1 and Milk are paired-type homeobox genes also expressed in the endodermal progenitors from blastula stages (Ecochard et al. 1998; Lemaire et al. 1998). Overexpression studies have indicated a role for Milk in endoderm differentiation, as well as for the related transcription factor Mixer (Henry and Melton 1998).

Early *Xenopus* endoderm development appears to proceed in two phases (Clements et al. 1999; Yasuo and Lemaire 1999). During the initial phase, maternal determinants such as VegT are able to induce expression of some endodermal genes (e.g., *Sox17α* and *Mix.1*) and some TGFβ family member gene (e.g., *derrière*, *Xnr1*, *Xnr2* and *Xnr4*) at the mid-blastula transition (Clements et al. 1999; Yasuo and Lemaire 1999). (There is some disagreement regarding whether Xnr1 and Xnr4 are both autonomously induced by vegetal determinants.) This first phase of expression occurs in the absence of protein synthesis indicating that these genes are direct targets of maternal factors. Cell disaggregation studies revealed a second phase during which cell signaling through TGFβ receptors increases the expression of *Sox17α* and *Mix.1* and induces the expression of other endodermal regulators such as *Mixer* and *Gata4* (Clements et al. 1999; Yasuo and Lemaire 1999). The dependence of *Mixer* expression on TGFβ signaling indicates that,

although autonomous maternal determinants are required for *Xenopus* endoderm development, cell signaling is also required.

TGFβ signaling and, more specifically, Nodal signaling are also required for zebrafish endoderm development. Embryos harboring mutations in *cyc* and *sqt*, two genes encoding Nodal factors, entirely lack endoderm and form little mesoderm (Feldman et al. 1998). Also, as mentioned above the extracellular EGF-CFC protein Oep is required for Nodal signaling and endoderm formation (Schier et al. 1997). Overexpression studies corroborate the genetic evidence that Nodal signaling is essential for endoderm formation; overexpression of low levels of *antivin/lefty2*, a secreted Nodal inhibitor, prevents endoderm formation (Meno et al. 1999; Thisse and Thisse 1999), and expression of a dominant interfering TGFβ receptor eliminates expression of *gata5* in endodermal progenitors (Rodaway et al. 1999). Taken together, these results argues that Nodal signaling performs a conserved role in the induction of vertebrate endodermal regulators.

Zebrafish *bon* mutants, like *oep* mutants, also fail to form endoderm (Kikuchi et al. 1999). *bon* encodes a zebrafish homolog of chick CMIX and *Xenopus* Mixer expressed during late blastula and early gastrula stages in the germ ring (Kikuchi et al. 1999). Like *Xenopus* Mixer, expression of *bon* is induced by Nodal signaling; *bon* expression is abolished in *cyc;sqt* double mutants, and almost absent in embryos lacking both maternal and zygotic *oep* (Alexander and Stainier 1999). Together with data on *Xenopus* endoderm development, these results indicate that *mix* genes have conserved roles in promoting vertebrate endoderm formation and function downstream of Nodal signaling. Expression of zebrafish *sox17* initiates approximately 80 minutes after *bon* begins to be expressed and is severely reduced in *bon* mutants, indicating that Bon functions upstream of *sox17* (Alexander and Stainier 1999). In contrast, during *Xenopus* development *Sox17* is expressed before *Mixer* (Henry and Melton 1998). However, *Xenopus Sox17* expression is not maintained in embryos overexpressing a dominant interfering engrailed repressor Mixer fusion, and co-expression of Mixer and Sox17-EnR in animal caps prevents Mixer

from inducing endodermal gene expression (Henry and Melton 1998), suggesting that *Xenopus* Mixer also functions through Sox17. As noted above, *Mixer* is just one of several *mix* genes that promote endoderm development in *Xenopus*. Whether multiple *mix* genes also function in endoderm differentiation in other vertebrate species remains unclear.

Although overexpression of *bon* can expand endodermal gene expression in *oep* mutants, *bon* overexpression in *cas* mutants fails to induce *sox17* expression, genetically placing *cas* between *bon* and *sox17* (Alexander and Stainier 1999). Together with our understanding of Nodal signaling in endoderm formation, these results suggest a pathway underlying vertebrate endoderm development (Alexander and Stainier 1999). Nodal signals, composed of Cyclops and Squint, require Oep function to induce the expression of *bon*. Bon, in turn, promotes the expression of endodermal regulators such as *sox17* in a manner dependent on *cas*.

As mentioned above, Gata factors are also expressed within the vertebrate endoderm and can activate transcription of endodermal genes in non-endodermal cells. During invertebrate development, Gata transcription factors are critical for the specification and differentiation of endoderm. Several Gata factors participate in *Caenorhabditis elegans* endoderm development; *elt-2* is required for gut differentiation (Fukushige et al. 1998), and *end-1* is implicated in the specification of E, the blastomere that gives rise to the entire endoderm (Zhu et al. 1997; Zhu et al. 1998). The *Drosophila gata* gene *serpent* is essential for development of the gut and the fat body, a mesodermal organ functionally analagous to the vertebrate liver.

During mouse development, *gata4* and *6* are expressed in the extraembryonic endoderm as early as gastrulation stages and *gata5* is expressed in the gut epithelium at embryonic day 18.5 (Morrisey et al. 1997). Although the early expression patterns have not been well described for other vertebrates, recent work by Weber et al. demonstrates that *Xenopus gata5* is expressed specifically in the endoderm from gastrula stages onward.

Moreover, *Xenopus* Gata5 can activate expression of the endodermal genes *Sox17α* and *HNF1β* ectopically in animal caps (Weber et al. 1999). As Sox17α is an early and essential regulator of endoderm development, *Xenopus* Gata5 is also implicated in early endodermal differentiation.

Wnt signaling and endoderm development

Induction of the endodermal progenitor, called E, is one of the earliest signaling events in *C. elegans* embryogenesis. After the second cycle of cell division, the EMS blastomere produces two daughters: MS principally gives rise to mesodermal tissues such as body wall muscle, and E produces the endoderm. If the EMS blastomere is removed from the embryo, both of its daughters behave like MS and fail to produce endoderm, indicating that a signal is required to induce EMS to form an endodermal progenitor (Goldstein 1993). Cellular recombination experiments revealed that the endoderm inducing signal originates from the neighboring P_2 blastomere (Goldstein 1993).

Genetic dissection has revealed that the signal from P_2 is a Wnt proteins. Members of the Wnt family are secreted, glycosylated signaling proteins associated with the extracellular matrix and cell surface (reviewed in Nusse 1999). Intense study over the last decade has identified many components of the Wnt signal transduction pathway. At its most skeletal, Wnt signaling entails reception of Wnt signals by transmembrane receptors of the Frizzled family. Frizzled receptors stabilize cytoplasmic and nuclear pools of β-catenin, which are normally quickly degraded by Glycogen Synthase Kinase 3 (GSK3) and Adenomatous Polyposis Coli (APC) (reviewed in Cox and Peifer 1998). In both *Drosophila* and vertebrates, β-catenin complexes with members of the TCF/LEF family of HMG transcription factors to activate Wnt responsive genes.

By screening for mutants that fail to produce endoderm, five *C. elegans* components of the Wnt signaling pathway were discovered and named *mom* for *mo*re *m*esoderm (Rocheleau et al. 1997; Thorpe et al. 1997). *mom-2* encodes a Wnt signaling

protein that functions in the signaling P_2 cell. *mom-5* encodes a Frizzled-like receptor and therefore is presumably expressed by the EMS cell and transduces the MOM-2 signal. RNA interference of *wrm-1* which encodes the *C. elegans* homolog of β-catenin produces a *mom*-like phenotype, indicating that β-catenin is an essential transducer of Wnt signals in both vertebrates and *C. elegans* (Rocheleau et al. 1997).

The TCF/LEF family of HMG domain transcription factors were initially discovered in vertebrates and subsequently shown to act as downstream effectors of Wnt signaling. The *C. elegans* gene *pop-1* encodes a TCF/LEF family member expressed in the EMS blastomere (Lin et al. 1995). Unexpectedly, loss-of-function mutations in *pop-1* create a phenotype opposite that of the *mom* mutants; two E-like cells are formed at the expense of the MS daughter (Lin et al. 1995). Thus, unlike TCF/LEF homologs in other systems, POP-1 opposes the function of Wnt signals and β-catenin. Cellular localization studies indicate that MS nuclei harbor higher levels of POP-1 than E nuclei (Lin et al. 1995). Therefore, Wnt signaling may induce endodermal fate in *C. elegans* by lowering nuclear levels of an inhibitory TCF/LEF transcription factor.

Wnt signaling is also implicated in the development of the sea urchin endoderm. β-catenin accumulates in the nuclei of the progenitors of the sea urchin endoderm and mesoderm (Logan et al. 1999). Modulating nuclear β-catenin levels through LiCl treatment or *cadherin* overexpression increases or decreases formation of the sea urchin endoderm and mesoderm, respectively (Logan et al. 1999). Thus, both endoderm and mesoderm development depend upon high levels of nuclear β-catenin. During *Xenopus* development, Xwnt8 is maternally supplied to the cells that give rise to the endoderm (Lemaire and Gurdon 1994), suggesting that Wnt signaling may also be critical for endoderm development in vertebrates.

The connection between Wnt signaling and vertebrate endoderm development is reinforced by the observation that Sox17β, a *Xenopus* homolog of Sox17, can bind β-catenin and repress activation of a β-catenin-inducible reporter of TCF/LEF

transcriptional activation (Zorn et al. 1999). The functional significance of this interaction is still unclear, but it raises the possibility that Wnt signaling may potentiate the ability of Sox17 to promote endodermal differentiation. Additionally, promoting endodermal differentiation may be related to inhibition of TCF/LEF activity, potentially mirroring the relationship between TCF/LEF function and *C. elegans* endoderm formation.

Notch signaling antagonizes sea urchin endoderm specification

The Notch pathway has been demonstrated to act in the segregation of cell types widely in both vertebrate and invertebrate embryos. Most famously, lateral signaling involving Notch and its ligand Delta executes the choice between neural and epidermal fates in *Drosophila* sensory bristle formation (reviewed in Simpson 1997).

As with Wnt signaling, research on the Notch signal transduction pathway has recently identified a profusion of previously unrecognized actors. However, at its most basic, the Notch signaling pathway involves a relatively small number of evolutionarily conserved participants. The Notch receptor is activated by a Delta or Serrate ligand, both of which are transmembrane proteins (reviewed in Artavanis-Tsakonas et al. 1999). Binding of ligand results in the cleavage and release of the Notch intracellular domain which translocates to the nucleus and associates with the transcription factor Suppressor of Hairless [Su(H)]. Despite accumulating evidence that Notch can perform some functions independently of Su(H), Su(H) appears to be the primary effector of Notch signaling. Through Su(H), Notch activates the expression of *hairy* and *Enhancer of split* (*her*) homologs. *her* genes encode bHLH transcription factors which cooperate with Groucho to repress the expression of *Achaete-Scute* proneural genes.

A sea urchin homolog of Notch, LvNotch, is dynamically expressed in the precursors of the endoderm and the secondary mesenchymal cells (SMCs) (Sherwood and McClay 1997). The SMCs give rise to most of the mesodermal cell types of sea urchin

larvae. Overexpression of active LvNotch expands SMC specification at the expense of endoderm formation (Sherwood and McClay 1999). Conversely, reducing LvNotch activity decreases or eliminates the SMCs (Sherwood and McClay 1999). Furthermore, mosaic studies indicate that LvNotch acts cell autonomously within SMCs (Sherwood and McClay 1999). Cumulatively, these results suggest that LvNotch signaling directly regulates the differential specification of SMC and endodermal fate.

Overview

The work described in this dissertation addresses the early development of the zebrafish myocardium and endoderm, with a focus on the role of Gata5 in these processes. In chapter 2, I show that the zebrafish *fau* locus encodes Gata5, and that *fau/gata5* mutants display dramatic defects in the differentiation, growth, patterning and morphogenesis of the myocardium. I also demonstrate that overexpression of *gata5* expands the expression of many myocardial genes. *gata5* overexpression can also lead to formation of ectopic myocardial tissue in vivo, and is the first gene demonstrated to have this capacity.

In chapter 3, I describe in detail the role of Gata5 in endodermal development. Gata5 acts during the blastula and gastrula stages to promote the formation of a normal number of endodermal progenitors and the expression of downstream endodermal genes. Without Gata5 activity, zebrafish develop dysmorphic guts and exhibit severe defects in the differentiation of endodermally derived organs such as liver, pancreas and thyroid. Furthermore, using complementary overexpression studies and mutant analyses, I place Gata5 in a pathway underlying formation of vertebrate endoderm.

swirl/bmp2b and *oep* mutants also exhibit severe defects in myocardial development. I present evidence in chapter 4 that these defects are due to the absence of mesodermal *gata5* expression. Forced expression of *gata5* is sufficient to restore at least

some aspects of myocardial differentiation to these mutants, suggesting that Gata5 is both necessary and sufficient for at least some aspects of myocardial differentiation.

In chapter 5, I examine the possible roles of Wnt and Notch signaling in endoderm specification. I demonstrate that overexpression of a Wnt signal expands endoderm formation while expression of an constitutively active Notch represses endoderm specification. While Wnt and Notch signaling have previously been implicated in invertebrate endoderm development, this study provides the first evidence that they may function in a similar capacity in vertebrates.

In addition to its roles in myocardial and endodermal development, Gata5 functions in the development of the pharyngeal endoderm. Both loss- and gain-of-function experiments demonstrating the role of Gata5 in pharyngeal endoderm development are detailed in appendix 1.

In appendix 2, I describe work reveal that Bon and Gata5 have overlapping function in the differentiation of the lateral plate and prechordal plate mesoderm.

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Chapter 2: Gata5 is required for the development of the heart and endoderm in zebrafish

SUMMARY

The mechanisms regulating vertebrate heart and endoderm development have recently become the focus of intense study. Here we present evidence from both loss- and gain-of-function experiments that the zinc finger transcription factor Gata5 is an essential regulator of multiple aspects of heart and endoderm development. We demonstrate that zebrafish Gata5 is encoded by the *faust* locus. Analysis of *faust* mutants indicates that early in embryogenesis Gata5 is required for the production of normal numbers of developing myocardial precursors and the expression of normal levels of several myocardial genes including *nkx2.5*. Later, Gata5 is necessary for the elaboration of ventricular tissue. We further demonstrate that Gata5 is required for the migration of the cardiac primordia to the embryonic midline and for endodermal morphogenesis. Significantly, overexpression of *gata5* induces the ectopic expression of several myocardial genes including *nkx2.5* and can produce ectopic foci of beating myocardial tissue. Together, these results implicate zebrafish Gata5 in controlling the growth, morphogenesis and differentiation of the heart and endoderm, and indicate that Gata5 regulates the expression of the early myocardial gene *nkx2.5*.

INTRODUCTION

Fusion of the cardiac primordia is the first in a complex series of morphogenetic steps required to form a functional heart. The cardiac primordia are contained within the anterior lateral plate mesoderm (ALPM). During segmentation stages, the bilateral populations of ALPM migrate medially and fuse to form a cell layer that crosses the embryonic midline. As part of this process, the two cardiac primordia meet and fuse at the embryonic midline to form the definitive heart tube. In the absence of migration, the two cardiac primordia develop in their original lateral positions, resulting in a phenotype called cardia bifida. Six zebrafish mutations causing cardia bifida were identified in the large-scale screens for genes regulating embryonic development; *faust* (*fau*), *casanova* (*cas*), *one-eyed pinhead* (*oep*), *bonnie and clyde*, *miles apart*, and *natter* (Chen et al. 1996; Stainier et al. 1996).

Two of these mutations, *oep* and *cas*, also disrupt endoderm formation (Schier et al. 1997; Alexander et al. 1999), underscoring the developmental relationship between the heart and endoderm. Classical embryological experiments initially identified the ability of endoderm to induce rhythmic contraction in cardiac explants (Jacobson and Sater 1988). More recent experiments have confirmed that the endoderm is required for avian cardiac myofibrillogenesis (Gannon and Bader 1995). The endoderm is also implicated in the earlier process of precardiac mesoderm induction; co-culture of *Xenopus* deep endoderm with the heart primordia enhances cardiogenesis (Nascone and Mercola 1995), and BMP2, which is secreted by the anterior endoderm, is sufficient to induce expression of myocardial genes in avian noncardiogenic mesoderm (Schultheiss et al. 1997).

Heart and endoderm development in the mouse both require the zinc finger transcription factor Gata4 (Kuo et al. 1997; Molkentin et al. 1997). While 35 percent of *gata4* null embryos arrest at the egg cylinder stage for unknown reasons, those that do gastrulate fail to form a fused heart or a foregut and develop partially outside the yolk sac

and amnion. Gene expression within the precardiac mesoderm is not significantly affected, suggesting that cardiac differentiation is not the primary defect. In support of this conclusion, chimera analyses indicate that Gata4 function in the visceral and/or anterior definitive endoderm restores ventral closure and cardiac fusion to otherwise *gata4* null embryos (Narita et al. 1997).

Gata4 is one of a family of six vertebrate Gata transcription factors, each of which contains two zinc fingers and binds to regulatory elements containing A/TGATAA/G or closely related sequences. The *gata* genes can be divided into two groups on the basis of sequence homology and expression pattern. The members of the first group, *gata1*, *2* and *3,* play unique roles in hematopoiesis (reviewed in Orkin and Zon 1997), while *gata4*, *5* and *6* are expressed in the ALPM and endoderm (reviewed in Evans 1997). Studies of *cis*-regulatory elements have suggested that *gata4*, *5* and *6* play important roles in promoting the expression of both myocardial and endodermal genes. Transfection of heterologous cells with Gata factors activates transcription of myocardial genes including *α–myosin heavy chain* (*α-MHC*), *cardiac troponin C,* and *B-type natriuretic peptide* (*BNP*; reviewed in Evans 1997), as well as endodermal genes such as *IFABP* and *HNF4* (Gao et al. 1998; Morrisey et al. 1998). Evidence that Gata factors directly activate the transcription of myocardial and endodermal genes has come from studies identifying functionally important Gata binding sites in the regulatory regions of the myocardial genes *α–MHC* and *BNP* (reviewed in Evans 1997) and the liver enhancer of *serum albumin* (Bossard and Zaret 1998). Recently, Gata binding sites have been identified in two different regulatory elements of *nkx2.5*, one of the earliest known markers of the myocardial precursors (Searcy et al. 1998; Lien et al. 1999). Each of these elements activates expression in a manner similar to the endogenous *nkx2.5* promoter and depends upon Gata sites for activity.

Experiments in chick and *Xenopus* provide further in vivo evidence for the role of *gata* factors in heart development. Inhibition of Gata activity through the addition of

three antisense oligonucleotides complementary to *gata4*, *5* and *6* produces variable abnormalities in avian cardiac morphogenesis (Jiang et al. 1998). Ectopic expression of *gata4* and *5* in *Xenopus* embryos leads to the premature expression of the myocardial genes *α-cardiac actin* and *α-MHC* (Jiang and Evans 1996). In contrast, sustaining the expression of *Xenopus gata6* after it has normally declined inhibits expression of markers of terminal myocardial differentiation, perhaps by maintaining the precardiac mesoderm in an undifferentiated state (Gove et al. 1997).

gata genes also play evolutionarily conserved roles in endoderm development, as the *Drosophila gata* gene *serpent* and the *Caenorhabditis elegans gata* gene *elt-2* are essential for differentiation of the embryonic gut (Rehorn et al. 1996; Fukushige et al. 1998). Another *C. elegans gata* gene, *end-1*, is implicated in the earlier process of endoderm specification (Zhu et al. 1997; Zhu et al. 1998).

Here, we show that the zebrafish *fau* locus is essential for multiple aspects of heart and endoderm development, and that *fau* encodes Gata5. Analysis of *fau* mutants reveals that Gata5 is required for the normal expression of *nkx2.5* and for other aspects of myocardial differentiation. Mutations in *gata5* also prevent the formation of normal amounts of cardiac and endodermal tissue and inhibit the normal morphogenesis of the heart, pharyngeal endoderm and gut. Furthermore, we demonstrate that the overexpression of *gata5* is sufficient to activate ectopic expression of *nkx2.5* and other cardiac genes, and to cause the formation of ectopic regions of rhythmically contracting tissue. Thus, our results establish Gata5 as a critical regulator of *nkx2.5* expression and of multiple aspects of cardiac and endoderm development.

MATERIALS AND METHODS

Zebrafish strains and maintenance

Zebrafish were maintained and staged as described (Westerfield 1995).

Wholemount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al. 1998); in certain cases embryos older than 28 hpf were incubated in 0.003% phenylthiourea to inhibit pigmentation. The *gata4* (GenBank accession # AF191577) and *gata6* (GenBank accession # AF191578; previously called *gata5* in (Pack et al. 1996)) genes were isolated in Leonard Zon's laboratory (Harvard Medical School; unpublished). Full-length cDNA was used as the template for the *gata6* riboprobe, but the *gata4* riboprobe was generated from a truncated cDNA comprised of the first 954 bp. The *gata5* riboprobe is described below. The *cmlc1* gene (GenBank accession # AF119163) was isolated as described (Yelon et al. 1999). As *fau* mutants younger than 24 hpf could not be phenotypically distinguished from wild-type siblings, earlier stage embryos were genotyped post-in situ hybridization to confirm that any defects in gene expression segregated with the *fau* mutation. Briefly, this entailed serial rehydration with PBST, proteinase K digestion at 55° C overnight, and PCR genotyping with a tightly linked marker.

Immunohistochemistry using the antibodies MF20 and S46 (generous gift of Dr. Frank Stockdale, Stanford University) was performed as described (Alexander et al. 1998). MF20 was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biological Studies, University of Iowa under contract NO1-HD-2-3144 from the NICHD.

β-galactosidase staining was performed as described (Rodaway et al. 1999).

Genetic mapping

The mapping strain was constructed by crossing a *fau* Tü male heterozygote to a WIK female. Genomic DNA extraction from zebrafish embryos was performed as described (Westerfield 1995), except that proteinase K digestion was done for 2-4 hours. By using only those embryos recombinant at SSRPs flanking *fau* in a search for amplified fragment length polymorphisms (AFLPs), we successfully restricted our identified markers to those that mapped within a useful genetic distance of *fau*. Otherwise, AFLP selection was performed as described (Vos et al. 1995). An AFLP marker linked to the *fau* mutation (primers: 5'-AAGAGAGCGAGCACTGCTACTAC-3' and 5'-CAGCTTTCCCATATTCTGGC-3') and an amplicon from the *gata5* 3' UTR (primers: 5'-TGATCCGAGACCTCCAC-3' and 5'-CTCCCGATCATTGGAAC-3') were placed on the Goodfellow zebrafish radiation hybrid panel (Research Genetics). These markers are separated by 14 cR (lod score 18.96). Human *gata5* was mapped using primers designed from the sequence of EST W00677 (primers: 5'-AGACCACCCACTGAGTCACC-3' and 5'-GGAGTCCCTTGCTGTACGTG-3') on the Stanford G3 radiation hybrid panel (Research Genetics). Human *gata5* is linked to marker SHGC-57473 with a lod score of 18.28.

PAC library screen and linkage analysis

A PAC library was screened by PCR as pools of clones using primers that amplify the *gata5* 3' UTR. Internal PAC sequencing was performed using the primer 5'-ACACTGTGGAGGAGAAACGCA-3'. A single strand conformational polymorphism identified at one end of a PAC clone containing *gata5* was linked to the fau^{tm236a} mutation in all of 272 gynogenetic haploid embryos examined. Additionally, a BsmFI RFLP at position 380 in the *gata5* gene was linked to fau^{tm236a} in all of 160 diploid embryos examined. The 400 bp region containing the RFLP was amplified by PCR (primers: 5'-GTACCAGCCATGCTCCAGAC-3' and 5'-CTCCTTCCCGGTAGAGTTCC-3'). The fau^{s26} mutation was linked to SSRP z4003 in

all of 40 gynogenetic haploid embryos examined. Amplification of SSRPs flanking *gata5* from *fau*^{s26} genomic DNA failed to reveal evidence of a deletion.

Isolation of *gata5* cDNA

Total RNA was isolated using Trizol (Gibco BRL). Reverse transcription was performed using Superscript II (Gibco BRL). The *gata5* ORF was amplified by PCR (primers: 5'-GGACGTTGACAAGGTTTTTACTAG-3' and 5'-GTCTGTCTACATTTGTGTAAAAGTCC-3'). The 1330 bp product was cloned into the pGEM-T vector (Promega) and sequenced using an ABI 377 DNA sequencer. We sequenced 20 independent clones of mutant-derived and 8 independent clones of wild-type-derived *gata5* cDNA (one of which was used as the template for the *gata5* riboprobe). Among the mutant-derived clones, the 31 bp insertion was represented by 17 clones, the 30 bp deletion was represented by 2 clones, and the wild-type cDNA was detected once. PCR analysis using a primer bridging the wild-type splice site (primer: 5'-GCTTCATGGGGTACCAAGG-3') confirmed that wild-type *gata5* message is detectable in *fau*^{tm236a} mutants.

Although the amount of *gata5* mRNA is profoundly diminished in *fau*^{s26} mutants, low levels were detectable by RT-PCR performed as above. Sequencing of the subcloned RT-PCR products indicated that *fau*^{s26} is not a mutation in the *gata5* coding sequence or untranslated regions.

***gata5* gene structure**

PCR of genomic amplicons and subsequent sequencing revealed that the first intron (723 bp) is present 3' of cDNA nucleotide 602, the second intron (1207 bp) is present 3' of cDNA nucleotide 771, the third intron (1.6 kb) is present 3' of cDNA nucleotide 901 and the fourth intron (343 bp) is present 3' of cDNA nucleotide 986. Additional internal PAC sequencing upstream of the transcriptional start site using the primer 5'-

GGCTAGTTAAAAACCTTGTCAACG-3' revealed a putative promoter sequence containing a consensus TATA box and several potential Gata binding elements. Sequencing of this region in *fau*^{s26} mutants failed to identify a mutation.

gata5 mRNA injection

Full length, capped mRNA was generated from pCS2:gata5, pCS2:tm236-1:MT, and pGST:GFP using the SP6 mMESSAGE mMACHINE system (Ambion). pCS2:tm236-1:MT was created by subcloning the coding sequence of the *gata5* cDNA species most frequently isolated from *fau*^{tm236a} mutants into pCS2:MT. For embryonic overexpression, 20 or 50 pg of mRNA was microinjected at the 1-4 cell stage. For autonomy studies, 100 pg *lacZ* mRNA was coinjected. To restrict overexpression to the YSL, mRNA was microinjected directly into the YSL at the 512 cell stage. Injected embryos were raised to 11-36 hpf for in situ hybridization or to 30 hpf for immunohistochemical analysis. After phenotypic analysis, embryos were genotyped as described above.

RESULTS

fau mutants display defects in heart development

The zebrafish *fau* locus is defined by two alleles; fau^{tm236a} is a previously reported, ENU-induced allele (Chen et al. 1996), while the second allele, fau^{s26}, arose spontaneously in an unrelated line. Both alleles segregate as fully penetrant, completely recessive mutations and cause equivalent phenotypes. The transheterozygous phenotype is also fully penetrant and is indistinguishable from the homozygous phenotypes.

Most conspicuously, *fau* mutations disrupt formation of the definitive heart tube, an early step in cardiac morphogenesis. Heart tube formation depends upon the medial migration and fusion of the bilateral cardiac primordia (Stainier et al. 1993). In *fau* mutants this step of cardiac morphogenesis usually fails to occur, resulting in cardia bifida. However, 0-45% of the *fau* mutant embryos in a given clutch produce fused, although morphologically abnormal, hearts. This variable expressivity is present in both alleles and also pertains to other aspects of the phenotype.

To examine when and how *fau* functions in heart development, we examined the expression of markers of the developing cardiac primordia. In *fau* mutants at 18.5 hours post-fertilization (hpf), myocardial precursors expressing *cardiac myosin light chain 2* (*cmlc2*; Yelon et al. 1999) and endocardial precursors expressing *tie2* (Lyons et al. 1998) fail to migrate to the embryonic midline (Fig. 1B,C), demonstrating that the morphogenesis of both tissues that comprise the zebrafish heart is affected. Development of more posterior lateral plate mesodermal derivatives, such as the fin bud and blood, are unaffected in *fau* mutants (data not included). Therefore, *fau* is essential for the proper morphogenesis of both of the cellular components of the zebrafish heart tube, but is not generally required for the development of lateral plate mesodermal derivatives.

Mutations in *fau* lead to the abnormal expression of genes encoding myocardial sarcomere components. *cardiac troponin T*, *tropomyosin*, *cmlc1* and *cmlc2* are all expressed in fewer cells in *fau* mutants than in wild-type siblings (Fig. 1B,D and data not included). Detailed examination of myocardial sarcomere gene expression in fau^{tm236a} mutants reveals a differential requirement for the *fau* gene product. For example, *cmlc1* expression is more profoundly reduced in fau^{tm236a} mutants than is the expression of *cmlc2* (Fig. 1B,D, middle panels). This distinction is less apparent in fau^{s26} mutants as too few cells express sarcomere genes in these mutants to allow easy comparison. The more severe defect in *cmlc2* and *cmlc1* expression observed in fau^{s26} mutants than in fau^{tm236a} mutants indicates that the fau^{s26} allele is the stronger of the two.

During earlier segmentation stages before the migration of the cardiac primordia to the midline, the ALPM exists as two parallel stripes of cells lateral to the head and anterior notochord. *gata6*, a gene expressed throughout the ALPM, is expressed normally in *fau* mutants (Fig. 1E). However, the expression of the related gene *gata4* in the ALPM is slightly reduced (Fig. 1F). Together, these results indicate that *fau* is not required for the formation of the ALPM, but is critical for the expression of cardiac genes within this tissue.

nkx2.5, an Nk homeobox gene required for heart development (reviewed in Evans 1999), is also expressed in part of the ALPM from early in development. Expression of *nkx2.5* is dramatically reduced in *fau* mutants (Fig. 1G), demonstrating that *fau* is a direct or indirect regulator of *nkx2.5* expression and is required at a very early stage of ALPM differentiation. fau^{tm236a} mutants display less severely diminished *nkx2.5* expression (Fig. 1G), further indicating that fau^{s26} is the stronger allele.

Later in development, the zebrafish heart differentiates into an anterior ventricle and a posterior atrium. Immunofluorescent staining using monoclonal antibodies that recognize myosin heavy chain epitopes differentially expressed in ventricular and atrial tissues (Stainier and Fishman 1992) reveals that ventricular and atrial differentiation does

not depend upon cardiac fusion. For example, a robust amount of both ventricular and atrial tissue forms in the cardia bifida mutant *cas* (Fig. 2A,B). However, *fau*^{tm236a} myocardia, in addition to being smaller than *cas* myocardia, contain proportionally much less ventricular tissue (Fig. 2B,C). This defect is also observable at earlier stages, before the cardiac primordia normally fuse. At the 18-somite stage the expression of *ventricular myosin heavy chain* (*vmhc*), a marker of ventricular myocardium (Yelon et al. 1999), is dramatically reduced in *fau*^{tm236a} mutants (Fig. 2D). *vmhc* expression is more severely reduced, or even absent, in *fau*^{s26} mutants (Fig. 2D).

fau mutants display defects in endoderm morphogenesis

As embryological experiments in chick and amphibia have suggested that the endoderm is a source of instructive signals for the precardiac mesoderm, we investigated the effects of the *fau* mutations on endoderm development. From 24 to 48 hpf, the zebrafish gut endoderm coalesces at the midline where it will form a tube, loop to the left and differentiate into the liver, pancreas, swim bladder and epithelium of the gastrointestinal tract (Warga and Nusslein-Volhard 1999). The anterior gut endoderm expresses *axial*, a homologue of *HNF3ß* (Strahle et al. 1996), while the posterior endoderm expresses *fkd2*, a homologue of *HNF3γ* (Odenthal and Nusslein-Volhard 1998). In *fau* mutants, *axial*-expressing cells fail to coalesce at the midline, disrupting anterior gut tube morphogenesis (Fig. 3A, arrow). The posterior endoderm displays more variable defects that range from the near absence of gut to the failure of gut looping (Fig. 3B).

fau mutations also disrupt the morphogenesis of the pharyngeal endoderm. In *fau* mutants, the pharyngeal pouches are separated by a larger mediolateral distance (Fig. 3C), a defect that is reminiscent of the inability of the gut endoderm and precardiac mesoderm to migrate to the embryonic midline. In addition, the anterior pharyngeal pouches are dysmorphic in *fau*^{tm236a} mutants (Fig. 3C, arrow), while the most anterior *nkx2.3*-expressing cells appear to be absent in *fau*^{s26} mutants (Fig. 3D, arrow).

***fau* encodes Gata5**

Initially, we mapped the *fau* locus 1.5 centimorgans proximal to the simple sequence repeat marker z20895 on linkage group 23 (Shimoda et al. 1998). Using an enriched selection for amplified fragment length polymorphisms, we identified a number of markers located within a centimorgan of *fau*. In a parallel approach, we mapped *gata5*, a gene dynamically expressed in both the endoderm and cardiac mesoderm (see below), to the same genomic region using the Goodfellow radiation hybrid panel (Kwok et al. 1998). Linkage analysis using a restriction fragment length polymorphism within the *gata5* gene demonstrated that the mutant *gata5* allele segregated with the fau^{tm236a} phenotype in over 300 meioses.

Sequencing of the fau^{tm236a} allele of *gata5* revealed a G to A substitution at the first base of the third intron (Fig. 4A). This mutation disrupts the highly conserved GT dinucleotide necessary for recognition of the splice site by the U1 snRNP (Krawczak et al. 1992). Sequencing of *gata5* cDNAs from *fau* mutants demonstrated that the fau^{tm236a} mutation leads to missplicing at flanking cryptic splice sites (Fig. 4B). The most frequently isolated cDNA results from the use of a downstream cryptic splice site and contains a 31 bp insertion predicted to create a translational frameshift (Fig. 4C,D). A less frequently isolated cDNA is produced by use of a cryptic splice site 30 bp upstream of the inactivated site (Fig. 4B). This mutant splice variant is predicted to produce a 10 amino acid deletion in the second zinc finger (Fig. 4C,D).

Structure/function studies have revealed that murine Gata5 contains two N-terminal transcriptional activation domains, as well as a basic domain required for nuclear localization (Morrisey et al. 1997b). Murine Gata4 also contains a C-terminal transcriptional activation domain required for synergistic activity with Nkx2.5 (Durocher et al. 1997; Lee et al. 1998; Sepulveda et al. 1998). As Gata5 shows a similar synergism with Nkx2.5 (Durocher et al. 1997), Gata5 may also contain a C-terminal activation

domain. Sequence comparison of murine Gata5 and zebrafish Gata5 suggests that the two proteins are similar in structure (Rodaway et al. 1999). The more prevalent 31 bp insertion caused by the *fau*^{tm236a} mutation disrupts the putative C-terminal activation domain and the basic nuclear localization domain of zebrafish Gata5 (Fig. 4C,D). The rarer splicing event deletes a 30 bp region that encodes part of the C-terminal zinc finger required for DNA binding and for interaction with the transcription factors NF-AT3 and Nkx2.5 (Durocher et al. 1997; Lee et al. 1998; Molkentin et al. 1998). Additionally, the C-terminal zinc finger has also been implicated in mediating heterotypic interactions between Gata factors (Charron et al. 1999). Therefore, we suspect that both mutant forms of Gata5 retain little, if any, activity. However, wild-type message can be detected in *fau*^{tm236a} mutants (see Materials and methods), indicating that the splicing machinery is still able to recognize the mutant splice site, albeit at a much reduced frequency. Taken together, these data indicate that *fau*^{tm236a} is a mutation in the *gata5* gene that substantially reduces, but does not eliminate, the production of wild-type Gata5.

Examination of the *fau*^{s26} *gata5* cDNA sequence did not identify a mutation. As *gata5* expression is dramatically diminished in *fau*^{s26} mutants (see below), we hypothesize that the *fau*^{s26} mutation disrupts the function of the *gata5* promoter. We sequenced 800 bp upstream of the transcriptional start site and have identified a putative core promoter without successfully identifying the *fau*^{s26} lesion (data not included). Therefore, we speculate that the *fau*^{s26} mutation disrupts a distal enhancer of the *gata5* gene that reduces *gata5* transcription to very low levels.

gata5 expression in wild-type and *fau* mutant embryos

In zebrafish, *gata5* is first expressed at the dome stage (late blastula) in the most marginal cells (Fig. 5A, arrowhead), a population that includes the endodermal progenitors and some mesodermal progenitors (Warga and Nusslein-Volhard 1999), and in the yolk syncytial layer (YSL), an extraembryonic tissue underlying the blastoderm which may be

equivalent to the mouse visceral endoderm (Fig. 5A, arrow). During gastrulation, *gata5*-expressing cells in the hypoblast adopt one of two different morphologies. Predominantly dorsally, *gata5*-expressing cells adopt the large, flat morphology characteristic of endoderm (Fig. 5C, arrowhead; Warga and Nusslein-Volhard 1999). Further evidence that these large *gata5*-expressing cells are endodermal progenitors comes from the very similar distribution of these cells and the *axial*- and *sox17*-expressing endodermal progenitors (Alexander and Stainier 1999). In a more ventral domain reflecting the regional propensity to become heart (Stainier et al. 1993), other *gata5* expressing cells adopt a rounder morphology characteristic of mesodermal progenitors (Fig. 5C, arrow). After completion of gastrulation, *gata5* expression persists in the endoderm and ALPM (Fig. 5D). ALPM expression of *gata5* is maintained throughout somitogenesis (10-24 hpf) while the level of endodermal expression diminishes (Fig. 5E). Later, from 20-22 hpf, cardiac expression of *gata5* becomes predominantly endocardial (Fig. 5F, arrow), as has been described for murine *gata5* (Morrisey et al. 1997a).

Expression of *gata5* in *fau*^{tm236a} mutants and wild-type siblings is indistinguishable until morphogenetic defects become apparent. In contrast, *fau*^{s26} mutants display markedly decreased *gata5* expression throughout development and in both the YSL and embryonic tissues (Fig. 5G,H). Very low levels of *gata5* expression can be detected in *fau*^{s26} mutants by in situ hybridization (Fig. 5G, arrow) and RT-PCR (data not included), indicating that while expression is strikingly diminished, the *fau*^{s26} mutation does not completely eliminate the production of *gata5* message.

Overexpression of wild-type *gata5* rescues heart fusion in *fau*^{tm236a} mutants

To confirm that the *fau* mutant phenotype is due to the absence of Gata5 activity, we injected mRNA encoding wild-type Gata5 into *fau*^{tm236a} mutants at the 1-4 cell stage. Although this approach leads to widespread misexpression, injection of 20 pg *gata5* mRNA restores cardiac fusion in *fau*^{tm236a} mutants (Fig. 6A; Table 1), confirming that

the *fau* phenotype is due to a loss-of-function mutation in *gata5*. *gata5*-injected *fau* mutants are not rescued to viability as *gata5* misexpression causes variable abnormalities in morphogenesis, including anterior truncations and compression of the anterior-posterior axis. Injection of *tm236a-1*, a *gata5* mRNA containing the same insertion and frameshift as most commonly caused by the fau^{tm236a} mutation, does not rescue cardiac fusion (Table 1). Moreover, *tm236a-1* overexpression does not disrupt morphogenesis, indicating that it has little if any biological activity.

To test whether the defects in organogenesis exhibited by *fau* mutants are due to reduced Gata5 activity in the extraembryonic YSL, we injected *gata5* mRNA directly into the YSL. Overexpression of *gata5* mRNA in the YSL does not restore cardiac fusion in fau^{tm236a} mutants (14/14 cardia bifida mutants observed in a clutch of 60 YSL-injected embryos), suggesting that *gata5* is required embryonically to promote heart fusion.

Ectopic expression of *gata5* leads to the ectopic expression of myocardial genes

Injection of wild-type embryos with 50 pg *gata5* mRNA expands the ALPM expression of *cmlc1*, *cmlc2*, *vmhc* and the MF20 and S46 myosin heavy chain proteins and leads to their ectopic expression (Fig. 6B,C, Table 2 and data not included). Occasionally, ectopic domains of myocardial gene expression both in the head and the tail were observed to contract rhythmically (Table 2). Control embryos injected with *gfp* mRNA did not manifest either expansion of myocardial gene expression or ectopic regions of beating tissue.

Because *nkx2.5* is downregulated in *fau* mutants, we investigated whether *gata5* overexpression was sufficient to induce the expression of zebrafish *nkx2.5* or other myocardial regulatory genes. Notably, we found that injection of 50 pg of *gata5* mRNA expands the domain of *nkx2.5*, *gata4* and *gata6* expression within the ALPM (Fig. 6D,E,F, Table 2) and causes them to be ectopically expressed (Fig. 6D, Table 2). Coinjection of *lacZ* and *gata5* mRNA allowed us to correlate the embryonic distribution

of the exogenous RNA with the increased expression of *nkx2.5*. In all cases but one (n=131), regions of expanded or ectopic *nkx2.5* expression overlapped with the distribution of exogenous *gata5* mRNA as indicated by β-galactosidase activity (Fig. 6D, Table 2), suggesting that Gata5 autonomously induces the expression of myocardial genes.

Surprisingly, the ectopic expression of these genes does not appear to be limited to the mesodermal germ layer. Ectopic expression of *nkx2.5, gata4* and *gata6* was observed in dorsal regions of the embryo normally fated to form neuroectoderm (Fig. 6D and data not included). Neuroectodermal expression of *nkx2.5* and *gata6* is never observed during normal zebrafish embryogenesis.

Human *gata5* maps to 20q13.2-q13.3

Mutations in human *NKX2-5* can cause congenital cardiac septation defects (Schott et al. 1998). As Gata4 and 5 physically interact and synergize with Nkx2.5 (Durocher et al. 1997; Lee et al. 1998; Sepulveda et al. 1998), and as zebrafish Gata5 is essential for the correct expression of *nkx2.5*, it is possible that mutations in *gata* genes also cause human congenital heart defects. In support of this hypothesis, *gata4* haploinsufficiency has been associated with congenital heart malformations (Pehlivan et al. 1999). Human *gata4* and *gata6* map to chromosome 8p23 (Pehlivan et al. 1999) and 18q11.1-q11.2 (Suzuki et al. 1996), respectively. Using the Stanford G3 radiation hybrid panel, we mapped human *gata5* to chromosome 20q13.2-q13.3. The study of appropriate pedigrees should determine whether *gata5* also plays an important role in human congenital heart disease.

DISCUSSION

gata5 is required for cardiac morphogenesis and differentiation, as well as the production of normal amounts of myocardial tissue

In this study, we establish that the *fau* locus encodes zebrafish Gata5, an evolutionarily conserved zinc finger transcription factor, and that mutations in *gata5* profoundly disrupt cardiac development. Most conspicuously, diminished Gata5 activity prevents the precardiac mesoderm from migrating to and fusing at the embryonic midline, resulting in cardia bifida.

Reduced Gata5 activity also disrupts the expression of several genes in the ALPM, including *nkx2.5*. The defect in *nkx2.5* expression is observable early in somitogenesis indicating that Gata5 is required very early for proper myocardial differentiation. As previous studies have demonstrated that two murine *nkx2.5* regulatory elements contain essential Gata binding sites (Searcy et al. 1998; Lien et al. 1999), Gata5 may be a direct endogenous regulator of *nkx2.5*. Consistent with this hypothesis, *gata5* is expressed during blastula stages in a domain that includes the myocardial precursors, while we first observe *nkx2.5* expression in a subset of *gata5*-expressing cells only after the completion of gastrulation (data not included). Similar relative timing of expression of these genes is seen during chick development (Todd Evans, personal communication).

Gata5 is capable of activating transcription from a wide range of myocardial promoters in vitro (Charron and Nemer 1999). Our results suggest that the same is true in vivo; *gata5* mutants display marked defects in the expression of many genes encoding components of the myocardial sarcomere (e.g., *cmlc1*, *cmlc2*, *vmhc*, *cardiac troponin T*, *tropomyosin*). However, it is not clear at this point which of these genes are direct targets of Gata5 and which require intermediate Gata5-dependent regulators. In fact, there may be no clear division between these cases as Gata5 may act both directly and indirectly on

a single promoter. For example, Gata5 may participate in the induction of *nkx2.5*, and also bind cooperatively with Nkx2.5 to regulatory elements of a wide range of myocardial genes. It is also interesting to note the different requirements various sarcomeric protein genes have for Gata5. For example, while the expression patterns of *cmlc1* and *cmlc2* are indistinguishable in wild-type embryos, *cmlc1* expression is more severely reduced than *cmlc2* expression in *fau* mutants. Perhaps these differences reflect different affinities of Gata5 for the respective *cis* regulatory elements.

In addition to exhibiting defects in myocardial differentiation, the hearts of *fau* mutants at 33 hpf are very small in comparison to those of wild-type siblings and other cardia bifida mutants such as *cas*. Similarly, the number of developing myocardial precursors is reduced in *fau* mutants throughout earlier segmentation stages. This deficit does not seem to reflect problems in ALPM specification or maintenance as *gata6* expression demonstrates that the ALPM is present and morphologically normal in *gata5* mutants. Instead, Gata5 appears to play a role in the induction, differentiation and/or proliferation of the myocardial precursors within the ALPM.

Differentiation of the myocardium into atrial and ventricular components is also defective in *fau* mutants. *fau* myocardia preferentially form atrial tissue, indicating that *gata5* is required for ventricular induction or differentiation and that the two chambers of the heart have different requirements for *gata5*. However, zebrafish *gata5* does not appear to be differentially expressed in the two chambers of the heart. Although we have not examined the distribution of Gata5 protein, the expression pattern of the *gata5* gene suggests that it is not directly involved in patterning the cardiac chambers. Instead, the defect in ventricular development may be secondary to the reduced amount of myocardial tissue produced in *gata5* mutants, if embryos must elaborate a threshold amount of myocardial tissue to sustain ventricular induction or differentiation. Alternatively, Gata5 may act with an unidentified ventricle-specific factor to promote ventricular development.

Taken together, these results demonstrate that Gata5 plays a critical role in multiple aspects of zebrafish heart development including the expression of *nkx2.5* and other myocardial genes, the production of a normal amount of myocardial precursors, the morphogenesis of both the myocardial and endocardial components of the heart, and the differentiation of the ventricular chamber.

gata5 is required for endoderm morphogenesis

Previous expression studies have suggested a role for Gata factors in the early development of the embryonic endoderm (Laverriere et al. 1994; Morrisey et al. 1997a; Bossard and Zaret 1998). Here, we have confirmed that zebrafish *gata5* is essential for the formation of normal amounts and morphogenesis of the endoderm. Although gut and pharyngeal endoderm lineages separate early during zebrafish development and have distinct genetic requirements (Alexander 1999), *gata5* is necessary for the morphogenesis of both tissues. Interestingly, Gata5 is required for the movement toward the midline of both the heart primordia and the gut and pharyngeal endoderm, suggesting a causative link between these defects or a common role of Gata5 in promoting the medial migration of several tissues.

gata5 overexpression causes the ectopic expression of myocardial genes

Here, we show that overexpression of zebrafish *gata5* leads to the expanded or ectopic expression of *cmlc1*, *cmlc2*, *vmhc*, *gata4*, *gata6*, *nkx2.5* and the MF20 and S46 myosin heavy chain proteins, indicating that zebrafish Gata5 can act as a positive regulator of many myocardial genes, even in noncardiogenic tissues. This is the first demonstration of the ability of a Gata factor to enhance cardiogenesis in the context of a developing embryo. In contrast, overexpression of *gata4* and *gata5* in *Xenopus* do not affect the spatial pattern of myocardial gene expression or heart morphogenesis (Jiang and Evans 1996). The apparently different capacities of *Xenopus* and zebrafish *gata5* may

reflect species-dependent differences in the plasticity of cell fate or differences in the activities of the two homologues. In fact, zebrafish Gata5 function may be more similar to that of murine Gata4 which can also potentiate cardiogenesis, at least in the P19 cell line (Grepin et al. 1997).

The ability of Gata5 to induce ectopic myocardial gene expression is not entirely unexpected, given that Gata5 regulates *nkx2.5* expression and overexpression of *nkx2.5* can induce some ectopic myocardial gene expression (Chen and Fishman 1996). It will be interesting to determine whether Nkx2.5 is an obligatory effector of Gata5 function in myocardial cells. Another intriguing issue concerns whether Gata5, when forcibly expressed in noncardiogenic tissues, simply activates the transcription of a subset of myocardial genes, or whether Gata5 can reprogram an otherwise noncardiac cell to become completely myocardial in character. The unique ability of *gata5* overexpression to produce ectopic beating tissue suggests that, at least in some contexts, these cells exhibit myocardial behavior. Therefore, both phenotypic analysis of *fau* mutants and gain-of-function experiments indicate that Gata5 plays an essential role in the induction, differentiation and/or proliferation of the myocardial precursors.

While the normal expression of *gata5* in *fau*^{tm236a} mutants suggests that Gata5 does not regulate its own expression, *fau* mutants exhibit modestly decreased expression of *gata4* and overexpression of *gata5* is able to induce the ectopic transcription of *gata4*. Therefore, Gata5 appears to positively regulate *gata4*, through which Gata5 may mediate some of its effects on cardiac differentiation. Although no defects in *gata6* expression were noted in *fau* mutants, *gata5* overexpression can cause ectopic *gata6* expression, suggesting that Gata5 may have a redundant role in regulating *gata6*. Perhaps embryos mutant for both *gata4* and *gata5* will reveal a nonessential role for Gata5 in *gata6* gene regulation. Previous work on mouse *gata4* and *gata6* mutants has demonstrated that Gata4 negatively regulates *gata6* in the ALPM while Gata6 positively regulates *gata4* in extraembryonic tissues (Kuo et al. 1997; Molkentin et al. 1997; Morrisey et al. 1998).

Thus, complex interregulation by Gata4, 5 and 6 may play an important role in controlling the early events of cardiac and endodermal development.

gata5 may act both tissue autonomously and non-autonomously in heart development

Previous studies in both mouse and zebrafish have demonstrated that cardia bifida can be secondary to defects in endoderm development (Narita et al. 1997; Peyrieras et al. 1998). This work leads us to hypothesize that the cardia bifida in *fau* mutants is also secondary to their severe endodermal defects. Moreover, the presence of endodermal defects in the cardia bifida mutants *fau*, *oep* and *cas* (Schier et al. 1997; Alexander et al. 1999), suggests that zebrafish cardia bifida may be a general consequence of defects in endodermal morphogenesis or differentiation. Conclusive experimental tests of the autonomy of the morphogenetic defects in *fau* mutants will require analyses of embryos in which interacting tissues are of distinct genotypes, the production of which is contingent on techniques not yet developed in zebrafish.

While the absence of cardiac fusion in *fau* mutants may be secondary to endoderm defects, other cardiac abnormalities may be due to reduced Gata5 activity in the ALPM. Despite the complete and early absence of gut endoderm in *cas* mutants, *cas* myocardia exhibit no defect in gene expression (Alexander et al. 1999), are larger than *fau* myocardia and contain a normal ratio of atrial to ventricular tissue. Thus, *fau* mutants display defects in gene expression, cardiac growth and ventricular development beyond what is attributable to the loss of endoderm, implicating ALPM *gata5* expression in these roles. The observation that *gata5* overexpression autonomously promotes the ectopic expression of *nkx2.5* further suggests that Gata5 acts within cells of the ALPM to regulate myocardial differentiation. We therefore propose that the defects in *fau* cardiac morphogenesis are secondary to the reduction of Gata5 activity in the endoderm, while

the additional defects in cardiac growth and differentiation are due to the reduction of Gata5 function in the ALPM.

Zebrafish Gata5 and murine Gata4 may function similarly during organogenesis

While mouse *gata5* null mutants have not been extensively described, they are viable (Morrisey et al. 1998), suggesting that some of the functions performed by zebrafish Gata5 may be fulfilled by Gata4 or 6 in the mouse. Unfortunately, mouse embryos deficient for Gata4 (Kuo et al. 1997; Molkentin et al. 1997) or Gata6 (Morrisey et al. 1998; Koutsourakis et al. 1999) arrest at E 7.0-9.5 and E 5.5-7.5, respectively, impeding the analysis of later developmental events such as cardiac chamber formation and the development of endoderm-derived organs. However, *gata4* null mice and *gata5* mutant zebrafish both display cardia bifida, prompting a comparison. *gata4* null mice do not undergo ventral closure, which has led to the idea that cardia bifida in *gata4* null mice is a direct consequence of the defect in ventral folding. Our study, indicating that zebrafish cardia bifida can result from *gata*-dependent defects in the migration of the cardiac primordia to the embryonic midline, suggests an alternative interpretation. In zebrafish, the cardiac precursors migrate on the yolk syncytial layer to reach the embryonic midline (Stainier et al. 1993). Perhaps murine precardiac mesoderm reaches the embryonic midline similarly, by actively migrating on the underlying visceral endoderm, and not through ventral folding of the body wall. In support of this hypothesis, in the mouse the midline cardiogenic plate appears to form before the beginning of ventral folding (Kaufman 1992; Plate 8d; Saga et al. 1999). Therefore, cardia bifida in *gata4* null mice may stem from a defect in the migration of the precardiac mesoderm to the embryonic midline in much the same way as cardia bifida arises in mice lacking MesP1 (Saga et al. 1999). We propose that mouse *gata4* is required for two early and distinct morphogenetic processes: *i*) the initial migration of the bilateral cardiac precursors to the embryonic midline where they form the cardiogenic plate and *ii*) the

subsequent ventral folding required for foregut and ventral body wall morphogenesis. Determining whether cardiac fusion is truly independent of ventral closure will require the identification of mouse mutations that inhibit ventral closure without affecting cardiac fusion.

In summary, we have shown that *gata5* mutants show reduced expression of *nkx2.5* and that *gata5* overexpression leads to ectopic *nkx2.5* expression. These data together with the demonstrated presence of Gata binding sites in *nkx2.5* regulatory elements indicate that *gata5* functions upstream of *nkx2.5* to initiate myocardial differentiation. Further studies will be required to identify additional regulators of heart and endoderm development that function downstream of or in combination with Gata5.

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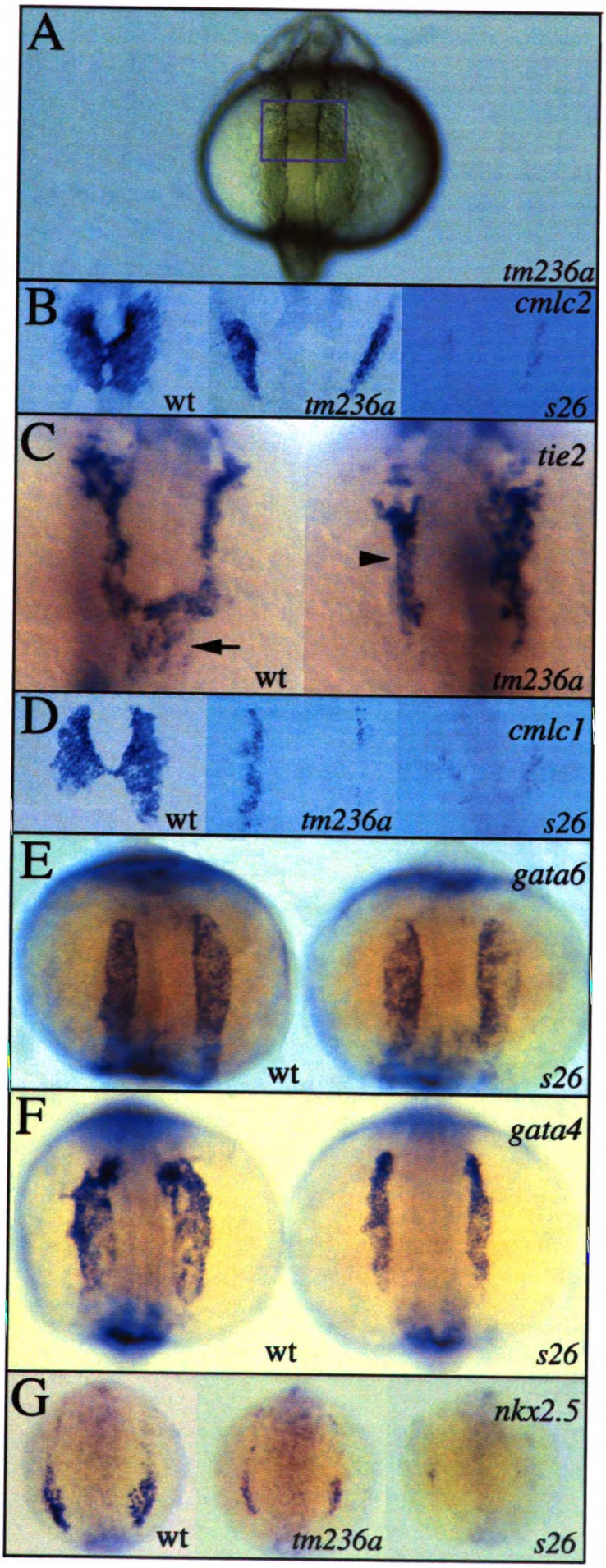
Figure 2.1 *fau* mutants display abnormal cardiac morphogenesis and differentiation. Expression of *cmlc2* (B), *tie2* (C), *cmlc1* (D), *gata6* (E) and *nkx2.5* (F) in wild-type embryos and *fau* mutant siblings at the 19-somite (18.5 hpf; A,B,C,D), 12-somite (15 hpf; E,F) and 6-somite (12 hpf; G) stages. All views are dorsal with anterior to the top. ***(A)*** At the 19-somite stage, both fau^{tm236a} and fau^{s26} mutants are morphologically indistinguishable from wild-type siblings. The blue box approximately outlines the cardiogenic region, which lies ventral and just posterior to the midbrain-hindbrain boundary. Panels B and D depict enlarged views of this region. ***(B)*** In wild-type embryos, the myocardial primordia express *cmlc2* and have fused at the embryonic midline (left). In contrast, the myocardial primordia of fau^{tm236a} mutants do not migrate to the embryonic midline, resulting in cardia bifida (middle). The myocardial primordia of fau^{s26} mutants also fail to migrate to the embryonic midline (right), and in addition display a severe reduction in the number of cells expressing *cmlc2*. ***(C)*** Formation of the endocardium (arrow) also depends upon the migration of precursors from the ALPM to the embryonic midline. In *fau* mutants, endocardial precursors expressing *tie2* are absent from the midline. *tie2* expression in endothelial cells (arrowhead) reveals that more anterior vasculature forms normally in *fau* mutants. ***(D)*** *cmlc1* is also expressed in the myocardial primordia. In the right fau^{tm236a} cardiac primordium (middle panel), fewer than 20 cells express *cmlc1* while more than 120 cells express *cmlc1* in each wild-type primordium (left). In contrast, fau^{tm236a} mutants and wild-type siblings contain approximately equal numbers of *cmlc2*-expressing cells (see Fig. 1B). ***(E)*** *gata6* is widely expressed throughout the ALPM throughout somitogenesis. *gata6* expression is not perturbed in *fau* mutants. ***(F)*** In contrast, ALPM expression of *gata4* is slightly reduced in *fau* mutants. ***(G)*** *nkx2.5* expression is also reduced in *fau* mutants. Although the number of cells expressing *nkx2.5* is only moderately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s26
C
tie2
wt
tm236a
D
cmlc1
wt
tm236a
s26
E
gata6
wt
s26
F
gata4
wt
s26
G
nkx2.5
wt
tm236a
s26

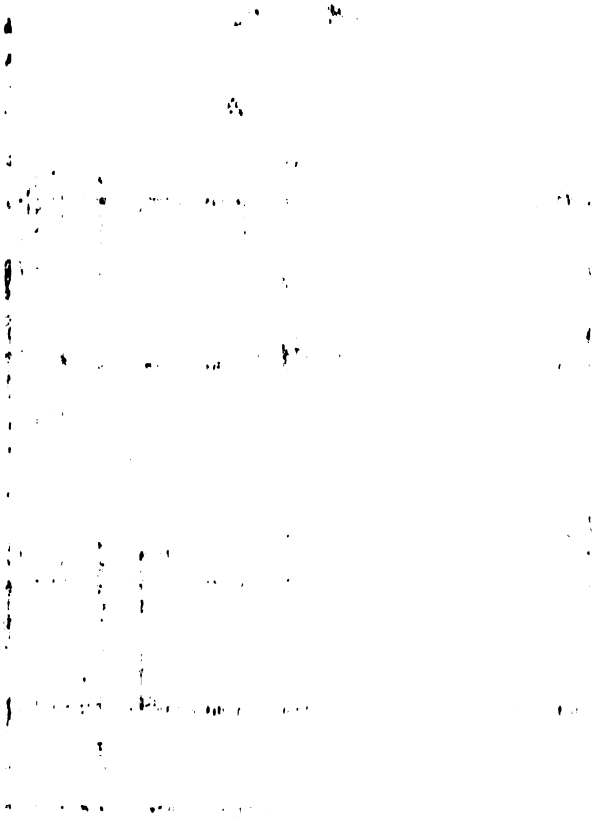
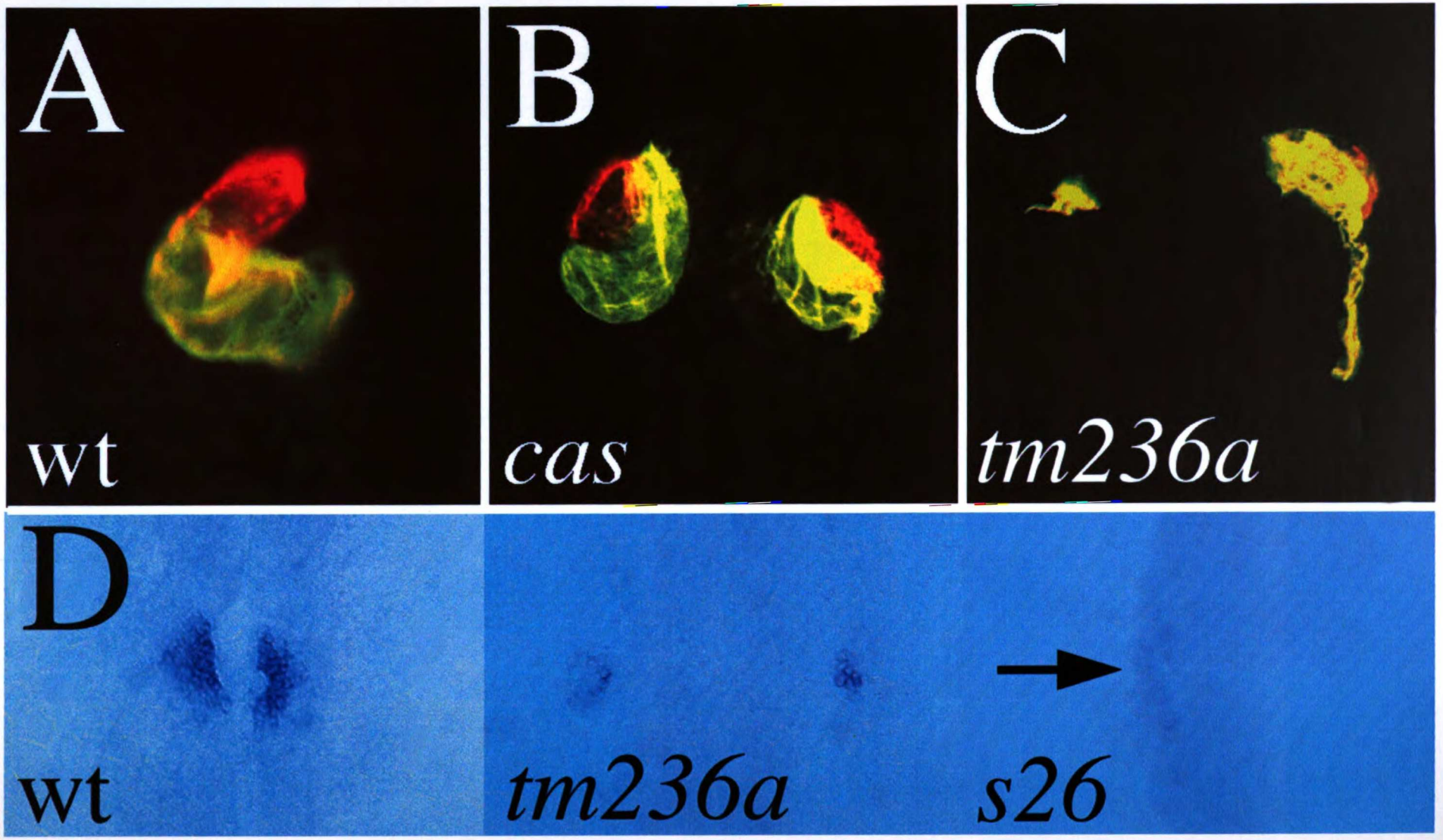


Figure 2.2 Formation of ventricular myocardial tissue is abnormal in *fau* mutants. 33 hpf mutants and wild-type siblings were stained with MF20 and S46 antibodies (A-C). Red fluorescence indicates MF20 (TRITC) staining of ventricular myocardium and yellow fluorescence indicates the overlap of MF20 (TRITC) and S46 (FITC) staining of atrial myocardium. Panels A-C show ventral views with anterior to the top. Panel D is a dorsal view of *vmhc* expression at the 18-somite stage (18 hpf). ***(A)*** Wild-type embryos develop a single, looped heart patterned into a ventricle (red) and atrium (yellow). ***(B)*** *cas* mutants display cardia bifida and form both ventricular and atrial tissue. ***(C)*** *fau* mutants also display cardia bifida, but form less total myocardium and only a very small amount of ventricular tissue. Here, one myocardium (left) has not formed any ventricular tissue. ***(D)*** *vmhc* is expressed in the preventricular component of the developing myocardium. *vmhc* expression is dramatically reduced in *fau* myocardial primordia even prior to cardiac fusion. fau^{s26} mutants display a variable loss of *vmhc* expression; the right panel shows a fau^{s26} mutant in which less than five cells weakly express *vmhc* in the left ALPM (arrow) while none do on the right.


A
wt
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cas
C
tm236a
D
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tm236a
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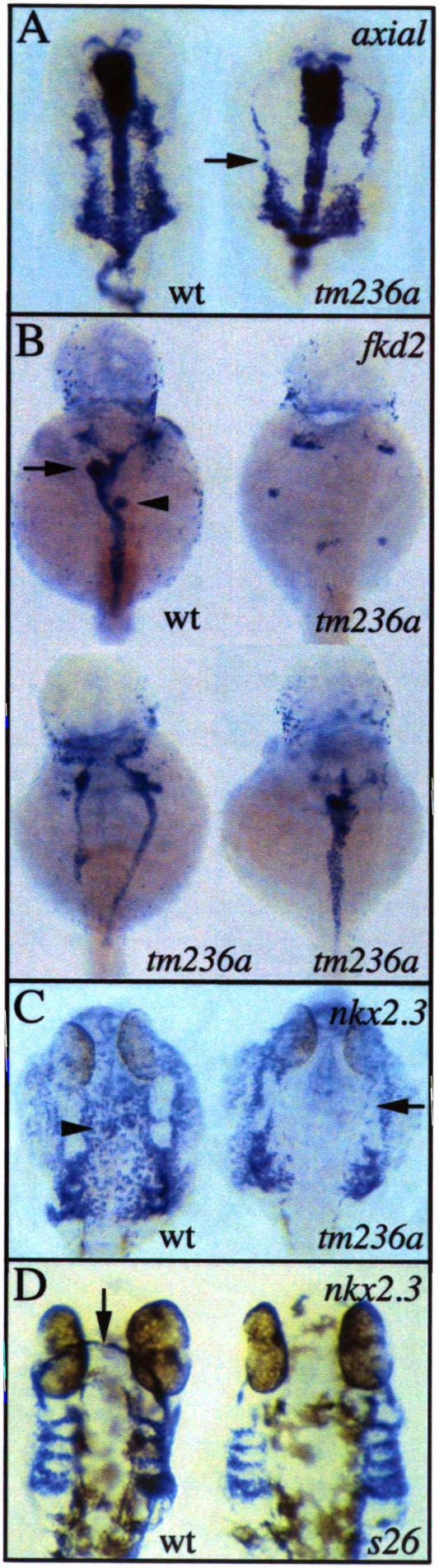
Figure 2.3 Endoderm development is abnormal in *fau* mutants. Expression of *axial* (A), *fkd2* (B) and *nkx2.3* (C,D) in wild-type embryos and *fau* mutant siblings at 44 hpf (A,B,D) and 28 hpf (C). All views are dorsal with anterior to the top. ***(A)*** *axial* is expressed in the anterior gut endoderm and the ventral neuroectoderm. At this stage, the anterior gut endoderm is coalescing at the embryonic midline in wild-type embryos. In *fau* mutants, gut endoderm is only present laterally (arrow). For purposes of orientation, the arrow is at the level of the midbrain-hindbrain boundary. ***(B)*** *fkd2* is expressed throughout the pancreas (arrowhead), liver (arrow) and posterior gut tube in wild-type embryos. *fau* mutants display variable abnormalities in the morphogenesis and amount of *fkd2*-positive tissue. These defects range from near absence of *fkd2*-positive endoderm (upper right), to formation of two lateral gut tubes (lower left), to the absence of gut looping (lower right). ***(C)*** Morphogenesis of the pharyngeal pouches is disrupted in *fau* mutants. The mediolateral distance between the pouches is greater in *fau* mutants and the anterior pouches (arrow) are disorganized. Also, midline *nkx2.3*-expressing cells including the putative thyroid primordium (arrowhead) appear to be absent in *fau* mutants. ***(D)*** In fau^{s26} mutants, the anterior domain of *nkx2.3* expression (arrow) is absent.


A
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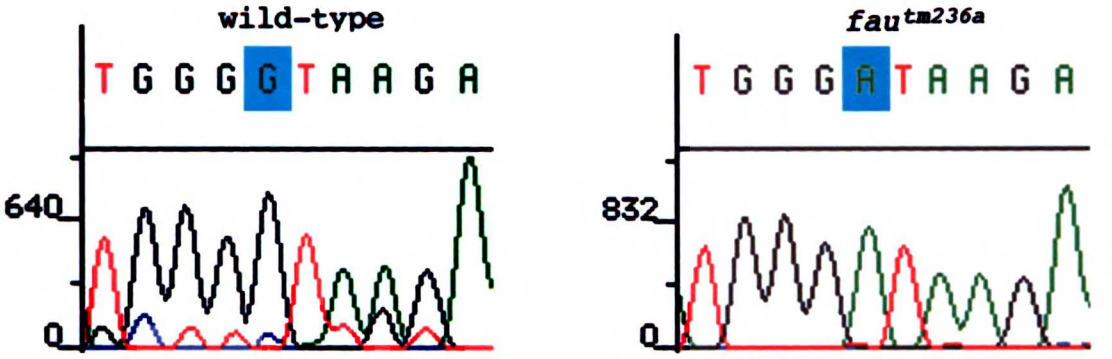
D
nkx2.3
wt
s26

Figure 2.4 *fau* encodes zebrafish Gata5. ***(A)*** Sequencing of *fau*^{tm236a} and wild-type genomic DNA reveals a G to A substitution at the +1 position of the third intron of *gata5* (shaded in blue). ***(B)*** Comparison of the vertebrate consensus 5' splice site (Krawczak et al. 1992) with the wild-type *gata5* intron 3 5' splice site, the mutant sequence, the cryptic downstream 5' splice site and the cryptic upstream 5' splice site. Subscripts indicate the frequency at which nucleotides occur in the consensus splice site. Blue shading marks the *fau*^{tm236a} mutation. ***(C)*** Comparison of the complete wild-type Gata5 protein sequence with the mutant sequences. "tm236a-1" is the sequence of the protein predicted to result from use of the downstream cryptic splice site and was most frequently isolated from *fau*^{tm236a} mutants. "tm236a-2" is the sequence of the protein predicted to result from use of the upstream cryptic splice site and was less frequently isolated from *fau*^{tm236a} mutants. The two zinc fingers are shaded green. The basic domain required for nuclear localization is shaded yellow. ***(D)*** Schematic of the expected effects of the *fau*^{tm236a} mutation on Gata5 structure. The purple C-terminal tail of *tm236a-1* represents the nonfunctional domain created by the frameshift mutation. The deletion of the C-terminal portion of the second zinc finger of *tm236a-2* is represented by the smaller size of the second green rectangle. The transcriptional activation domains depicted in red are predicted based on comparison of zebrafish Gata5 domains with identified murine Gata4 and Gata5 activation domains (Durocher et al. 1997; Morrisey et al. 1997b).

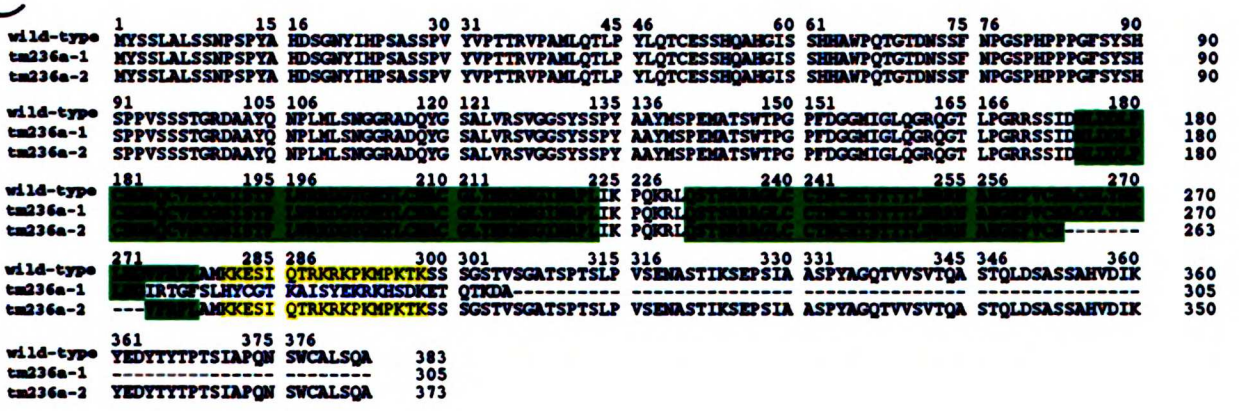
A



B

	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
				Exon	Intron					
vertebrate consensus 5' splice site	C_{29}/A_{34}	C_{38}/A_{35}	A_{62}	G_{77}	G_{100}	T_{100}	A_{60}	A_{74}	G_{84}	T_{50}
wild-type intron 3 5' splice site	T	G	G	G	G	T	A	A	G	A
fau^{tm236a} intron 3 5' splice site	T	G	G	G	A	T	A	A	G	A
cryptic downstream 5' splice site	T	G	T	G	G	T	A	C	C	C
cryptic upstream 5' splice site	C	A	A	T	G	C	A	T	G	T

C



D

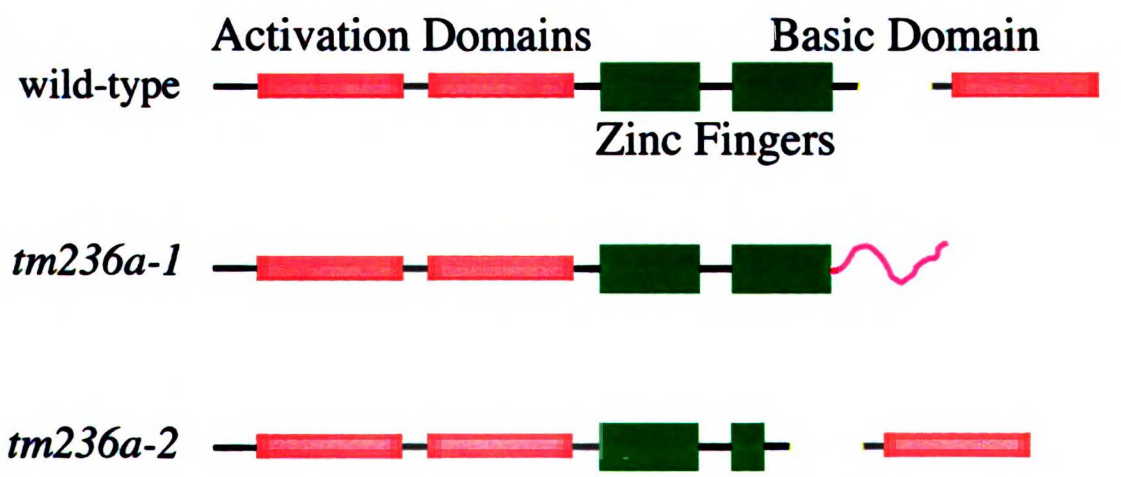


Figure 2.5 *gata5* expression in zebrafish embryos at dome (4.3 hpf; A), germ ring (5.7 hpf; B), 90% epiboly (9 hpf; C), bud (10 hpf; D), 4-somite (11.3 hpf; E), 28 hpf (F, H) and 80% epiboly (8.3 hpf, G) stages. Panels A, B, C and G show lateral views with dorsal to the right. Panels D, E, F and H show dorsal views with anterior to the top. ***(A)*** At dome stage, *gata5* is expressed in the yolk syncytial layer (arrow) and the most marginal tiers of the blastoderm (arrowhead). ***(B)*** During early gastrulation, *gata5* expression is maintained in the marginal zone (bracket) and yolk syncytial layer (arrow). ***(C)*** Late in gastrulation, *gata5* is expressed by cells of two different morphologies. Predominantly dorsally, endodermal cells displaying a characteristically large and flat morphology (arrowhead) express *gata5*. Ventrally, hypoblast cells with a rounder, more mesodermal appearance (arrow) also express *gata5*. ***(D)*** After the completion of gastrulation, *gata5* is expressed in the endoderm (stippled staining) and ALPM (arrow). ***(E)*** During early somitogenesis, both the endoderm and lateral plate mesoderm (arrow) continue to express *gata5*. ***(F)*** At 28 hpf, *gata5* is expressed most strongly in the endocardium (arrow) and weakly in the surrounding noncardiogenic ALPM. ***(G)*** *fau*^{s26} mutants display markedly reduced *gata5* expression. At 80% epiboly, *gata5* mRNA is undetectable in the YSL and observed only faintly (arrow) in the blastoderm of *fau*^{s26} mutants. ***(H)*** *gata5* expression is undetectable in *fau*^{s26} mutants at 28 hpf.

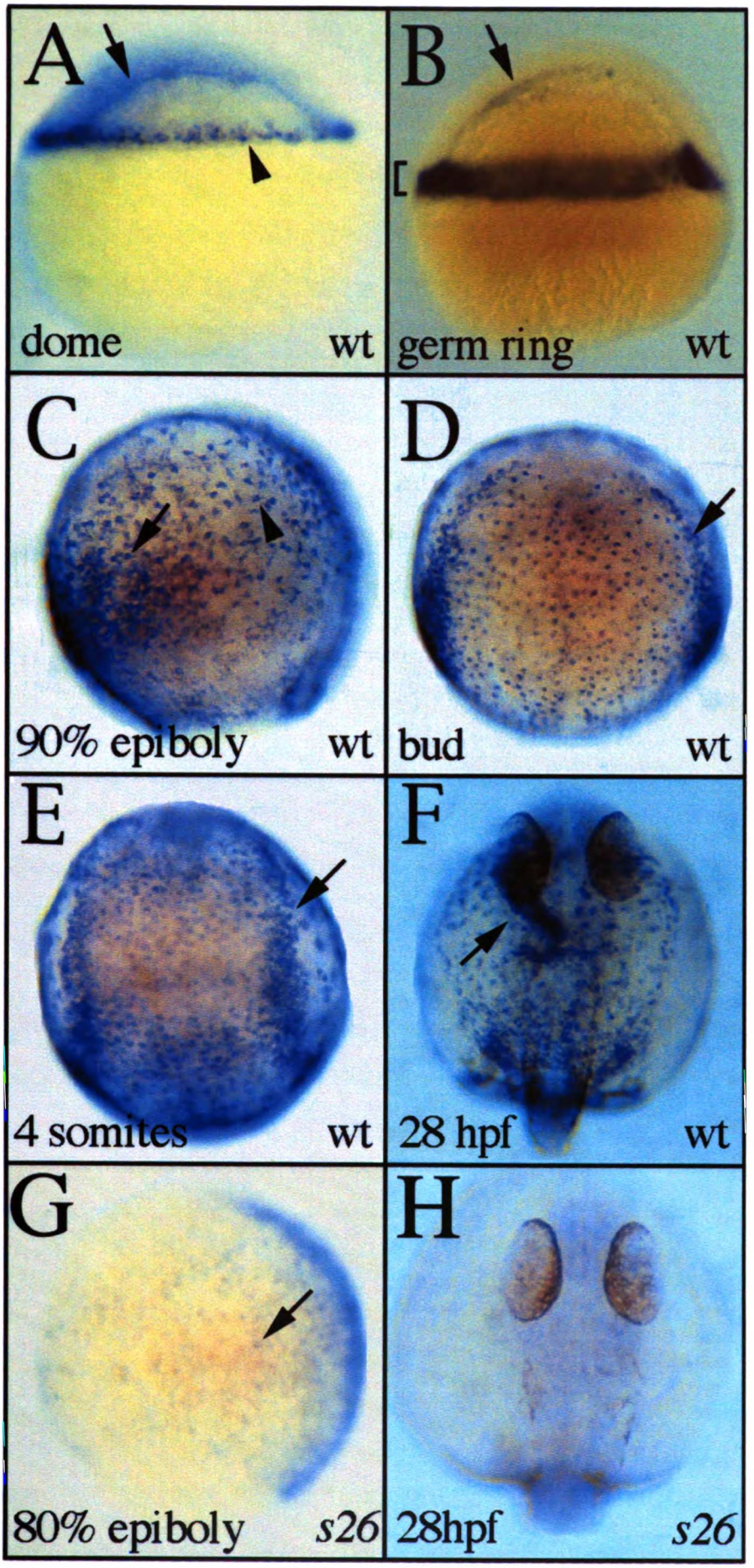

A
dome
wt
B
germ ring
wt
C
90% epiboly
wt
D
bud
wt
E
4 somites
wt
F
28 hpf
wt
G
80% epiboly
s26
H
28hpf
s26

Figure 2.6 Overexpression of *gata5* rescues cardiac fusion in *fau*^{tm236a} mutants and leads to ectopic expression of myocardial genes. Expression of *cmlc1* (A, C), *cmlc2* (B), *nkx2.5* (D), *gata4* (E) and *gata6* (F) in wild-type embryos and *fau* mutant siblings at 36 hpf (A), 25 hpf (B,C), 5-somite stage (11.7 hpf; D) and 12-somite stage (14.5 hpf; E,F). Views are anterior (A), lateral (B,C,D [lower right]) and dorsal (D [upper left and right, and lower left],E,F). ***(A)*** Injection of 20 pg of *tm236a-1* mRNA has no demonstrable effect on development (upper left) and does not restore cardiac fusion in *fau*^{tm236a} mutants (upper right, arrows). In contrast, injection of an equivalent amount of wild-type *gata5* mRNA causes variable defects in head and body morphology (lower left) and can restore cardiac fusion in *fau*^{tm236a} mutants (lower right, arrowhead). ***(B)*** Injection of a higher dose of *gata5* mRNA (50 pg) can cause expanded expression of *cmlc2* in a domain contiguous to the ALPM. ***(C)*** *gata5* overexpression can also cause ectopic expression of *cmlc1*. Here, *gata5* overexpression has led to ectopic *cmlc1* expression in cells overlying the ventral side of the yolk (arrow). A heart tube has also formed in the expected location (arrowhead). ***(D)*** Injection of 50 pg of *gata5* mRNA can increase the number of cells expressing *nkx2.5* (upper right), and cause ectopic *nkx2.5* expression (lower panels). The lower panels show dorsal and lateral views of the same embryo, which exhibits a dorsal midline region of ectopic *nkx2.5* expression (arrows). β-galactosidase staining produces a turquoise color marking the distribution of coinjected *lacZ* mRNA. ***(E)*** *gata5* overexpression can also lead to increased expression of *gata4* and ***(F)*** *gata6.*

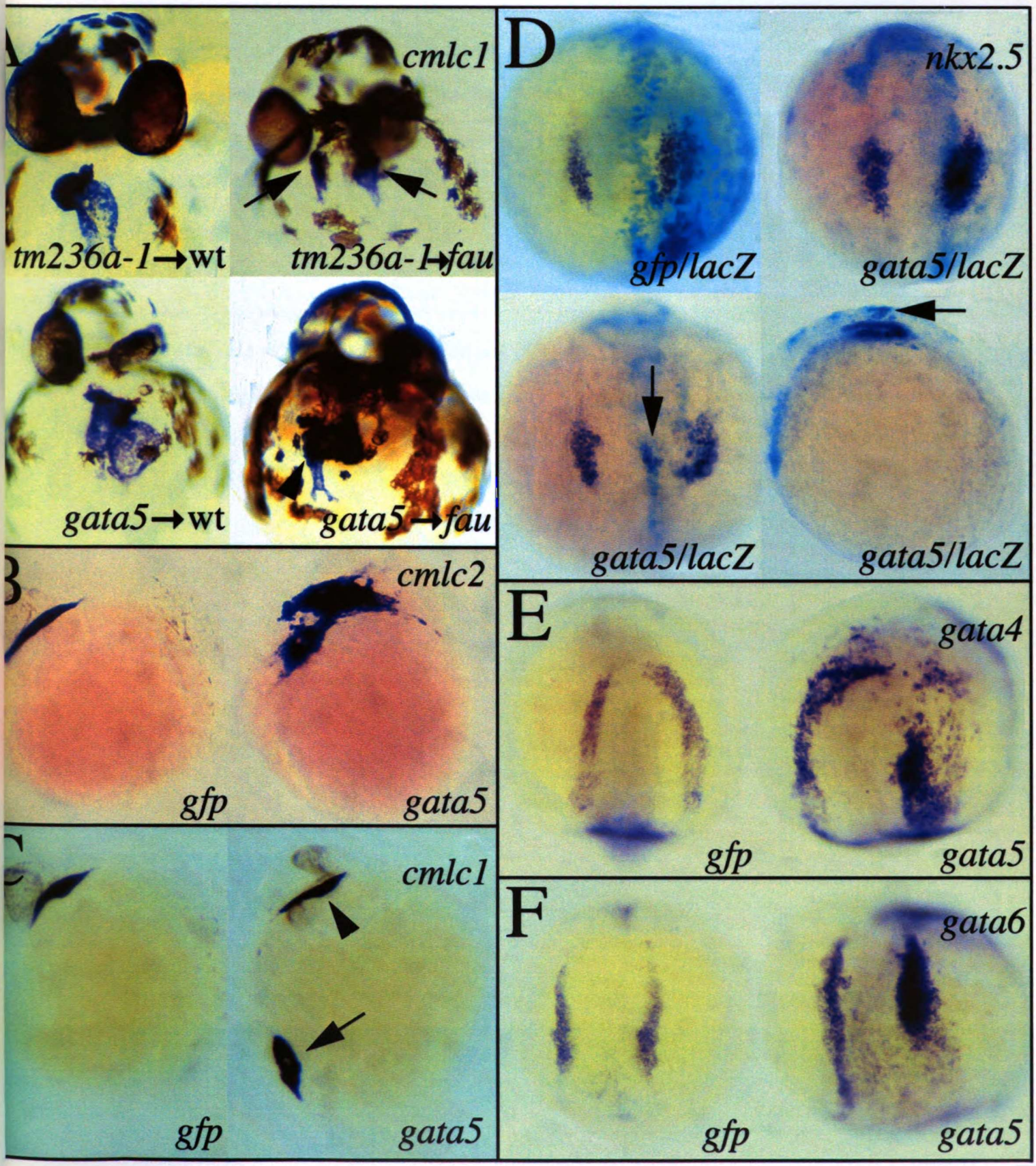

A
cmlc1
tm236a-1→wt
tm236a-1→fau
gata5→wt
gata5→fau
B
cmlc2
gfp
gata5
C
cmlc1
gfp
gata5
D
gfp/lacZ
nkx2.5
gata5/lacZ
gata5/lacZ
gata5/lacZ
E
gata4
gfp
gata5
F
gata6
gfp
gata5

Table 2.1. Overexpression of wild-type *gata5* restores cardiac fusion in *fau*^{tm236a} mutants

Injected mRNA	Number of *fau*^{tm236a} mutants (by genotype)	Number with cardia bifida
gata5	39	9 (23%)
tm236a-1	25	19 (76%)
Uninjected	35	23 (70%)

The observed value of the chi-squared test $\chi^2(1)$, 17.33, indicates that the increased rate of cardiac fusion following overexpression of *gata5* in *fau*^{tm236a} mutants is statistically significant.

Table 2.2. *gata5* overexpression causes ectopic expression of myocardial genes and beating tissue

Injected mRNA	Riboprobe	Expanded expression	Ectopic expression
gata5	*cmlc1*	32/95	10/95
gata5	*cmlc2*	20/64	7/64
gata5	*vmhc*	13/22	4/22
gata5	*nkx2.5*	11/18	4/18
gata5	*nkx2.5*	0/6	0/6
gata5 and *lacZ*	*nkx2.5*	84/237*	47/237*
gfp and *lacZ*	*nkx2.5*	0/36	0/36
gata5	*gata4*	9/16	6/16
gfp	*gata4*	0/6	0/6
gata5	*gata6*	14/22	10/22
gfp	*gata6*	0/5	0/5

Injected mRNA	Ectopic beating tissue
gata5	4/52
gfp	0/7

Embryos were scored as displaying expanded expression if expression was noted in cells adjacent to the normal domain (see Fig. 6B,D [upper right],E,F for examples). Embryos

were scored as displaying ectopic expression or ectopic beating tissue if tissue was noted dorsally or posteriorly within the embryo in a domain discontinuous with the ALPM or heart (see Fig. 6C,D [lower] for examples).

*Of the *gata5*-overexpressing embryos exhibiting expanded or ectopic *nkx2.5* expression, 84/84 and 46/47 of the embryos, respectively, displayed increased expression within a domain of β-galactosidase activity.

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Chapter 3: Specification and differentiation of the zebrafish endoderm requires Gata5

SUMMARY

Background: Formation of the zebrafish endoderm requires Nodal signals, the EGF-CFC protein One-eyed pinhead and the product of the *bonnie and clyde* (*bon*) locus, a homeobox transcription factor homologous to Mixer. Nodal signaling induces *bon/mixer*. Bon/Mixer activates the endodermal expression of two other transcription factor genes, *sox17* and *axial*, in a way that requires the product of the *casanova* locus. In *C. elegans* and *Drosophila,* Gata zinc finger transcription factors are required for endoderm specification and differentiation. However, whether vertebrate Gata homologs function during early endoderm development is unknown.

Results: We demonstrate an essential role for zebrafish Gata5 in several aspects of vertebrate endoderm development, and place Gata5 relative to other proteins in a pathway underlying endoderm formation. Zebrafish Gata5 is expressed from the late blastula stage in the cells that give rise to the endoderm. Loss-of-function mutations in *gata5* reduce the number of *sox17*- and *axial*-expressing cells while *gata5* overexpression expands number of *sox17*- and *axial*-expressing cells. Analysis of *gata5* mutants also reveals that Gata5 is required for normal levels of *sox17* and *axial* expression during gastrulation and for the subsequent differentiation of endoderm-derived organs. Through mutant and overexpression analyses, we demonstrate that Nodal signaling induces *gata5* expression in a manner dependent upon Oep, and that Gata5 acts in parallel to Bon/Mixer and upstream of the *casanova* gene product to regulate early endoderm development.

Conclusions: Cumulatively, these results indicate that Nodal and Oep signaling, but not Bon/Mixer activity, is essential for the expression of *gata5*. Gata5, together with Bon/Mixer, regulates the number of endodermal progenitors in a way that requires Casanova. Within the endodermal progenitors, Gata5 promotes the expression of *sox17* and *axial*. These results demonstrate that, as in invertebrates, a vertebrate Gata transcription factor plays a central and early role in endoderm development.

INTRODUCTION

All triploblastic organisms must partition the developing blastoderm into three germ layers, the ectoderm, mesoderm and endoderm. Our understanding of how the endoderm forms in vertebrates has been recently advanced by the identification of several gene products required for endoderm formation and the ordering of these factors into a pathway (Hudson et al. 1997; Henry and Melton 1998; Alexander and Stainier 1999; Casey et al. 1999; Clements et al. 1999; Yasuo and Lemaire 1999).

In zebrafish, the endoderm arises from the most marginal four tiers of the late blastula stage embryo (Warga and Nusslein-Volhard 1999). Before gastrulation, single marginal cells can give rise to both endoderm and mesoderm (Warga and Kimmel 1990). By the time they involute, however, blastomeres have committed to either an endodermal or a mesodermal fate (Warga and Kimmel 1990). Druing gastrulation, the large, flat endodermal progenitors are distinguished from mesodermal progenitors in that they directly overlie the yolk syncytial layer (YSL), and express *axial*, a zebrafish homolog of the forkhead transcription factor gene *HNF3β,* and *sox17*, a high mobility group (HMG) domain transcription factor gene (Strahle et al. 1993; Alexander and Stainier 1999; Warga and Nusslein-Volhard 1999). In addition to their expression in the endodermal progenitors, *axial* is expressed in the axial mesoderm and *sox17* is expressed in the forerunner cells (Strahle et al. 1993; Alexander and Stainier 1999).

Nodal proteins are members of the TGFβ family of signaling molecules that are implicated in the induction of both mesoderm and endoderm in several vertebrate systems. Zebrafish embryos mutant for both *cyclops* (*cyc*) and *squint* (*sqt*), two genes that encode Nodal proteins, produce no endoderm and little mesoderm (Feldman et al. 1998). Similarly, overexpression of a dominant negative Nodal in *Xenopus laevis* embryos suppresses expression of endodermal and mesodermal genes (Osada and Wright 1999), and mouse *nodal* mutants fail to form either a node or a primitive streak (Conlon et

al. 1994). When overexpressed, Nodal proteins can induce endodermal gene expression in both *Xenopus* animal caps and zebrafish embryos (Erter et al. 1998; Osada and Wright 1999). Therefore, Nodal signaling is both necessary and sufficient for at least some aspects of endodermal differentiation.

Zebrafish Nodal signaling requires *one-eyed pinhead* (*oep*), a maternally and zygotically expressed gene encoding an EGF-CFC protein (Zhang et al. 1998; Gritsman et al. 1999). Embryos lacking both maternal and zygotic Oep resemble *cyc;sqt* mutants in that they form no endoderm and essentially no mesoderm (Gritsman et al. 1999). Mutants lacking only zygotically-encoded Oep (*Zoep* mutants) produce mesoderm, yet still display a complete absence of endoderm (Schier et al. 1997), suggesting that endoderm induction requires a higher level of Nodal signaling than does mesoderm formation. Consistent with this hypothesis, overexpression of low levels of Antivin/Lefty2, an inhibitor of Nodal signaling (Meno et al. 1999), appears to selectively inhibit endoderm formation (Thisse and Thisse 1999).

Nodal signaling induces the expression of zebrafish *mixer*, a Mix-type homeobox gene most closely related to chick *CMIX* and *Xenopus Bon/Mixer* (Henry and Melton 1998; Peale et al. 1998; Alexander et al. 1999). Recently, the zebrafish *bonnie and clyde* (*bon*) locus has been shown to encode Bon/Mixer (Kikuchi et al. 1999). *bon/mixer* mutants produce only a few endodermal progenitors, as assessed by *sox17* and *axial* expression, and do not form guts (Kikuchi et al. 1999). Overexpression of *bon/mixer* can induce endodermal *sox17* expression in *Zoep* mutants, further indicating that Bon/Mixer functions in endoderm formation downstream of Nodal signaling (Alexander et al. 1999). Mix-type transcription factors are also required for endoderm development in other vertebrates, as overexpression of a dominant interfering form of *Bon/Mixer* in *Xenopus* embryos prevents endoderm differentiation and represses the expression of *Xsox17β*, a homolog of *sox17* (Henry and Melton 1998).

Another zebrafish gene, *casanova* (*cas*), is also essential for endoderm formation; *cas* mutants do not express *sox17* and do not form any gut endoderm (Alexander et al. 1999). Although *bon/mixer* overexpression can induce *sox17* expression in *Zoep* mutants, *bon/mixer* overexpression in *cas* mutants does not induce *sox17*, suggesting that *cas* functions between *bon/mixer* and *sox17*(Alexander and Stainier 1999). Thus, complementary loss- of gain-of-overexpression studies and mutant analyses have organized the genes required for endoderm formation into a coherent pathway. Nodal signaling induces the expression of *bon/mixer* in a manner dependent on Oep (Alexander et al. 1999). Bon/Mixer then functions through *cas* to promote the expression of downstream endodermal regulators such as *sox17* (Alexander and Stainier 1999).

This framework for vertebrate endoderm formation is likely to be incomplete. Studies of endoderm formation in invertebrates suggest the involvement of another class of transcription factors, the Gata factors. Gata factors are zinc finger transcriptional activators that bind to the consensus sequence (A/T)GATA(A/G). The *Caenorhabditis elegans* genome contains several *gata* genes, one of which, *elt-2*, is essential for differentiation of the embryonic gut (Fukushige et al. 1998), and another of which, *end-1*, is implicated in specification of the endodermal progenitor (Zhu et al. 1997). *end-1* is sufficient to induce endodermal fate, as ubiquitous expression compels all blastomeres to produce endoderm instead of ectoderm and mesoderm (Zhu et al. 1998). Similarly, the *Drosophila gata* gene *serpent* is expressed in, and required for the development of, the endoderm and the fat body, a mesoderm-derived organ functionally analogous to the vertebrate liver (Abel et al. 1993; Reuter 1994; Rehorn et al. 1996).

Vertebrate genomes are known to contain six evolutionarily conserved *gata* homologues. While *gata1*, *2* and *3* are implicated predominantly in hematopoietic development (reviewed in Orkin and Zon 1997), *gata4*, *5* and *6* are expressed in extraembryonic tissues, heart and endoderm (reviewed in Charron and Nemer 1999). Gene inactivation studies in mouse have revealed critical roles for *gata4* in endoderm-

dependent body movements and for *gata6* in the development of the extraembryonic visceral endoderm (Kuo et al. 1997; Molkentin et al. 1997; Morrisey et al. 1998; Koutsourakis et al. 1999). Studies of the regulation of endodermal genes have also suggested a role for Gata factors in the differentiation of the definitive endoderm; transfection of heterologous cells with *gata* genes induces the transcription of endodermal genes such as *IFABP*, *gastric H^+/K^+-ATPase* and *HNF4* (Maeda et al. 1996; Gao et al. 1998; Morrisey et al. 1998). Also, overexpression of *Xenopus gata4* and *gata5* has been shown to efficiently activate expression of *Sox17α* and *HNF1β* in animal caps (Weber et al. 1999). The *gata* motif of an albumin gene regulatory element is occupied in vivo suggesting that Gata factors directly regulate endodermal gene expression (Bossard and Zaret 1998). However, genetic evidence that Gata factors are involved in vertebrate definitive endoderm development has been elusive, perhaps because mouse *gata4* and *gata6* mutants arrest before differentiation of the definitive endoderm can be easily assessed (Kuo et al. 1997; Molkentin et al. 1997; Morrisey et al. 1998; Koutsourakis et al. 1999)..

Recently, we have shown that the zebrafish *faust* (*fau*) locus encodes Gata5 (Reiter et al. 1999). *fau/gata5* mutants display prominent defects in the differentiation and morphogenesis of heart and gut. Here, we demonstrate that zebrafish *gata5* is required for the differentiation of endoderm-derived organs such as the liver, pancreas and thyroid. Both loss- and gain-of-function experiments further demonstrate several roles for Gata5 much earlier during blastula and gastrula stages. First, Gata5 regulates the number of endodermal progenitors formed. Second, Gata5 promotes the expression of *sox17* and *axial* within the endodermal progenitors. We also show through a combination of mutant analysis and overexpression studies that Nodal and Oep signaling induces *gata5*, and that Gata5 acts in parallel to Bon/Mixer, but upstream of *cas* to regulate early endoderm development. These results identify Gata5 as a critical early regulator of

MATERIALS AND METHODS

Zebrafish strains

Zebrafish were maintained and staged as described (Westerfield 1995). All *fau/gata5* mutants depicted are of the *fau^{tm236a}* allele (Chen et al. 1996), except Figure 3.3b which depicts a *fau^{s26}* mutant (Reiter et al. 1999). Other mutant alleles used were *bon^{m425}* (Stainier et al. 1996), *cas^{ta56}* (Chen et al. 1996), *oep^{z1}* (Schier et al. 1996), *cyc^{b16}* (Hatta et al. 1991), and *sqt^{cz35}* (Feldman et al. 1998).

Wholemount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al. 1998); embryos older than 28 hpf were incubated in 0.003% phenylthiourea to inhibit pigmentation. Gastrulation stage embryos were genotyped post-in situ hybridization to confirm their identity. Briefly, this entailed serial rehydration with PBS + 0.1% Tween, proteinase K digestion at 55° C for 10 hours, and PCR genotyping. The *oep^{z1}* deletion was detected using primers 5'-GTGAGGGGTCAGAATGTGTG-3' and 5'-TCAGTCCAACGAACGGTAAC-3', *bon^{m425}* mutants were identified using a restriction fragment length polymorphism described by Kikuchi et al. (Kikuchi et al. 1999), and *cas^{ta56}* mutants were identified using a tightly linked simple sequence repeat polymorphism.

Immunohistochemistry using anti-human Insulin (Sigma) was performed using a previously described protocol (Alexander et al. 1998).

mRNA injection

Full length, capped *gata5*, *gfp*, *TARAM-A*, *TARAM-A** and *β-galactosidase* messages were synthesized from previously described templates (Renucci et al. 1996; Reiter et al. 1999) using the SP6 mMessage Machine system (Ambion). Embryos were injected at the 1-4

cell stage with 60 pg of *gata5* or *gfp* mRNA, or 170 pg of *TARAM-A* or *TARAM-A** mRNA. Some *gata5* and *gfp* injected embryos were coinjected with 150 pg *β-galactosidase* mRNA. β-Galactosidase staining was performed as described (Rodaway et al. 1999).

RESULTS

gata5 is required for the differentiation of endoderm-derived organs

We have previously demonstrated that *fau/gata5* mutants display variable defects in the morphogenesis of both the gut and the pharyngeal pouches (Reiter et al. 1999). These morphogenetic defects led us to investigate whether the differentiation of endoderm-derived organs was also affected. Even in *fau/gata5* mutants displaying minor morphogenetic defects (Figure 3.1a), expression of markers of liver (e.g., *gata4*, *gata6*, *hnf1ß*, *hex*; Figure 3.1b and data not included) and pancreas differentiation (e.g., *pdx1*, *islet1*, Insulin; Figure 3.1c and data not included) is dramatically reduced.

fau/gata5 mutants also exhibit defects in the differentiation of derivatives of the pharyngeal endoderm. In zebrafish, the pharyngeal endoderm gives rise to the thyroid, the thymic stroma and the epithelial components of the gills (Kimmel et al. 1995). In *fau/gata5* mutants, the thyroid primordium fails to express *nkx2.1* (Lazzaro et al. 1991; Figure 3.1d, arrow), and thymic differentiation is abnormal as assessed by the presence of *rag1*-expressing thymocytes (Willett et al. 1997; Figure 3.1e, arrow). Thymocytes do accumulate in other zebrafish circulation mutants such as *silent heart* (data not included), excluding the possibility that the absence of thymocytes in *fau/gata5* mutants is secondary to the lack of circulation. We therefore hypothesize that the *fau/gata5*-specific defect in thymic differentiation is secondary to abnormalities in the development of the endodermally-derived thymic stroma.

gata5 is expressed in the endodermal progenitors before *sox17*, *axial*, *gata4* or *gata6*

In zebrafish, *gata5* is first expressed at the dome stage (late blastula) in the most marginal cells (Figure 3.2a, arrowhead), a population that gives rise to the endoderm and some mesoderm (Reiter et al. 1999; Rodaway et al. 1999; Warga and Nusslein-Volhard 1999). *gata5* is also expressed in the yolk syncytial layer (Figure 3.2a, arrow), an

extraembryonic tissue underlying the blastoderm that may be equivalent to the mouse visceral endoderm (Ho et al. 1999). *gata5* expression is maintained in the endodermal progenitors throughout gastrulation (Figure 3.2c,g,k). Evidence that the large, flat *gata5*-expressing cells are endodermal progenitors comes from the similar morphology and distribution of these cells and the *axial*- and *sox17*-expressing endodermal progenitors (Figure 3.2k-m). Furthermore, these *gata5*-expressing cells also express *fkd2*, another endodermally expressed gene (Rodaway et al. 1999). Endodermal *gata5* expression persists during early segmentation stages but diminishes from the 5- to 10-somite stages. However, endodermal expression of *gata5* in the epithelium of the gut tube is again clearly evident at four days of development (Rodaway et al. 1999).

Comparison of *gata5* expression to that of other endodermally expressed genes reveals significant differences. Although both *bon/mixer* and *gata5* are initially expressed in the germ ring at late blastula stages, *gata5* expression is more marginally restricted than is *bon/mixer* expression (Figure 3.2a,b; Alexander et al. 1999)). Also, *bon/mixer*, unlike *gata5*, is not expressed after early gastrulation (Alexander et al. 1999).

The endodermal progenitors do not express *sox17* and *axial* until they have involuted, approximately 80 minutes after they have begun to express *bon/mixer* and *gata5* (Figure 3.2e,f,i,j; Alexander and Stainier 1999). During early gastrula stages, *gata5* and *bon/mixer* are uniformly expressed in the germ ring (Figure 3.2c,d,g,h). In contrast, *axial* and *sox17* are expressed only in a small number of the cells that comprise the germ ring (Figure 3.2e,f,i,j). Comparison of the expression patterns of *gata5* and *bon/mixer* with those of *sox17* and *axial* suggest that the endodermal progenitors are a subset of the *gata5*- and *bon/mixer*-expressing marginal cells. Fate mapping studies have demonstrated that, in addition to the endoderm, the marginal domain also gives rise to mesoderm such as the prechordal plate and myocardial precursors (Kimmel et al. 1990; Stainier et al. 1993).

In further contrast to *gata5*, zebrafish *gata4* and *gata6* are not expressed embryonically before segmentation stages. During gastrulation, *gata4* is not expressed

and *gata6* is expressed only extraembryonically in the YSL (Figure 3.2n and data not included). Only during segmentation stages do *gata4* and *6* begin to be expressed in the endoderm. Interestingly, comparison of the *gata4* and *6* expression with that of *fkd2*, a homolog of *HNF3γ* expressed throughout the posterior endoderm (Odenthal and Nusslein-Volhard 1998), reveals that *gata4* and *6* are not expressed throughout the endoderm, but in a subset of the *fkd2*-expressing endoderm (Figure 3.2o,p,q). Therefore, *gata5* is the first zebrafish *gata* gene to be expressed in the endoderm, and the only *gata* gene to be expressed throughout the endoderm. In contrast, mouse *gata4*, *5* and *6* are all expressed embryonically during gastrulation (Morrisey et al. 1997), raising the possibility that the functions performed by zebrafish *gata5* during gastrulation are shared by multiple *gata* genes in mouse.

gata5 regulates endoderm specification and differentiation during gastrulation

The early expression pattern of *gata5* led us to investigate whether *gata5* might be required not only for the late morphogenesis and differentiation of the gut, but also for early endoderm development. Analysis of *fau/gata5* mutants reveals two striking defects in the *sox17* and *axial* expression patterns during gastrulation. First, *fau/gata5* mutants display a reduction in the number of *axial*- and *sox17*-expressing endodermal progenitors (Figure 3.3). Although the severity of this defect is somewhat variable, the number of endodermal progenitors in *fau/gata5* mutants is reduced by approximately 40% at bud stage. Second, the endodermal progenitors that do form in *fau/gata5* mutants express variably reduced levels of both *sox17* and *axial* (Figure 3.3). Mutants of both *fau* alleles (fau^{tm236a} and fau^{s26}) exhibit very similar endodermal defects. Therefore, *gata5* is required for the development of a normal number of endodermal progenitors and for the expression of wild-type levels of *sox17* and *axial* within those endodermal progenitors.

gata5 overexpression expands endodermal gene expression

We have previously demonstrated that overexpression of *gata5* leads to the expanded and ectopic expression of myocardial genes and the occasional formation of ectopic, rhythmically beating myocardial tissue (Reiter et al. 1999). The requirement for *gata5* in endoderm development led us to investigate whether *gata5* overexpression was also able to increase endodermal gene expression. Injection of 60 pg of *gata5* mRNA into zebrafish embryos at the 1-4 cell stage leads to an increased number of cells expressing *sox17* (75/148 embryos show an increase in four independent experiments) and *axial* (25/66 embryos show an increase in two independent experiments; Figure 3.4a-c). Moreover, coinjection of *β-galactosidase* mRNA with *gata5* allowed us to detect the presumptive distribution of injected mRNA. The parallel distribution of β-galactosidase staining and expanded *sox17* and *axial* expression implies that Gata5 may autonomously induce the expression of these endodermal genes (Figure 3.4b,c). Interestingly, although the number of endodermal progenitors in the normal position directly overlying the YSL is increased, *sox17*- and *axial*-expressing cells are also observed more superficially within the embryo (Figure 3.4d; arrow). Similar superficial expression of *sox17* or *axial* are never observed in control *gfp*-overexpressing embryos outside of the axis. Despite their increased numbers, most of the *sox17*- and *axial*-overexpressing cells in *gata5*-overexpressing embryos display the large, flat morphology characteristic of endodermal progenitors.

The ability of Gata5 to induce endoderm is more limited in *cas* mutants than in *bon/mixer* or *oep* mutants

Other studies have demonstrated that zebrafish Bon/Mixer is required for early endoderm formation (Kikuchi et al. 1999), and that *bon/mixer* overexpression can expand the expression of *sox17* (Alexander and Stainier 1999). However, Bon/Mixer cannot induce *sox17* in *cas* mutants, arguing that induction of *sox17* expression by Bon/Mixer requires *cas* (Alexander and Stainier 1999). Overexpression of *gata5* in zebrafish *oep*,

bon/mixer and *cas* mutants allows us to also place *gata5* within this epistatic pathway. When overexpressed in *oep* or *bon/mixer* mutants, Gata5 is able to increase the number of *sox17*-expressing endodermal progenitors (Figure 3.5a,b, Table 1). Because Gata5 does not require either Oep or Bon/Mixer to activate expression of *sox17*, Gata5 must function downstream of, or in parallel to, Oep and Bon/Mixer.

Overexpression of *gata5* in *cas* mutants gave a qualitatively different result. In most cases examined, *gata5* overexpression does not restore *sox17* expression to *cas* mutants, indicating that *cas* is absolutely required for the regulation of *sox17* by Gata5 (Figure 3.5c, right upper panel). However, in 6 of 41 *gata5*-overexpressing *cas* mutants, a very few *sox17*-expressing cells were noted (Figure 3.5 lower panels, Table 1), indicating that Gata5 functions differently than other potent endoderm inducers such as *bon/mixer* and the constitutively active type I TGFβ receptor *TARAM-A** which cannot induce *sox17* expression in *cas* mutants (Alexander and Stainier 1999). Together, these results argue that Gata5 normally requires *cas* to induce expression of *sox17*, but when overexpressed, Gata5 can occasionally circumvent the requirement for *cas* in a few cells.

Nodal and Oep activate *gata5* expression

Zebrafish embryos mutant for both *cyc* and *sqt*, two *nodal* homologs, form no endoderm and very little mesoderm (Feldman et al. 1998). Mutants lacking both the maternal and zygotic contributions of *oep* (*MZoep*) display a very similar phenotype (Gritsman et al. 1999). Embryos lacking only the zygotic contribution of *oep* (*Zoep*) show much less profound mesodermal defects, but still form little or no endoderm (Schier et al. 1997).

Recently, Rodaway et al. (Rodaway et al. 1999) have shown that *cyc;sqt* double mutants do not express *gata5* in the blastoderm (also, Figure 3.6a). Here, we extend these results by demonstrating that *gata5* expression is also dependent on the Nodal cofactor Oep. Like *cyc;sqt* double mutants, *MZoep* mutants do not express *gata5* in the

blastoderm (Figure 3.6b). *Zoep* mutants initially express *gata5* normally within the germ ring (data not included). However, by midgastrulation stages, *Zoep* mutants show markedly diminished *gata5* expression in both the endoderm and mesoderm (Figure 3.6c). In contrast, embryos lacking only the maternal component of *oep* (*Moep*) develop normally, and do not exhibit defects in *gata5* expression (Figure 3.6b). Therefore, maternally provided *oep* is only sufficient to support *gata5* expression through blastula stages whereupon zygotic transcription of *oep* becomes required for *gata5* expression during gastrulation. Additionally, these results show that *gata5* expression in the extraembryonic YSL is not dependent on Nodal proteins or Oep, and therefore must be regulated by a distinct mechanism.

Nodal signaling is thought to be transduced by TGFβ-type receptors. Overexpression of the constitutively active type I activin receptor ALK-4 (ALK-4*) has been shown to increase *gata5* expression (Rodaway et al. 1999). TARAM-A*, another constitutively active type I TGFβ–type receptor, autonomously directs cells to an endodermal fate and can induce expression of endodermal genes such as *axial* and *sox17* (Peyrieras et al. 1998; Alexander and Stainier 1999). Therefore, we analyzed the effects of *TARAM-A** overexpression on *gata5*. Overexpression of wild-type *TARAM-A* had negligible effects on *gata5* expression (Figure 3.6d). However, overexpression of *TARAM-A**, in addition to inhibiting epiboly, increased *gata5* expression throughout the blastoderm (Figure 3.6d). Taken together with the reduced expression of *gata5* in *cyc;sqt* mutants and *oep* mutants, these results indicate that TGFβ signaling is both necessary and instructive for the expression of *gata5*.

Gata5 and Bon/Mixer do not regulate each other's expression

TGFβ signaling also induces expression of *bon/mixer* at a time and location similar to *gata5* (Alexander and Stainier 1999). To test for regulatory relationships between Bon/mixer and Gata5, we examined the expression of the two genes in the respective

mutants. *bon/mixer* is expressed normally in *faw/gata5* mutants (Figure 3.6e), and similarly, *gata5* is expressed normally during late blastula stages in *bon/mixer* mutants (Figure 3.6f). These results indicate that Bon/Mixer and Gata5 are not required for each other's expression and, therefore, that they function in parallel. Consistent with the hypothesis that Bon/Mixer and Gata5 jointly promote endoderm formation without regulating each other, overexpression of *gata5* does not expand *bon/mixer* expression (data not shown), but *gata5* overexpression can increase endodermal gene expression in *bon/mixer* mutants (Figure 3.5b).

Embryos mutant for both *bon/mixer* and *faw/gata5* produce very few endodermal progenitors (Figure 3.6g). *bon/mixer* mutants produce, on average, 45 *sox17*-positive endoderm cells (n=13) and 31 *axial*-positive endoderm cells (n=7) at 90% epiboly, while *bon/mixer;faw/gata5* double mutant siblings produce 13 *sox17*-positive cells (n=6) and 5 *axial*-positive cells (n=7). Occasionally, *bon/mixer;faw/gata5* double mutants display no endodermal gene expression (2/13). These results indicate that Gata5 and Bon/Mixer act in concert to regulate the formation of most of the endodermal progenitors.

faw/gata5 interacts genetically with *cas* and *bon/mixer*

Mutations in *cas*, *bon/mixer* and *faw/gata5* are completely recessive; heterozygous embryos develop normally into healthy, viable adults indistinguishable from their non-heterozygous siblings. However, when fish heterozygous for a mutation in either *cas* or *bon/mixer* are crossed to fish heterozygous for a *faw/gata5* mutation, a small percentage of their progeny exhibit morphologically abnormal hearts (Table 2). Similar defects are noted among the progeny of *bon/mixer* and *cas* heterozygote intercrosses. The cardiac defects of affected embryos range in severity from dilated hearts that fail to pump blood to cardia bifida. However, not all genes involved in endoderm formation genetically interact; the progeny of *oep* heterozygotes and *cas*, *bon/mixer* or *faw/gata5* heterozygotes

do not display similar defects in cardiac morphogenesis (Table 2), and similar cardiac defects are never observed among the progeny of wild-type fish (n>500).

As each of these mutations affect endoderm development, we investigated whether the transheterozygous embryos also exhibited defects in endoderm development. We analyzed intercrosses between fish heterozygous for mutations in *cas*, *bon/mixer* or *fau/gata5* for defects in *sox17* expression. In each clutch, a few embryos displayed a reduced number of endodermal progenitors, reduced expression of *sox17* within endodermal progenitors or a combination thereof (Figure 3.7). Once genotyped, these embryos were found to be transheterozygous for the mutations carried by their parents. These results reveal that *cas*, *bon/mixer* and *fau/gata5* all interact with each other genetically, and that embryos transheterozygous for mutations in *cas*, *bon/mixer* or *fau/gata5* exhibit endodermal and cardiac defects at low penetrance.

DISCUSSION

Gata5 is required for the development of endoderm-derived organs

Our analysis of *fau/gata5* mutants demonstrates a requirement for *gata5* in the development of the liver, pancreas, thyroid and thymus, indicating that *gata5* is essential for the progression of endoderm to a differentiated state. Although some of the defects in endodermal organ differentiation may be secondary to abnormal morphogenesis, it is unlikely that this is true for liver and pancreas development, as liver and pancreas differentiation are profoundly affected in *fau/gata5* mutants even when the gut forms normally at the embryonic midline.

The defects in organ differentiation in *fau/gata5* mutants may be due to both autonomous and non-autonomous influences. Although our studies have emphasized the autonomous role of Gata factors in promoting endodermal gene expression, tissue recombination experiments in mouse and chick have demonstrated that the precardiac mesoderm can induce the endoderm to differentiate as liver (LeDouarin 1975; Gualdi et al. 1996). We have previously demonstrated that *fau/gata5* mutants display severe defects in the differentiation of the precardiac mesoderm (Reiter et al. 1999). Therefore, it is possible that the liver differentiation defects observed in *fau/gata5* mutants are due to the combination of loss of inductive signals from the anterior lateral plate mesoderm and the autonomous reduction of Gata5 function in the endoderm. In addition, the proximity of the developing pharyngeal endoderm to the anterior lateral plate mesoderm leads us to hypothesize that pharyngeal endoderm differentiation is also dependent on signals from the anterior lateral plate mesoderm.

Gata5 induces endodermal progenitors and promotes endodermal differentiation

Analysis of both loss- and gain-of-function experiments reveals that Gata5 plays several roles during early endoderm development. First, Gata5 regulates the number of

endodermal progenitors that form during gastrulation; *gata5* mutants have on average 40% fewer endodermal progenitors than do their wild-type siblings at bud stage, and overexpression of *gata5* expands the number of endodermal progenitors as assessed by the expression of *sox17* and *axial*. *gata5* is expressed during late blastula stages, before blastomere fate has been restricted to a single germ layer. Therefore, we hypothesize that Gata5 functions during late blastula stages to help define the number of cells that will become the endoderm. Alternatively, Gata5 could affect the proliferation or survival of the endoderm during gastrulation. In either case, the sensitivity of the endoderm to the level of *gata5* expression indicates that Gata5 is an early and critical regulator of the number of endodermal progenitors.

In the normal embryo, *sox17*- and *axial*-expressing endoderm cells are always closely apposed to the underlying YSL. However, in *gata5*-overexpressing embryos, *sox17*- and *axial*-expressing cells are observed superficial to the normal position of the endoderm, implying that *gata5* overexpression either prevents endodermal progenitors from assuming their proper position within the hypoblast, or induces ectopic endodermal gene expression.

Coinjection of *β-galactosidase* mRNA with *gata5* allows monitoring of the distribution of the exogenous messages within the embryo. Increased endodermal gene expression is observed only within areas of β-galactosidase activity, suggesting that Gata5 induces endodermal fate either cell autonomously or at a short distance. Previously, we have shown that overexpression of *gata5* appears to also autonomously expand the myocardial precursor population (Reiter et al. 1999). How some *gata5*-expressing cells become endoderm and others myocardium remains an interesting, but answered, question.

Additionally, we have demonstrated that Gata5 promotes the expression of *sox17* and *axial* within the endodermal progenitors. Expression of *sox17* and *axial* is reduced within the endoderm of *fau/gata5* mutants, while expression of *sox17* in the forerunner cells and *axial* in the axial mesoderm is unaffected. This aspect of the phenotype is

qualitatively different from the endoderm defect of *bon/mixer* mutants. While *bon/mixer* mutants also form few endodermal progenitors, those that do form express wild-type levels of *sox17* and *axial* (for example, see Figure 3.7g). As *gata5* expression is maintained in the endodermal progenitors throughout gastrulation, we hypothesize that *gata5* acts autonomously within the endodermal progenitors to promote the expression of downstream endodermal regulators such as *sox17* and *axial*.

Nodal and Oep signaling induces *gata5* expression

With Rodaway et al. (Rodaway et al. 1999), we have shown that the Nodal proteins Cyc and Sqt and their cofactor Oep are necessary for the induction of *gata5* expression. Nodal signaling also appears to be sufficient to induce *gata5*, as overexpression of the constitutively active TGFβ receptors ALK-4* (Rodaway et al. 1999) and TARAM-A* increases *gata5* expression. Rodaway et al. have also demonstrated that overexpression of *activin* also can induce *gata5* expression (Rodaway et al. 1999). However, endogenous Activin is not able to activate *gata5* expression in zebrafish mutants lacking Cyc and Sqt, indicating that if Activin does have a role in *gata5* regulation, it must act through or cooperate with required Nodal signals.

The observation that *gata5* overexpression can induce endodermal gene expression in *Zoep* mutants further supports the idea that Gata5 functions downstream of Nodal signaling. Although *gata5* overexpression can nearly triple the number of endodermal progenitors present in *Zoep* mutants, overexpression of *gata5* does not restore a wild-type number of *sox17*-expressing cells, even at higher injected doses of *gata5* (data not included). *bon/mixer*, much like *gata5*, is initially expressed normally in the germ ring of *Zoep* mutants, but then quickly diminishes during gastrulation. Perhaps Gata5 is limited in its ability to induce endoderm in *Zoep* mutants by the reduction in *bon/mixer* expression. Alternatively, there could be one or more other Oep-dependent factors (denoted X in Figure 3.8) that promote endoderm development together with Bon/Mixer

and Gata5. Several paired-type homeobox transcription factors other than Bon/Mixer have been implicated in *Xenopus* early endoderm development, and it may be that a zebrafish homolog of Milk (Ecochard et al. 1998), Mix.1 (Rosa 1989) or one of the Bix factors (Tada et al. 1998; Casey et al. 1999) also functions in endoderm development downstream of Nodal signaling.

Bon/Mixer and Gata5 independently promote endoderm development

Zebrafish *bon/mixer* and *gata5* show striking functional similarity. Both genes encode transcription factors induced in the margin of late blastula stage embryos by Nodal signaling (Alexander et al. 1999). Mutations in both genes reduce the number of endodermal progenitors (Kikuchi et al. 1999). Overexpression of either gene can increase the number of endodermal progenitors (Alexander and Stainier 1999). These similarities raise the possibility that Bon/Mixer and Gata5 act linearly within a pathway regulating endoderm induction. However, our results demonstrate that expression of *bon/mixer* does not depend upon Gata5 activity and, conversely, expression of *gata5* does not depend upon Bon/Mixer activity. The absence of regulation between these two gene products indicates that the endoderm induction pathway bifurcates, such that Nodal signaling induces both *gata5* and *bon/mixer*, and that Gata6 and Bon/Mixer subsequently act in parallel to promote endodermal specification (Figure 3.8). Consistent with this hypothesis, overexpression of *gata5* in *bon/mixer* mutants is able to expand the number of endodermal progenitors, indicating that Gata5 does not act through Bon/Mixer.

Analysis of *bon/mixer;fau/gata5* double mutants further supports this model of cooperative specification of endoderm. Although embryos mutant for either *gata5* or *bon/mixer* form fewer endodermal progenitors than wild-type siblings, double mutants form very few or none. Thus, most of the endoderm that does form in either *fau/gata5* or *bon/mixer* single mutants is due to the activity of the other factor, arguing that Bon/Mixer and Gata5 together act as effectors by which Nodal signaling induces the endoderm. The

very small number of endodermal progenitors that do arise in *bon/mixer;fau/gata5* double mutants may reflect residual activity of the mutant Gata5 and Bon/Mixer proteins, or may be due to a Nodal-dependent factor that functions alongside Gata5 and Bon/Mixer to promote endoderm development (i.e., X in Figure 3.8).

gata5 and *bon/mixer* are also expressed together in germ ring cells that become mesoderm, indicating that expression of these genes is not sufficient to induce endodermal fate. It remains unclear how the embryo segregates marginal blastomeres into committed mesoderm and endoderm. Resolution of this problem will require further study of the complex interactions in the zebrafish germ ring.

Gata5 acts upstream of *cas*

Overexpression of *gata5* increases the number of endodermal progenitors in wild-type embryos and in *oep* and *bon/mixer* mutants. In contrast, overexpression of *gata5* in *cas* mutants usually does not induce expression of *sox17*, placing *cas* function between Gata5 and Sox17. Similarly, *bon/mixer* and *TARAM-A** overexpression can increase endodermal gene expression, but not in *cas* mutants (Alexander and Stainier 1999). Therefore, Gata5, like Bon/Mixer and TARAM-A*, appears to function upstream of *cas*.

However, Gata5 is distinguished from Bon/Mixer and TARAM-A*, in that overexpression of *gata5* in *cas* mutants is occasionally able to induce the expression of *sox17* in a small number of cells. Therefore, in rare instances Gata5 can bypass the endodermal requirement for *cas*. As *gata5* continues to be expressed in the endodermal progenitors after their induction, the process in which *cas* presumably functions, perhaps Gata5 can also act downstream of the *cas* gene product.

Although these results demonstrate that endodermal specification by Gata5 requires *cas*, they do not indicate the mechanism by which *cas* and *gata5* interact. *cas* could encode an essential transactivator of *sox17* and *axial* through which Gata5 and Bon/Mixer must function. Alternatively, the *cas* gene product could antagonize a

repressor of *sox17* and *axial* expression (i.e., Y in Figure 3.8). This hypothesis predicts that in the absence of *cas* activity, the repressor Y prevents Gata5 and Bon/Mixer from promoting endodermal gene expression except in cases of profound *gata5* overexpression. Identification of the *cas* gene will help to discriminate between these models.

Transheterozygous genetic interactions in the zebrafish

The *fau/gata5*, *bon/mixer* and *cas* mutations show transheterozygous interactions with each other significant enough to conspicuously perturb development, although less severely than homozygous mutations. One quarter of progeny of heterozygote intercrosses are predicted to be transheterozygous. However, the incidence of obvious defects among such progeny is only 3 to 18 percent. Therefore, cardiac defects in transheterzygous embryos is semipenetrant.

Interestingly, this phenomenon of transheterozygous genetic interaction is not applicable to all genes involved in endoderm development, as *oep* does not interact with *fau/gata5*, *bon/mixer* or *cas* in the same manner. Why mutations in some genes interact genetically while others do not is unclear. Perhaps the embryo is especially sensitive to changes in the dosage of *fau/gata5*, *bon/mixer* and *cas*, but less so to changes in *oep* dosage.

The occurrence of transheterozygous phenotypes raises the possibility of using genetic interactions to expedite screens for new mutations. The F_1 progeny of a mutagenized fish could be crossed to fish heterozygous for mutations previously demonstrated to have the capacity to interact with a range of mutations of interest. If the progeny of such an intercross displayed defects in the developmental process of interest (for example, heart morphogenesis), the F_1 fish could be outcrossed, and its progeny rescreened for recessive mutations affecting heart development. By selecting F_1 fish most likely to provide relevant mutations, the amount of work and space required for effective screening could be dramatically reduced. Although this strategy is generally applicable to

any developmental process for which transheterozygous mutants display a clear phenotype, there are several caveats. First, such a strategy is likely to identify only a small subset of the total number genes involved in the events of interest. To extend the heart development example, although *oep* is required for heart morphogenesis, because oep mutations do not interact with *bon/mixer*, *fau/gata5* or *cas* mutations, such a screen would not identify alleles of *oep*. Second, the productivity of such a screen would critically depend upon the robustness of the genetic interaction. Less robust interactions would be unlikely to identify weak alleles of relevant mutations or mutations that do not have strongly affect the process under study.

CONCLUSIONS

Gata transcription factors are required for endoderm induction and differentiation in both *Drosophila* and *C. elegans*. Here, we have demonstrated that the vertebrate Gata factor, Gata5, is required for early endoderm development in zebrafish. During blastula and gastrula stages, Gata5 regulates the number of endodermal progenitors and promotes the transcription of downstream transcription factor genes such as *sox17* and *axial*. Later in development, *gata5* mutants exhibit widespread defects in the morphogenesis and differentiation of endoderm-derived organs, indicating that Gata5 is required for the proper maturation of the endoderm.

Also, we have placed Gata5 within a previously described pathway that underlies zebrafish endoderm formation (Alexander and Stainier 1999; Figure 3.8). We demonstrated through both loss- and gain-of-function experiments that *gata5*, like *bon/mixer*, is induced by Nodal signaling and is dependent upon Oep activity. Within the germ ring, Gata5 and Bon/Mixer do not regulate each other, but rather function in parallel to specify endodermal progenitors and promote the expression of *sox17* and *axial*. Gata5, like Bon/Mixer, functions upstream of or parallel to the *cas* gene product, but may circumvent the requirement for *cas* when overexpressed.

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Figure 3.1

Differentiation of endoderm-derived organs requires *gata5*. Expression of (a) *fkd7*, (b) *gata4*, (c) Insulin, (d) *nkx2.1*, and (e) *rag1* in wild-type embryos and *fau* mutant siblings at (a,b,d) 44 hours post-fertilization (hpf), (c) 72 hpf, and (e) 5.5 days post-fertilization. All views are dorsal with anterior to the top, except (d) which shows an anterior view. **(a)** *fkd7* is expressed in the floorplate and gut tube. The anterior gut of the *fau/gata5* mutant has failed to migrate toward the embryonic midline (arrow), and the posterior gut has failed to loop (arrowhead). **(b)** *gata4* is expressed in the wild-type liver (arrow), but in few cells in *fau/gata5* mutants (arrowhead). **(c)** Similarly, production of Insulin by the pancreas is profoundly reduced in *fau/gata5* mutants. **(d)** The thyroid (arrow), a derivative of the pharyngeal endoderm, does not form or does not express *nkx2.1* in *fau/gata5* mutants. *nkx2.1* is also expressed dorsally in the ventral forebrain (arrowhead). **(e)** *rag1* expression, which identifies thymocytes in the bilateral thymic primordia (arrow), is absent in *fau* mutants.

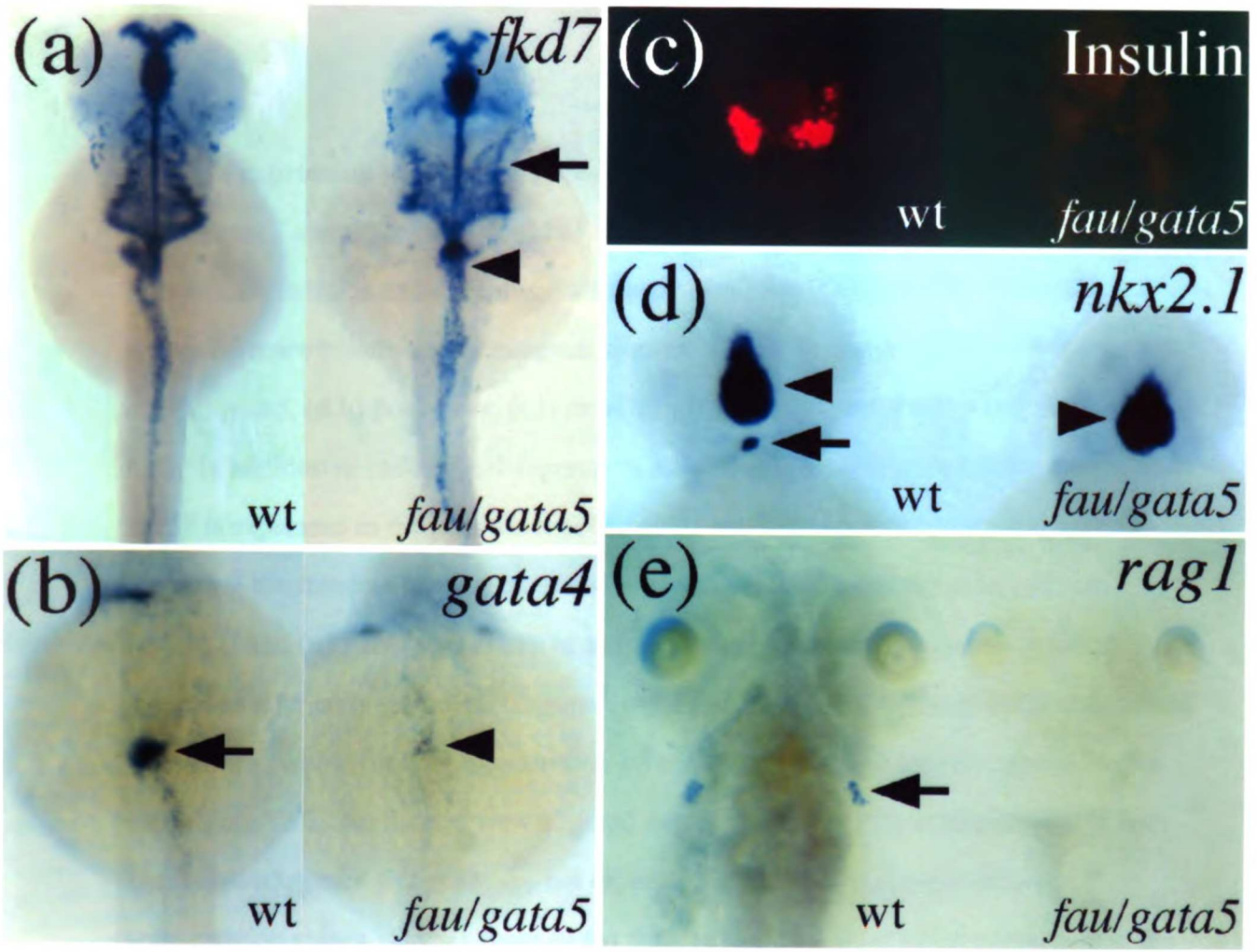
(a)
fkd7
wt
fau/gata5
(b)
gata4
wt
fau/gata5
(c)
Insulin
wt
fau/gata5
(d)
nkx2.1
wt
fau/gata5
(e)
rag1
wt
fau/gata5

Figure 3.2

Relative expression of *gata5*, *bon/mixer*, *axial*, *sox17*, *gata4* and *gata6* in wild-type endoderm. Lateral views of **(a)** *gata5* and **(b)** *bon/mixer* expression at dome stage. *gata5* is expressed in the marginal blastomeres (arrowhead) and YSL (arrow). *bon/mixer* is expressed more broadly in the marginal domain. Dorsal (c-f) and animal pole (g-j) views of **(c,g)** *gata5*, **(d,h)** *bon/mixer*, **(e,i)** *axial* and **(f,j)** *sox17* expression at late shield stage (6 hpf). In addition to endodermal expression, *axial* is expressed in the axial mesoderm and *sox17* is expressed in the forerunner cells. *gata5* and *bon/mixer* are expressed in more germ ring blastomeres than are *axial* and *sox17*. Dorsal views of **(k)** *gata5*, **(l)** *bon/mixer*, **(m)** *axial* and **(n)** *sox17* expression at early bud stage (9.5 hpf). bon/mixer is not expressed after early gastrulation. *gata5*, *axial* and *sox17* are expressed in the endodermal progenitors, which form a discontinuous layer of large, flat cells closely apposed to the underlying YSL. **(o)** Lateral view of *gata6* expression in the YSL at shield stage (6 hpf). Unlike *gata5*, *gata4* and *gata6* are not expressed in the embryo proper before segmentation stages. Dorsal views of **(p)** *fkd2*, **(q)** *gata4* and **(r)** *gata6* expression in the posterior endoderm at the 12 somite stage (15 hpf). *fkd2* is expressed throughout the posterior endoderm, while endodermal expression of *gata4* and *gata6* is more limited.

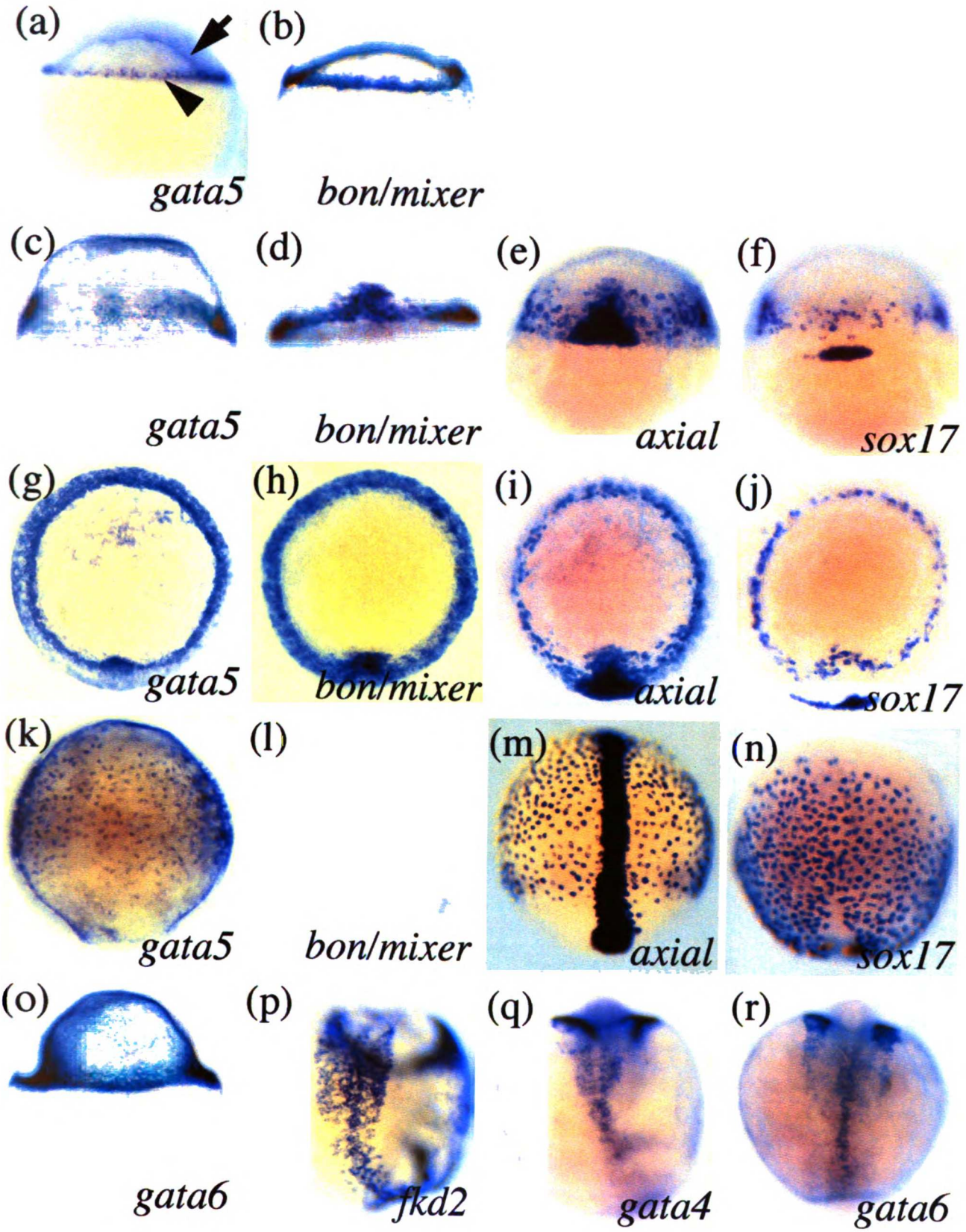
(a)
(b)
gata5
bon/mixer
(c)
(d)
(e)
(f)
gata5
bon/mixer
axial
sox17
(g)
(h)
(i)
(j)
gata5
bon/mixer
axial
sox17
(k)
(l)
(m)
(n)
gata5
bon/mixer
axial
sox17
(o)
(p)
(q)
(r)
gata6
fkd2
gata4
gata6

Figure 3.3

Gata5 is required for early endoderm development. Dorsal views of **(a)** *sox17* and **(b)** *axial* expression in *faw/gata5* mutants and wild-type siblings at early bud stage (9.5 hpf). The identity of mutants was confirmed by genotyping (see Materials and methods). *faw/gata5* mutants form fewer endodermal progenitors than their wild-type siblings. While this defect is variable in its severity, the number of progenitors is reduced 40% on average at early bud stage. Also, *faw/gata5* mutants express *sox17* and *axial* at lower levels in the endodermal progenitors.

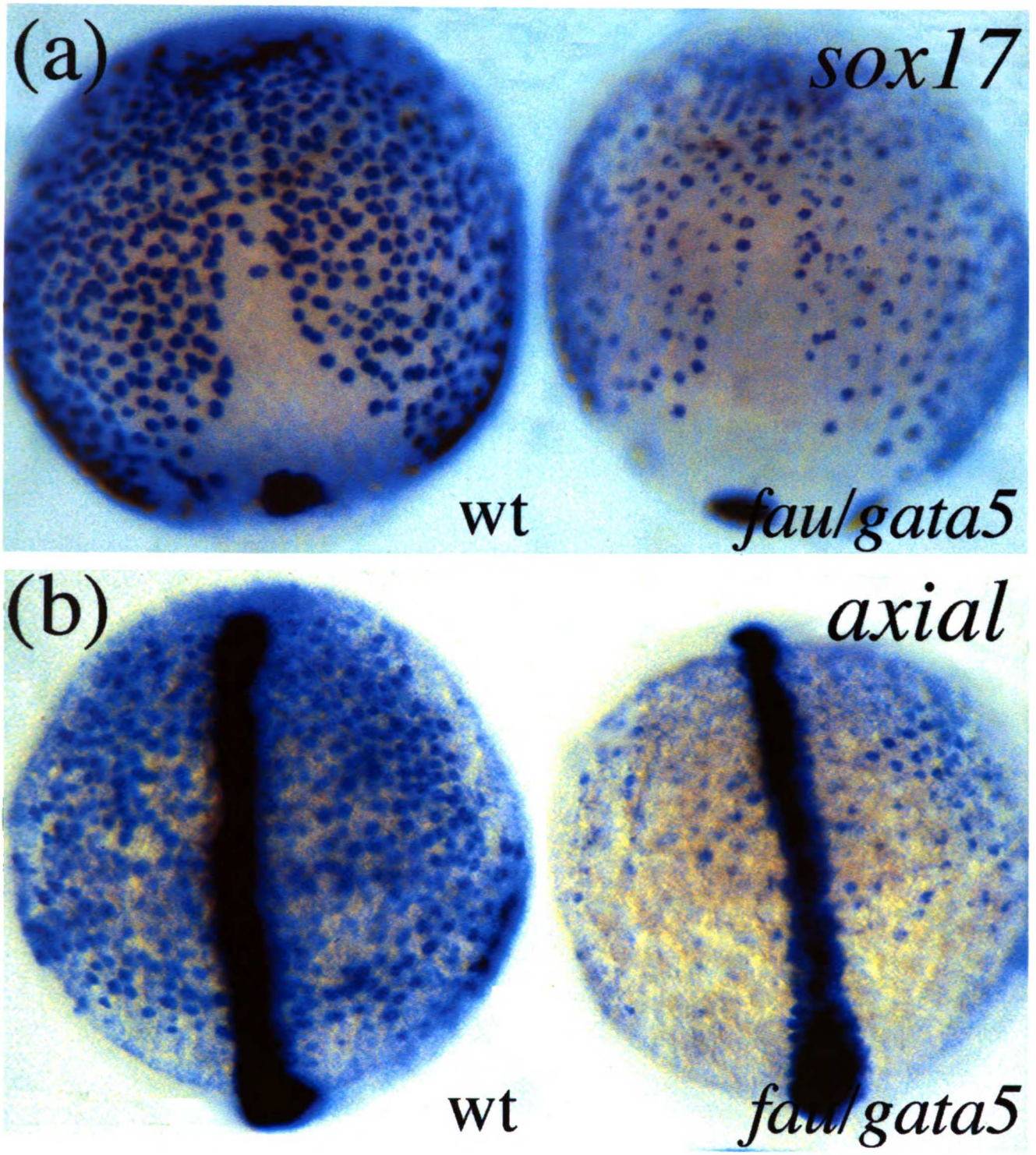
(a)
sox17
wt
faul/gata5
(b)
axial
wt
faul/gata5

Figure 3.4

***gata5* overexpression expands the expression of *sox17* and *axial*.** At the two cell stage, one blastomere was injected with either 60 pg *gfp* or 60 pg *gata5* mRNA. Dorsal views of (a,b,d) *sox17* and (c) *axial* at (a-c) 90% epiboly (9 hpf) and 75% epiboly (8 hpf). **(a)** While *gfp* overexpression has no effect on *sox17* expression, overexpression of *gata5* increases the number of cells expressing *sox17*. **(b,c)** Embryos were co-injected with 150 pg *β-galactosidase* mRNA and analyzed by β-galactosidase staining (turquoise) before in situ hybridization (dark blue). Overexpression of *gata5* also expands expression of *axial*. Regions of increased *sox17* and *axial* expression always overlapped with β-galactosidase staining (n>100). **(d)** Optical section of *gfp*- and *gata5*-overexpressing embryos. While all the *sox17*-expressing cells at the dorsal margin of the control embryo directly overlie the YSL, many *sox17*-expressing cells in the *gata5*-overexpressing embryo are superficial to the deep layer of the hypoblast (arrow).

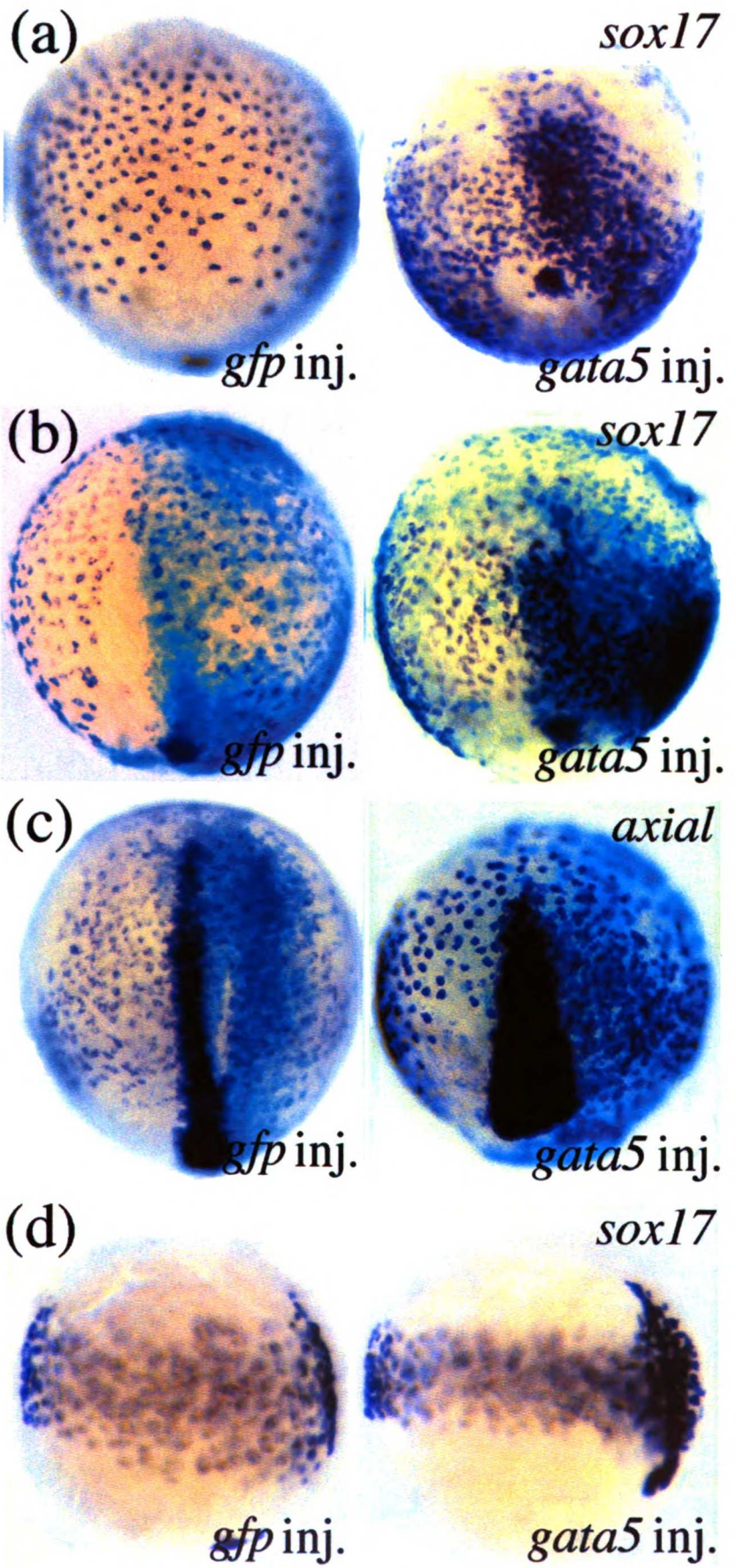
(a)
sox17
gfp inj.
gata5 inj.
(b)
sox17
gfp inj.
gata5 inj.
(c)
axial
gfp inj.
gata5 inj.
(d)
sox17
gfp inj.
gata5 inj.

Figure 3.5

***gata5* induction of *sox17* expression in *oep*, *bon/mixer* and *cas* mutants.** Embryos were injected with 60 pg *gfp* or *gata5* mRNA at the 1-4 cell stage, and examined for *sox17* expression at 90% epiboly (9 hpf). *gfp* injection did not affect *sox17* expression. Overexpression of *gata5* increased the number of *sox17*-expressing cells in **(a)** *oep* mutants and **(b)** *bon/mixer* mutants. **(c)** In *cas* mutants, *gata5* overexpression did not usually induce any *sox17* expression (for example, right upper panel). However, in 6 of 41 cases, *sox17* expression was observed in *gata5*-overexpressing *cas* mutants (lower panels).

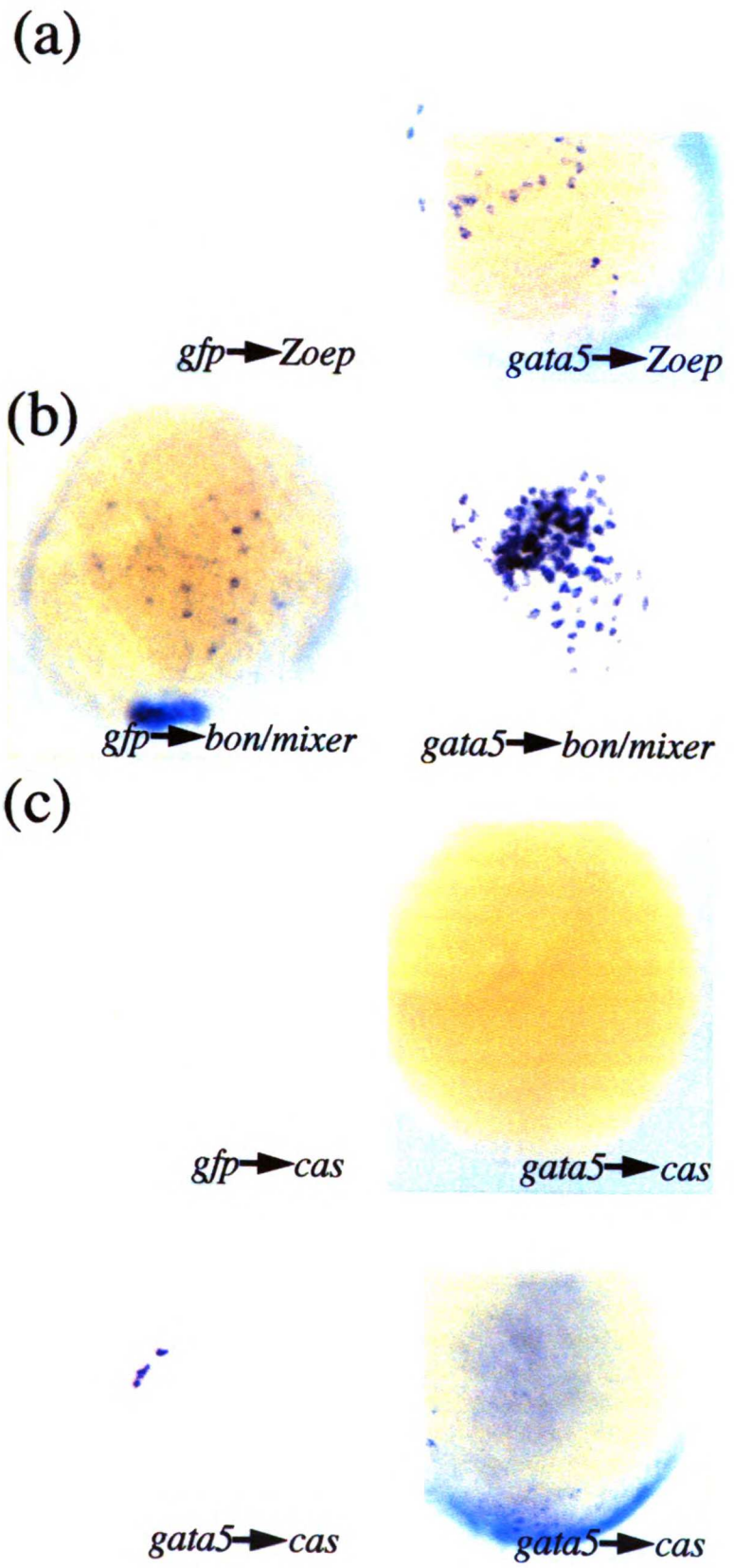
(a)
gfp→Zoep
gata5→Zoep
(b)
gfp→bon/mixer
gata5→bon/mixer
(c)
gfp→cas
gata5→cas
gata5→cas

Figure 3.6

Regulation of *gata5* expression. Dorsal views of (a-e) *gata5*, (f) *bon/mixer* and (g) *sox17* expression at (a,b,f) 50% epiboly (5.3 hpf), (c,d) 80% epiboly (8.5 hpf), (e) germ ring (5.7 hpf) and (g) 90% epiboly (9 hpf). **(a)** In *cyc;sqt* double mutants, *gata5* is expressed normally in the YSL, but is not expressed in the blastoderm. **(b)** Similarly, *gata5* is not expressed in the blastoderm of *MZoep* mutants. **(c)** Expression of *gata5* in the blastoderm is not maintained during gastrulation in *Zoep* mutants. **(d)** Injection of 170 pg of wild-type *TARAM-A* mRNA does not affect *gata5* expression. Injection of an equal amount of *TARAM-A** mRNA inhibits epiboly, and expands *gata5* expression. **(e)** *gata5* expression in the germ ring is indistinguishable in *bon/mixer* mutants and their wild-type siblings. **(f)** Similarly, *fau/gata5* mutants and wild-type siblings express *bon/mixer* equally. **(g)** While endodermal expression of *sox17* is reduced in *fau/gata5* and *bon/mixer* mutants, it is nearly absent in *fau/gata5;bon/mixer* double mutants.

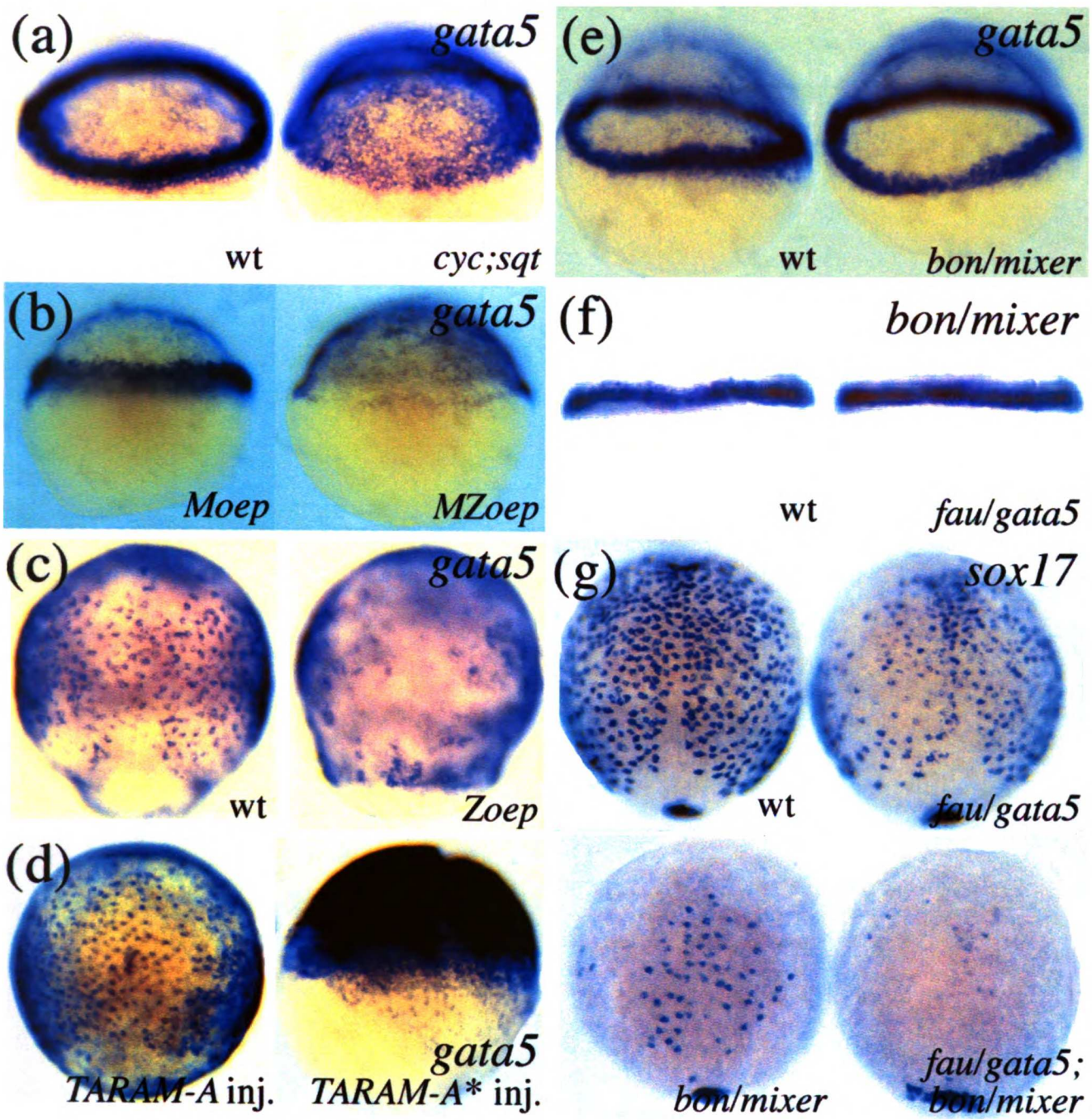
(a)
gata5
wt
cyc;sqt
(e)
gata5
wt
bon/mixer
(b)
gata5
Moep
MZoep
(f)
bon/mixer
wt
fau/gata5
(c)
gata5
wt
Zoep
(g)
sox17
wt
fau/gata5
(d)
TARAM-A inj.
gata5
TARAM-A* inj.
bon/mixer
fau/gata5;
bon/mixer

Figure 3.7

Embryos transheterozygous for *fau/gata5* and *bon/mixer* or *cas* mutations exhibit defects in early endoderm development. Dorsal views of *sox17* expression at early bud stage (9.5 hpf). Compared to **(a)** wild-type, embryos transheterozygous for **(b)** *fau/gata5* and *bon/mixer*, **(c)** *fau/gata5* and *cas*, and **(d)** *bon/mixer* and *cas* exhibit reduced expression of *sox17*.

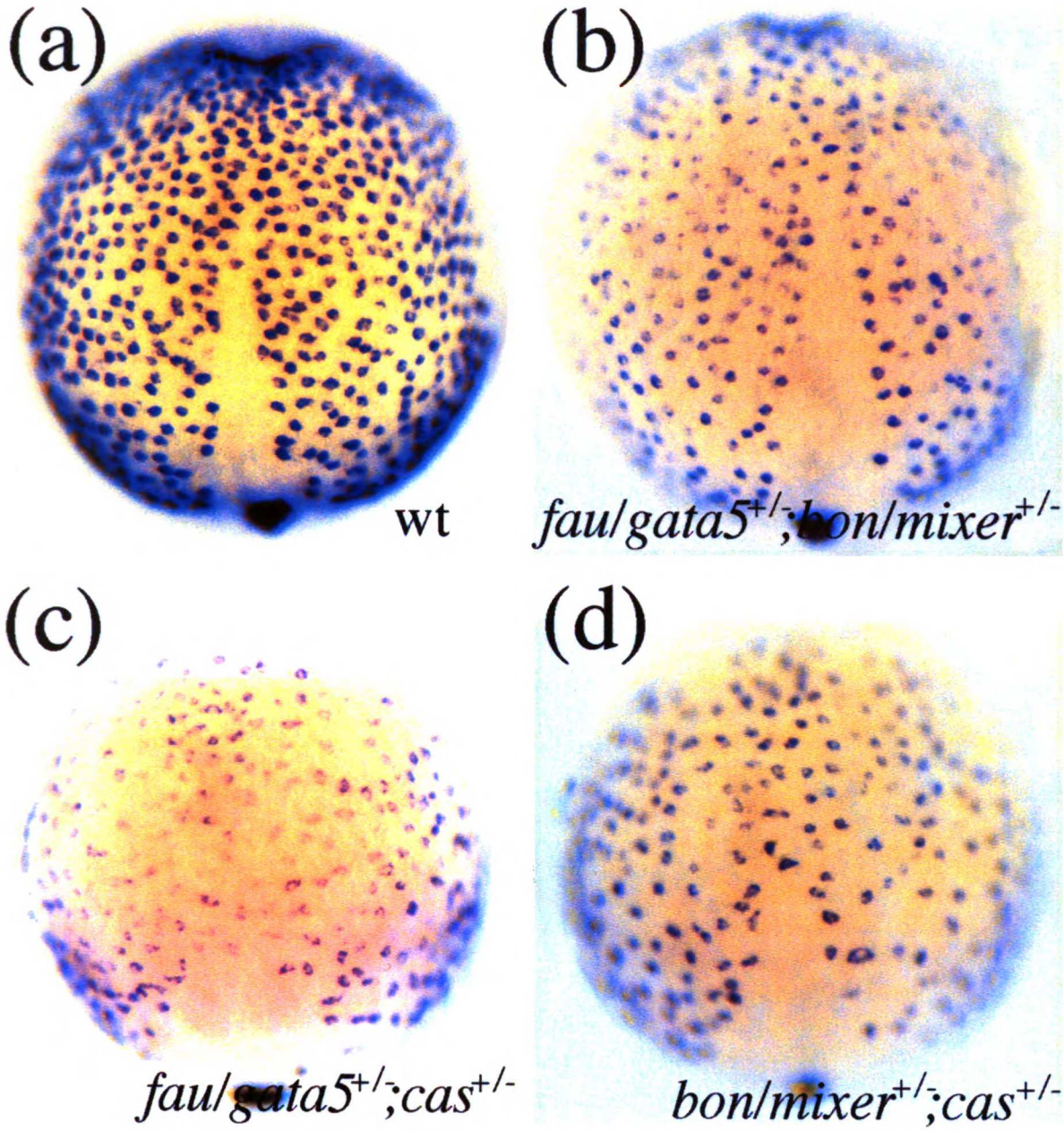
(a)
wt
(b)
fau/gata5+/-;bon/mixer+/-
(c)
fau/gata5+/-;cas+/-
(d)
bon/mixer+/-;cas+/-

Figure 3.8

A model of zebrafish endoderm development, based upon that of Alexander et al. (Alexander and Stainier 1999). The Nodal proteins encoded by *cyc* and *sqt* act through TGFβ-type receptors. Oep is also essential for Nodal signaling. Nodal signaling induces the expression of *bon/mixer* and *gata5*. We hypothesize that other Nodal- and Oep-dependent factors, represented here by X, may also be required for endoderm development. Bon/Mixer and Gata5 both promote the expression of *sox17* and *axial,* but do not regulate each other's expression. Although *cas* is required for *sox17* expression and appears to function downstream of, or in parallel to, Bon/Mixer and Gata5, it is not yet clear how it interacts with other members of the pathway. Here, we have placed it at two possible positions. *cas* may encode an obligate downstream effector of Bon/Mixer and Gata5, or the *cas* gene product may antagonize a repressor of *axial* and *sox17* expression represented by Y.

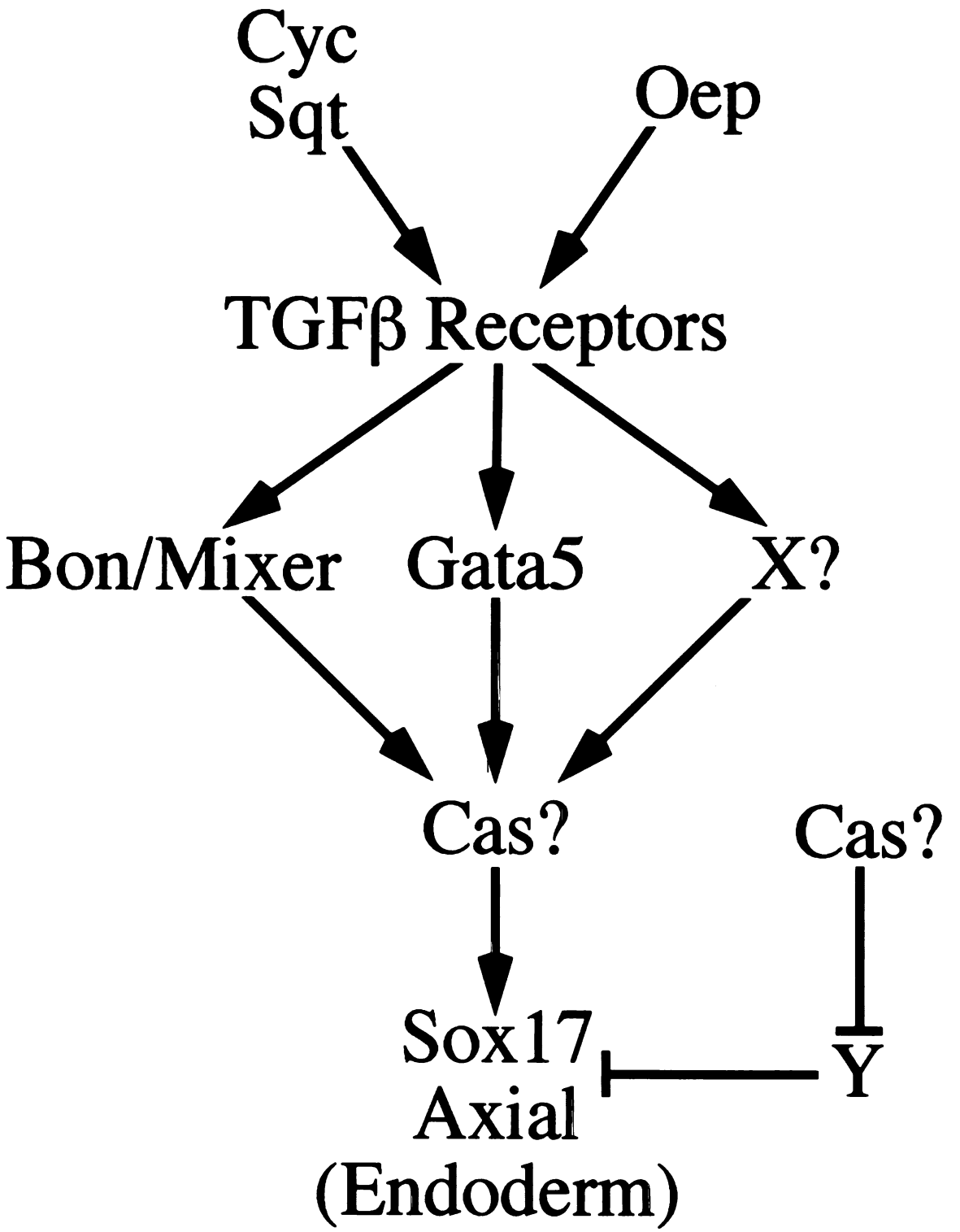
Cyc
Sqt
Oep
TGFβ Receptors
Bon/Mixer
Gata5
X?
Cas?
Cas?
Sox17
Axial
(Endoderm)
Y

Table 3.1

Overexpression of *gata5* increases endodermal gene expression in endoderm mutants

	Average number of *sox17*-expressing cells		
Injected mRNA	*oep*	*bon/mixer*	*cas*
gfp	7.3 (*n*=15)	21.5 (*n*=6)	0 (*n*=12)
gata5	19.6 (*n*=9)	62.5 (*n*=4)	0.5 (*n*=41)

Embryos were injected with 60 pg *gfp* or *gata5* mRNA at the 1-4 cell stage and examined for *sox17* expression at 90% epiboly (9 hpf). After in situ hybridization, embryos were genotyped to unambiguously identify mutants.

Table 3.2

Transheterozygous interactions between *fau/gata5*, *bon/mixer* and *cas* mutations

	bon/mixer	*cas*	*oep*
fau/gata5	6.4% (36/557, 9 cardia bifida)	18% (18/101, 2 cardia bifida)	0% (0/>200)
bon/mixer		3.2% (3/96)	0% (0/>200)
cas			0% (0/>200)

Table shows the percentage of progeny exhibiting cardiac defects with respect to parental genotype. Hearts were scored as defective if they failed to pump blood at 36 hpf and 72 hpf. The number of embryos in which the defective heart was also bifid is noted. Each total is the sum of the observations for at least two clutches.

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Chapter 4: Bmp2b and Oep promote myocardial differentiation through their regulation of *gata5*

SUMMARY

Members of both the bone morphogenetic protein (BMP) and EGF-CFC families are implicated in vertebrate myocardial development. Zebrafish *swirl* (*swr*) encodes *bmp2b*, a member of the BMP family required for patterning of the dorsal-ventral axis. Zebrafish *one-eyed pinhead* (*oep*) encodes a maternally- and zygotically-expressed member of the EGF-CFC family essential for Nodal signaling. Both *swr/bmp2b* and *oep* mutants exhibit severe defects in myocardial development. *swr/bmp2b* mutants exhibit reduced or absent expression of *nkx2.5*, the earliest known myocardial marker. Embryos lacking zygotic *oep* display cardia bifida and, as we show here, also display reduced or absent *nkx2.5* expression. Recently, we demonstrated that the zinc finger transcription factor Gata5 is an essential regulator of *nkx2.5* expression, and that overexpression of *gata5* can lead to ectopic expression of myocardial genes. Here, we show that both *swr/bmp2b* and *oep* mutants fail to express *gata5* in the myocardial precursors. Forced expression of *gata5* in *swr/bmp2b* and *oep* mutants restores robust *nkx2.5* expression. Moreover, overexpression of *gata5* in *oep* mutants can also restore expression of *cmlc1*, a marker of myocardial maturation, as well as cardiac fusion. These results indicate that both Bmp2b and Oep are essential for the induction of *gata5* in the myocardial precursors, and that Gata5 does not require Bmp2b or Oep to promote myocardial differentiation.

INTRODUCTION

Congenital defects in cardiac development are a significant source of morbidity and mortality in humans, occurring in one percent of live births (Hoffman 1995). The lack of understanding of the causes of cardiac defects provides a rationale for the identification of genes required for proper heart development. Recent studies of vertebrate myocardial differentiation have implicated bone morphogenetic protein (Bmp) signaling and EGF-CFC proteins in myocardial differentiation. However, how these genes promote myocardial differentiation has not been fully elucidated.

The myocardium arises from primordia within the anterior lateral plate mesoderm (ALPM), paired regions of mesoderm lateral to the somites. In mice, amphibia, birds and zebrafish, the ALPM is in close contact with the endoderm from the time of gastrulation (reviewed in Nascone and Mercola 1996). Removal of the endoderm apposed to the ALPM from amphibian and avian embryos prior to neurulation stages prevents the subsequent formation of beating myocardial tissue (Jacobson 1960; Orts-Llorca 1963; Sugi and Lough 1994).

Molecular characterization of chick embryos from which anterior endoderm has been removed indicates that the endoderm is not required for the specification of myocardium, but rather is a source of signals that promote myocardial differentiation (Gannon and Bader 1995). Co-culture of anterior endoderm with regions of the embryo that normally do not give rise to myocardium have revealed that these signals are sufficient to induce a wide range of myocardial genes, including *nkx2.5* (Schultheiss et al. 1995). *nkx2.5* is a homolog of the *Drosophila tinman* gene and the earliest known marker of the myocardial precursors (Bodmer 1993; Komuro and Izumo 1993; Lints et al. 1993). *tinman* is induced by Dpp, a member of the TGFβ family (Frasch 1995). Remarkably, regulation of *tinman*-type genes appears to have been conserved over large phylogenetic distances, as Bmp2, a vertebrate homolog of Dpp, is able to induce the ectopic expression

of *nkx2.5* in chick embryos (Schultheiss et al. 1997). Conversely, Noggin, a secreted inhibitor of Bmp2, 4 and 7 (Zimmerman et al. 1996), inhibits the expression of *nkx2.5* expression (Schultheiss et al. 1997). Thus, Bmp signaling appears to instruct the expression of *nkx2.5* in the ALPM.

The requirement for Bmp2 in heart development is further emphasized by analysis of *bmp2* mutants. Mouse *bmp2* null mutants exhibit variable cardiac defects ranging from development of myocardial tissue within abnormal locations in the embryo to apparent absence of myocardial tissue (Zhang and Bradley 1996). Similarly, *nkx2.5* expression in mouse *bmp2* mutants varies from reduced to absent (Zhang and Bradley 1996). Zebrafish *bmp2b* is encoded by the *swirl* (*swr*) locus (Kishimoto et al. 1997). *swr/bmp2b* mutants are dorsalized, reflecting the role of Bmps in early dorsoventral patterning, and exhibit little to no expression of *nkx2.5* (Mullins et al. 1996; Kishimoto et al. 1997). Whether the defect in *nkx2.5* expression reflects a specific requirement for Bmp2b or the absence of myocardial specification secondary to dorsalization remains unclear.

The EGF-CFC family of proteins are also implicated in early myocardial differentiation. Mice mutant for one member of the family, *cripto*, in addition to failing to form the anterior-posterior axis, do not express myocardial genes at embryonic day 8.7, suggesting a role for Cripto in myocardial differentiation (Ding et al. 1998; Xu et al. 1999). Zebrafish *one-eyed pinhead* (*oep*) also encodes a member of the EGF-CFC family, thought to be an essential permissive cofactor for Nodal signaling (Zhang et al. 1998; Gritsman et al. 1999). Mutants lacking zygotic *oep* (*Zoep*) do not form endoderm, prechordal plate mesoderm, or ventral neuroectoderm, and display cardia bifida resulting from the failure of the paired myocardial primordia to fuse at the embryonic midline (Schier et al. 1997). *oep* is expressed broadly during gastrulation and segmentation stages (Zhang et al. 1998), allowing for the possibility that Oep is also required for myocardial differentiation.

Recently, we have demonstrated an early and essential role for Gata5, a zinc finger transcription factor, in zebrafish myocardial differentiation (Reiter et al. 1999). *gata5* is one of six known evolutionarily-conserved vertebrate *gata* genes. *gata1*, *2* and *3* are implicated in hematopoietic development (reviewed in Orkin and Zon 1997), while *gata4*, *5* and *6* function in the development of endoderm, myocardium and extraembryonic tissues (Kuo et al. 1997; Molkentin et al. 1997; Morrisey et al. 1998; Koutsourakis et al. 1999; Reiter et al. 1999). Zebrafish *gata5* is encoded by the *faust* (*fau*) locus (Reiter et al. 1999). *fau/gata5* mutants exhibit cardia bifida and greatly diminished expression of several myocardial genes, including *nkx2.5* (Reiter et al. 1999). Gata5 regulation of *nkx2.5* may be direct, as two separate regulatory elements of mouse *nkx2.5* depend upon Gata-binding motifs (Searcy et al. 1998; Lien et al. 1999). Overexpression of *gata5* can lead to the ectopic expression of *nkx2.5* and other myocardial genes, and to the formation of ectopic, rhythmically-contracting myocardial tissue (Reiter et al. 1999). Therefore, zebrafish Gata5 plays an essential and central role in the early events of myocardial development.

In this study, we investigated the relationship of *swr/bmp2b*, *oep* and *fau/gata5* in zebrafish myocardial development through both mutant analysis and overexpression studies. Using molecular markers, we describe defects in myocardial development in *swr/bmp2b* and *Zoep* mutants, and demonstrate that both mutants show markedly reduced expression of both *nkx2.5* and *gata5*. To investigate the regulatory relationship between these defects, we overexpressed *gata5* in *swr/bmp2b* and *Zoep* mutants. *gata5* overexpression can restore *nkx2.5* expression in both mutants, indicating that Gata5 does not function through either Bmp2b or Oep to induce *nkx2.5*. Taken together, these results also suggest that a major cause of defects in myocardial development in both *swr/bmp2b* and *Zoep* mutants is the failure to express *gata5* in the myocardial precursors.

MATERIAL AND METHODS

Zebrafish strains

Zebrafish were maintained and staged as described (Westerfield 1995). Mutant alleles used were *swr*^{ta72}, *snh*^{ty68a} (Mullins et al. 1996), *oep*^{m134} (Schier et al. 1996) and *oep*^{z1} (Schier et al. 1996).

Wholemount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al. 1998). Immunohistochemistry using the antibodies MF20 and S46 (generous gift of Dr. Frank Stockdale, Stanford University) was performed as described (Alexander et al. 1998). MF20 was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biological Studies, University of Iowa under contract NO1-HD-2-3144 from the NICHD.

mRNA injection

Full length, capped *gata5*, *tm236a* and *β-galactosidase* messages were synthesized from previously described templates (Reiter et al. 1999); using the SP6 mMessage Machine system (Ambion). *tm236a* is a mutant version of *gata5* that includes a frameshift in the critical second zinc finger domain (Reiter et al. 1999). Embryos were injected at the 1-4 cell stage with 60 pg of *gata5* or *tm236a* mRNA. Some *gata5* injected embryos were coinjected with 150 pg *β-galactosidase* mRNA. β-Galactosidase staining was performed as described (Rodaway et al. 1999).

RESULTS AND DISCUSSION

Oep and Bmp2b are essential for *nkx2.5* expression

Zoep mutants belong to the cardia bifida class of mutants. Cardia bifida represents a failure of the myocardial primordia to execute one of their earliest morphogenetic steps, the migration to the embryonic midline. In addition to *oep*, the cardia bifida mutants include *casanova* (*cas*), *bonnie and clyde* (*bon*), *fau/gata5, miles apart* and *natter*. Both *cas* and *bon* mutants form an approximately normal amount of myocardial tissue, despite the defects in morphogenesis. *Zoep* mutants, in contrast, form relatively little myocardial tissue (Figure 4.1a). In this way, *Zoep* myocardia resemble those of *fau/gata5* mutants more than those of other cardia bifida mutants (Figure 4.1a).

nkx2.5 is expressed by the myocardial precursors from the onset of segmentation. Examination of *nkx2.5* expression reveals that *Zoep* mutants usually fail to express *nkx2.5* (Figure 4.1b). When *Zoep* mutants do express *nkx2.5*, *nkx2.5* expression is very reduced compared to wild-type siblings. Loss of Oep function results in a more profound defect in *nkx2.5* expression than is observed in any of the other cardia bifida mutants (data not included).

As previously demonstrated, *swr/bmp2b* mutants also usually fail to express *nkx2.5* (6 of 8; Figure 4.1c and Kishimoto et al. 1997). The absence of *nkx2.5* expression in *swr/bmp2b* mutants may reflect a general loss of ventral fates including myocardium. However, other dorsalized mutants such as *snailhouse* (*snh*) robustly express *nkx2.5* (Figure 4.1d), suggesting that *bmp2b* may be specifically required for induction of *nkx2.5* expression. It would be interesting to test whether ventralization of *swr/bmp2b* mutants by overexpressing other Bmp family members can restore myocardial gene induction. Zebrafish *bmp2b* is not expressed in or near the ALPM during early segmentation when *nkx2.5* is first expressed (data not included), suggesting that if Bmp2b does induce *nkx2.5*,

it must act indirectly. *swr/bmp2b* mutants do not survive past the 14-somite stage, prohibiting analysis of the expression of myocardial genes such as *cmlc1* that are not expressed until late segmentation stages.

To determine whether Gata5 reciprocally regulates *bmp2b* or *oep*, we analyzed *fau/gata5* mutants and found that neither *bmp2b* nor *oep* expression depends on Gata5 at any stage examined (data not included).

Myocardial expression of *gata5* requires Bmp2b and Oep

gata5 is first expressed at late blastula stages in the most marginal blastomeres and the yolk syncytial layer (YSL), an extraembryonic tissue underlying the blastoderm (Reiter et al. 1999; Rodaway et al. 1999). During gastrulation, *gata5* expression is maintained in two distinct populations. Predominantly dorsally, *gata5* is expressed in large, flat endodermal precursors (Reiter et al. 1999). More ventrally, *gata5* is expressed in rounder, more superficial mesodermal cells thought to be the precursors of the ALPM (Reiter et al. 1999). During segmentation stages, the endodermal expression of *gata5* diminishes, while *gata5* expression persists in the ALPM (Reiter et al. 1999).

To further characterize myocardial differentiation in *swr/bmp2b* and *Zoep* mutants, we assessed their expression of *gata5*. *swr/bmp2b* mutants initiate and maintain *gata5* expression normally through blastula stages (Figure 4.2a). However, during gastrulation, the *gata5* expression pattern of *swr/bmp2b* mutants is easily distinguished from that of their wild-type siblings; instead of being maintained in cells of endodermal morphology dorsally and cells of mesodermal morphology ventrally, endodermal progenitors are found at the exclusion of mesoderm (Figure 4.2b). Fate map studies have indicated that more endoderm forms dorsally than ventrally, and that the myocardial precursors arise more ventrally (Stainier et al. 1993; Warga and Nusslein-Volhard 1999). Also, excess endoderm formation has previously been described in dorsalized mutants

(Alexander 1999). Thus, in the absence of Bmp2b, endoderm formation is expanded ventrally and mesodermal *gata5* expression is abolished. Mesodermal *gata5* expression is observed in *snh* mutants (data not included), further discriminating between *swr/bmp2b* and *snh* in the induction of myocardial gene expression.

After the completion of gastrulation, wild-type embryos maintain *gata5* expression in the ALPM. Analysis of *swr/bmp2b* mutants during segmentation reveals that *gata5* expression is severely reduced and not organized into two lateral stripes (Figure 4.2c). Expression of *gata4* and *6* is similarly reduced in *swr/bmp2b* mutants (data not included).

As in *swr/bmp2b* mutants, *gata5* is initially expressed normally in *Zoep* mutants. However, by late blastula stages, expression of *gata5* has begun to diminish (Figure 4.2d). During gastrulation, *gata5* expression essentially disappears from the blastoderm of *Zoep* mutants; the only remaining *gata5* expression is in a few scattered cells and in the YSL (Figure 4.2e). Because zygotic *oep* is required for the formation of endoderm, it is not surprising that *Zoep* mutants do not express *gata5* dorsally. However, the additional absence of the ventral *gata5*-expressing mesodermal cells in *Zoep* mutants reveals a novel function for Oep; zygotic Oep is essential for the maintenance of *gata5* expression in the presumptive ALPM precursors past early gastrula stages. ALPM expression of *gata5* does not recover later in *Zoep* mutants (Figure 4.2f).

Gata5 can restore *nkx2.5* expression to *swr/bmp2b* and *Zoep* mutants

We have previously demonstrated that overexpression of *gata5* can increase *nkx2.5* in wild-type embryos (Reiter et al. 1999; also, Figure 4.3a). To explore the relationship between the loss of *nkx2.5* expression and the loss of myocardial *gata5* expression, we examined the effects of *gata5* overexpression on myocardial differentiation in *swr/bmp2b* and *Zoep* mutants.

Injection of *swr/bmp2b* mutants with 50 pg of *gata5* mRNA restored robust *nkx2.5* expression (9 of 18; Figure 4.3b). We have noted previously that following *gata5* overexpression in wild-type embryos, *nkx2.5* expression was usually expanded in a restricted anterior-posterior domain similar to the region of endogenous *nkx2.5* expression (Reiter et al. 1999). While occasionally, *gata5* overexpression in *swr/bmp2b* mutants induced diffuse *nkx2.5* expression, in most cases (7 of 9), *nkx2.5* expression was induced in two lateral stripes similar to the wild-type pattern. *gata5* overexpression does not increase expression of *swr/bmp2b* in wild-type embryos (data not included), further indicating that *gata5* does not conversely regulate *swr/bmp2b*. Consistent with these data, *gata5* overexpression does not affect dorsoventral patterning of wild-type embryos (data not included).

Overexpression of *gata5* can also restore *nkx2.5* expression in *Zoep* mutants (3 of 13; Figure 4.3c). By co-injecting mRNA encoding β-galactosidase, it is possible to monitor the distribution of the exogenous mRNA within the embryo. Gata5 also can induce endodermal gene expression in *Zoep* mutants (Reiter and Stainier 1999), raising the possibility that increased *nkx2.5* expression in *Zoep* mutants could reflect increased endoderm formation. However, restored *nkx2.5* expression always overlapped with the turquoise β-galactosidase staining in *Zoep* mutants, suggesting that Gata5 acts autonomously, and not through endodermal induction, to induce *nkx2.5*. Moreover, *nkx2.5* expression is normal in *cas* mutants, which entirely lack endoderm from an early stage (Alexander et al. 1999).

Furthermore, as *Zoep* mutants survive to primordial stages, we were also able to assess other aspects of myocardial differentiation in *gata5*-overexpressing *Zoep* mutants. *Zoep* mutants exhibit severely reduced or absent expression of *cmlc1* (Figure 4.3e), a marker of myocardial maturation (Reiter et al. 1999). Analysis of *gata5*-overexpressing *Zoep* mutants revealed that Gata5 can restore *cmlc1* expression (6 of 6) and formation of a midline heart to *Zoep* mutants (7 of 20; Figure 4.3e). These hearts were observed to beat

normally. Cumulatively, these results suggest that Oep is not required downstream of *gata5* for myocardial differentiation.

A pathway underlying early myocardial development

Previously, we have shown that Gata5 functions as a critical regulator of myocardial differentiation. Here, we have demonstrated that both Bmp2b and Oep are required for the expression of *gata5* within the myocardial precursors. Through gain-of-function experiments we have further demonstrated that Gata5 is sufficient to induce expression of *nkx2.5* in *swr/bmp2b* and *Zoep* mutants. Therefore, the loss of *gata5* expression exhibited by *swr/bmp2b* and *Zoep* mutants is sufficient to explain why they fail to express *nkx2.5*. Cumulatively, these results further underscore the central and essential role Gata5 performs in myocardial differentiation.

Additionally, these results begin to elucidate in vivo a pathway underlying early myocardial development: Bmp2b and Oep are required for *gata5* expression in the myocardial precursors. Gata5 functions downstream of Bmp2b and Oep in the myocardial precursors to induce *nkx2.5* expression. Other work has demonstrated that Gata5 and Nkx2.5 subsequently cooperate to activate downstream myocardial genes such as *atrial natriuretic factor* and *cardiac α-actin* (Durocher et al. 1997; Lee et al. 1998; Sepulveda et al. 1998). This pathway begins the work of connecting the signaling events of gastrulation to the process of myocardial differentiation.

Figure 4.1

***Zoep* and *swr/bmp2b* mutants exhibit defective myocardial development. (a)** Ventrolateral views of embryos at 36 hours post-fertilization (hpf) stained with MF20 and S46 antibodies. Red fluorescence indicates MF20 (TRITC) staining of ventricular myocardium and somites and yellow fluorescence indicates the overlap of MF20 (TRITC) and S46 (FITC) staining of atrial myocardium. Both *faw/gata5* and *Zoep* mutants exhibit cardia bifida and produce less myocardial tissue than wild-type siblings. **(b-d)** *nkx2.5* expression at the (b) 15-somite stage (16.5 hpf) and (c,d) the 11-somite stage (14.5 hpf). All views are dorsal, except the view of the *snh* mutant in (d) which is ventral. *nkx2.5* expression is severely reduced or absent in *Zoep* and *swr/bmp2b* mutants. Although *nkx2.5* expression is severely disorganized in *snh* mutants, *nkx2.5* is expressed in approximately the same number of cells as in wild-type siblings.

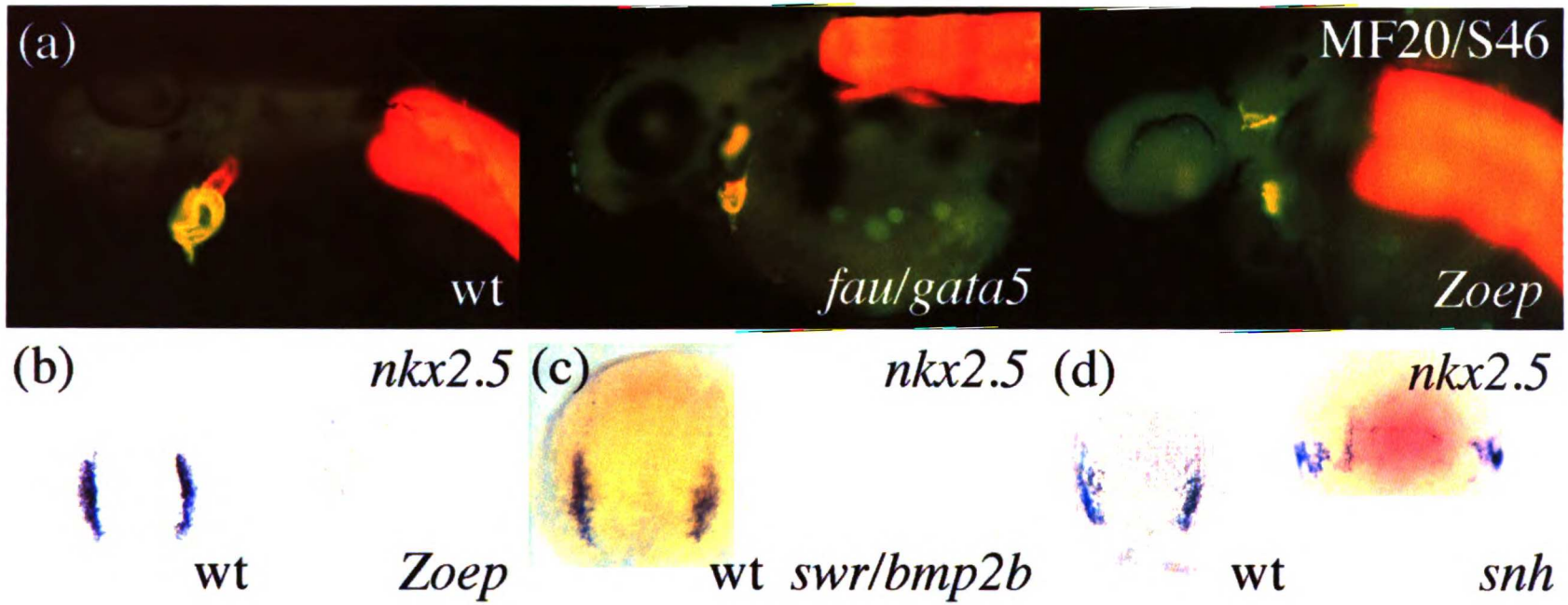

(a)
MF20/S46
wt
faul/gata5
Zoep
(b)
nkx2.5
wt
Zoep
(c)
nkx2.5
wt
swr/bmp2b
(d)
nkx2.5
wt
snh

Figure 4.2

***swr/bmp2b* and *Zoep* mutants do not express *gata5* in myocardial precursors.**

(a,b,d,e) Lateral and (c,f) dorsal views of *gata5* expression. **(a)** At 50% epiboly (5.3 hpf), *gata5* is expressed normally in the marginal cells of *swr/bmp2b* mutants. **(b)** In wild-type embryos at 80% epiboly (8.3 hpf), *gata5* is expressed in large, flat endodermal precursors predominantly dorsally and smaller, rounder mesodermal cells ventrally. *swr/bmp2b* mutants lack ventral, mesodermal expression of *gata5*, but display a ventral expansion of *gata5*-expressing endodermal precursors. **(c)** At the 12-somite stage (15 hpf), *gata5* is normally expressed in the ALPM. *swr/bmp2b* mutants express *gata5* in a reduced number of cells underlying the head. **(d)** *Zoep* mutants exhibit decreased *gata5* expression in the germ ring by 50% epiboly. **(e)** At 80% epiboly, *gata5* expression in *Zoep* mutants is limited to the YSL and a very few cells within the blastoderm. **(f)** *Zoep* mutants do not express *gata5* in the ALPM at the 12-somite stage.

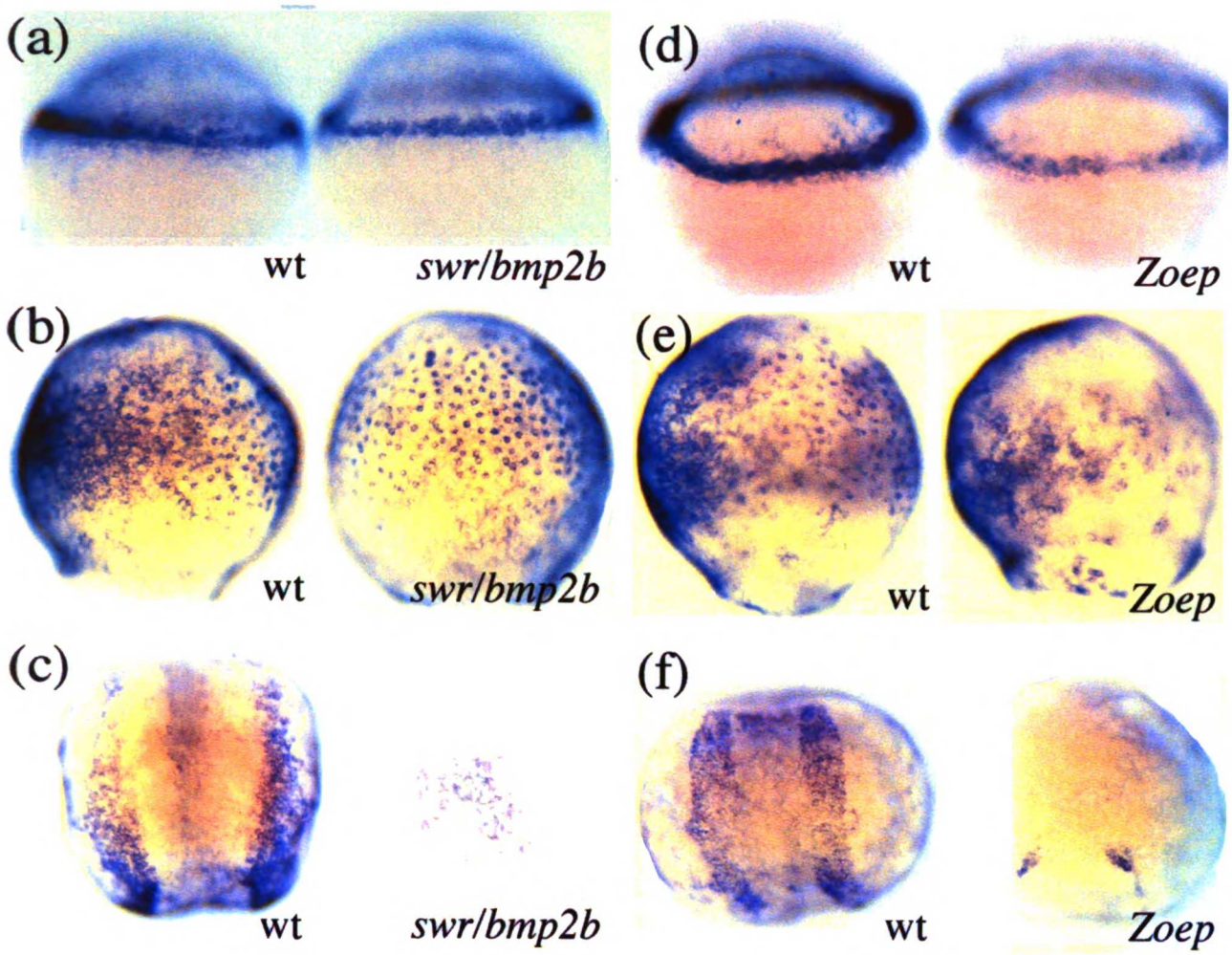
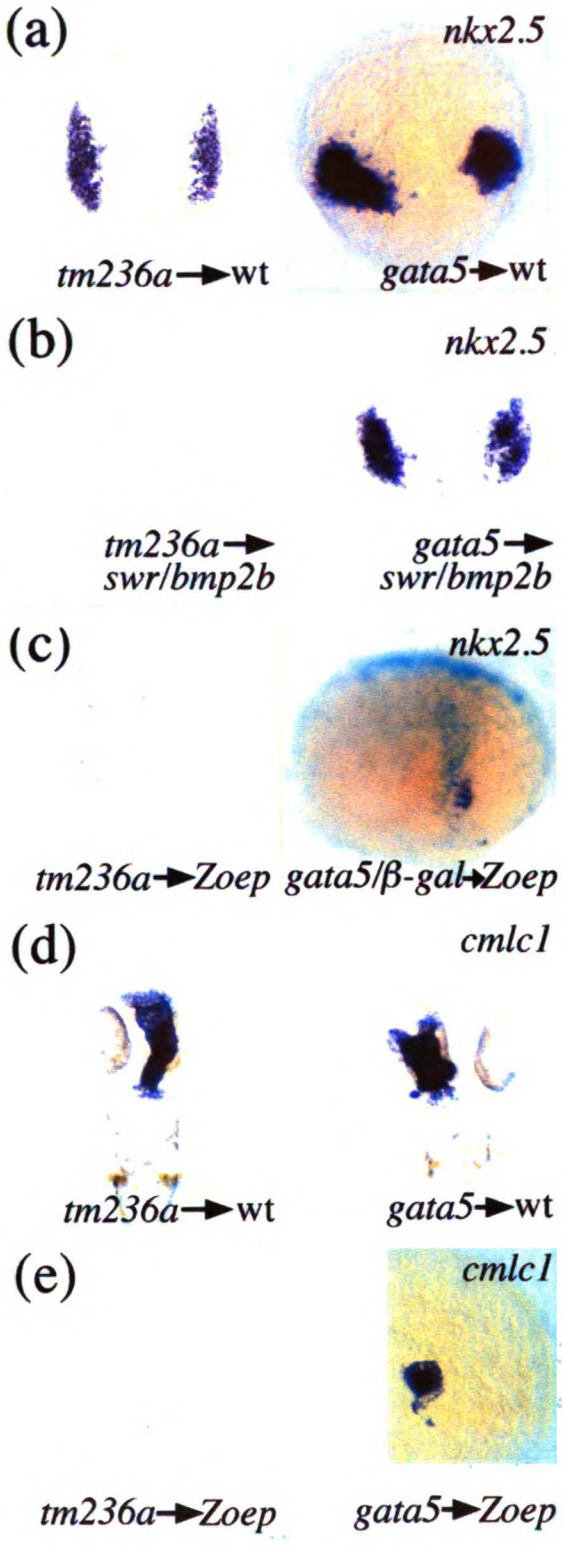

(a)
wt
swr/bmp2b
(d)
wt
Zoep
(b)
wt
swr/bmp2b
(e)
wt
Zoep
(c)
wt
swr/bmp2b
(f)
wt
Zoep

Figure 4.3

Forced expression of *gata5* can restore *nkx2.5* expression to *swr/bmp2b* and *Zoep* mutants. (a-c) Dorsal views of *nkx2.5* expression at the (a,b) 9-somite (13.5 hpf) and (c) 15-somite stages (16.5 hpf). **(a)** Injection of 50 pg of *tm236a* mRNA does not perturb *nkx2.5* expression. *tm236a* encodes a mutant version of Gata5 (Reiter et al. 1999). Injection of 50 pg of wild-type *gata5* mRNA leads to expanded expression of *nkx2.5* in wild-type embryos. **(b)** Overexpression of *gata5* can restore *nkx2.5* expression to *swr/bmp2b* mutants. **(c)** *gata5* overexpression can also restore *nkx2.5* expression to *Zoep* mutants. Coinjection of mRNA encoding β-galactosidase allows visualization of the distribution of the injected messages. **(d,e)** Dorsal views of *cmlc1* expression at 26 hpf. Overexpression of *gata5* restores *cmlc1* expression to *Zoep* mutants.


(a)
nkx2.5
tm236a→wt
gata5→wt
(b)
nkx2.5
tm236a→
swr/bmp2b
gata5→
swr/bmp2b
(c)
nkx2.5
tm236a→Zoep
gata5/β-gal→Zoep
(d)
cmlc1
tm236a→wt
gata5→wt
(e)
cmlc1
tm236a→Zoep
gata5→Zoep

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Chapter 5: Notch and Wnt signaling can regulate formation of the vertebrate endoderm

SUMMARY

Wnt and Notch signaling are implicated in the regulation of endoderm specification during *C. elegans* and sea urchin development, respectively. Here, we demonstrate that Wnt and Notch signaling also regulate endoderm formation in zebrafish. Genes encoding signals for both pathways including Wnt8, Wnt11, DeltaC and DeltaD are expressed in the germ ring during early gastrulation when the zebrafish endoderm is specified. TOPGAL, a reporter of β-catenin-inducible TCF/LEF transcriptional activation, is expressed in endodermal progenitors, indicating that Wnt signaling is active in the endodermal progenitors. A functional role for Wnt signals is demonstrated through the overexpression of Wnt antagonists, such as dnXwnt8 and Xfrzb, both of which inhibit endoderm formation. Conversely, overexpression of zebrafish Wnt8 increases the number of endodermal progenitors formed. Together, these results suggest that, as in *C. elegans*, Wnt signals stimulate endoderm formation in vertebrates. Similar overexpression experiments demonstrate that Notch signaling can inhibit endodermal specification; overexpression of DeltaC or downstream effectors of Notch signaling inhibits endoderm formation. However, antagonists of Notch signaling do not increase endodermal specification. Therefore, Notch signaling represses endoderm formation, but the absence of Notch signaling is not sufficient to induce endodermal gene expression. Cumulatively, these results suggest that Wnt and Notch signals function antagonistically to regulate specification of the vertebrate endoderm.

INTRODUCTION

During *C. elegans* embryogenesis, the EMS blastomere produces two daughters: MS which principally gives rise to mesodermal tissues such as body wall muscle, and E which gives rise to the endoderm. Without a signal from its neighbor, the P_2 blastomere, EMS produces two MS-like daughters at the expense of the endodermal progenitor (Goldstein 1993). The endoderm-inducing signal produced by P_2 is a Wnt protein encoded by the *mom-2* gene (Rocheleau et al. 1997; Thorpe et al. 1997). Wnt proteins are secreted, glycosylated signaling proteins associated with the extracellular matrix and cell surface and widely implicated in both invertebrate and vertebrate developmental processes (reviewed in Nusse 1999).

Wnt proteins are also implicated in the development of the sea urchin endoderm. Nuclear β-catenin, a downstream effector of Wnt signaling, is found at high levels in the endodermal and mesodermal progenitors (Logan et al. 1999). Activation of Wnt signaling through LiCl treatment expands endoderm and mesoderm formation, while inhibition of Wnt signaling through cadherin overexpression suppresses endoderm formation (Logan et al. 1999), revealing that Wnt signaling positively regulates endodermal specification during sea urchin embryogenesis.

Sea urchin endoderm formation is also regulated by Notch signaling. LvNotch, a sea urchin Notch homolog, is dynamically expressed in the endoderm and the secondary mesenchymal cells (SMCs), a population of cells that gives rise to mesodermal tissues (Sherwood and McClay 1997). Expression of a constitutively active version of LvNotch expands SMC specification at the expense of endoderm, while a dominant interfering version of LvNotch represses SMC specification (Sherwood and McClay 1999). Moreover, chimeric analysis reveals that Notch signaling autonomously directs SMC specification (Sherwood and McClay 1999). Thus, Wnt and Notch signaling exert

opposing influences on the specification of endoderm during early sea urchin development.

Formation of the endodermal germ layer is also one of the first developmental events of vertebrate embryogenesis. The endoderm gives rise to the epithelial lining of the gut tube and respiratory tract and gut-associated organs including the pancreas and liver. In zebrafish, the endoderm, as well as some mesoderm, forms from the most marginal blastomeres (Warga and Nusslein-Volhard 1999). Fate map studies have demonstrated that before involution, single marginal blastomeres can still produce both endoderm and mesoderm (Warga and Kimmel 1990). However, after involution, cells are fated to a single germ layer (Warga and Kimmel 1990), suggesting that blastomeres are specified as endodermal or mesodermal around the time they gastrulate. Endodermal expression of *sox17*, an HMG-domain transcription factor gene (Alexander and Stainier 1999), and *axial*, a forkhead transcription factor gene homologous to *HNF3β* (Strahle et al. 1993), initiates soon after involution, further suggesting that zebrafish endoderm specification occurs during early gastrulation.

Genetic analysis of zebrafish development has begun to identify some of the genes required for endoderm formation. Embryos mutant for *cyclops* and *squint*, two genes that encode members of the Nodal family of TGFβ signaling proteins, form no endoderm and very little mesoderm (Feldman et al. 1998). Similarly, embryos lacking maternal and zygotic Oep, an EGF-CFC protein required for Nodal signaling, also form no endoderm and very little mesoderm (Zhang et al. 1998; Gritsman et al. 1999). Together these results demonstrate that Nodal signaling is essential for the formation of both endoderm and mesoderm. However, endoderm formation appears to require a higher level of Nodal signaling than does mesoderm formation; mutants lacking only the zygotic contribution of Oep form some mesoderm, but completely lack endoderm (Schier et al. 1997), and overexpression of low levels of Antivin/Lefty2, a secreted inhibitor of Nodal signaling, selectively inhibits endoderm formation (Thisse and Thisse 1999).

Nodal signaling induces the expression of *gata5,* a zinc finger transcription factor gene, and *bonnie and clyde (bon),* a paired-type homeodomain transcription factor gene homologous to chick *CMIX* (Alexander et al. 1999; Reiter et al. 1999; Rodaway et al. 1999). Both *gata5* and *bon* mutants exhibit reduced endoderm formation as assessed by *sox17* and *axial* expression (Kikuchi et al. 1999; Reiter and Stainier 1999). Embryos mutant for both *gata5* and *bon* exhibit almost no endodermal gene expression (Reiter and Stainier 1999). Taken together, these results indicate that Nodal signaling induces the expression of *gata5* and *bon*, and that Gata5 and Bon subsequently cooperate to promote the formation of the endoderm.

As Wnt and Notch signals are also expressed in the germ ring during early gastrulation, we investigated whether they may participate in endodermal specification. Here, we demonstrate through use of the TOPGAL reporter (DasGupta and Fuchs 1999) that Wnt signals activate TCF/LEF-dependent transcription in the endodermal progenitors. Furthermore, overexpression of Wnt proteins and Wnt antagonists indicate that Wnt signaling promotes endodermal specification. In contrast, activation of Notch signaling inhibits endoderm formation. Together, these results suggest that Wnt and Notch exert opposing influences on vertebrate endodermal specification.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish were maintained and staged as described (Westerfield 1995).

Wholemount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al. 1998). Immunohistochemistry using MF20 and S46 was performed as described (Alexander et al. 1998).

mRNA and DNA injection

Capped mRNA was synthesized using the SP6 mMessage Machine system (Ambion) from the following previously described templates: dnXwnt8 (Hoppler et al. 1996), Frzb-1 (Wang et al. 1997), wnt8 (Kelly et al. 1995), X-Su(H)1/ANK, X-Su(H)1^{DBM} (Wettstein et al. 1997), deltaC (Takke and Campos-Ortega 1999), deltaD, deltaD^{Pst}, Notch1(ICD) (Takke et al. 1999), X-Delta-1 and X-Delta-1^{dn} (Chitnis et al. 1995). Notch3(ICD) was created by truncating the extracellular and transmembrane domains from zebrafish *notch3* cDNA and cloning the region encoding the intracellular domain into pCS2+. Embryos were injected at the 1-4 cell stage with RNA. 20 pg TOPGAL was injected at the one cell stage and β-galactosidase staining was performed as described (Haddon et al. 1998).

RESULTS AND DISCUSSION

delta homolog expression and Wnt signaling in the zebrafish germ ring during endodermal specification

The endodermal progenitors are among the first cells to involute (Warga and Kimmel 1990) and, soon after involuting, begin to express *sox17* and *axial*. Examination of *axial* or *sox17* expression during early gastrulation (shield stage) reveals that these cells are distributed around the germ ring in a salt-and-pepper distribution (Figure 5.1a,b). *deltaC* and *deltaD*, two genes encoding Notch ligands (Haddon et al. 1998), begin to be expressed during late blastula stages. Later, as the endodermal progenitors involute and begin to express endoderm-specific genes, *deltaC* and *deltaD* are widely expressed in the germ ring (Figure 5.1c,d). Additionally, *deltaC* is expressed in a separate domain in the dorsal epiblast. Although expressed throughout the germ ring, *deltaD* is expressed at lower levels at the dorsal midline.

Like *deltaC* and *deltaD*, expression of zebrafish *wnt8* and *wnt11* initiates in the margin of late blastula stage embryos and is maintained in the germ ring during early gastrulation (Kelly et al. 1995; Makita et al. 1998); also, Figure 5.1e). *lef1* encodes an HMG domain transcription factor that functions as a downstream effector of Wnt signaling and is highly expressed in the germ ring during early gastrulation (data not included; Behrens et al. 1996; Dorsky et al. 1999). Comparison of the *deltaC*, *deltaD*, *wnt8*, *wnt11* and *lef1* expression patterns to those of *sox17* and *axial* indicate that Notch and Wnt signals are present in the germ ring as the endoderm becomes specified.

Wnt signaling stabilizes the cytosolic and nuclear pools of β-catenin by inhibiting the activity of GSK-3β (reviewed in Cox and Peifer 1998). To activate the transcription of Wnt-responsive genes, nuclear β-catenin cooperates with Lef1 and other members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors. TOPGAL is a β-catenin-inducible β-galactosidase reporter of TCF/LEF transcriptional

activation (DasGupta and Fuchs 1999). To identify which blastomeres activate TCF/LEF-dependent transcription, presumably in response to Wnt signals, we injected 20 pg TOPGAL DNA into zebrafish embryos at the 1-4 cell stage and examined β-galactosidase activity during gastrulation. Because of the mosaic distribution of exogenous DNA within the zebrafish embryo, injected embryos exhibit different patterns of TOPGAL expression. In 5 of 21 injected embryos, β-galactosidase was expressed in the marginal cells of the germ ring at shield stage (Figure 5.1f). Comparison of TOPGAL expression to *axial* expression in the dorsal midline reveals that TCF/LEF transcription is widely active in the germ ring (Figure 5.1f). Later in gastrulation, TOPGAL expression is observed in cells expressing endodermal genes such as *sox17* and *axial* (Figure 5.1g and data not included). Together, these results indicate that TCF/LEF transcription is active in at least some of the endodermal progenitors. Determining whether all endodermal progenitors activate TCF/LEF transcription will require generation of TOPGAL transgenic fish.

Wnt signaling antagonists inhibit endoderm specification

To investigate Wnt function during endoderm development, we expressed antagonists of Wnt signaling. *dnXwnt8* encodes a truncated Wnt protein that can dominantly interfere with both endogenous and ectopic Wnt signals during *Xenopus* development (Hoppler et al. 1996). Embryos injected with 100 pg of *dnXwnt8* mRNA at the 1 to 4 cell stage exhibit decreased gut as assessed by the expression of *shh* at 36 hours post-fertilization (hpf; 3 of 8; Figure 5.2a). Also, dnXwnt8 prevents fusion of the cardiac primordia (2 of 18), a defect associated with problems in zebrafish endoderm formation (Figure 5.2b; Alexander et al. 1999; Reiter et al. 1999).

To investigate how inhibition of Wnt signaling interferes with endodermal development, we analyzed endodermal gene expression during gastrulation; dnXwnt8 dramatically reduces the formation of *sox17*-expressing endodermal progenitors during

gastrulation (Figure 5.2c; Table 1). Overexpression of *Xenopus* Frzb-1, another secreted antagonist of Wnt signaling (Leyns et al. 1997; Wang et al. 1997), similarly suppresses endoderm formation (Table 1). To address the specificity of the effects of dnXwnt8, we also examined the expression of *ntl*, a zebrafish homolog of *brachyury* expressed in the germ ring and axial mesoderm (Schulte-Merker et al. 1994) and *myoD*, a bHLH transcription factor gene expressed in the precursors of the paraxial mesoderm (Weinberg et al. 1996). dnXwnt8 did not affect the expression of either *ntl* or *myoD* (Figure 5.2d,e), arguing that dnXwnt8 does not disrupt mesoderm formation. Cumulatively, these results suggest that endogenous Wnt signals are required for the formation of the vertebrate endodermal germ layer.

Wnt8 overexpression increases endoderm formation

fkd7 encodes a forkhead transcription factor expressed in the gut, hypochord and floorplate. By 24 hpf, the endoderm is a thin, ventral sheet of cells that is in the process of accumulating at the embryonic midline to form the gut tube (Figure 5.2f, arrow). Embryos injected with 250 pg of zebrafish *wnt8* RNA exhibit increased *fkd7* expression in the endodermal domain (Figure 5.2f, arrowhead), but show normal *fkd7* expression in the hypochord and floorplate. Although Wnt overexpression inhibits *bmp4* expression in *Xenopus* embryos (Baker et al. 1999), expression of neither zebrafish *bmp2b* nor *bmp4* is reduced at the doses at which *fkd7* expression is expanded (Figure 5.2g and data not included). Correspondingly, Wnt8-overexpressing embryos are not visibly dorsalized, although tail and head morphogenesis are frequently perturbed.

To further investigate how Wnt8 overexpression affects endoderm development, we examined the effects of Wnt8 overexpression on endodermal gene expression during gastrulation. Normally, the endodermal progenitors are found in a discontinuous layer over the dorsal half of the embryo at late gastrulation (Warga and Nusslein-Volhard 1999; Figure 5.2h, left). An animal pole view of Wnt8-overexpressing embryos reveals that the

endodermal progenitors are found over both the dorsal and ventral portions of the embryo (Figure 5.2h, right). By measuring the area of the embryo from which endodermal progenitors are normally absent, we estimate that Wnt8 overexpression expands the number of endodermal progenitors at 90% epiboly by 35-40%. Injection of larger amounts of *wnt8* RNA (400 pg) causes ectopic *sox17* expression within the epiblast (16 of 32), but also severely disrupts epiboly, making interpretation of this ectopic expression difficult.

Wnt8-overexpressing embryos express normal levels of *ntl*, although in a pattern reflecting abnormal germ ring morphogenesis (Figure 5.2i), and Wnt8 overexpression frequently suppresses *myoD* expression (6 of 16; Figure 5.2j), suggesting that Wnt8 does not promote mesoderm formation as well as endoderm formation.

Notch signaling antagonizes endoderm specification

Notch signaling inhibits endodermal specification during sea urchin embryogenesis (Sherwood and McClay 1999). Because *deltaC* and *deltaD* are expressed in the germ ring during endodermal specification, we investigated whether Notch signaling also regulates vertebrate endoderm formation. Notch signaling is transduced by the intracellular domain of Notch which, upon receptor activation, is cleaved and translocates to the nucleus (reviewed in Chan and Jan 1998). Ligand-independent activation of the Notch signaling pathway can be accomplished by overexpressing the intracellular domain of zebrafish Notch3 [Notch3(ICD)]. In addition to producing anterior truncations, injection of 50 pg of *Notch3(ICD)* RNA dramatically decreases endodermal expression of *fkd7* to a few scattered patches (5 of 5; Figure 5.3a, arrowhead), and like dnXwnt8 produces cardia bifida (5 of 22; Figure 5.3b). Furthermore, Notch3(ICD) inhibits formation of the *sox17*-expressing endodermal progenitors (Figure 5.3c; Table 1), indicating that Notch signaling blocks endoderm formation very early in its development.

Various vertebrate Notch homologs differently activate downstream signaling pathways (Beatus et al. 1999). To determine whether suppression of endoderm formation was specific to Notch3(ICD), we injected 100 pg of RNA encoding the intracellular domain of zebrafish Notch1 [Notch1(ICD); Bierkamp and Campos-Ortega 1993]. Notch1(ICD) does appear to function differently than Notch3(ICD), as Notch1(ICD) does not cause anterior truncations (data not included). However, like Notch3(ICD), Notch1(ICD) inhibits formation of the endodermal progenitors (Figure 5.3d; Table 1).

The transcription factor Suppressor of Hairless [Su(H)] is a downstream effector of Notch signaling (reviewed in Greenwald 1998). *X-Su(H)1/ANK* encodes a constitutively active form of *Xenopus* Su(H) (Wettstein et al. 1997). Like Notch1(ICD) and Notch3(ICD), X-Su(H)1/ANK also inhibits endoderm formation (Table 1). Neither Notch1(ICD) nor X-Su(H)1/ANK prevent expression of *myoD* (Figure 5.3d and data not included), suggesting that Notch signaling does not suppress mesoderm formation, but specifically affects the endoderm.

As we described above, two zebrafish *delta* genes, *deltaC* and *deltaD*, are expressed in the germ ring during endodermal specification. Overexpression of *deltaC*, like overexpression of other effectors of Notch signaling, inhibits formation of the endoderm (Figure 5.3f; Table 1). However, overexpression of *deltaD* does not affect endoderm formation (Table 1), suggesting that DeltaC and DeltaD may function differently during endoderm formation.

To test whether blocking Notch signaling would have the opposite effect on endodermal specification, we injected RNA encoding X-Delta-1^{dn}, a Delta protein that lacks most of its intracellular domain and dominantly interferes with Notch signaling (Chitnis et al. 1995). Although X-Delta-1^{dn} perturbs the organization of the nascent endodermal progenitors, it increases the number of endodermal progenitors to a small extent and only rarely (Figure 5.3g; Table 1). Other antagonists of Notch signaling, such

as deltaD^{Pst} (Takke and Campos-Ortega 1999) and XSu(H)^{DBM} (Wettstein et al. 1997) similarly fail to increase endoderm formation. Also, *mindbomb* (*white tail*) mutants which exhibit a neurogenic phenotype consistent with defects in Notch signaling (Jiang et al. 1996; Schier et al. 1996) form endoderm normally (data not included). Therefore, although Notch signaling inhibits endoderm formation, the absence of Notch signaling is not sufficient to specify endoderm.

Conclusions

Here, we have investigated the involvement of Wnt and Notch signaling in endodermal specification. We have found evidence that Wnt signaling, possibly through Wnt8 or Wnt11, activates TCF/LEF-dependent transcription in the endodermal progenitors. Antagonists of Wnt signaling suppress formation of the endoderm, and conversely, increasing Wnt signaling by overexpressing Wnt8 expands the number of endodermal progenitors. Cumulatively, these results suggest that Wnt signaling acts during late blastula or early gastrula stages of vertebrate development to promote the formation of the endodermal germ layer.

Like Wnt antagonists, effectors of Notch signaling inhibit endoderm formation, suggesting that Notch signaling normally suppresses endodermal gene expression. During *Drosophila* development, Wnt and Notch signaling pathways are known to interact during several developmental processes (reviewed in Arias 1998). The opposing influences of Wnt and Notch signaling on endoderm specification indicate that these two signaling pathways may similarly interact during formation of the vertebrate endoderm.

During vertebrate hematopoietic development, Notch signaling maintains stem cells in an undifferentiated state (reviewed in Milner and Bigas 1999). Possibly, Notch signaling functions similarly during gastrulation to prevent the premature differentiation of involuting endodermal progenitors. However, expression of Notch agonists does not

inhibit *myoD* expression, indicating that Notch signaling does not inhibit the differentiation of all involuting blastomeres.

Alternatively, the salt-and-pepper distribution of endodermal progenitors in the germ ring suggest that lateral specification may regulate their formation. Notch-mediated lateral specification is most well known in *Drosophila* sensory bristle and neuroblast selection, but lateral specification also functions in the selection of the mesodermal myoblast founder. Perhaps the zebrafish germ ring represents an equivalence domain for the hypoblast, within which mesodermal or endodermal fate is assigned through Notch-mediated lateral specification. Test of this hypothesis will require perturbation of Notch signaling with single cell resolution.

Figure 5.1. *delta* gene expression and Wnt signaling in the zebrafish germ ring. *A-F* show lateral and animal views with dorsal to the right. Embryos are at shield (6 hpf) stage. ***(A)*** *axial* is expressed in the axial mesoderm and, like *sox17*, in the endodermal progenitors. ***(B)*** *sox17* is expressed in the large, flat endodermal progenitors and dorsally in the forerunner cells. ***(C)*** *deltaC* is expressed in the germ ring and dorsal epiblast. ***(D)*** *deltaD* is expressed at high levels in the germ ring and lower levels at the dorsal midline. ***(E)*** *wnt11* is expressed throughout the germ ring. ***(F)*** TOPGAL is a β-galactosidase reporter under the regulation of a TCF/LEF inducible promoter. β-galactosidase staining of TOPGAL injected embryos produces a turquoise color. Embryos are costained for *axial* expression to indicate dorsal. ***(G)*** Lateral view of an 80% epiboly stage (8.3 hpf) TOPGAL-injected embryo costained for *sox17* expression with close-up. TOPGAL activation produces a turquoise color (for example, black arrowhead). *sox17* expression is indicated by blue staining (for example, white arrowhead). Cells expressing both TOPGAL and *sox17* appear dark purple (for example, arrow).

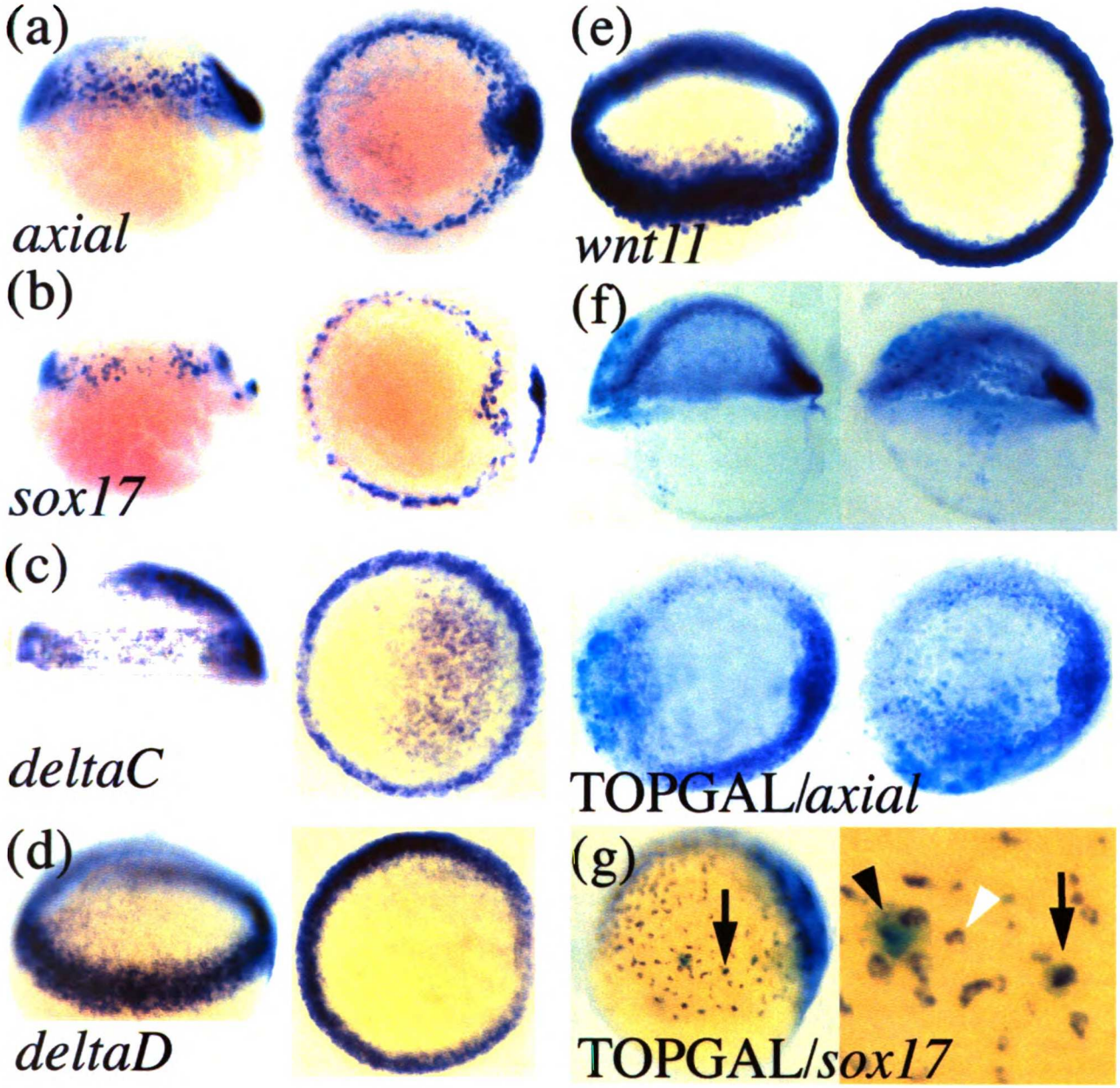
(a)
axial
(b)
sox17
(c)
deltaC
(d)
deltaD
(e)
wnt11
(f)
TOPGAL/axial
(g)
TOPGAL/sox17

Figure 5.2. Wnt signaling promotes endoderm formation. ***(A)*** Dorsal view of 36 hpf dnXwnt8-expressing embryo and uninjected sibling. *shh* is expressed in the floorplate, fin buds and endoderm (arrow). The dnXwnt8-expressing embryo exhibits reduced and dysmorphic endoderm (arrowhead). ***(B)*** Ventral views of 36 hpf dnXwnt8-expressing embryo and uninjected control immunohistochemically stained with MF20 (red) and S46 (green), two antibodies that recognize myosin heavy chain isoforms differentially expressed in the heart chambers. Although the control embryo has formed a single, midline heart tube, the heart primordia of the dnXwnt8-expressing embryo have not fused at the embryonic midline. *C-E* show dorsal views of 90% epiboly (9 hpf) dnXwnt8-expressing embryos and GFP-expressing controls. ***(C)*** dnXwnt8 suppresses formation of *sox17*-expressing endoderm progenitors. ***(D,E)*** dnXwnt8 does not disturb expression of *ntl* or *myoD*. ***(F)*** Lateral view of a 24 hpf Wnt8-overexpressing embryo and an uninjected sibling. *fkd7* expression in the floorplate and hypochord is not affected by Wnt8 overexpression, but endodermal expression of *fkd7* is expanded (arrow). The embryos shown in *G-J* are at 90% epiboly (9 hpf). ***(G)*** Lateral view of *bmp2b* expression reveals that Wnt8 overexpression does not reduce *bmp2b* expression. ***(H)*** Animal pole view of a Wnt8-expressing embryo and a GFP-expressing control. The ventral half of the animal pole is normally free of *sox17*-expressing endodermal progenitors. Embryos overexpressing Wnt8 form endodermal progenitors both dorsally and ventrally. ***(I)*** Wnt8 overexpression affects germ ring morphogenesis but does not expand *ntl* expression. ***(J)*** Wnt8 overexpression suppresses expression of *myoD*.

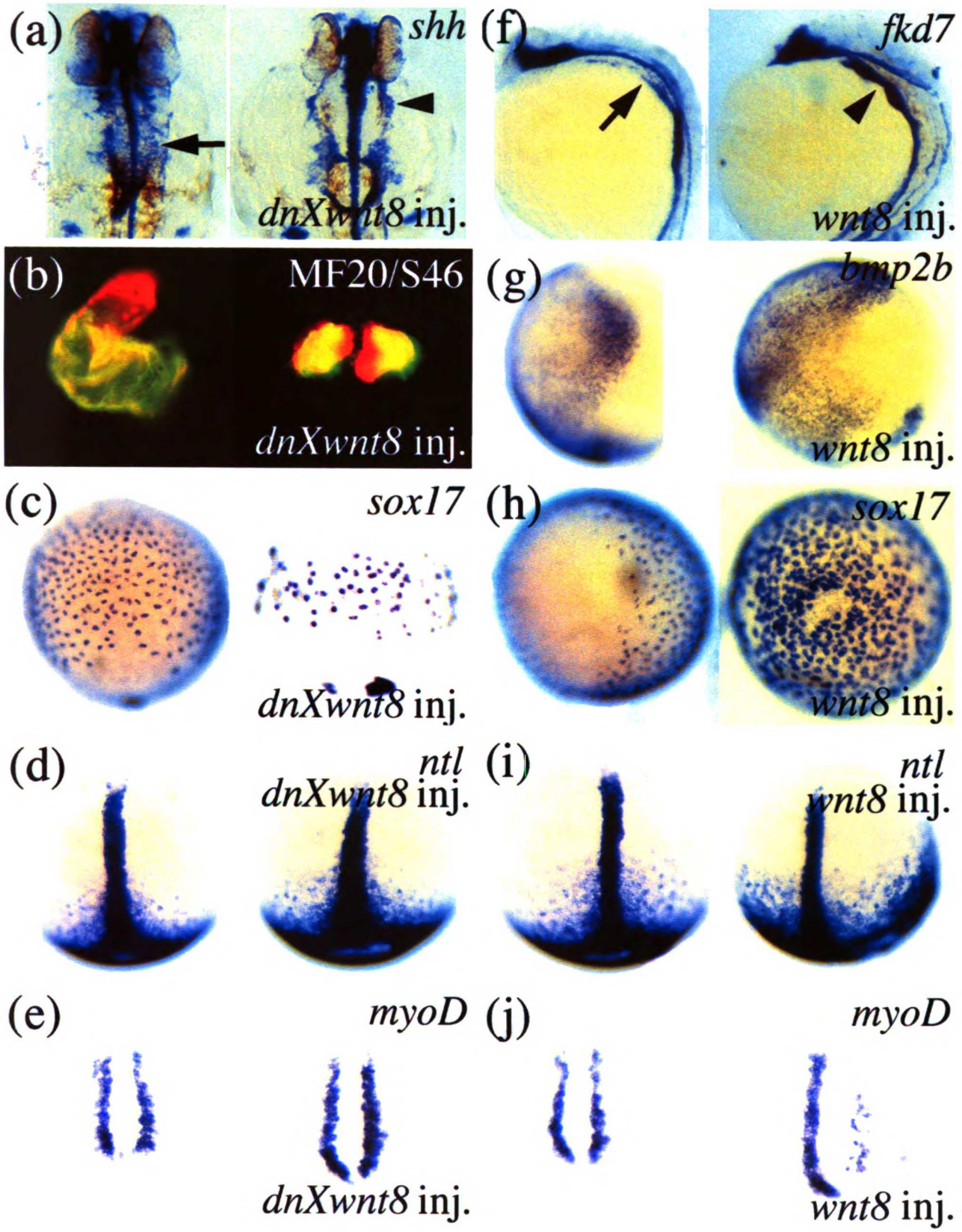
(a)
shh
dnXwnt8 inj.
(f)
fkd7
wnt8 inj.
(b)
MF20/S46
dnXwnt8 inj.
(g)
bmp2b
wnt8 inj.
(c)
sox17
dnXwnt8 inj.
(h)
sox17
wnt8 inj.
(d)
ntl
dnXwnt8 inj.
(i)
ntl
wnt8 inj.
(e)
myoD
dnXwnt8 inj.
(j)
myoD
wnt8 inj.

Figure 5.3. Notch signaling suppresses endoderm formation. ***(A)*** *fkd7* expression in the endoderm at 36 hpf (arrow). Injection of *Notch3(ICD)* RNA prevents the formation of anterior structures and reduces endodermal expression of *fkd7* (arrowhead). ***(B)*** *cmlc1* is expressed in the myocardium (Reiter et al. 1999). The myocardial primordia of Notch3(ICD)-expressing embryos fail to fuse at the embryonic midline. *C-F* show dorsal views of embryos at 90% epiboly (9 hpf). ***(C)*** Notch3(ICD) suppresses formation of *sox17*-expressing endodermal progenitors. ***(D)*** Like Notch3(ICD), Notch1(ICD) suppresses endodermal formation. ***(E)*** Notch1(ICD) does not inhibit expression of *myoD*. ***(F)*** Overexpression of DeltaC, like other effectors of Notch signaling, suppresses endodermal formation. ***(G)*** A lateral view of X-Delta-1^{dn}-expressing embryo and a control sibling. X-Delta-1^{dn} minimally increases formation of the endoderm.

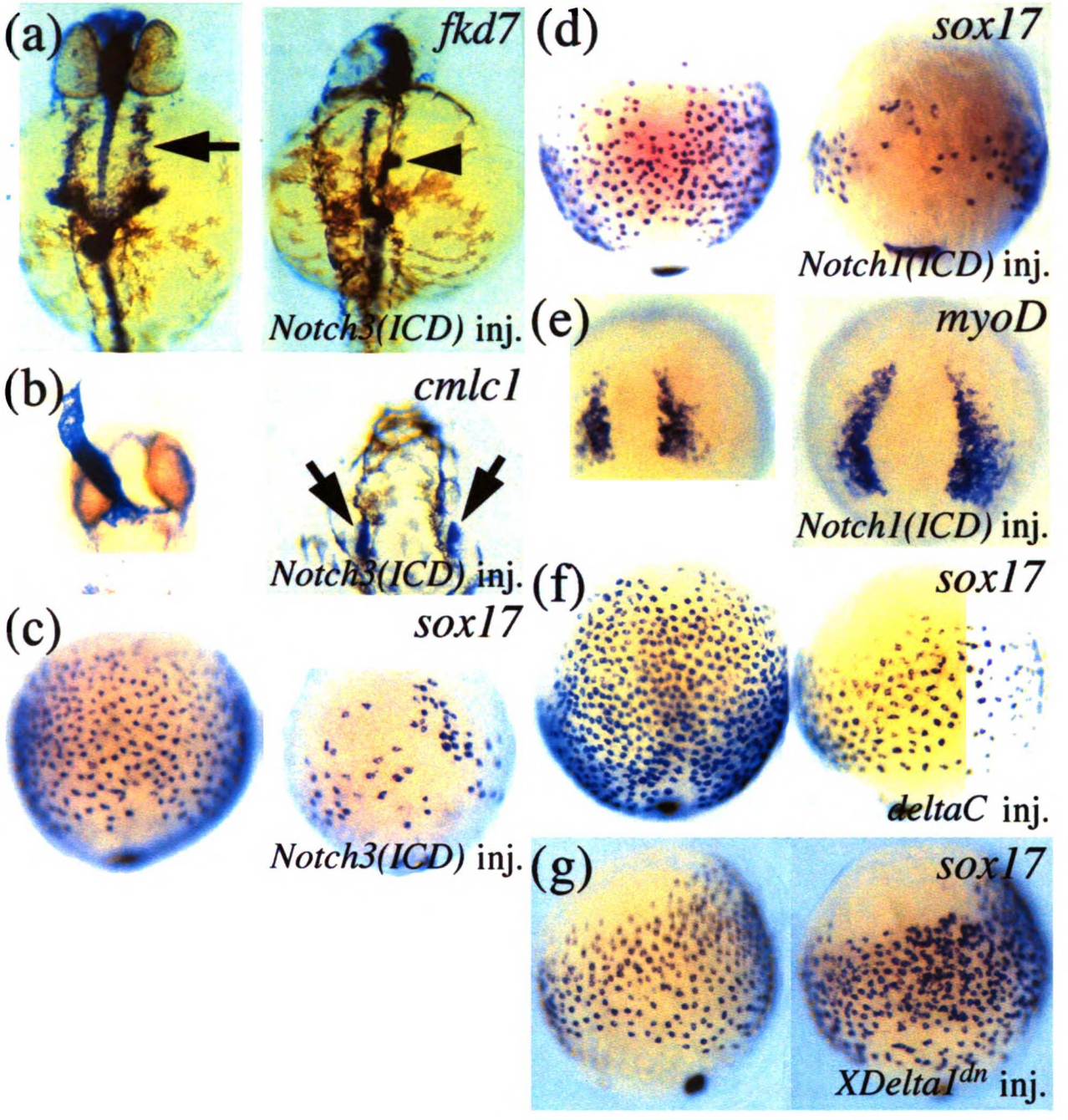
(a)
fkd7
Notch3(ICD) inj.
(b)
cmlc1
Notch3(ICD) inj.
(c)
sox17
Notch3(ICD) inj.
(d)
sox17
Notch1(ICD) inj.
(e)
myoD
Notch1(ICD) inj.
(f)
sox17
deltaC inj.
(g)
sox17
XDelta1dn inj.

Table 1. Effects of Wnt and Notch signaling on *sox17* expression

RNA Injection	Dose	Number of embryos	Decreased *sox17* expression	Increased *sox17* expression
dnXwnt8	100 pg	60	37 (62%)	0
Frzb-1	200 pg	40	14 (35%)	0
wnt8	250 pg	140	0	94 (67%)
Notch3(ICD)	50 pg	57	45 (79%)	0
Notch1(ICD)	100 pg	21	21 (100%)	0
X-Su(H)1/ANK	50 pg	100	92 (92%)*	0
deltaC	100 pg	52	17 (33%)	0
deltaD	400 pg	35	0	0
deltaD^{PST}	400 pg	38	0	0
X-Delta-1	300 pg	32	14 (44%)	0
X-Delta-1^{dn}	300 pg	42	0	4 (10%)

Embryos were scored as demonstrating reduced *sox17* expression if they formed less than half as many *sox17*-expressing endodermal cells as control siblings, and scored as demonstrating increased *sox17* expression if they exhibited a greater than 15% increase in endodermal progenitors.

*X-Su(H)1/ANK also inhibited endodermal *axial* expression in 17 of 18 injected embryos.

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Chapter 6: Unanswered questions

Functions of Gata5 during blastula and gastrula stages

We have identified aspects of myocardial and endodermal development that require Gata5 activity. As a transcriptional activator, it seems likely that Gata5 promotes the differentiation of endoderm and myocardium by transactivating downstream genes. However, how Gata5 achieves specificity to activate either endodermal or myocardial genes in different cells remains unclear. We know that Gata5 is a promiscuous partner, capable of physically interacting with several other proteins. Perhaps Gata5 is redirected to endodermal and myocardial targets by differentially expressed interacting proteins. Previously identified Gata5 partners include Nkx2.5, Gata4, Gata6, NF-AT3 and FOG2. However, while Nkx2.5, Gata4 and Gata6 may cooperate with Gata5 during segmentation stages, none are expressed within the embryo during late blastula or early gastrula stages when *gata5*-expressing blastomeres are restricted to an endodermal or mesodermal fate. The zebrafish homologs of NF-AT3 and FOG2 have not been identified and therefore remain attractive candidates as early partners that may restrict the specificity of Gata5.

All of the aforementioned Gata5 interactors have been investigated for their roles in myocardial development. As partners of Gata5, they may also cooperate with promoting the differentiation of the endoderm. Alternatively, Gata5 may cooperate with a distinct set of undiscovered endodermal partners unrelated to its myocardial-specific partners. A yeast two hybrid screen employing an endodermal library and Gata5 as bait may be a fruitful method of uncovering new endoderm-specific partners of Gata5.

Another means of gaining insight into the early roles of Gata5 is through the isolation of downstream Gata5 target genes. This has been previously accomplished only on an ad hoc candidate basis. A less biased method is to screen for genes differentially expressed in wild-type and *fau/gata5* mutants, either by differential display or subtractive hybridization. Alternatively, screening expressed sequence tag (EST) arrays takes

advantage of existing large zebrafish EST libraries. As identification of *fau/gata5* mutants during gastrulation would be laborious, isolating cDNA from *gata5*-overexpressing embryos is an easier means of creating a cDNA pool enriched for Gata5 target genes. Comparison of the hybridization patterns of probes made from enriched cDNA and cDNA from uninjected embryos allows for the identification of differentially expressed clones. Screening of a shield stage EST macroarray has revealed numerous differentially expressed clones which await further analysis. Additionally, cDNA has been sent to Matthew Clark in Tübingen, Germany for hybridization to a shield stage EST microarray.

A potential complication of this approach is the identification of genes that are not endogenous targets for Gata5. Determining whether putative targets are expressed in *fau/gata5* mutants will quickly clarify which genes critically depend upon Gata5 activity. Genes that do not require Gata5 activity for expression may represent genes such as dHand which are induced by Gata5 (see Appendix 2), but whose expression is normal in fau/gata5 mutants. Alternatively, widespread *gata5* overexpression may activate target genes of other Gata factors. As other Gata factors are involved in hematopoietic development, such genes may be of interest for other reasons.

Appendix 2 describes how double mutant analysis has revealed previously undetected functions for Gata5 and Bon. Analysis of other double mutants may similarly reveal additional aspects of Gata5 function. Fgf8 and Gata5 have been demonstrated to be required for many of the same aspects of myocardial differentiation . However, Fgf8 and Gata5 do not regulate each other's expression (data not included), suggesting that they represent distinct pathways that cooperate to promote myocardial differentiation. Preliminary analysis indicates that *ace/fgf8;fau/gata5* double mutants display more severe defects in *nkx2.5* and *cmlc1* expression than either single mutant (data not included). It would be interesting to extend the analysis of myocardial differentiation of *ace/fgf8;fau/gata5* mutants. For example, because ALPM expression of *gata6* is unaffected in *fau/gata5* and *ace/fgf8* mutants, a defect in *gata6* expression in

ace/fgf8;fau/gata5 mutants would reveal novel roles for Fgf8 and Gata5 in the regulation of *gata6*. Similarly, examination of *fgf8* and *gata5* expression in *ace/fgf8;fau/gata5* mutants may uncover examples of autoregulation.

cas^{s4}*;fau*^{tm236a} double mutants also currently reside in our zebrafish facility. While preliminary analysis suggests that endodermal defects of *cas*^{s4}*;fau*^{tm236a} mutants are additive (data not included), the absence of novel phenotypes has not been rigorously established.

Wnt signaling and vertebrate endoderm development

To address the question of how endoderm and mesoderm segregate from blastomeres that express *gata5*, we must identify the signals that regulate this diversification. As mentioned previously, during *C. elegans* development a Wnt signal from the P_2 blastomere is required to induce the neighboring EMS blastomere to form the endodermal progenitor (reviewed in Han 1997). As we have demonstrated, Wnt signals are present in the germ ring during formation of the endodermal progenitors and the canonical Wnt signaling pathway is active in at least some endodermal progenitors. Furthermore, inhibition of Wnt signaling prevents endoderm formation and *wnt8* overexpression expands endodermal gene expression. These results suggest a positive role for Wnt signals in endoderm development. However, overexpression of a stabilized version of β-catenin during late blastula stages does not seem to expand endodermal gene expression in either wild-type embryos or *cas* mutants (data not included). Therefore, it remains unclear how Wnt signaling affects endoderm development.

It is possible that Wnt signals act indirectly, perhaps through the induction of secondary Nodal signals. Test of this hypothesis will require assessment of the expression of the *Nodal*-related genes *cyc* and *sqt* in *wnt8*- and *dnXwnt8*-overexpressing embryos. Alternatively, Wnt signals may induce endodermal gene expression in a manner that does not require β-catenin, perhaps through the JNK subclass of MAPK proteins

(Boutros et al. 1998; Li et al. 1999). MAPK signaling is required for endoderm formation in *C. elegans* (Meneghini et al. 1999). Another possibility is that Wnt signals are permissive and not instructive for endoderm formation. Wnt signals are maintained in the germ ring throughout gastrulation. In contrast, *cyc* and *sqt* are expressed in the germ ring only during early gastrulation when the endoderm is involuting. Therefore, it is possible that Wnt signals function collaboratively with Nodal signals to induce endoderm. There is ample precedent for Wnt signals affecting development in cooperation with unrelated signals such as Activin (Crease et al. 1998) and Vg1 (Cui et al. 1996). Such a model may explain the differences that *wnt8* RNA and *hsp:β-catenin* DNA injection have on endoderm development, as RNA injection is likely to affect more blastomeres than heat-shock regulation.

Another connection between endoderm development and Wnt signaling is reflected by the observation that the *Xenopus* HMG domain transcription factor Sox17β can bind to β-catenin, and that this interaction represses transactivation of a TCF/LEF reporter (Zorn et al. 1999). While Sox17 transcription factors are required for *Xenopus* endoderm development, how this interaction relates to endoderm development is unclear. Perhaps the presence of Sox17 in the endodermal progenitors represses the canonical transcriptional response to Wnt signaling by blocking transcriptional activation by TCF/LEF transcription factors, and that this repression is important for the function of Sox17 in promoting endodermal differentiation. Certainly, a critical function of Wnt signaling in *C. elegans* endoderm development is the downregulation of nuclear POP-1, a TCF/LEF homolog (Rocheleau et al. 1997; Thorpe et al. 1997). Thus, at least in *C. elegans*, TCF/LEF transcription factors appear to inhibit formation of the endodermal progenitor. Alternatively, Sox17 may require β-catenin for its transcriptional activity in much the same way as TCF/LEF transcription factors do (A. Zorn, personal communication). The observation that *Sox17* overexpression in animal cap explants can induce ectopic endodermal gene expression (Hudson et al. 1997) does not necessarily

indicate that Sox17 acts independently of β-catenin, as animal cap cells contain a high level of nuclear β-catenin (A. Zorn, personal communication). It will be interesting to learn whether Sox17 can induce endodermal gene expression in animal caps derived from β-catenin-depleted embryos.

If Sox17 does require β-catenin for its transcriptional activity, an inconsistency between Sox17 function in *Xenopus* and zebrafish may be resolved. In contrast to its function in *Xenopus* animal cap explants, overexpression of *Xenopus Sox17β* and zebrafish *sox17* in zebrafish embryos does not expand endodermal gene expression (J. Alexander, personal communication). Perhaps different prevalences of stabilized β-catenin in blastula stage zebrafish embryos and *Xenopus* animal caps explains this discrepancy. If so, co-injection of stabilized β-catenin may reveal a potent endoderm-inducing capacity for Sox17 in zebrafish embryos.

Notch signaling in the zebrafish germ ring

During sea urchin development, Notch signaling promotes formation of the secondary mesenchymal cells and inhibits formation of the endoderm (Sherwood and McClay 1999). We have demonstrated that overexpression of active Notch receptor similarly inhibits the development of vertebrate endoderm (Chapter 5). However, dominant interfering constructs (e.g., *XSu(H)^{DBT}*, *delta D^{Pst}*, and *XDelta1^{STU}*) do not expand endodermal gene expression (Chitnis et al. 1995; Wettstein et al. 1997; Takke and Campos-Ortega 1999; Takke et al. 1999; data not included). Also, cells expressing activated *notch1* can express both *sox17* and *axial* (data not included). Therefore, it remains unclear how Notch signaling functions in endodermal development.

The salt-and-pepper distribution of the endodermal progenitors in the germ ring raises the possibility that Notch-regulated lateral specification selects endodermal progenitors from within a germ ring equivalency domain. Blastomeres receiving a low level of Notch signaling may upregulate their expression of a Notch ligand and be directed

toward an endodermal fate. Neighboring blastomeres may receive a higher level of Notch signaling, upregulate receptor expression, and become mesoderm. In fact, mesodermal expression of *myoD* is expanded in some embryos overexpressing active *notch1* (data not included). Although lateral specification is sufficient to explain the salt-and-pepper distribution of endoderm and mesoderm, examination of the expression of Notch signaling pathway members (e.g., *notch1b*, *notch3*, *deltaA*, *B*, *C* and *D*, *serrateB*, *zash1a* and *1b* and *her1*, *4*, *5*, *6*, *A*, *B*, *C*, *D* and *E*; data not included) have not revealed differential expression in the cells of the germ ring. Perhaps in situ hybridization is insufficiently sensitive to detect subtle differences in expression level, or perhaps the signaling pathway members that are differentially expressed in the germ ring have not yet been identified.

Pannier, a *Drosophila* Gata factor, directly activates expression of the proneural genes *achaete* and *scute* in the dorsal mesothorax (Ramain et al. 1993). Thus, Pannier appears to be a key regulator of the formation of the dorsocentral proneural cluster. By extension, one role of Gata5 in the germ ring may be the creation of an equivalence domain for marginal fates such as endoderm, pharyngeal endoderm, myocardium and prechordal plate. In support of this theory, *her5* is not expressed in the germ ring of *fau/gata5* mutants (Appendix 2). This theory would be further buoyed by the identification of a Gata5-dependent *achaete-scute* homolog (*ash*). Unfortunately, *ash* genes are bHLH transcription factors with a low degree of conserved homology outside of the bHLH domain. Perhaps the array-based screens for Gata5 target genes will identify an *ash* gene expressed in the germ ring.

Notch signaling has also been implicated in the maintenance of stem cell populations in the retina, the skeletal muscle, and the blood (Kopan et al. 1994; Shawber et al. 1996; Henrique et al. 1997; Jones et al. 1998; Li et al. 1998; Vargesson et al. 1998; Varnum-Finney et al. 1998). Instead of acting in lateral specification, Notch signaling in the germ ring may prevent blastomeres from differentiating prematurely by maintaining them in an undifferentiated stem cell-like state. Overexpressing active Notch may then

maintain blastomeres in an undifferentiated state and blastomeres expressing dominant interfering constructs may differentiate prematurely. It would be interesting to test this hypothesis by determining whether *XDelta1*^{STU} overexpression can activate the precocious expression of genes such as *sox17* and *axial* in blastomeres that have not involuted. However, this model may be an oversimplification as *myoD* expression in differentiating paraxial mesoderm initiates normally in embryos overexpressing active Notch.

Although I have focused on the expression of Notch homologs during gastrulation, the Notch signaling pathway is widely deployed throughout vertebrate development. I have produced a heat shock promoter construct that controls expression of an active *notch1* with a C-terminal Myc tag. Transgenic fish carrying this construct would be a useful tool for unraveling the diverse roles of Notch signaling in development. For example, the Notch pathway is implicated in both valve formation and chamber patterning during heart development (Li et al. 1997; Oda et al. 1997). Application of in situ hybridization and immunohistochemistry to an *hsp:Notch1(ICD)-myc* transgenic embryo may reveal how Notch signaling functions in these processes. Transplantation experiments may further indicate autonomy. Similarly, the *hsp:β-catenin(stabilized)-myc* construct allows for the creation of transgenic fish that may facilitate analysis of the many roles performed by Wnt signaling during development.

TOPGAL is a β-galactosidase reporter of TCF/LEF and β-catenin transcriptional activity (DasGupta and Fuchs 1999). Transgenic TOPGAL fish would allow anatomical description of Wnt activation of TCF/LEF transcription throughout development. Furthermore, TOPGAL fish would facilitate analysis of how Wnt signaling is perturbed by mutations of interest. Finally, TOPGAL fish would be an ideal substrate for a future screen for genes involved in the regulation or transduction of Wnt signals.

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Appendix 1: Gata5 is required for the development of the zebrafish pharyngeal endoderm

SUMMARY

During vertebrate development, the pharyngeal endoderm gives rise to the epithelium of the pharynx, the thyroid, the thymus and, in aquatic vertebrates, the gills. Recent work has identified a number of genes required for development of the non-pharyngeal endoderm, but little is known about the development of the pharyngeal endoderm. Here, we demonstrate that, unlike other zebrafish endoderm mutants such as *casanova* and *bonnie and clyde*, *faust* mutants form less pharyngeal endoderm than wild-type siblings and exhibit defects in pharyngeal endoderm differentiation. *faust* encodes Gata5, a zinc finger transcription factor gene also required for development of the myocardium and the non-pharyngeal endoderm. Overexpression of *gata5* leads to expanded expression of *nkx2.3*, a gene expressed throughout the pharyngeal endoderm. Therefore, both loss- and gain-of-function experiments demonstrate a central role for Gata5 in the regulation of the amount and differentiation of pharyngeal endoderm.

INTRODUCTION

During vertebrate development, epithelia line the pairs of mesodermally-derived pharyngeal arches. Embryologists have named these epithelia the pharyngeal pouches. In mammals, the pharyngeal pouches give rise to part of the pharynx, a common chamber for the alimentary and respiratory tubes. Outpockets of the pharynx produce the epithelium of the tonsils, middle ear and thymus as well as the thyroid and parathyroid glands. Although fish lack parathyroid glands, the fish pharyngeal endoderm contributes to one organ absent in mammals; the gills. While the pharyngeal pouches give rise to much of the epithelial lining of the pharynx, a distinct endodermal population that gives rise to the remainder of the epithelial lining of the gut appears to contribute to the dorsal aspect of the pharynx (Moody and Kline 1990). To distinguish between these populations, in this text we refer to them as gut endoderm and pharyngeal endoderm.

Despite the importance of the organs derived from the pharyngeal endoderm to human health, little is known about pharyngeal endoderm development. Expression studies have identified some of the genes potentially important for pharyngeal endoderm differentiation. In zebrafish, the pharyngeal endodermal precursors express two Nk homeobox genes, *nkx2.3* and *nkx2.7*, from late segmentation stages (Lee et al. 1996). Neither gene is specific for the pharyngeal endoderm; *nkx2.3* is also expressed in the posterior visceral mesoderm and *nkx2.7* is also expressed in the myocardial primordia (Lee et al. 1996 and J.F. Reiter and D.Y.R. Stainier, unpublished data). Mouse *nkx2.3* is also expressed within the pharyngeal endoderm, but pharyngeal defects have not been described for mouse *nkx2.3* null mutants. Another transcription factor gene, *pax9a*, is expressed in the medial pharyngeal endoderm as well as in the medial somites (Neubuser et al. 1995).

Recent work has identified a number of genes required for formation of the gut endoderm, including *one-eyed pinhead* (*oep*), *casanova* (*cas*) and *bonnie and clyde* (*bon)*

(Schier et al. 1997; Alexander et al. 1999; Kikuchi et al. 1999). However, pharyngeal endoderm formation is relatively unaffected in the *cas* and *bon* mutants, leading to the proposal that, from a genetic perspective, the pharyngeal endoderm may be more akin to mesoderm than endoderm (Alexander 1999).

We have previously shown that the zebrafish *faust* (*fau*) locus encodes Gata5, a zinc finger transcription factor, and that Gata5 is essential for the development of the myocardium and the gut endoderm (Reiter et al. 1999; Reiter and Stainier 1999). Additionally, *fau/gata5* mutants exhibit defects in the morphogenesis of the pharyngeal endoderm and the differentiation of two of its derivatives, the thyroid and the thymus (Reiter et al. 1999; Reiter and Stainier 1999). Here, we show that *gata5* is expressed in the precursors of the pharyngeal endoderm and that expression of *nkx2.3* and *pax9a* is diminished in *fau/gata5* mutants, suggesting that Gata5 autonomously promotes the differentiation of pharyngeal endoderm. Furthermore, we demonstrate that overexpression of *gata5* dramatically expands *nkx2.3* expression, further arguing that Gata5 plays a critical role in pharyngeal endoderm development.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish were maintained and staged as described (Westerfield 1995). The *tm236a* allele of *fau* was used (Chen et al. 1996).

Wholemount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al. 1998). Photographs were taken on a Leica MZ12 stereomicroscope and processed using Adobe Photoshop 4.0.

mRNA injection

Full length, capped *gata5* and *gfp* messages were synthesized from previously described templates (Reiter et al. 1999) using the SP6 mMessage Machine system (Ambion). Embryos were injected at the 1-4 cell stage with 20 pg of *gata5* or *gfp* mRNA.

RESULTS AND DISCUSSION

The precursors of the pharyngeal endoderm express *gata5*

During segmentation stages, *gata5* is expressed throughout the anterior lateral plate mesoderm (ALPM; Figure A1.1a). Comparison with other genes reveals that expression of *gata5* extends more anteriorly than does that of *nkx2.5*, an Nk homeobox gene expressed in the myocardial precursors during early segmentation stages (Figure A1.1a). Expression of *nkx2.7* overlaps with that of *nkx2.5*, but extends more anteriorly to include the pharyngeal endoderm precursors (Lee et al. 1996); Figure A1.1a). By extension, the *gata5*-expressing cells anterior to the domain of *nkx2.5* co-expression are most probably the precursors of the pharyngeal endoderm.

Microscopic examination of these pharyngeal endoderm precursors reveals that they are small, round cells indistinguishable from the more posterior myocardial precursors. In contrast, the precursors of the gut endoderm are large, flat cells (Warga and Nusslein-Volhard 1999). This distinction further strengthen the assertion that the pharyngeal endodermal precursors are more similar to the anterior lateral plate mesoderm than to the precursors of the gut endoderm (Alexander 1999).

nkx2.3 is not expressed in the pharyngeal endoderm precursors until late segmentation stages. At these and at pharyngula stages, there is little overlap of *nkx2.3* and *gata5* expression (Figure A1.1b), indicating that *gata5* is not expressed throughout the pharyngeal endodermal precursors after early segmentation stages.

Expression of *nkx2.3* and *pax9a* is reduced in *fau/gata5* mutants

We have previously demonstrated that *fau/gata5* mutants exhibit defects in the morphogenesis of the pharyngeal endoderm and the differentiation of organs derived from the pharyngeal endoderm (Reiter et al. 1999; Reiter and Stainier 1999). Two other cardia bifida mutants, *cas* and *bon*, also display defects in pharyngeal endoderm morphogenesis

(Alexander et al. 1999; Kikuchi et al. 1999). All three genes are required for the development of the gut endoderm, suggesting that the gut endoderm may play a role in the morphogenesis of the pharyngeal endoderm (Alexander et al. 1999).

However, comparison of the pharyngeal endoderm defects of these three mutants reveals important differences. Despite defects in morphogenesis, both *cas* and *bon* mutants produce an approximately wild-type amount of pharyngeal endoderm. In contrast, *fau/gata5* mutants also exhibit a decrease in the number of cells expressing *nkx2.3* (Figure A1.2a). A similar defect is observed in *oep* mutants (data not included), which is not surprising given that Oep is required to maintain expression of *gata5* past early gastrulation (see Chapter 4). The dependence of pharyngeal endoderm gene expression on Gata5 is even more apparent in the *cas*e of *pax9a*; *fau/gata5* mutants express *pax9a* normally in somites, but do not express *pax9a* in the pharyngeal endoderm (Figure A1.2b). *dHand* expression in the pharyngeal mesoderm is relatively normal in *fau/gata5* mutants (data not included), suggesting that Gata5 is not required for the development of other pharyngeal components.

Overexpression of *gata5* expands expression of *nkx2.3*

Gene overexpression experiments have provided a means of complementing the findings of loss-of-function studies. Earlier, we demonstrated that overexpression of *gata5* can expand myocardial and gut endoderm gene expression (Reiter et al. 1999; Reiter and Stainier 1999). Here, we show that injection of a small amount of *gata5* mRNA (20 pg) dramatically expands expression of *nkx2.3* (22 of 36; Figure A1.3). Increased *nkx2.3* expression was observed in cells found overlying the anterior and ventral yolk, and not at more dorsal positions within the embryo. Microscopic examination reveals that the corresponding region of uninjected embryos is largely free of cells, indicating that *gata5* overexpression does not simply expand *nkx2.3* expression to regions that normally do not express *nkx2.3*, but leads to abnormal positioning of *nkx2.3*-expressing cells within the

embryo. Whether these supernumerary *nkx2.3*-expressing cells overlying the yolk are due to altered cell migration or increased proliferation is unclear.

Gata5 is also necessary and sufficient for expression of *nkx2.5* (Reiter et al. 1999), and it is interesting to speculate how Gata5 may regulate both *nkx2.3* and *nkx2.5* expression. Perhaps Gata5 promotes the expression of *nkx2.5* in the posterior ALPM and *nkx2.3* in the anterior ALPM, suggesting the presence of an anterior or posterior ALPM-specific cofactor of Gata5 capable of directing Gata5 to different targets. Alternatively, Gata5 may promote expression of *nkx2.7* which in turn would regulate expression of both *nkx2.5* and *nkx2.3* in some context-dependent manner.

Gata5 appears to regulate *nkx2.5* in a complex manner at both the transcriptional and post-translational levels. Gata5 is thought to promote *nkx2.5* expression through functionally important gata motifs in *cis*-regulatory elements (Searcy et al. 1998; Lien et al. 1999). Additionally, Gata5 binds to the Nkx2.5 protein to potentiate its transcriptional activity (Durocher et al. 1997; Lee et al. 1998; Sepulveda et al. 1998). Perhaps this paradigm of dual regulation is applicable to other Nk homeobox transcription factors as well. For example, while we have shown that Gata5 promotes the expression of *nkx2.3*, Gata5 may prove to also cooperate with the Nkx2.3 protein to execute pharyngeal endoderm differentiation. Regardless, it appears that Gata5 is essential not only to myocardial differentiation, but is also critical for multiple aspects of pharyngeal endoderm development.

Future directions

The connection between Gata5 and pharyngeal endoderm differentiation would be strengthened by more careful analysis of the previously described observations. For example, definitive proof that *gata5* is expressed in the *nkx2.7*-expressing precursors of the pharyngeal endoderm will require double in situ hybridization analysis. Similarly, analysis of the expression of *nkx2.7* and other pharyngeal endoderm genes in *faw/gata5*

mutants and *gata5*-overexpressing embryos will provide a better understanding of whether Gata5 functions in the induction, proliferation or differentiation of the pharyngeal endoderm.

nkx2.7 is expressed with *gata5* in the ALPM, before *nkx2.5* and *nkx2.3*. It has been suggested that Nkx2.7 promotes the expression of *nkx2.5* and *nkx2.3* (Lee et al. 1996). While analysis of the effects of mutations in *nkx* genes would be the preferred method of establishing regulatory relationships between these genes, overexpression provides a more immediately available means.

A larger question concerns how the ALPM becomes patterned along the anterior-posterior axis. The anterior domain expresses *nkx2.3* and presumably gives rise to the pharyngeal endoderm, while the posterior domain expresses *nkx2.5* and encompasses the myocardial precursors. Retinoic acid and Hox proteins suggest themselves as candidate participants, but certainly other regionally expressed factors deserve investigation.

Here, we have reasserted the proposal that the pharyngeal endoderm is genetically more similar to the mesoderm than the endoderm (Alexander 1999). Determination of whether the pharyngeal endoderm is derived from the endodermal progenitors or from the ALPM would require fate mapping studies. Such studies would be facilitated by previous mapping of the pharyngeal endoderm precursors at late blastula stages (Warga and Nusslein-Volhard 1999). Proof that the pharyngeal endoderm is in fact mesodermal in origin would require demonstrating that the pharyngeal endoderm precursors do not become large, flat endodermal progenitors during gastrulation, but in fact become small, round cells that become part of the ALPM. Such evidence would incontrovertibly establish that, like *C. elegans*, the anterior portion of the zebrafish alimentary tract is of mesodermal origin.

Figure A1.1

Comparison of *gata5*, *nkx2.3*, *nkx2.5* and *nkx2.7* expression patterns. Views are dorsal with anterior up. **(a)** Expression of *nkx2.7* and *nkx2.5* at the 1-somite stage (10.3 hours post-fertilization; hpf) and *gata5* at the bud stage (10 hpf). Both *nkx2.7* and *gata5* are expressed throughout the anterioposterior extent of the ALPM. *nkx2.5* expression in the ALPM is more restricted. **(b)** Expression of *nkx2.3* at the 12-somite stage (15 hpf) and *gata5* at the 15-somite stage (16.5 hpf). Both embryos were co-stained for *ntl* which marks the notochord, the anterior tip of which is indicated. At these stages, *nkx2.3* and *gata5* are expressed by largely distinct cell populations.

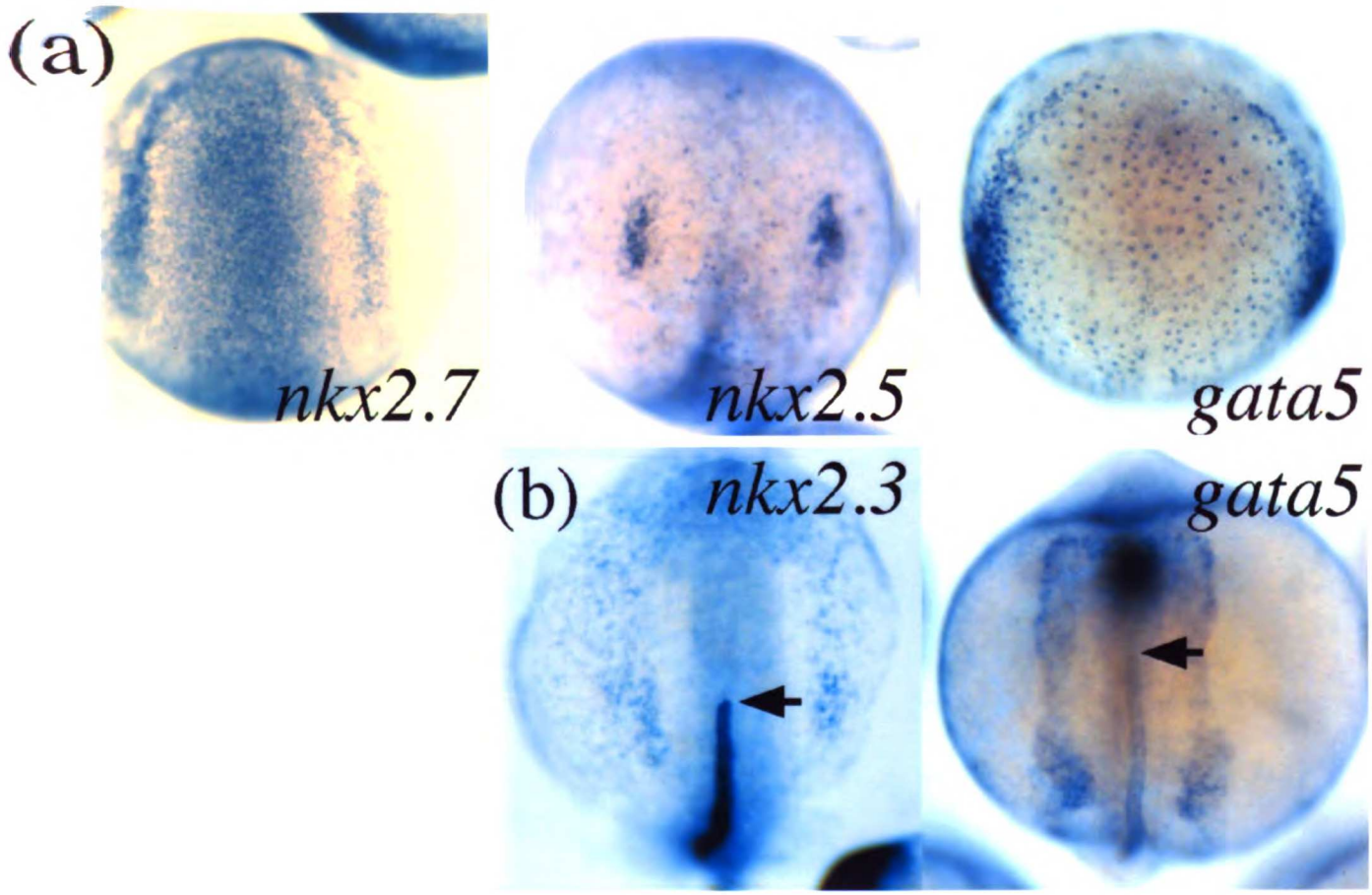

(a)
nkx2.7
nkx2.5
gata5
(b)
nkx2.3
gata5

Figure A1.2

***fau/gata5* mutants display reduced expression of pharyngeal endoderm genes.** Views are dorsal with anterior up. **(a)** Fewer cells appear to express *nkx2.3* in *fau/gata5* mutants than in wild-type siblings at the 25-somite stage (21.5 hpf). **(b)** At the 20-somite stage (19 hpf), *pax9a* is not expressed in the medial pharyngeal endoderm of *fau/gata5* mutants.

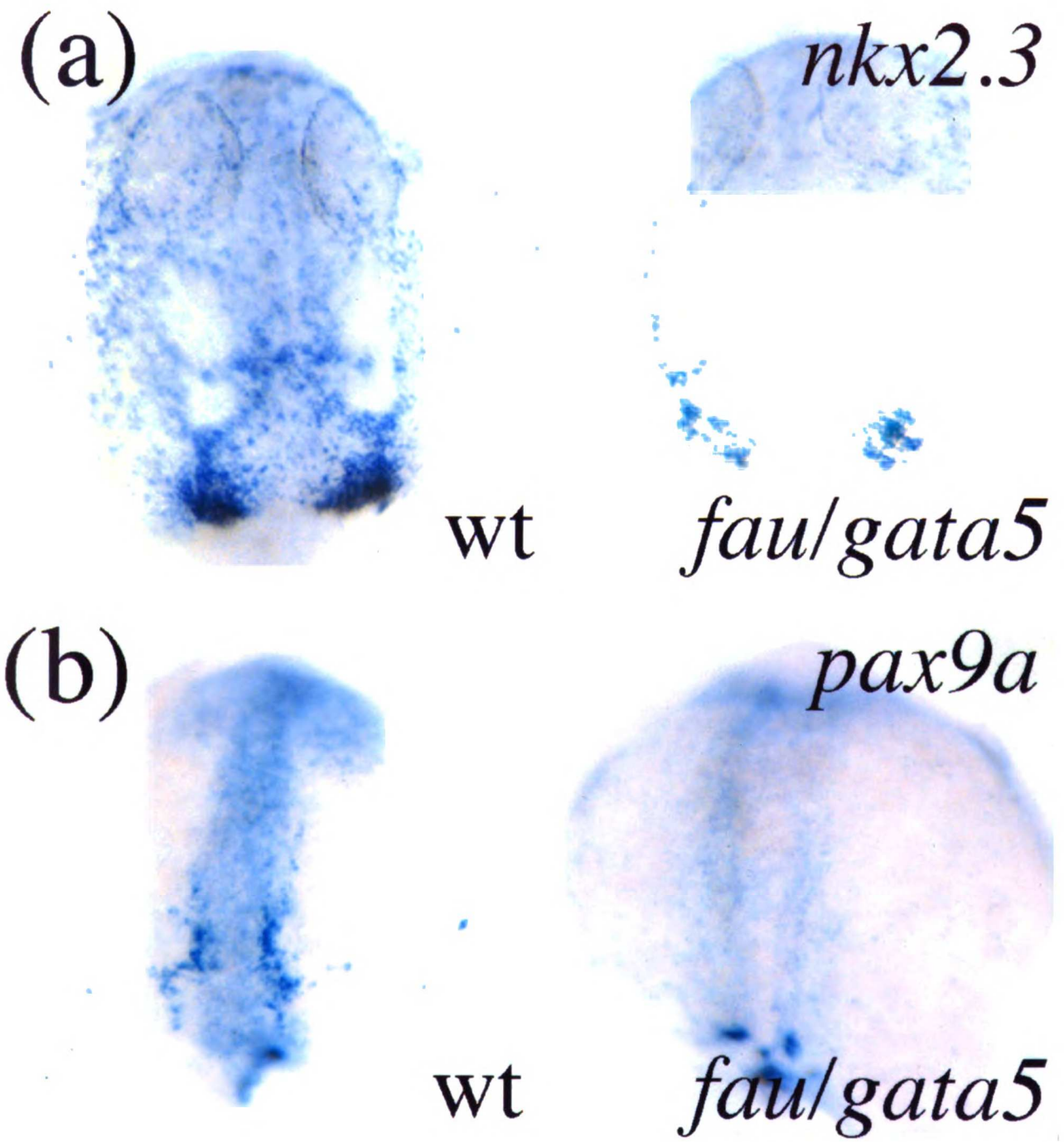
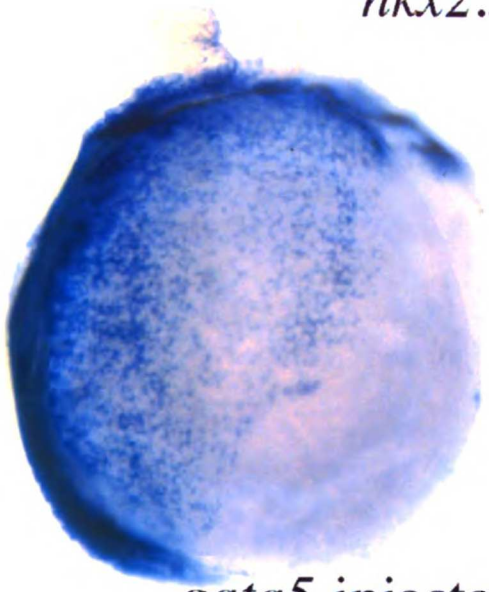

(a)
nkx2.3
wt
fau/gata5
(b)
pax9a
wt
fau/gata5

Figure A1.3

Overexpression of *gata5* expands *nkx2.3* expression. Views are lateral with dorsal to the right. Although injection of 20 pg of *gfp* mRNA does not perturb *nkx2.3* expression, injection of an equal amount of *gata5* mRNA leads to expanded expression of *nkx2.3*, manifested by an increased number of *nkx2.3*-expressing cells overlying the yolk.

nkx2.3



gfp injected

gata5 injected

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Appendix 2: Overlapping functions for Gata5 and Bon in prechordal plate and lateral plate mesoderm development

SUMMARY

The zebrafish *faust* (*fau*) locus encodes Gata5 and the *bonnie and clyde* (*bon*) locus encodes a homolog of Mixer, two transcription factors that perform early and essential roles in myocardial and endodermal development. Analysis of embryos mutant for both *fau/gata5* and *bon* (*fau/gata5*;*bon* mutants) reveals novel, overlapping functions for their gene products. Although *fau/gata5* and *bon* mutants both display only minor defects in the expression of *dHand*, a bHLH transcription factor gene expressed in the lateral plate mesoderm, *fau/gata5*;*bon* double mutants display almost no lateral plate *dHand* expression, revealing that Gata5 and Bon are critical for *dHand* expression and function in the differentiation of posterior lateral plate mesoderm. Also, although neither *fau/gata5* or *bon* mutants exhibit defects in the development of the anterior neuroectoderm, *fau/gata5*;*bon* mutants have small, misshapen eyes and dysmorphic brains. We demonstrate that during gastrulation, *fau/gata5*;*bon* mutants display diminished expression of *goosecoid*, a *bicoid*-related gene expressed in the prechordal plate. As the prechordal plate is implicated in patterning of the neuroectoderm, we propose that the defects in eye and brain development observed in *fau/gata5*;*bon* mutants reflect an underlying defect in prechordal plate differentiation. Furthermore, the anterior axial mesoderm of *fau/gata5*;*bon* mutants expresses *flh*, a marker of more posterior axial mesoderm, indicating that without Gata5 and Bon function, the prechordal plate adopts some characteristic of the notochord precursors. Cumulatively, these results suggest that Gata5 and Bon cooperate to promote prechordal plate differentiation dorsally and myocardial differentiation ventrally.

INTRODUCTION

The prechordal plate arises from a subset of the dorsal equatorial cells of the blastula stage embryo that form the shield, the zebrafish equivalent of the Spemann organizer (Kimmel et al. 1990; Ho 1992). The shield also gives rise to more posterior axial mesoderm which forms the notochord. Patterning of the axial mesoderm into the prechordal plate and notochord precursors is apparent as early as gastrulation stages, when the bicoid-type homeobox gene *goosecoid* (*gsc*) is expressed specifically in the prechordal plate and *floating head* (*flh*), the zebrafish homolog of the *Xenopus laevis* transcription factor gene *Xnot*, is expressed in the notochord progenitors (Stachel et al. 1993; von Dassow et al. 1993; Talbot et al. 1995). In fish, the prechordal plate differentiates into head and eye muscles and hatching gland (Jacob et al. 1984; Wachtler et al. 1984; Kimmel et al. 1995).

While the function of the prechordal plate in patterning the overlying neuroectoderm has been the focus of much study (Li et al. 1997; Pera and Kessel 1997), there is little known about the development of the prechordal plate itself. Prechordal plate formation requires Nodal signaling; embryos mutant for *cyclops* or *squint*, two genes that encode Nodal-related signaling proteins, lack prechordal plate (Feldman et al. 1998; Rebagliati et al. 1998). Similarly, embryos lacking zygotic Oep (*Zoep*), an EGF-CFC protein required for Nodal signaling, also lack prechordal plate (Schier et al. 1997; Zhang et al. 1998). Moreover, overexpression of low levels of Antivin/Lefty2, a putative antagonist of Nodal signaling, inhibits formation of the prechordal plate (Thisse and Thisse 1999). Further reduction of Nodal signaling suppresses formation of other types of mesoderm, as observed in embryos mutant for both *cyclops* and *squint*, in embryos lacking both maternal and zygotic Oep, and in embryos overexpressing high levels of Antivin/Lefty2 (Feldman et al. 1998; Gritsman et al. 1999; Thisse and Thisse 1999). Taken together, these results suggest that prechordal plate formation requires a higher

level of Nodal signaling than does formation of other mesoderm. However, the molecular mechanism by which Nodal signaling is interpreted to achieve prechordal plate formation is not understood.

Nodal signaling is also essential for development of the myocardial precursors. As shown in Chapter 5, *Zoep* mutants fail to maintain *gata5* expression in the myocardial precursors, suggesting that, as for the prechordal plate, a high level of Nodal signaling is required for myocardial development. Gata5 is required for multiple aspects of myocardial differentiation including the expression of *nkx2.5*, the earliest known marker of the myocardial precursors (Reiter et al. 1999). However, Gata5 is not essential for the expression of other myocardial genes such as *gata6* (Reiter et al. 1999) and, as we show here, *dHand*, a bHLH transcription factor essential for ventricular development in the mouse (Srivastava et al. 1997).

In addition to inducing *gata5*, Nodal signaling induces the expression of *bonnie and clyde* (*bon*) , which encodes a zebrafish paired-type homeobox transcription factor similar to chick CMIX (Kikuchi et al. 1999). *gata5* and *bon* show striking functional similarities. Both genes are initially expressed during late blastula stages in the marginal cells of the germ ring, a domain that encompasses the precursors of the endoderm and prechordal plate (Kimmel et al. 1990; Alexander et al. 1999; Reiter et al. 1999; Rodaway et al. 1999). Bon, like Gata5, is required for endoderm formation and for the early expression of *nkx2.5* in the myocardial precursors (Kikuchi et al. 1999; L. Trinh, D.Y.R. Stainier, unpublished results). Previously, we have reported that embryos mutant for both *fau/gata5* and *bon* (*fau/gata5*;*bon* mutants) display more profound defects in endoderm formation than exhibited by either single mutant (Reiter and Stainier 1999). Here, we show that *fau/gata5*;*bon* double mutants also display more severe defects in the differentiation of the lateral plate mesoderm than observed in either single mutant. *fau/gata5*;*bon* mutants fail to express *dHand* in the lateral plate mesoderm and exhibit

profoundly reduced expression of *cmlc1*, a marker of myocardial maturation (Reiter et al. 1999).

Furthermore, *fau/gata5;bon* double mutants exhibit novel defects in eye and brain development not exhibited by either single mutant. We hypothesize that these defects are secondary to defects in prechordal plate formation, and demonstrate that expression of *gsc*, *antivin* and *shh* in prechordal plate precursors, is diminished in *fau/gata5;bon* mutants. Conversely, expression of *flh*, a gene specifically expressed in the notochord precursors, is extended anteriorly in *fau/gata5;bon* mutants. Cumulatively, these results suggest that Gata5 and Bon are essential for the differentiation of prechordal plate, and that without Gata5 and Bon function, the prechordal plate assumes some characteristics of more posterior mesoderm.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish were maintained and staged as described (Westerfield 1995). Mutant alleles used were *bon*^{m425} (Stainier et al. 1996) and *fau*^{tm236a} (Chen et al. 1996).

Wholemount in situ hybridization

We performed in situ hybridization as described (Alexander et al. 1998). Photographs were taken on a Leica MZ12 stereomicroscope and processed using Adobe Photoshop 4.0. Embryos were genotyped post-in situ hybridization to confirm their identity. Briefly, this entailed serial rehydration with PBS + 0.1% Tween, proteinase K digestion at 55° C for 10 hours, and PCR genotyping. The *bon*^{m425} mutation was identified using a restriction fragment length polymorphism described by Kikuchi et al. (Kikuchi et al. 1999), and the *fau*^{tm236a} mutation was identified using a single stranded conformational polymorphism (primers: 5'-TGCAATGCATGTGGACTTTAC-3' and 5'-CATGCAGCCTCTCAAAATTC-3'). However, embryos stained for *dHand* expression were not successfully genotyped for the *fau*^{tm236a} mutation.

RESULTS AND DISCUSSION

Gata5 and Bon have overlapping functions in the differentiation of the lateral plate mesoderm

Both *fau/gata5* and *bon* mutants exhibit defects in the differentiation of the anterior lateral plate mesoderm (ALPM), the domain of the lateral plate mesoderm containing the myocardial precursors. Both mutants display decreased expression of *nkx2.5*, a gene encoding an Nk homeobox transcription factor required for murine heart development and *hrt*, an evolutionarily conserved T-box containing gene (Kikuchi et al. 1999; Reiter et al. 1999; J. Reiter, K. Griffin, D.Y.R. Stainier, unpublished data).

dHand, a bHLH transcription factor gene required for murine heart development, is expressed in the ALPM as well as more posterior lateral plate mesoderm which gives rise to the fin bud and the cells that line the yolk extension. Additionally, *dHand* is expressed in a few cells dorsal to the ALPM thought to be developing neurons (D. Yelon, D.Y.R. Stainier, unpublished data). In contrast to *nkx2.5* and *hrt*, *dHand* is expressed relatively normally in *fau/gata5* and *bon* mutants (Figure A2.1a). However, analysis of embryos putatively mutant for both *fau/gata5* and *bon* reveals dramatically decreased expression of *dHand* (Figure A2.1a). Moreover, decreased *dHand* expression is observed not only in the ALPM, but throughout the lateral plate mesoderm (Figure A2.1b), revealing that Gata5 and Bon also play roles in the differentiation of the *dHand*-expressing cells of the posterior lateral plate mesoderm. Neuronal expression of *dHand* is unaffected in *fau/gata5;bon* mutants, arguing that Gata5 and Bon regulation of *dHand* expression is limited to the mesoderm. The role of Gata5 in *dHand* regulation is further supported by gain-of-function experiments; overexpression of *gata5* mRNA leads to expanded and ectopic *dHand* expression (Figure A2.1c).

In accordance with the increased defects in ALPM differentiation, expression of *cmlc1* is more profoundly diminished in *fau/gata5;bon* mutants than in either single mutant (Figure A2.1d).

fau/gata5;bon mutants exhibit defects in eye and brain development

The morphogenesis of the anterior neuroectoderm is normal in both *fau/gata5* and *bon* mutants. *fau/gata5;bon* double mutants, however, display striking defects in both brain and eye development (Figure A2.2). The eyes of *fau/gata5;bon* mutants are small, hypopigmented and poorly separated in comparison to those of wild-type siblings. Also, the forebrains of *fau/gata5;bon* mutants are small and dysmorphic. Thus, analysis of double mutants reveals overlapping roles for Gata5 and Bon in the development of the anterior neuroectoderm.

A role for Gata5 and Bon in prechordal plate differentiation

The prechordal plate is implicated in the induction and patterning of the anterior neuroectoderm. As both *gata5* and mixer are expressed in the prechordal plate precursors, but not in the neuroectoderm, we examined whether the defects in eye and brain development were secondary to defects in prechordal plate formation.

gsc encodes a bicoid-related transcription factor expressed specifically in the prechordal plate precursors during gastrulation. *gsc* is expressed normally in *fau/gata5* and may be expressed normally in *bon* mutants (L. Trinh, D.Y.R. Stainier, unpublished results). In contrast, *gsc* expression in *fau/gata5;bon* mutants is either greatly diminished or absent at shield stage (Figure A2.3a). By late gastrulation, *fau/gata5;bon* mutants express *gsc*, but at reduced levels (Figure A2.3b). Similarly, prechordal plate expression of *antivin*, a zebrafish homolog of mouse Lefty2, is reduced in *fau/gata5;bon* mutants (Figure A2.3c).

sonic hedgehog (*shh*) is required for the induction of ventral neuroectodermal fates (Roelink et al. 1995). Examination of *shh* expression in *fau/gata5;bon* mutants reveals that, while expression in the posterior axial mesoderm is not affected, the prechordal plate precursors fail to express *shh* (Figure A2.3d). Therefore, defects in eye and brain development may be secondary to the absence of Shh signaling from the underlying prechordal plate.

In contrast, *cyclops* (*cyc*), a gene encoding a Nodal-related signaling protein expressed predominantly in the prechordal plate during gastrulation and implicated in patterning of the neuroectoderm, is expressed normally in *fau/gata5;bon* mutants (Figure A2.3e). Therefore, prechordal plate is formed in *fau/gata5;bon* mutants, but its differentiation is defective. Also, these results suggest that *gsc* expression is dispensable for *cyc* expression in the prechordal plate.

As mentioned previously, *fau/gata5;bon* mutants display defects in brain morphogenesis. Transient defects in the ventral forebrain expression of *nkx2.1*, an Nk homeodomain transcription factor gene, are apparent in *bon* mutants during early segmentation stages (L. Trinh, D.Y.R. Stainier, unpublished results). Defects in brain development are apparent in *fau/gata5;bon* mutants as early as 90% epiboly; embryos mutant for *fau/gata5* and *bon* fail to induce *nkx2.1* (Figure A2.3f). Taken together, these results indicate that the failure of the prechordal plate differentiation caused by loss of Gata5 and Bon function prevents the normal differentiation of the overlying anterior neuroectoderm, possibly through the loss of Shh signaling.

The axial mesoderm is patterned by gastrulation stages into the anterior prechordal plate precursors which express *gsc* and the posterior notochord precursors which express *flh*. *fau/gata5;bon* mutants exhibit an anterior expansion of *flh* expression commensurate with the decrease of *gsc* expression (Figure A2.3g). This result suggests that not only are Gata5 and Bon required for the proper differentiation of the prechordal plate, but that without Gata5 and Bon, the anterior axial mesoderm assumes characteristics of more

posterior notochord precursors. However, the transformation from prechordal plate to notochord is not total, as the anterior axial mesoderm fails to express *shh* in *fau/gata5;bon* mutants.

Subtle defects in the development of the axial mesoderm are apparent even in *fau/gata5* mutants. *her5*, a zebrafish homolog of *Drosophila Enhancer of split*, is expressed in cells of the deep shield early in gastrulation. This feature of *her5* expression does not depend upon *casanova* (data not included), arguing that these deep *her5*-expressing cells are not endodermal and are distinct from the *her5*-expressing cells of late gastrulation. However, the deep shield expression of *her5* requires Gata5 (Figure A2.3h).

Conclusions and future directions

Taken with previous work, these results indicate that Gata5 and Bon are required for the differentiation of many of the tissues that form from marginal blastomeres: the endoderm, the myocardium ventrally, and the prechordal plate dorsally. Moreover, we have demonstrated that Gata5 and Bon have some functions in common. Although development of the endoderm and myocardium is abnormal in each single mutant, *fau/gata5;bon* double mutants form very little or no endoderm (Reiter and Stainier 1999) and display more profound defects in myocardial differentiation, as demonstrated by the near absence of lateral plate mesodermal expression of *dHand* expression. Together, these results suggest two models for specification of fate at the zebrafish margin. We know that high levels of Nodal signaling induce expression of *gata5* and *bon* within the germ ring (Alexander et al. 1999; Reiter and Stainier 1999; Rodaway et al. 1999). Gata5 and Bon could promote prechordal plate formation by maintaining the expression of Nodal-related genes. Alternatively, Gata5 and Bon could cooperate with regionally expressed factors to induce prechordal plate dorsally and myocardium more ventrally. These models may be easily distinguished by examining *cyclops* and *squint* expression in *fau/gata5;bon* mutants.

If Gata5 and Bon do cooperate with dorsoventrally restricted factors to direct the differentiation of different tissues, the identification of these partners would be a worthwhile goal. Studies of *Xenopus* dorsoventral patterning have suggested several candidates. The homeobox transcription factors Xvent-1 and -2 participate in Bmp signaling to pattern the dorsal-ventral axis (Onichtchouk et al. 1996; Onichtchouk et al. 1998). As the myocardium is derived from a ventrolateral domain, zebrafish homologs of Xvent-1 and -2 may cooperate with Gata5 and Bon to specify the portion of the germ ring that will give rise to the myocardium. Dorsally, nuclear localized β-catenin or its downstream target, the homeobox transcription factor Siamois, may distinguish the future axis from the rest of the germ ring (Lemaire et al. 1995; Brannon et al. 1997).

Analysis of *fau/gata5;bon* double mutants may prove to be a powerful tool in the discovery of roles for Gata5 and Bon not immediately apparent from the analysis of the single mutants. For example, further characterization of ALPM and prechordal plate differentiation in *fau/gata5;bon* mutants may expand the implications of this work. For instance, expression of neither *gata4* or *gata6* is profoundly affected in *fau/gata5* and *bon* mutants (Kikuchi et al. 1999; Reiter et al. 1999). However, as with *dHand*, *gata5* overexpression is able to expand the expression of both genes (Reiter et al. 1999). Therefore, it would be interesting to determine whether *gata4* and *gata6* expression is severely diminished in *fau/gata5;bon* mutants. Similarly, analysis of the expression of other genes expressed in the prechordal plate may further demonstrate how Gata5 and Bon promote its differentiation. Like, myocardium and prechordal plate, the fin bud mesoderm also originates from marginal blastomeres that express both *gata5* and *bon*. Analysis of early markers of the fin bud mesoderm in *fau/gata5;bon* mutants may also reveal roles for these genes in limb development.

Another possibly fruitful area of investigation would be the further characterization of the interaction of Gata5 and Bon. Gata5 is known to physically interact with several transcription factors including Nkx2.5, NF-AT3, Gata4 and Gata6.

Similarly, paired-type transcription factors, such as Bon, are known to interact with caudal-like homeodomain proteins and members of the pRB family (Wiggan et al. 1998; Ritz-Laser et al. 1999). It would be interesting to test whether the genetic interaction of Gata5 and Bon corresponds to a biochemical one.

Figure A2.1

Lateral plate defects in *fau/gata5;bon* mutants. ***(a)*** Dorsal views of *dHand* expression at the 22-somite stage (20 hours post-fertilization [hpf]). The ALPM of both *bon* and *fau/gata5* mutants does not migrate to the embryonic midline. Additionally, *fau/gata5* mutants exhibit reduced *dHand* expression in the ALPM. *fau/gata5;bon* mutants exhibit almost no *dHand* expression in the ALPM, although neuronal expression is normal (arrows). ***(b)*** Lateral views of *dHand* expression at 22-somite stage (20 hpf). *fau/gata5;bon* mutants exhibit reduced *dHand* expression in both the anterior and posterior (arrow) lateral plate mesoderm. ***(c)*** Compared to injection of *gfp* mRNA, injection of 50 pg *gata5* mRNA expands *dHand* expression by the 12-somite stage (14.5 hpf). ***(d)*** Dorsal views of *cmlc1* expression at 40 hpf. Compared to *fau/gata5* mutants and *bon* mutants, *fau/gata5;bon* mutants exhibit reduced expression of *cmlc1*. For example, while *cmlc1* expression is reduced in the *fau/gata5* mutant, the *fau/gata5;bon* mutant expresses *cmlc1* in only one myocardial primordia (arrows).

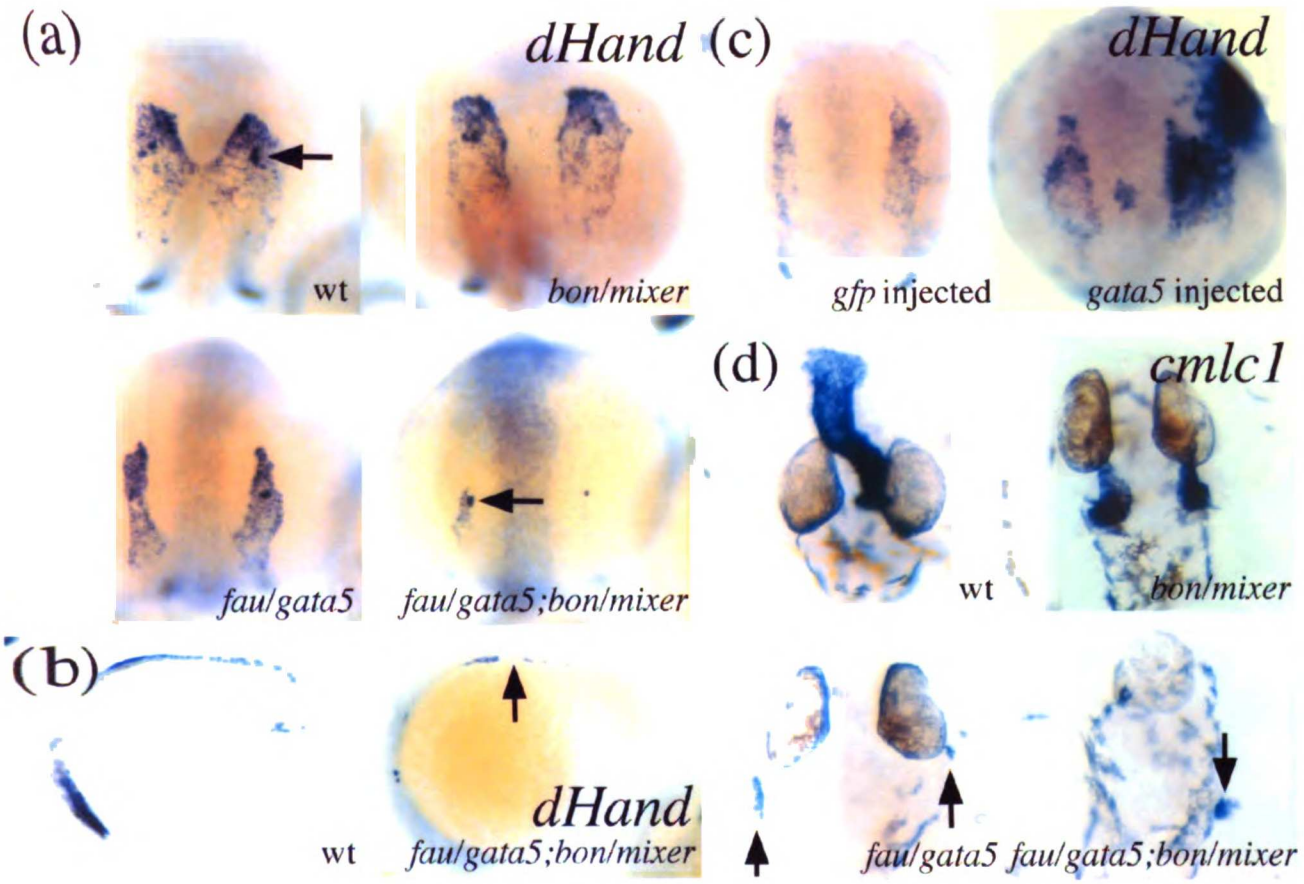

(a)
dHand
wt
bon/mixer
fau/gata5
fau/gata5;bon/mixer
(b)
wt
dHand
fau/gata5;bon/mixer
(c)
dHand
gfp injected
gata5 injected
(d)
cmlc1
wt
bon/mixer
fau/gata5
fau/gata5;bon/mixer

Figure A2.2

Eye and brain formation is disrupted in *fau/gata5;bon* mutants. *(a)* Lateral and *(b)* anterior views at 32 hpf. ***(a)*** Compared to *fau/gata5* mutants and wild-type siblings, *fau/gata5;bon* mutants form small, hypopigmented eyes and small, disorganized brains. ***(b)*** Wild-type embryos form two, pigmented eyes well separated by the forebrain. In contrast, *fau/gata5;bon* mutants display dysmorphic eyes and reduced brain size, in addition to pericardial edema secondary to cardia bifida.

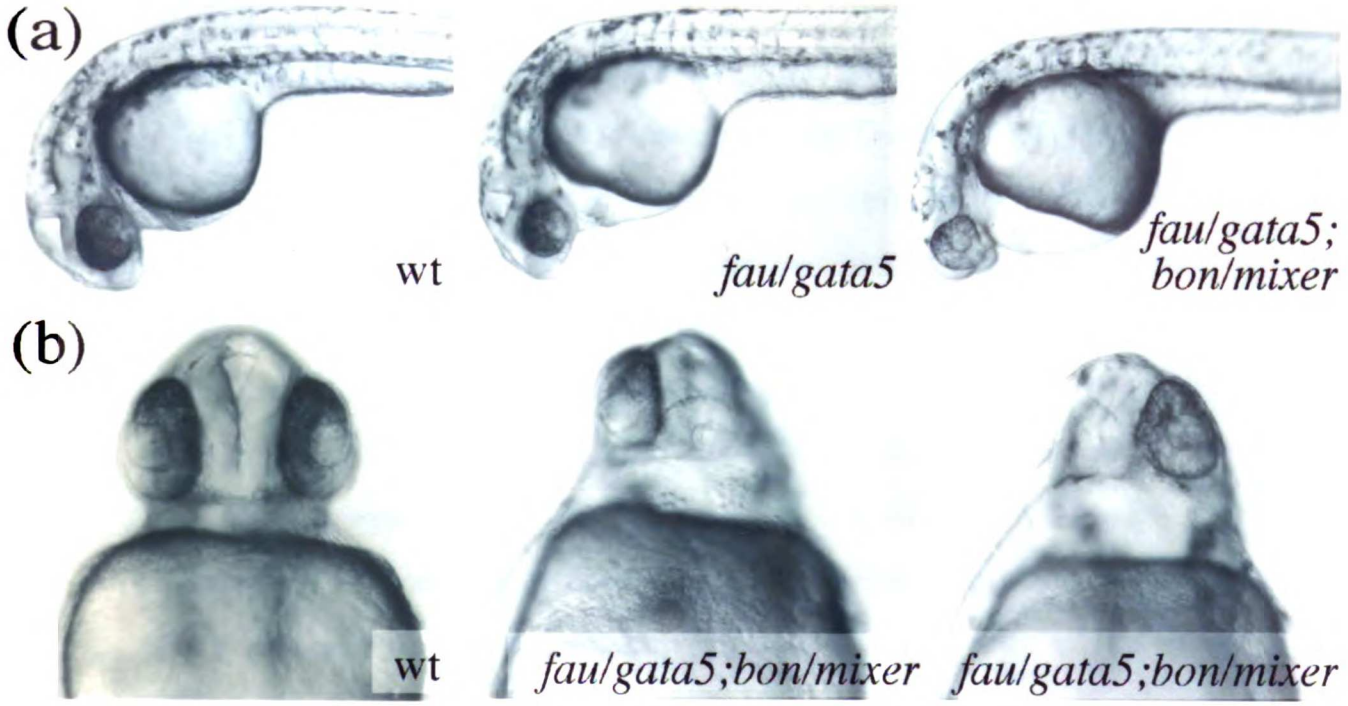
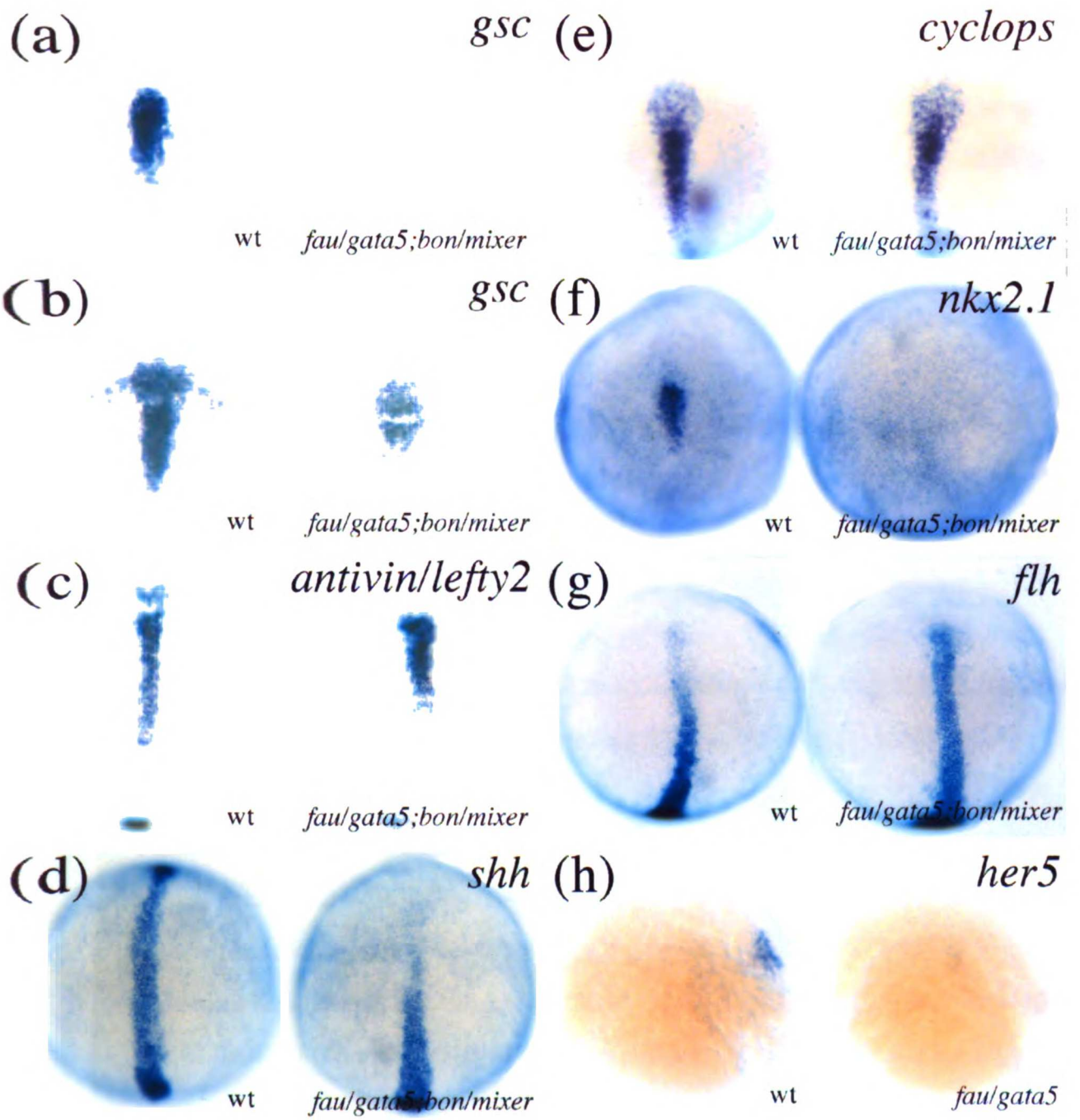

(a)
wt
fau/gata5
fau/gata5;
bon/mixer
(b)
wt
fau/gata5;bon/mixer
fau/gata5;bon/mixer

Figure A2.3

Prechordal plate precursors fail to express *gsc* and adopt characteristics of notochord precursors in *fau/gata5;bon* mutants. ***(a)*** Dorsal view of *gsc* expression in the prechordal plate precursors at late shield stage (6.2 hpf). *fau/gata5;bon* mutants exhibit profoundly reduced or absent *gsc* expression. ***(b)*** Animal pole view of *gsc* expression at 90% epiboly (9 hpf). At the end of gastrulation, *fau/gata5;bon* mutants exhibit decreased *gsc* expression. *c-g* show dorsal views at 90% epiboly (9 hpf). ***(c)*** Like *gsc* expression, *antivin/lefty2* expression is also reduced at late gastrulation stages. ***(d)*** *shh* is normally expressed throughout the axial mesoderm. In *fau/gata5;bon* mutants, *shh* is not expressed anteriorly. ***(e)*** In contrast, *cyclops* is expressed normally in the prechordal plate precursors of *fau/gata5;bon* mutants. ***(f)*** *nkx2.1*, a marker of the nascent ventral forebrain, is not expressed during gastrulation stages in *fau/gata5;bon* mutants. ***(g)*** *flh* is expressed in the posterior axial mesoderm, the precursors of the notochord. *flh* expression is anteriorly expanded in *fau/gata5;bon* mutants. ***(h)*** At shield stage (6 hpf), *her5* is expressed in a population of cells within the deep shield. Lateral views demonstrate that *her5* is not expressed at shield stage in *fau/gata5* mutants.


(a)
gsc
wt
fau/gata5;bon/mixer
(b)
gsc
wt
fau/gata5;bon/mixer
(c)
antivin/lefty2
wt
fau/gata5;bon/mixer
(d)
shh
wt
fau/gata5;bon/mixer
(e)
cyclops
wt
fau/gata5;bon/mixer
(f)
nkx2.1
wt
fau/gata5;bon/mixer
(g)
flh
wt
fau/gata5;bon/mixer
(h)
her5
wt
fau/gata5

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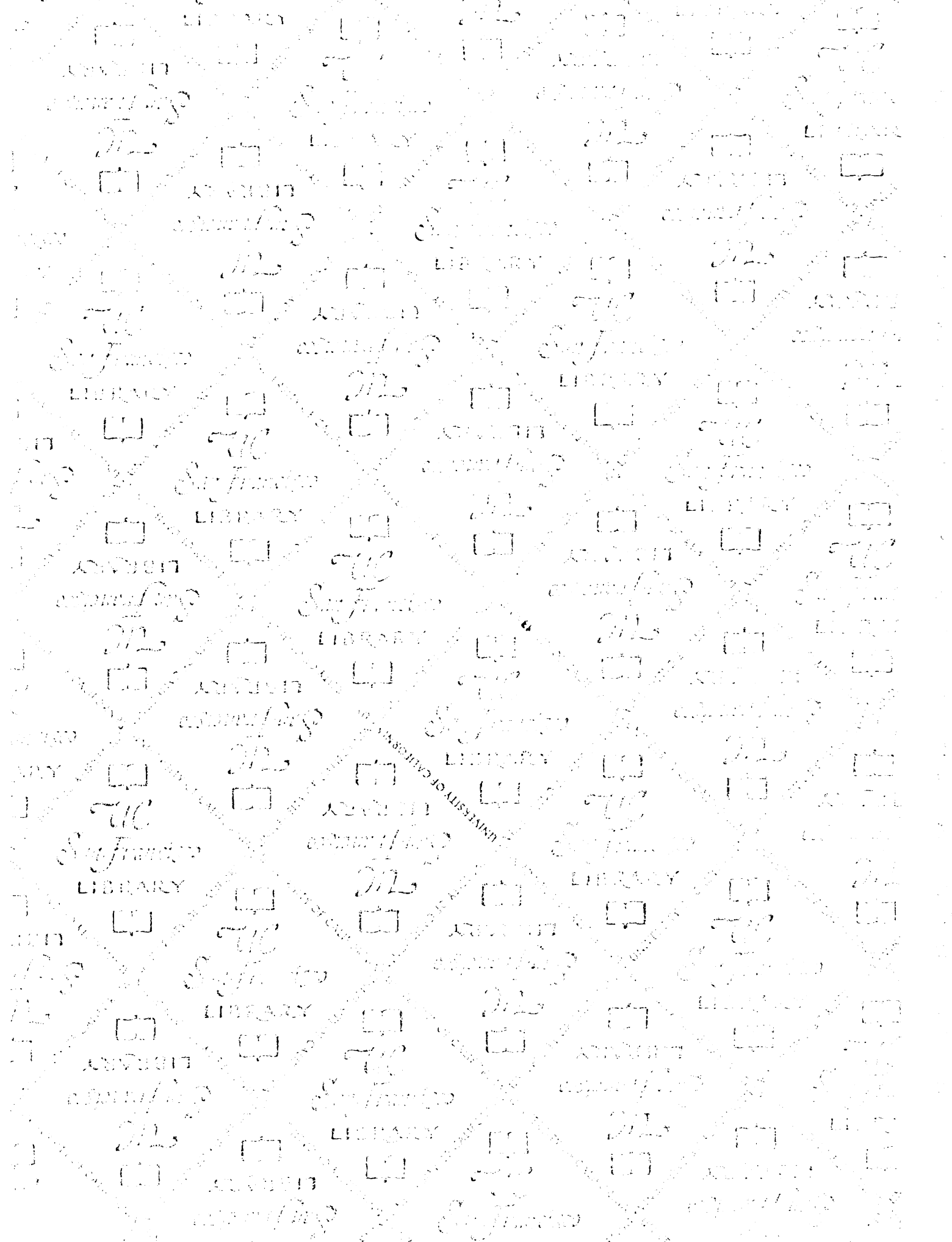

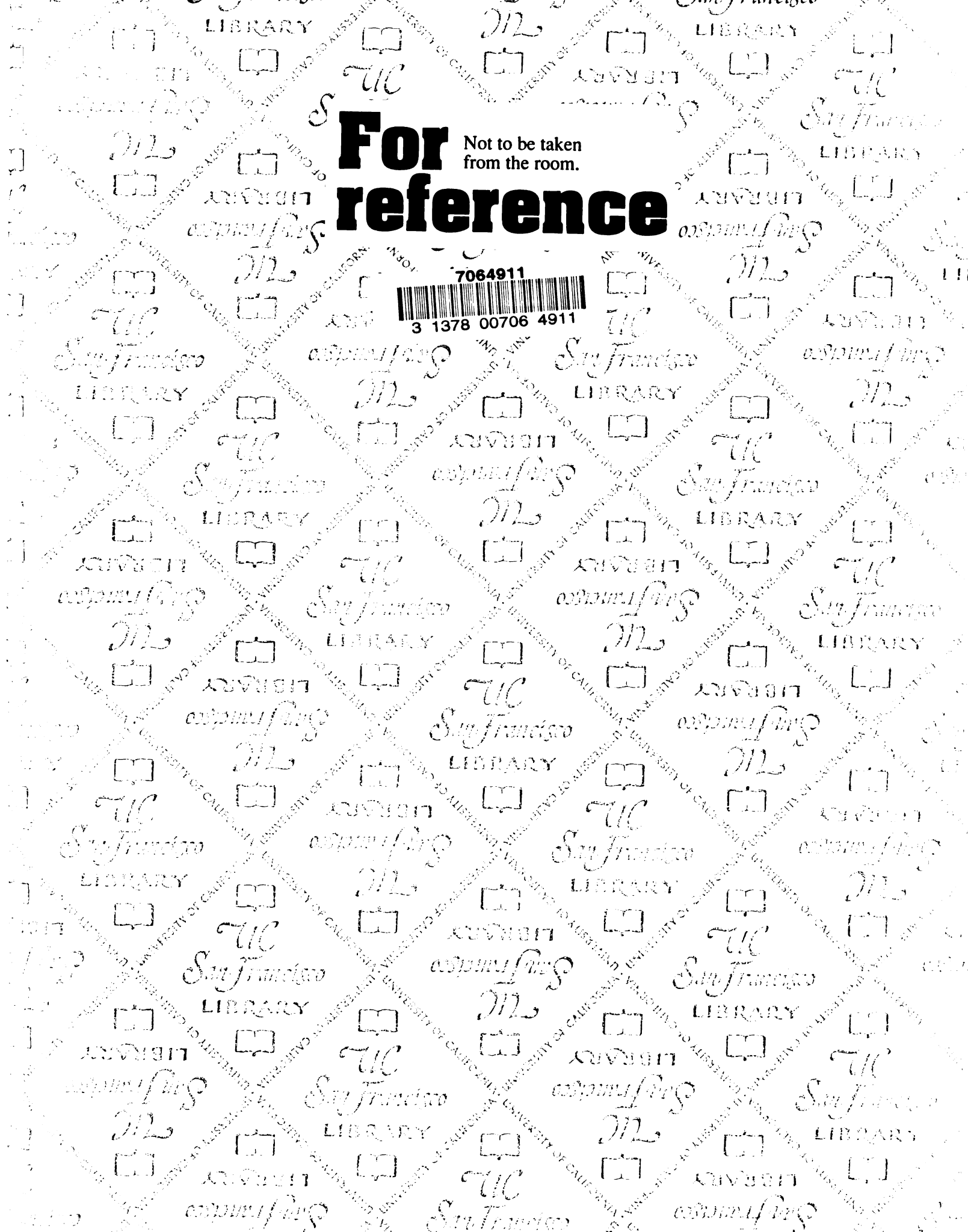
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