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Multiple roles for Gata5 in zebrafish endoderm formation

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SUMMARY

Previous studies have indicated that *gata5*, a zinc-finger transcription factor gene, is required for the development of the zebrafish gut tube. Here, we show that *gata5* mutants also display defects in the development of other endodermal organs such as the liver, pancreas, thyroid and thymus. *gata5* is expressed in the endodermal progenitors from late blastula stages, suggesting that it functions early during endoderm development. We indeed find that during gastrulation stages, *gata5* mutants form fewer endodermal cells than their wild-type siblings. In addition, the endodermal cells that form in *gata5* mutants appear to express lower than wild-type levels of endodermal genes such as *sox17* and *axial/foxA2*. Conversely, overexpression of *gata5* leads to expanded endodermal gene expression. These data indicate that Gata5 is involved both in the generation of endodermal cells at late blastula stages and in the maintenance of endodermal *sox17* expression during gastrulation.

We have also analyzed the relationship of Gata5 to other factors involved in endoderm formation. Using complementary mutant and overexpression analyses, we show that Gata5 regulates endoderm formation in cooperation with the Mix-type transcription factor Bon, that Gata5 and Bon function downstream of Nodal signaling, and that *cas* function is usually required for the activity of Gata5 in endoderm formation. Finally, we show that *fau/gata5*, *bon* and *cas* exhibit dominant genetic interactions providing additional support that they function in the same pathway. Together, these data demonstrate that Gata5 plays multiple roles in endoderm development in zebrafish, and position Gata5 relative to other regulators of endoderm formation.

Key words: *faust*, *bonnie and clyde*, *casanova*, *sox17*, *one-eyed pinhead*, Zebrafish

INTRODUCTION

Triploblastic organisms partition the developing blastoderm into three germ layers, the ectoderm, mesoderm and endoderm. In zebrafish, the endoderm arises from the four most marginal blastomere tiers of the late blastula stage embryo (Warga and Nüsslein-Volhard, 1999). These blastomeres involute early during gastrulation and occupy the deep hypoblast, directly overlying the yolk syncytial layer (YSL) (Warga and Kimmel, 1990). The endodermal cells are characteristically large and flat, distinct from the rounder mesodermal cells (Warga and Nüsslein-Volhard, 1999). The endoderm is further distinguished from the mesoderm by the expression of *sox17*, a high mobility group (HMG) domain transcription factor gene, and *foxA2*/*axial*/*HNF3β* (*foxa2* – Zebrafish Information Network), a winged helix/forkhead transcription factor gene (Strahle et al., 1993; Alexander and Stainier, 1999; Warga and Nüsslein-Volhard, 1999; Kaestner et al., 2000).

Our understanding of vertebrate endoderm development has recently been advanced by the isolation and analysis of several genes involved in endoderm formation (Hudson et al., 1997; Henry and Melton, 1998; Casey et al., 1999; Clements et al., 1999). These genes have now been assembled into regulatory pathways in zebrafish and *Xenopus* (Alexander and Stainier, 1999; Yasuo and Lemaire, 1999). At the top of this pathway are Nodal-related proteins, members of the TGFβ family of signaling molecules (reviewed by Schier and Shen, 2000). In zebrafish, two Nodal-related proteins, Cyclops and Squint, as well as Oep, a transmembrane protein essential for Nodal signaling, are required for the formation of both mesoderm and endoderm (Feldman et al., 1998; Gritsman et al., 1999). Nodal signaling has also been implicated in mouse and *Xenopus* endoderm formation (Conlon et al., 1994; Osada and Wright, 1999).

Nodal signaling induces the expression of zebrafish *bonnie and clyde* (*bon*), a Mix-type homeobox gene (Alexander and Stainier, 1999; Kikuchi et al., 2000). Bon is essential for early endoderm formation, as revealed by the reduced number of *sox17*- and *foxA2*-expressing endodermal cells in *bon* mutants (Kikuchi et al., 2000). Similarly, a Mix-type transcription factor known as Mixer is required for *Xenopus* endoderm formation and regulates the expression of the *Xenopus Sox17* genes (Henry and Melton, 1998).

casanova (*cas*), another zebrafish gene, is essential for endoderm formation and functions downstream of Nodal signaling and Bon, but upstream of *sox17* and *foxA2* (Alexander et al., 1999; Alexander and Stainier, 1999). Thus, the framework of a molecular pathway regulating zebrafish

endoderm formation has begun to emerge: Nodal signaling induces the expression of *bon*; Bon then functions through *cas* to promote the expression of downstream endodermal genes such as *sox17* and *foxA2* (Alexander and Stainier, 1999).

This molecular pathway is likely to be incomplete. Studies of invertebrate endoderm formation suggest the involvement of another class of transcriptional regulators, the Gata factors. Gata factors are zinc-finger transcriptional activators that bind to the consensus sequence (A/T)GATA(A/G). The *Drosophila* Gata gene *serpent* is expressed in, and required for the development of, the endoderm and the fat body, a mesodermal organ functionally similar to the vertebrate liver (Abel et al., 1993; Rehorn et al., 1996; Reuter, 1994). Similarly, the *Caenorhabditis elegans* Gata gene, *elt-2*, is essential for the formation of the embryonic gut (Fukushige et al., 1998). Another *C. elegans* Gata gene, *end-1*, is implicated in the specification of E, the endodermal progenitor (Zhu et al., 1997). *end-1* is sufficient to induce endodermal fate, as ubiquitous expression drives normally non-endodermal blastomeres to produce gut tissue (Zhu et al., 1998). In addition, *end-1* is a potent activator of *Sox17α* expression in *Xenopus* animal caps (Shoichet et al., 2000), suggesting that Gata genes also play an important role in vertebrate endoderm formation.

Vertebrate genomes are known to contain six evolutionarily conserved Gata genes. While *gata1*, *gata2* and *gata3* function predominantly in hematopoietic development (reviewed by Orkin and Zon, 1997), *gata4*, *gata5* and *gata6* are expressed in extra-embryonic tissues, heart and endoderm (reviewed by Charron and Nemer, 1999). Studies of endodermal gene regulation have suggested the involvement of Gata factors; transfection of non-endodermal cells with Gata genes induces the transcription of endodermal genes such as *Ifabp*, *gastric H^+/K^+-ATPase* and *Hnf4* (Gao et al., 1998; Maeda et al., 1996; Morrisey et al., 1998). Also, overexpression of *Xenopus gata4* and *gata5*, but not *gata6*, has been shown to efficiently activate expression of *Sox17α* and *HNF1β* in animal caps (Weber et al., 2000). Direct regulation of endodermal genes by Gata factors has been suggested by a study demonstrating occupation of an albumin gene Gata motif in mouse embryonic endodermal cells (Bossard and Zaret, 1998).

Gene inactivation studies in mouse have revealed essential roles for *Gata4* in endoderm-dependent embryonic movements, for *Gata5* in the development of the female genitourinary tract, and for *Gata6* in the development of the extra-embryonic visceral endoderm (Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998; Koutsourakis et al., 1999; Molkentin et al., 2000). However, genetic evidence that Gata factors are involved in definitive endoderm development in vertebrates has been elusive.

Recently, we have shown that the zebrafish *faust* (*fau*) locus encodes Gata5 (Reiter et al., 1999). *fau/gata5* mutants display prominent defects in myocardial differentiation and gut morphogenesis (Reiter et al., 1999). Here, we show that zebrafish *gata5* is required for the development of endoderm-derived organs such as the liver, pancreas, thyroid and thymus. Using both loss- and gain-of-function experiments, we further show that Gata5 plays several roles in endoderm formation during the late blastula and gastrula stages. First, Gata5 regulates the amount of endoderm formed. Second, Gata5 promotes the expression of *sox17* and *foxA2* within the endoderm. We also show through a combination of mutant analyses and overexpression studies that Gata5 cooperates with Bon downstream of Nodal signaling and upstream of *cas* to regulate endoderm formation. These results identify Gata5 as an essential regulator of early zebrafish endoderm development and define how Gata5 functions relative to other factors known to participate in vertebrate endoderm formation.

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 Fig. 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), and oep^{z1} (Schier et al., 1996).

Whole-mount in situ hybridization and immunohistochemistry

We performed in situ hybridization as described (Alexander et al., 1998); embryos older than 28 hours postfertilization (hpf) were incubated in 0.003% phenylthiourea to inhibit pigmentation. Gastrulation stage embryos were genotyped after 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′-TCAGTCCAACGAA-CGGTAAC-3′. bon^{m425} mutants were identified using an *Mse*I restriction fragment length polymorphism (Kikuchi et al., 2000). 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*, *bon* (originally called *mixer*), *gfp* and *β-galactosidase* messages were synthesized from previously described templates (Alexander et al., 1999; Reiter et al., 1999) using the SP6 mMessage Machine system (Ambion). Embryos were injected at the one to four-cell stage with 60 pg of *gata5*, 100 pg of *bon* mRNA, or both. Control embryos were injected with equivalent amounts of *gfp* mRNA. Some *gata5* and *gfp* injected embryos were co-injected with 150 pg of *lacZ* mRNA. β-galactosidase staining was performed as described (Takke et al., 1999).

RESULTS

gata5 is required for the development of the liver, pancreas, thyroid and thymus

We have previously shown that *fau/gata5* mutants display defects in the morphogenesis of the gut and pharyngeal pouches that range from lack of gut looping to severe reduction in the amount of gut tissue (Reiter et al., 1999). These morphogenetic defects led us to examine the differentiation of other endodermal organs in mildly affected mutants (Fig. 1A). The embryonic liver expresses genes such as *gata4*, *gata6*, *hnf1β* and *hhex*. The expression of each of these genes is dramatically diminished in *fau/gata5* mutants (Fig. 1A and data not included). Similarly, pancreatic expression of *pdx1*, *islet1* and insulin is also diminished in *fau/gata5* mutants (Fig. 1B and data not included).

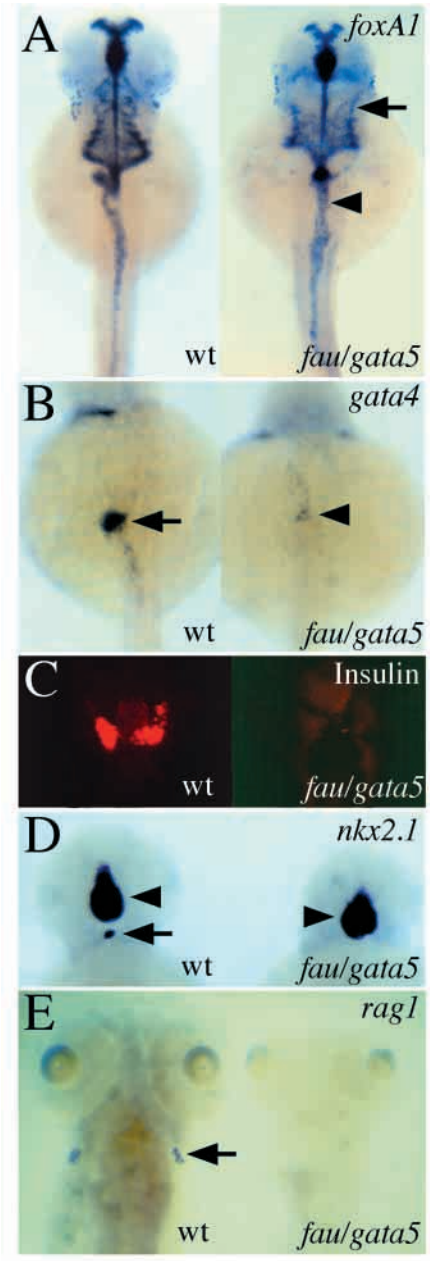

Fig. 1. Differentiation of endoderm-derived organs requires Gata5. Expression of (A) *foxA1* (*foxa1* – Zebrafish Information Network), (B) *gata4*, (C) insulin, (D) *nkx2.1* and (E) *rag1* in wild-type embryos (left) and *fau/gata5* mutant siblings (right) at (A,B,D) 44 hpf, (C) 72 hpf and (E) 5.5 days postfertilization. All views are dorsal with anterior towards the top, except (D), which shows an anterior view. (A) *foxA1* is expressed in the floorplate and gut tube. The anterior gut tissue (arrow) of the *fau/gata5* mutant has failed to migrate toward the embryonic midline and the posterior gut (arrowhead) has failed to loop. (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/gata5* mutants.

In addition, we found that *fau/gata5* mutants also exhibit defects in the differentiation of derivatives of the pharyngeal endoderm, which include the thyroid and the stroma of the thymus (Kimmel et al., 1995). In *fau/gata5* mutants, the thyroid primordium fails to express *nkx2.1* (Lazzaro et al., 1991; Fig. 1D, arrow). Thymus development in *fau/gata5* mutants is also abnormal as ascertained by the absence of *rag1*-expressing thymocytes (Willett et al., 1997; Fig. 1E, arrow). The thymus develops normally in other zebrafish cardiac mutants such as *silent heart* (data not included), suggesting that the absence of thymocytes in *fau/gata5* mutants is not due to the lack of circulation, but reflects a more specific defect in the development of the thymic stroma. Together these data indicate that the differentiation of endodermal cells is affected in *fau/gata5* mutants.

gata5 and *bon* are expressed in the endoderm before *sox17*, *foxA2* or *gata4*

In zebrafish, *gata5* is first expressed at the dome stage (late blastula) in the most marginal cells (Fig. 2A, arrowhead), a population that gives rise to the endoderm and some mesoderm (Reiter et al., 1999; Rodaway et al., 1999; Warga and Nüsslein-Volhard, 1999). *gata5* is also expressed in the YSL (Fig. 2A, arrow), an extra-embryonic tissue underlying the blastoderm. *gata5* expression is maintained in the endoderm throughout gastrulation (Fig. 2C,G,K). Evidence that the large, flat *gata5*-expressing cells are endoderm comes from the similar morphology and distribution of these cells and the *foxA2*- and *sox17*-expressing endodermal cells (Fig. 2K,M,N). Furthermore, these *gata5*-expressing cells also express *foxA3*/*fkd2* (*foxa3* – Zebrafish Information Network), another endodermal marker (Odenthal and Nüsslein-Volhard, 1998; Rodaway et al., 1999), and are absent in *cas* mutants (Alexander et al., 1999). Endodermal *gata5* expression persists during early somitogenesis stages but diminishes from the 5- to 10-somite stages. However, endodermal expression of *gata5* is again evident at four days of development in the epithelium of the gut tube (Rodaway et al., 1999).

Comparison of *gata5* expression with that of other endodermal genes reveals significant differences. Expression of *bon* and *gata5* initiates at around the same time during blastula stages in the marginal domain of the embryo (Fig. 2A,B). However, *gata5* expression is more marginally restricted than is *bon* expression (Fig. 2A,B, and Alexander et al., 1999). In addition, *gata5* expression persists after early gastrulation, while *bon* expression does not (Alexander et al., 1999).

Endodermal cells do not express *sox17* and *foxA2* until they have involuted, approximately 80 minutes after they have begun to express *gata5* and *bon* (Alexander and Stainier, 1999) (Fig. 2E,F,I,J). During early gastrula stages, *gata5* and *bon* are expressed throughout the germ ring (Fig. 2C,D,G,H). In contrast, at this stage *foxA2* and *sox17* are expressed only in scattered cells of the germ ring (Fig. 2E,F,I,J). Comparison of the expression patterns of *foxA2* and *sox17* with those of *gata5* and *bon* suggests that the endoderm originates from a subset of the *gata5*- and *bon*-expressing marginal cells. Fate-mapping studies have demonstrated that, in addition to the endoderm, marginal cells also give rise to the prechordal plate and myocardium (Kimmel et al., 1990; Stainier et al., 1993), indicating that progenitors of these tissues likely also express *gata5* before they involute.

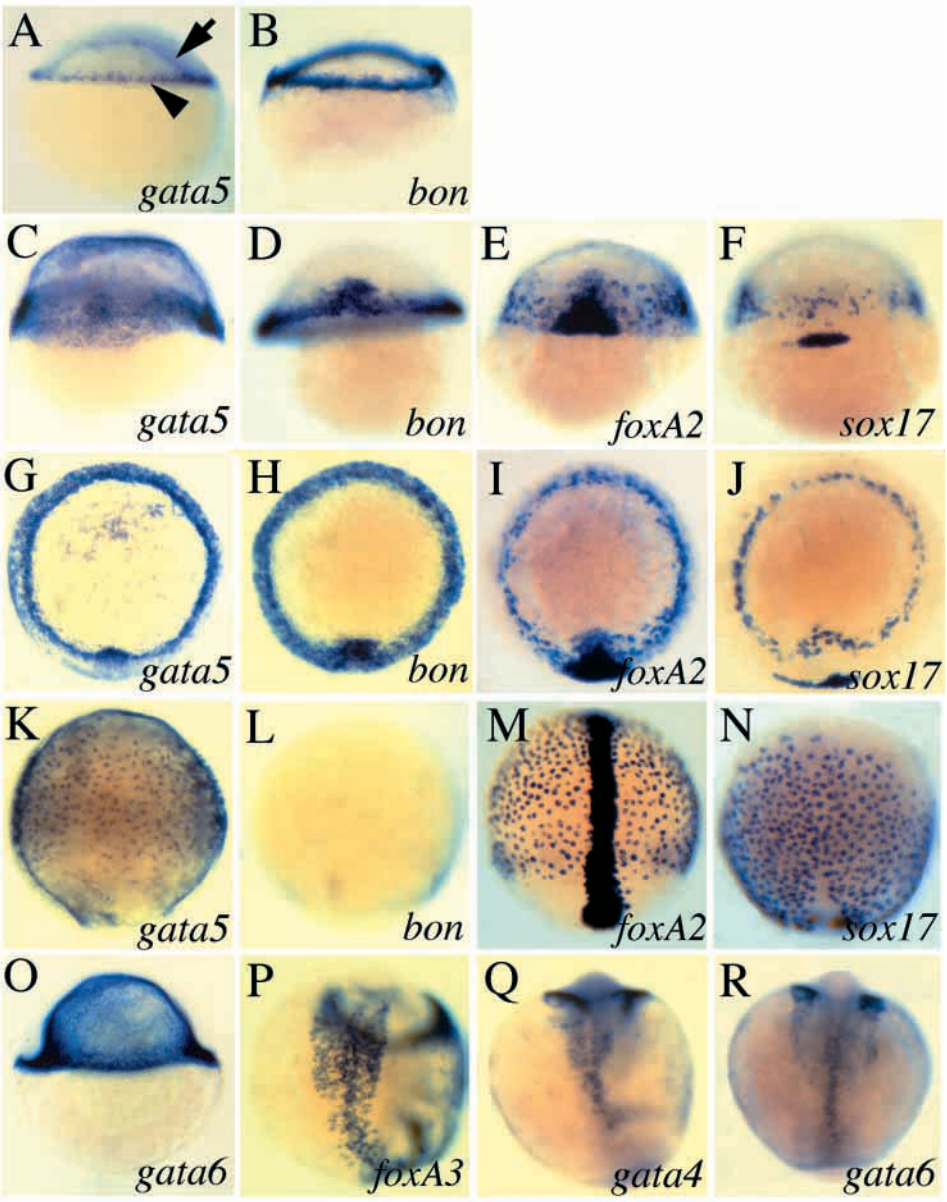

Fig. 2. Comparison of the expression of *gata5*, *bon*, *foxA2*, *sox17*, *foxA3*, *gata4* and *gata6* in wild-type endoderm. Lateral views of (A) *gata5* and (B) *bon* expression at dome stage (4.3 hpf). *gata5* is expressed in the marginal blastomeres (arrowhead) and YSL (arrow). *bon* is expressed more broadly in the marginal domain. Dorsal (C-F) and animal pole (G-J) views of (C,G) *gata5*, (D,H) *bon*, (E,I) *foxA2* and (F,J) *sox17* expression at late shield stage (6 hpf). *gata5* and *bon* are expressed in more germ ring blastomeres than are *foxA2* and *sox17*. In addition to expression in the endoderm, *foxA2* is expressed in the axial mesoderm and *sox17* is expressed in the forerunner cells. Dorsal views of (K) *gata5*, (L) *bon*, (M) *foxA2* and (N) *sox17* expression at early bud stage (9.5 hpf). *bon* is not expressed after early gastrulation. *gata5*, *foxA2* and *sox17* are expressed in the endoderm, a discontinuous layer of large, flat cells closely apposed to the underlying YSL. (O) Lateral view of *gata6* expression in the YSL at late shield stage (6 hpf). *gata4* is not expressed in the embryo proper before segmentation stages. Dorsal views of (P) *foxA3*, (Q) *gata4* and (R) *gata6* expression in the posterior endoderm at the 12-somite stage (15 hpf). *foxA3* is expressed throughout the posterior endoderm, while endodermal expression of *gata4* and *gata6* is more restricted.

In contrast to *gata5*, zebrafish *gata4* is not expressed during gastrulation. *gata6* is expressed during early gastrulation in the most marginal tier of blastomeres (A. Rodaway, personal communication), but during later gastrulation is expressed only in the YSL (Fig. 2O and data not included). Only during somitogenesis stages do *gata4* and *gata6* begin to be strongly expressed in the endoderm. Interestingly, comparison of *gata4* and *gata6* expression with that of *foxA3*, a marker of the posterior endoderm, reveals that *gata4* and *gata6* are expressed in a subset of the posterior endoderm (Fig. 2P,Q,R). Therefore, *gata5* appears to be the first zebrafish *gata* gene to be expressed and the only zebrafish Gata gene to be expressed throughout the entire endoderm.

gata5 regulates early endoderm formation and differentiation during gastrulation

The early expression pattern of *gata5* led us to investigate whether *gata5* is required not only for the late morphogenesis and differentiation of endoderm-derived organs, but also for early endoderm development. Analysis of *fau/gata5* mutants reveals two striking defects in the *sox17* and *foxA2* expression patterns during gastrulation. First, *fau/gata5* mutants display a reduction in the number of *sox17*- and *foxA2*-expressing endodermal cells. The number of endodermal cells in *fau/gata5* mutants is reduced by approximately 40% at bud stage (Fig. 3A). Second, the endodermal cells that do form in *fau/gata5* mutants express variably reduced levels of both *sox17* and *foxA2* (Fig. 3B). In contrast to the endodermal defects, expression of *sox17* in the forerunner cells and of *foxA2* in the axial mesoderm appear unaffected in *fau/gata5* mutants. Mutants of both *fau* alleles (fau^{tm236a} and fau^{s26}) exhibit very similar endodermal phenotypes. In summary, these data show that *gata5* is required both for the generation of a normal number of endodermal cells as well as for the expression of wild-type levels of *sox17* and *foxA2* within these cells.

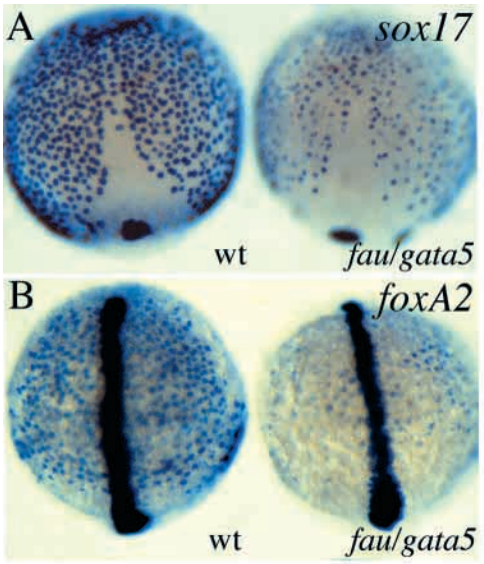

Fig. 3. Gata5 is required for early endoderm development. Dorsal views of (A) *sox17* and (B) *foxA2* expression in wild-type embryos and *fau/gata5* mutant siblings at early bud stage (9.5 hpf). The identity of mutants was confirmed by genotyping (see Materials and Methods). *fau/gata5* mutants form fewer endodermal cells than their wild-type siblings. In addition, *fau/gata5* mutants express *sox17* and *foxA2* at lower levels in the endoderm.

Gata5 and Bon function in parallel in endoderm formation

Because *gata5* and *bon* are co-expressed in the marginal domain, we tested for regulatory relationships between Bon and Gata5 by examining the expression of the two genes in the respective mutants. *bon* is expressed normally in *fau/gata5* mutants (Fig. 4A), and similarly, *gata5* is expressed normally during late blastula stages in *bon* mutants (Fig. 4B). These results indicate that Bon and Gata5 are not required for each other's expression.

Embryos mutant for both *fau/gata5* and *bon* exhibit more profound endodermal defects than either *fau/gata5* or *bon* single mutants (Fig. 4C). *bon* mutants contain, on average, 45 *sox17*-positive endodermal cells (n=13) and 31 *foxA2*-positive endodermal cells (n=7) at 90% epiboly, while *fau/gata5;bon* double mutant siblings contain an average of 13 *sox17*-positive endodermal cells (n=6) and 5 *foxA2*-positive endodermal cells (n=7). Occasionally, *fau/gata5;bon* double mutants lack all endodermal gene expression (2/13). Together, these results indicate that Gata5 and Bon function in parallel during early endoderm formation.

gata5 overexpression expands endodermal gene expression

fau/gata5 mutants exhibit defects in endoderm formation. In order to analyze the role of *gata5* in this process further, we investigated the effects of *gata5* overexpression. Injection of 60 pg of *gata5* mRNA into zebrafish embryos at the one to four-cell stage led to an increased number of cells expressing *sox17* (75/148 embryos showed an increase in four independent experiments) and *foxA2* (25/66 embryos showed an increase in two independent experiments) (Fig. 5A-C). Co-injection of *lacZ* mRNA with *gata5* allowed us to detect the

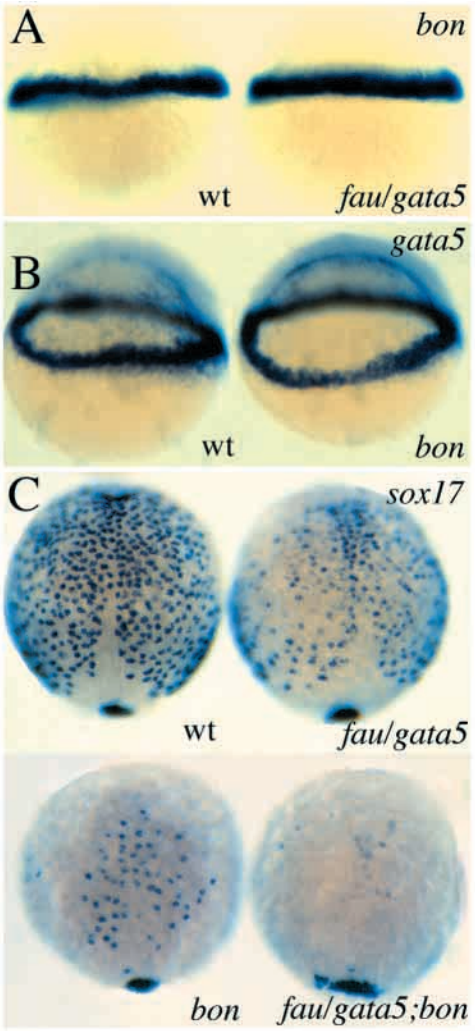

Fig. 4. *gata5* and *bon* function in parallel in endoderm formation. Dorsal views of (A) *bon*, (B) *gata5* and (C) *sox17* expression at (A,B) 50% epiboly (5.3 hpf) and (C) 90% epiboly (9 hpf). (A) *bon* expression is indistinguishable in *fau/gata5* mutants and their wild-type siblings. (B) Similarly, *gata5* expression is indistinguishable in *bon* mutants and their wild-type siblings at this stage. (C) While endodermal expression of *sox17* is reduced in *fau/gata5* (top right) and *bon* mutants (bottom left), it is nearly absent in *fau/gata5;bon* double mutants (bottom right).

distribution of injected mRNA within the embryo. The parallel distribution of β-galactosidase staining and expanded *sox17* and *foxA2* expression suggests that Gata5 autonomously induces the expression of these endodermal genes (Fig. 5B,C).

Interestingly, while the number of endodermal cells in the normal position directly overlying the YSL was increased, *sox17*- and *foxA2*-expressing cells were also observed more superficially in *gata5*-overexpressing embryos (Fig. 5D; arrow, and data not included). Similar superficial expression of *sox17* or *foxA2* was never observed in control *gfp*-injected embryos. Despite the fact that they do not lie in the deep hypoblast, many of these ectopic *sox17*- and *foxA2*-expressing cells seen in *gata5*-overexpressing embryos appear to display the large, flat morphology characteristic of endoderm. In order to start addressing the origin of the additional *sox17*- and *foxA2*-

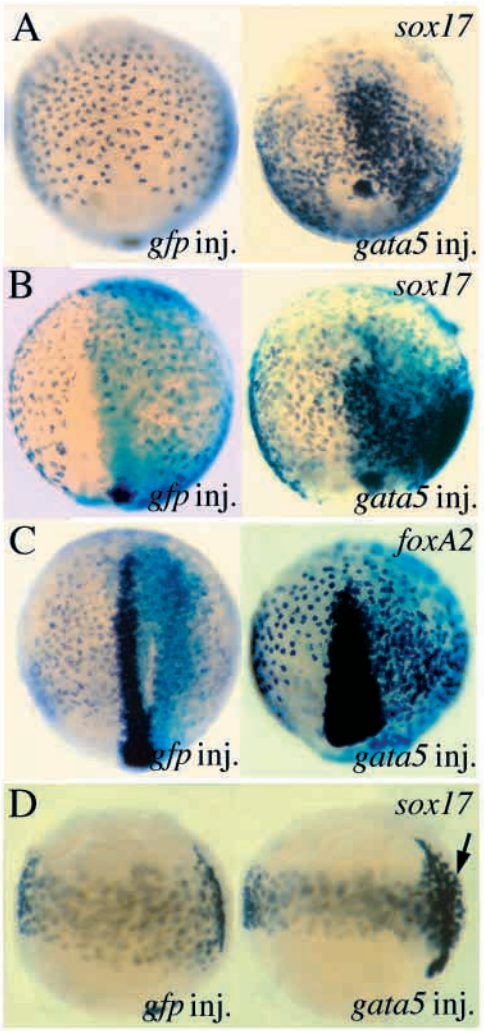

Fig. 5. *gata5* overexpression expands the expression of *sox17* and *foxA2*. At the two-cell stage, one blastomere was injected with either 60 pg of *gfp* or *gata5* mRNA. Dorsal views of (A,B) *sox17* and (C) *foxA2* expression at 90% epiboly (9 hpf). (A) Injection of *gfp* mRNA has no effect on *sox17* expression. Overexpression of *gata5* appears to increase the number of *sox17*-expressing cells. (B,C) Embryos were co-injected with 150 pg of *lacZ* mRNA and stained for β-galactosidase activity (light blue) before in situ hybridization (dark blue). Overexpression of *gata5* expands the expression of both *sox17* and *foxA2*. Regions of increased *sox17* and *foxA2* expression always overlapped with β-galactosidase staining (n>100). (D) Optical sagittal section of *sox17* expression in *gfp*- and *gata5*-overexpressing embryos at 75% epiboly (8 hpf). Dorsal is towards the right. 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).

expressing endodermal cells, we examined the expression pattern of *otx2* and *myoD* (*myod* – Zebrafish Information Network) in *gata5*-injected embryos. We found no decrease in the extent of *otx2* expression but a pronounced reduction in *myod* expression (data not included), suggesting that the endoderm was expanded at the expense of the mesoderm. Alternatively, or in addition, *gata5* could function to downregulate *myod* expression cell-autonomously. Further studies using germ layer- or tissue-specific promoters will allow us to address whether *gata5* expression in these cells is sufficient to convert them to endoderm.

Table 1. Overexpression of *gata5* increases endodermal gene expression in endoderm mutants

	Average number of *sox17*-expressing cells			
Injected mRNA	wt	*oep*	*bon*	*cas*
gfp	500-600 (n=5)	7.3 (n=15)	21.5 (n=6)	0 (n=12)
gata5	>850 (n=10)	19.6 (n=9)	62.5 (n=4)	0.5 (n=41)

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

At 36 hpf, embryos injected with 60 pg of *gata5* mRNA displayed variable defects in head development (e.g. small eyes, dysmorphic brain) and tail morphogenesis (e.g. kinked tails, dysmorphic somites) as well as ectopic myocardium (Reiter et al., 1999).

gata5 overexpression induces endodermal gene expression in *oep* and *bon* mutants, but is less effective in *cas* mutants

Nodal signaling is required both for endoderm formation and for *gata5* expression (Feldman et al., 1998; Rodaway et al., 1999). Oep, an EGF-CFC (epidermal growth factor-cripto, FRL-1, cryptic) family member essential for Nodal signaling, is also required for endoderm formation and *gata5* expression (Schier et al., 1997; Gritsman et al., 1999; data not included). To test whether Gata5 can promote endoderm formation in embryos in which Nodal signaling is reduced, we injected mRNA encoding Gata5 into embryos lacking zygotically expressed *oep* (*Zoep* mutants). Forced expression of *gata5* in these mutants led to an increase in the number of *sox17*-expressing endodermal cells (Fig. 6A, Table 1). This 2.7-fold increase, although modest, is comparable with that seen in *bon*-injected *Zoep* mutants (3.7-fold, Alexander and Stainier, 1999).

Overexpression of *gata5* in *bon* mutants also led to an increase in the number of *sox17*-expressing endodermal cells (Fig. 6B, Table 1). This 2.9-fold increase is the same as that seen in *bon*-injected *bon* mutants (Kikuchi et al., 2000). However, we observed qualitative differences between *gata5*- and *bon*-injected *bon* mutants: *gata5* overexpression in *bon* mutants usually led to a patch of *sox17*-expressing endodermal cells that did not exhibit the regularly spaced distribution seen in wild-type or *bon*-injected *bon* mutant embryos (Fig. 6B; Kikuchi et al., 2000). Co-injections of *gata5* and *bon* into *bon* mutants led to a greater increase in the number of *sox17*-expressing endodermal cells which was difficult to quantitate, owing to the piling up of the cells (Fig. 6B).

gata5 overexpression in *cas* mutants did not restore *sox17* expression, suggesting that Gata5 requires *cas* to activate *sox17* expression (Fig. 6C, right upper panel). However, in 6 of 41 *gata5*-overexpressing *cas* mutants, a very small number of *sox17*-expressing cells were noted (Fig. 6C lower panels, Table 1). These latter data indicate that Gata5 functions differently from other potent endoderm inducers such as Bon and the constitutively active type I TGFβ-type receptor Taram-a*

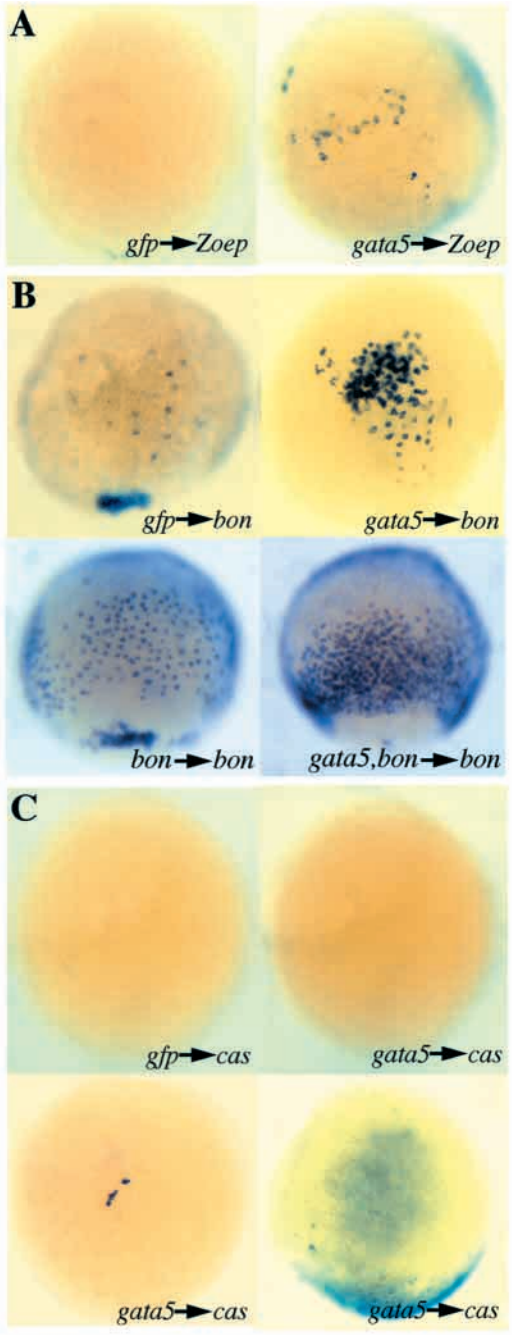

Fig. 6. *gata5* and *bon* induction of *sox17* expression in *Zoep*, *bon* and *cas* mutants. Embryos were injected at the one to four-cell stage and examined for *sox17* expression at 90% epiboly (9 hpf). *gfp* injection did not affect *sox17* expression. Overexpression of *gata5* increased the number of *sox17*-expressing cells in (A) *Zoep* mutants and (B) *bon* mutants. (B) Comparison of *sox17* expression in *gata5*-injected (top right), *bon*-injected (bottom left) and *gata5*-*bon* co-injected *bon* mutants (bottom right). *gata5* overexpression usually led to a piling up of the endodermal cells, rather than the regularly spaced distribution seen in wild-type and *bon*-injected *bon* mutant embryos. Co-injected embryos showed a greater number of endodermal cells than single injected ones, although this increase was difficult to quantitate because of the pronounced piling up of the cells. (C) In *cas* mutants, *gata5* overexpression usually did not induce any *sox17* expression (top right panel). However, in six of 41 cases, *sox17* expression was observed in *gata5*-overexpressing *cas* mutants (bottom panels).

(Renucci et al., 1996), which never induced *sox17* expression in *cas* mutants (Alexander and Stainier, 1999).

fau/gata5 interacts genetically with *bon* and *cas*

Because *fau/gata5* and *bon* appear to play similar roles in endoderm formation, we wanted to examine whether they interact genetically. Mutations in *fau/gata5*, *bon* and *cas* are completely recessive: heterozygous embryos are indistinguishable from wild-type siblings. However, when *fau/gata5* heterozygotes are crossed to either *bon* or *cas* heterozygotes, some of the progeny exhibit morphological defects that are most easily scored by examining their hearts (Table 2). These cardiac defects range in severity from dilated hearts that fail to pump blood to cardia bifida. Similar defects are noted among the progeny of *bon* and *cas* heterozygote intercrosses. However, not all genes involved in endoderm formation exhibit dominant genetic interactions: the progeny of *oep* heterozygotes and *fau/gata5*, *bon* or *cas* heterozygotes do not display defects in heart morphogenesis (Table 2).

DISCUSSION

Gata5 is required for the development of endoderm-derived organs

Our analysis of *fau/gata5* mutants shows that Gata5 is essential for the development of the liver, pancreas, thyroid and thymus. These defects in endodermal organ development are observed even in *fau/gata5* mutants with a near normal amount of endodermal tissue. These data suggest that the defects in endodermal organ development do not reflect an absence of endoderm, but true defects in organ-specific endodermal differentiation.

Differentiation defects in *fau/gata5* mutants seem likely to be autonomous, as *gata5* is expressed in the endoderm. However, it is also possible that some endodermal defects in *fau/gata5* mutants are non-autonomous. For example, tissue recombination experiments in mouse and chick have demonstrated that the precardiac mesoderm can induce the endoderm to differentiate as liver (Gualdi et al., 1996; LeDouarin, 1975). We have previously shown that *fau/gata5* mutants display severe defects in the differentiation of the precardiac mesoderm (Reiter et al., 1999). It is therefore

Table 2. Dominant genetic interactions between *fau/gata5*, *bon* and *cas* mutations

	bon	*cas*	*oep*
fau/gata5	6.4% (36/557, nine with cardia bifida)	18% (18/101, two with cardia bifida)	0% (0/200+)
bon		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 exhibiting cardia bifida is also noted. Each total is the sum of the observations from at least two clutches. Similar cardiac defects were never observed among the progeny of wild-type fish (n>500).

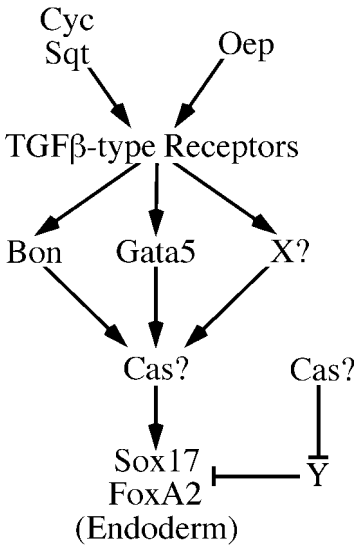

Fig. 7. A model of zebrafish endoderm formation, elaborated upon that of Alexander and Stainier (1999). The Nodal-related proteins Cyc and Sqt act through TGFβ-type receptors. Oep is also essential for Nodal signaling and is thought to act upstream of TGFβ-type receptors (Gritsman et al., 1999). Nodal signaling induces the expression of *bon* and *gata5*. Other Nodal- and Oep-dependent factors, represented here by X, may also be required for endoderm formation. Bon and Gata5 cooperatively regulate the expression of *sox17* and *foxA2*, 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 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 and Gata5, or the *cas* gene product may antagonize a repressor of *foxA2* and *sox17* expression represented by 'Y'. *cas* may also represent all or part of the functions ascribed to 'X'.

possible that the liver differentiation defects observed in *fau/gata5* mutants are partly due to the loss of inductive signals from the anterior lateral plate mesoderm. In addition, the occurrence of defects in pharyngeal endoderm differentiation in *fau/gata5* mutants and the proximity of the developing pharyngeal endoderm to the anterior lateral plate mesoderm lead us to hypothesize that pharyngeal endoderm differentiation may also be regulated by signals from the anterior lateral plate mesoderm.

Gata5 is required for the formation and early differentiation of the endoderm

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 cells that form during gastrulation. As indicated by *sox17* and *foxA2* expression, *gata5* mutants produce 40% fewer endodermal cells by bud stage than do their wild-type siblings. Conversely, overexpression of *gata5* increases the number of endodermal cells formed. *gata5* is expressed during late blastula stages, before blastomere fate has been restricted to a single germ layer. Therefore, we propose that Gata5 functions during late blastula stages to help define the number of blastomeres that will become endoderm. Alternatively, Gata5 could affect the proliferation or survival of 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 endoderm formation.

Additionally, we have shown that Gata5 promotes the expression of *sox17* and *foxA2* within the endoderm, as expression of *sox17* and *foxA2* is reduced in *fau/gata5* mutant endodermal cells, while non-endodermal expression of *sox17* and *foxA2* is unaffected. This aspect of the phenotype is qualitatively different from the endoderm defect of *bon* mutants. While *bon* mutants also form fewer endodermal cells, those that do form express wild-type levels of *sox17* and *foxA2* (for examples, see Fig. 4C and Kikuchi et al., 2000). As *gata5* expression is maintained in the endoderm throughout gastrulation, we hypothesize that *gata5* acts autonomously within the endoderm to promote the expression of downstream endodermal genes such as *sox17* and *foxA2*. To summarize these data, we propose that Gata5 functions early to determine endodermal cell fate and later to maintain endodermal *sox17* and *foxA2* expression.

In wild-type embryos, *sox17*- and *foxA2*-expressing endodermal cells are always closely apposed to the underlying YSL. However, in *gata5*-overexpressing embryos, *sox17*- and *foxA2*-expressing cells are also observed superficial to the deep hypoblast. These data imply that *gata5* overexpression either prevents endodermal cells from assuming their proper position within the hypoblast, or induces ectopic endodermal gene expression. These data are reminiscent of the ectopic expression of myocardial genes in *gata5*-injected embryos (Reiter et al., 1999), and of the ectopic expression of endothelial and blood genes in *hhex*-injected embryos (Liao et al., 2000). In these cases, various lines of evidence indicated that only part of the myocardial, endothelial or blood program was activated in these ectodermal cells. In addition, cell transplantation experiments performed by Ho and Kimmel (1993) predict that if these *sox17*-expressing cells were true endodermal cells, they would migrate and incorporate into the endodermal layer. Thus, it is most likely that, in these *sox17*-expressing cells that lie outside the deep hypoblast, only part of the endodermal program has been activated. This interpretation is in agreement with the work of Weber et al. (2000) who found that while Gata5 was sufficient to activate endodermal gene expression in prospective ectoderm, the conversion to endoderm was incomplete as the majority of injected cells did not contribute to the endodermal cell mass.

Gata5 functions downstream of Oep

Rodaway et al. (1999) have previously shown that the Nodal-related proteins Cyc and Sqt are essential for the initiation of embryonic *gata5* expression. We have found that the EGF-CFC protein Oep, which is essential for Nodal signaling, is also essential for the initiation of embryonic *gata5* expression (J. Alexander and D. Y. R. S., unpublished observations). The observation that *gata5* overexpression can induce endodermal gene expression in *Zoep* mutants indicates that Gata5 can promote endoderm formation in embryos where Nodal signaling is reduced. Together, these data suggest that Gata5 does not require Nodal signaling to activate expression of *sox17* and that Gata5 acts downstream of Oep and Nodal signaling. Although *gata5* overexpression can nearly triple the number of endodermal cells present in *Zoep* mutants, *gata5* overexpression does not restore a wild-type number of *sox17*-expressing cells, even at higher doses (data not included). *bon*, much like *gata5*, is initially expressed normally in the germ ring of *Zoep* mutants, but then quickly diminishes during gastrulation (Alexander and Stainier, 1999), indicating that Bon function is reduced in *Zoep* mutants. However, co-injection of *gata5* and *bon* did not restore a wild-type number of endodermal cells in *Zoep* embryos (Y. K. and D. Y. R. S.,

unpublished observations), suggesting that one or more other Oep-dependent factors (denoted X in Fig. 7) promote endoderm development together with Gata5 and Bon. Several paired-type homeobox transcription factors other than Bon have been implicated in endoderm formation in *Xenopus* (Rosa, 1989; Ecochard et al., 1998; Tada et al., 1998; Casey et al., 1999). It is possible that another zebrafish Mix or Bix homolog also functions in endoderm formation downstream of Nodal signaling. Alternatively, the presence of a Smad-interaction domain (Germain et al., 2000) in Bon indicates that X could represent a nuclear Smad that transduces the Nodal signal.

Bon and Gata5 cooperatively regulate endoderm formation

Zebrafish *bon* and *gata5* show striking similarities: both genes encode transcription factors induced in the margin of late blastula stage embryos by Nodal signaling; mutations in either gene limit endoderm formation; and overexpression of either gene can increase endoderm formation (this paper; Alexander et al., 1999; Alexander and Stainier, 1999; Kikuchi et al., 2000). These similarities raise the possibility that Bon and Gata5 act linearly within a pathway regulating endoderm formation. However, our results show that expression of *bon* does not depend upon Gata5 activity and, conversely, early expression of *gata5* does not depend upon Bon activity. The absence of transcriptional regulation between these two gene products argues that the endoderm formation pathway bifurcates, such that Nodal signaling induces both *gata5* and *bon* independently (Fig. 7). Consistent with this hypothesis, overexpression of *gata5* in *bon* mutants is able to expand endoderm formation, indicating that Bon is not essential for Gata5 activity.

Analysis of *fau/gata5;bon* double mutants reveals that Gata5 and Bon play unique, non-overlapping roles. Although embryos mutant for either *gata5* or *bon* form fewer endodermal cells than wild-type siblings, double mutants form very little or no endoderm. Thus, most of the endoderm that does form in either *fau/gata5* or *bon* single mutants is due to the activity of the other factor, suggesting that Bon and Gata5 act in similar but distinct ways to promote endoderm formation. The very small amount of endoderm that does form in *fau/gata5;bon* double mutants may reflect residual activity of the mutant Gata5 and/or Bon proteins, or may be due to an additional Nodal-dependent factor that promotes endoderm development in parallel to Gata5 and Bon (i.e., X in Fig. 7).

While both *gata5* and *bon* when overexpressed restore some endoderm in *bon* mutants, their respective activities appear different. *gata5*-injected *bon* mutants usually contained a patch of *sox17*-expressing endodermal cells that did not exhibit the regularly spaced distribution seen in wild-type or *bon*-injected *bon* mutant embryos (Fig. 6B). In addition, injections into wild-type embryos of *gata5*, but not *bon*, mRNA led to an increased density of *sox17*-expressing endodermal cells (Fig. 5A,B). While *bon* is expressed transiently around the onset of gastrulation, *gata5* expression is maintained in the endoderm. Finally, while endodermal cells in *bon* mutants express wild-type levels of *sox17*, those in *fau/gata5* mutants express a reduced level. All these data are consistent with a model where both Bon and Gata5 function to regulate the number of endodermal cells that form. However, Gata5, but not Bon, also functions to maintain *sox17* expression in gastrulating endodermal cells. While this second role of Gata5 in maintaining *sox17* expression may well be direct, the role of Gata5 and Bon in initiating *sox17* expression may occur via *cas* (see below). Analysis of the *cas* and *sox17* promoters should allow further investigation of these questions, as well as the question of additive versus synergistic activities of Gata5 and Bon on these promoters.

gata5 and *bon* are also expressed together in marginal cells that become mesoderm, indicating that coexpression of these genes is not sufficient to induce endodermal fate. It remains unclear how the embryo segregates marginal blastomeres into mesoderm and endoderm. Resolution of this problem will require further study of the complex molecular and cellular interactions in the zebrafish germ ring. In addition, *gata5* is also expressed in the myocardial lineage before and during gastrulation and *gata5* overexpression can lead to ectopic myocardial gene expression (Reiter et al., 1999). How a single transcription factor, Gata5, can play such critical roles in two distinct lineages is a fascinating question that warrants further study.

Gata5 acts upstream of *cas*

Overexpression of *gata5* increases the number of endodermal cells in wild-type embryos as well as in *Zoep* and *bon* mutants. In contrast, overexpression of *gata5* in *cas* mutants usually does not induce expression of *sox17*, placing the function of the *cas* gene product between Gata5 and *sox17*.

bon and *taram-a** overexpression cannot increase endodermal gene expression in *cas* mutants (Alexander and Stainier, 1999). Gata5 function is distinct from the functions of Bon and Taram-a* in that overexpression of *gata5* in *cas* mutants can occasionally 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 endodermal cells after their induction, the process in which *cas* presumably functions, Gata5 also likely acts downstream of, or in parallel to, Cas to regulate *sox17* expression.

Although these results show that Gata5 usually requires *cas* to promote endoderm formation, they do not indicate the mechanism by which *gata5* and *cas* interact. *cas* could encode an essential transactivator of *sox17* and *foxA2* through which both Gata5 and Bon function in a linear pathway. Alternatively, Cas could regulate *sox17* expression in combination with Gata5 and Bon, and be essential for this process. Biochemically, this scenario would position Cas parallel to Gata5 and Bon. A third possibility is that Cas antagonizes a repressor of *sox17* and *foxA2* expression (i.e., Y in Fig. 7). This latter hypothesis predicts that in the absence of *cas* activity, the repressor Y prevents Gata5 and Bon from promoting endodermal gene expression, except in cases of profound *gata5* overexpression. The isolation of *cas* will help to discriminate between these models.

Dominant genetic interactions between *fau/gata5*, *bon* and *cas*

The *fau/gata5*, *bon* and *cas* mutations show dominant genetic interactions with each other significant enough to perturb development conspicuously, although less severely than the homozygous mutations. One quarter of the progeny of

heterozygote intercrosses are predicted to be transheterozygous. However, the incidence of obvious cardiac defects among such progeny is only 3-18%. Therefore, the phenotypes we observe in transheterozygous embryos are only partially penetrant.

Interestingly, this phenomenon of dominant genetic interaction does not apply to all genes involved in endoderm formation, as *oep* does not interact with *fau/gata5*, *bon* 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* and *cas*, but less so to changes in *oep* levels, as might be the case if Gata5, Bon and Cas physically interact to form a regulatory complex, but do not interact directly with Oep. Given that Fau and Bon are transcription factors, while Oep can function as an extracellular secreted factor, this model is certainly plausible.

The existence of transheterozygous phenotypes raises the possibility of using genetic interactions to expedite screens for new mutations. By screening F_1 progeny for dominant interactions, a screen could be focused on identifying the mutations that affect a specific process of interest with less effort than is required for a traditional F_2 screen. However, such a strategy would identify only a subset of the total number of pertinent genes, for example in this case alleles of *cas* and *bon*, but not *oep*, and would critically depend upon the robustness of the genetic interactions.

It is likely that many human congenital abnormalities are also due to the cumulative effect of mutations in several genes. Therefore, partially penetrant, transheterozygous interactions in zebrafish may provide good genetic models for some complex, medically significant human defects.

Conclusions

Gata transcription factors are required for endoderm development in both *Drosophila* and *C. elegans*. Here, we have demonstrated that a vertebrate Gata factor, Gata5, is required for endoderm formation in zebrafish. During blastula and gastrula stages, Gata5 regulates the number of endodermal cells and the transcription of endodermal genes such as *sox17* and *foxA2*. 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.

In addition, we have placed Gata5 within a molecular pathway regulating zebrafish endoderm formation. Nodal signaling induces the expression of both *gata5* and *bon*. Within the germ ring, Gata5 and Bon do not regulate each other, but rather function to specify endoderm and promote the expression of *sox17* and *foxA2* in a manner dependent on Cas function.

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REFERENCES

Abel, T., Michelson, A. M. and Maniatis, T. (1993). A Drosophila GATA family member that binds to Adh regulatory sequences is expressed in the developing fat body. *Development* **119**, 623-633.

Alexander, J., Rothenberg, M., Henry, G. L. and Stainier, D. Y. (1999). *casanova* plays an early and essential role in endoderm formation in zebrafish. *Dev. Biol.* **215**, 343-357.

Alexander, J. and Stainier, D. Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. *Curr. Biol.* **9**, 1147-1157.

Alexander, J., Stainier, D. Y. R. and Yelon, D. (1998). Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. *Dev. Genet.* **22**, 288-299.

Bossard, P. and Zaret, K. S. (1998). GATA transcription factors as potentiators of gut endoderm differentiation. *Development* **125**, 4909-4917.

Casey, E. S., Tada, M., Fairclough, L., Wylie, C. C., Heasman, J. and Smith, J. C. (1999). Bix4 is activated directly by VegT and mediates endoderm formation in *Xenopus* development. *Development* **126**, 4193-4200.

Charron, F. and Nemer, M. (1999). GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10**, 85-91.

Chen, J.-N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P. et al. (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* **123**, 293-302.

Clements, D., Friday, R. V. and Woodland, H. R. (1999). Mode of action of VegT in mesoderm and endoderm formation. *Development* **126**, 4903-4911.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-1928.

Ecochard, V., Cayrol, C., Rey, S., Foulquier, F., Caillol, D., Lemaire, P. and Duprat, A. M. (1998). A novel *Xenopus mix*-like gene *milk* involved in the control of the endomesodermal fates. *Development* **125**, 2577-2585.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* **395**, 181-185.

Fukushige, T., Hawkins, M. G. and McGhee, J. D. (1998). The GATA-factor *elt-2* is essential for formation of the *Caenorhabditis elegans* intestine. *Dev. Biol.* **198**, 286-302.

Gao, X., Sedgwick, T., Shi, Y. B. and Evans, T. (1998). Distinct functions are implicated for the GATA-4, -5, and -6 transcription factors in the regulation of intestine epithelial cell differentiation. *Mol. Cell Biol.* **18**, 2901-2911.

Germain, S., Howell, M., Esslemont, G.M. and Hill, C.S. (2000). Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev.* **14**, 435-451.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J. R. and Zaret, K. S. (1996). Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev.* **10**, 1670-1682.

Henry, G. L. and Melton, D. A. (1998). *Mixer*, a homeobox gene required for endoderm development. *Science* **281**, 91-96.

Ho, R.K. and Kimmel, C.B. (1993). Commitment of cell fate in the early zebrafish embryo. *Science* **261**, 109-11.

Hudson, C., Clements, D., Friday, R. V., Stott, D. and Woodland, H. R. (1997). Xsox17α and -β mediate endoderm formation in *Xenopus*. *Cell* **91**, 397-405.

Kaestner, K., Knöchel, W. and Martínez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* **14**, 142-146.

Kikuchi, Y., Trinh, L. A., Reiter, J. F., Alexander, J., Yelon, D. and Stainier, D. Y. (2000). The zebrafish *bonnie and clyde* gene encodes a mix family homeodomain protein that regulates the generation of endodermal precursors. *Genes Dev.* **14**, 1279-1289.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling,

T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kimmel, C. B., Warga, R. M. and Schilling, T. F. (1990). Origin and organization of the zebrafish fate map. *Development* **108**, 581-594.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723-732.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.

Lazzaro, D., Price, M., de Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.

LeDouarin, N. (1975). An experimental analysis of liver development. *Med. Biol.* **53**, 427-55.

Liao, W., Ho, C.-H., Yan, Y.L., Postlethwait, J. and and Stainier, D.Y.R. (2000). Hhex and Scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development* **127**, 4303-4313.

Maeda, M., Kubo, K., Nishi, T. and Futai, M. (1996). Roles of gastric GATA DNA-binding proteins. *J. Exp. Biol.* **199**, 513-520.

Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.

Molkentin, J. D., Tymitz, K. M., Richardson, J. A. and Olson, E. N. (2000). Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol. Cell. Biol.* **20**, 5256-60.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.

Odenthal, J. and Nüsslein-Volhard, C. (1998). fork head domain genes in zebrafish. *Dev. Genes Evol.* **208**, 245-258.

Orkin, S. H. and Zon, L. I. (1997). Genetics of erythropoiesis: induced mutations in mice and zebrafish. *Annu. Rev. Genet.* **31**, 33-60.

Osada, S. I. and Wright, C. V. (1999). *Xenopus* nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. *Development* **126**, 3229-3240.

Rehorn, K. P., Thelen, H., Michelson, A. M. and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. *Development* **122**, 4023-4031.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y. R. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13**, 2983-2995.

Renucci, A., Lemarchandel, V. and Rosa, F. (1996). An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling pathway involved in fish head organiser formation. *Development* **122**, 3735-3743.

Reuter, R. (1994). The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the *Drosophila* gut. *Development* **120**, 1123-1135.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R. and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell- derived TGFβ family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126**, 3067-3078.

Rosa, F. M. (1989). Mix.1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965-974.

Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z. et al. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.

Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S. and Driever, W. (1997). The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail*. *Development* **124**, 327-342.

Schier, A. F. and Shen, M. M. (2000). Nodal signalling in vertebrate development. *Nature* **403**, 385-389.

Shoichet, S.A., Malik, T.H., Rothman, J.H. and Shivdasani, R.A. (2000). Action of the *Caenorhabditis elegans* GATA factor END-1 in *Xenopus* suggests that similar mechanisms initiate endoderm development in ecdysozoa and vertebrates. *Proc. Natl. Acad. Sci. USA* **97**, 4076-4081

Stainier, D. Y. R., Fouquet, B., Chen, J. N., Warren, K. S., Weinstein, B. M., Meiler, S. E., Mohideen, M. A., Neuhauss, S. C., Solnica-Krezel, L., Schier, A. F. et al. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**, 285-292.

Stainier, D. Y. R., Lee, R. K. and Fishman, M. C. (1993). Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development* **119**, 31-40.

Strahle, U., Blader, P., Henrique, D. and Ingham, P. W. (1993). *Axial*, a zebrafish gene expressed along the developing body axis, shows altered expression in *cyclops* mutant embryos. *Genes Dev.* **7**, 1436-1446.

Tada, M., Casey, E. S., Fairclough, L. and Smith, J. C. (1998). Bix1, a direct target of Xenopus T-box genes, causes formation of ventral mesoderm and endoderm. *Development* **125**, 3997-4006.

Takke, C., Dornseifer, P., v. Weizsacker, E. and Campos-Ortega, J. A. (1999). *her4*, a zebrafish homologue of the *Drosophila* neurogenic gene *E(spl)*, is a target of NOTCH signalling. *Development* **126**, 1811-1821.

Warga, R. M. and Kimmel, C. B. (1990). Cell movements during epiboly and gastrulation in zebrafish. *Development* **108**, 569-580.

Warga, R. M. and Nüsslein-Volhard, C. (1999). Origin and development of the zebrafish endoderm. *Development* **126**, 827-838.

Weber, H., Symes, C., Walmsley, M., Rodaway, A. and Patient, R. (2000). A role for GATA5 in *Xenopus* endoderm specification. *Development* **127**, 4345-4360.

Westerfield, M. (1995). *The Zebrafish Book*. Eugene, OR: University of Oregon Press.

Willett, C. E., Zapata, A. G., Hopkins, N. and Steiner, L. A. (1997). Expression of zebrafish *rag* genes during early development identifies the thymus. *Dev. Biol.* **182**, 331-341.

Yasuo, H. and Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in Xenopus embryos. *Curr. Biol.* **9**, 869-879.

Zhu, J., Hill, R. J., Heid, P. J., Fukuyama, M., Sugimoto, A., Priess, J. R. and Rothman, J. H. (1997). *end-1* encodes an apparent GATA factor that specifies the endoderm precursor in *Caenorhabditis elegans* embryos. *Genes Dev.* **11**, 2883-2896.

Zhu, J., Fukushige, T., McGhee, J. D. and Rothman, J. H. (1998). Reprogramming of early embryonic blastomeres into endodermal progenitors by a *Caenorhabditis elegans* GATA factor. *Genes Dev.* **12**, 3809-3814.