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Gata4 regulates the formation of multiple organs

Audrey Holtzinger and Todd Evans *Development* **132**, 4005-4014.

This article contains two errors that have come to light after recent updates to the Zebrafish Information Network and the Ensemble databases.

The sequence of the translation blocker morpholino MO detailed on p. 4006 is 5′-TCCACAGG…-3′ and MO1 actually targets the intron 2/exon 3 boundary of the *gata4* pre-mRNA, although this does not affect its function.

The authors apologise to readers for these mistakes.

Gata4 regulates the formation of multiple organs

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Summary

We have developed a loss-of-function model for Gata4 in zebrafish, in order to examine broadly its requirement for organogenesis. We show that the function of Gata4 in zebrafish heart development is well conserved with that in mouse, and that, in addition, Gata4 is required for development of the intestine, liver, pancreas and swim bladder. Therefore, a single transcription factor regulates the formation of many organs. Gata6 is a closely related transcription factor with an overlapping expression pattern. We show that zebrafish depleted of Gata6 show defects in liver bud growth similar to mouse Gata6 mutants and zebrafish Gata4 morphants, and that zebrafish embryos depleted of both Gata4 and Gata6 display an earlier block in liver development, and thus completely lack liver buds. Therefore, Gata4 and Gata6 have distinct non-redundant functions in cardiac morphogenesis, but are redundant for an early step of liver development. In addition, both Gata4 and Gata6 are essential and non-redundant for liver growth following initial budding.

Key words: Organogenesis, Heart, Liver, Pancreas, Zebrafish

Introduction

Gata transcription factors encode zinc-finger DNA-binding proteins that regulate diverse pathways associated with embryonic morphogenesis and cellular differentiation (Patient and McGhee, 2002). Gata4 was described initially as an early cardiogenic marker, although the first requirement of mammalian Gata4 is within extra-embryonic visceral endoderm for embryonic morphogenesis (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997b). The gene is not required in cardiomyocytes (Narita et al., 1997a) yet studies have implicated Gata4 in regulating various steps of heart formation, including morphogenesis, proliferation, cell survival, differentiation and growth (Pu et al., 2004; Watt et al., 2004; Zeisberg et al., 2005). A genetic mosaic analysis showed the gene is also required in gastric epithelium (Jacobsen et al., 2002), and a few target genes for Gata4 are described for differentiated cells of the intestine (Gao et al., 1998; Her et al., 2004), liver (Cirillo et al., 2002; Dame et al., 2004) and pancreas (Ritz-Laser et al., 2005). Further progress in evaluating the function of mammalian Gata4 has been limited, in large part, because of the early embryonic lethality of the knockout mouse embryos (E7.0-9.5).

However, recent progress has been made by taking advantage of tetraploid embryo complementation assays, whereby wild-type cells rescue the requirement for Gata4 in extra-embryonic endoderm, with mutant cells constituting the embryo proper (Watt et al., 2004). These experiments revealed essential functions for Gata4 in heart tube morphogenesis (probably related specifically to pro-epicardial development). Gata6 is a closely related gene that is also required for extra-embryonic endoderm development (Koutsourakis et al., 1999; Morrisey et al., 1998), and similar rescue experiments revealed a required role for Gata6 in liver development subsequent to hepatic specification (Zhao et al., 2005). Because Gata4 and Gata6 are expressed in overlapping patterns in both heart and gut tissues, it raises the question of whether they might play redundant roles in some aspects of heart or liver development. The murine Gata4 'embryo-specific' mutant embryos still die too early to evaluate a role in gut-derived organ development. In the case of Gata6 mutants, the heart appears to be unaffected. It was suggested that Gata4 might play a redundant role for hepatic specification, but unlike Gata6 might not be required for subsequent organ growth (Zhao et al., 2005).

Here, we describe a loss-of-function analysis for Gata4 in the zebrafish. A primary advantage of this model is that, unlike mouse, fish embryos are not dependent upon support from a primitive (visceral) extra-embryonic tissue. Furthermore, the mutant zebrafish survive beyond the cardiomyopathy, as embryos do not require a functional heart for several days, including during the time for normal gut development. Using the morpholino approach, it is possible to block the expression of both Gata4 and Gata6 in order to reveal potential functional redundancies. We show that, in zebrafish, Gata4 is essential for the generation of endoderm-derived organs, including the intestine, liver, pancreas and swim bladder. In addition, our results show that there is a functional redundancy between Gata4 and Gata6 at an early stage of liver development.

Materials and methods

Zebrafish strains

Zebrafish embryos were maintained at 28°C and staged as described (Westerfield, 1995). The *gata4:GFP* reporter strain (line 39) has been described previously (Heicklen-Klein and Evans, 2004). The *fli1:GFP* line (Lawson and Weinstein, 2002) was obtained from the Zebrafish International Research Center (Eugene, OR). The *tie2:GFP* line was provided by Didier Stainier (UCSF, CA). The *gata6:GFP* line was generated by inserting the GFP gene into an isolated PAC clone at the

gata6 translational start site (representing the proximal ATG or 'short form' of Gata6), using recombination in *Escherichia coli* (Jessen et al., 1998). The recombinant PAC clone was injected into fertilized eggs and potential founders screened by crosses, to establish a germ-line transmitting strain. The recombination step and establishment of the *gata6:GFP* line was performed with the assistance of Dr Shuo Lin and colleagues (UCLA, CA), and this line will be described in full detail in a subsequent report.

Morpholino design and microinjections

A Pac clone containing the *gata4* gene was isolated and used to define the 5′UTR and intron/exon structure (Heicklen-Klein and Evans, 2004). Two morpholino oligomers were designed against the *gata4* sequences (Gene Tools, LLC). MO (5-TCCAGGTGAGCGATTAT-TGCTCC-3′) is complementary to the 5′UTR of the mature *gata4* transcript (a translation blocker), whereas MO1 (5′-TGGACGCAGA-CTGAGAGAAAGAGAG-3′) was designed to bind to sequences at the boundary between intron 1 and exon 2 of the *gata4* pre-mRNA (a splicing blocker). Blast searches predicted that the morpholinos should be specific for Gata4. Morpholinos were injected into fertilized eggs in various doses to establish an optimal dose. Embryos presented a consistent phenotype with injections of between 5 and 20 ng MO. All the experiments shown (except for Fig. 2C) used 10 ng MO per injection. MO1 caused early embryonic death at high doses, but generated an identical phenotype to that generated by MO when using less than 1 ng, and a typical dose used was 0.3 ng per injection. The morpholino designed to target Gata6 was previously described and characterized with respect to specificity (Peterkin et al., 2003). This morpholino targets the 'long form' of Gata6, and the sequence is present therefore in the RNA derived from the *gata6:GFP* transgene.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed essentially as described (Alexander et al., 1998). Briefly, embryos were treated with 0.003% phenylthiourea (PTU) to prevent pigmentation. After fixation, embryos older than 24 hours were treated with 10 μg/ml proteinase K. Hybridization was performed at 70°C, in 50% formamide buffer with digoxigenin-labeled RNA anti-sense probes. The probes used for in situ hybridization were prepared and used as described: Gata4/5/6 (Heicklen-Klein and Evans, 2004), Nkx2.5 (Alexander et al., 1998), Cmlc2 and Amhc (Yelon et al., 1999). The probe for transferrin RNA was derived from the RT-PCR product described below.

Histology

Embryos at 5 days post-fertilization (dpf) were fixed in paraformaldehyde, processed under standard conditions and embedded in paraffin wax. Cross sections at 5 μm were cut with a cryostat, mounted onto slides, and subjected to Hematoxylin and Eosin staining. Sections were observed with brightfield inverted light, and photographed at 20× magnification.

Table 1. Sequence of primers for PCR

Gene	Sequence	Product length (base pairs)
β-Actin	F: 5′-AAGCAGGAGTACGATGAGTCTG-3′ R: 5′-GGTAAACGCTTCTGGAATGAC-3′	278
Elastase B	F: 5′-TTGCGGTGGAAGCCTTATTGACA-3′ R: 5′-AGCGCAGTTTAGAGGACCACCAGA-3′	487
foxA2	F: 5′-CATCGCAAGCTCCAAATCT-3′ R: 5′-TGCAGATCCAGATGGTGCAT-3′	151
i-fabp	F: 5′-AAGTCGACCGCAATGAGAAC-3′ R: 5′-GTTTGACATTGGGAGTGCAG-3′	405
sox17	F: 5′-ACCCGCTCAGTCCGCTCTCAG-3′ R: 5′-ACTCAGTTGTCCCGGCATCCATAG-3′	516
Transferrin	F: 5′-GAGAAAATCAAGCGCAAAGAAGC-3′ R: 5′-AGCCCCATCATAGCCATAATACTG-3′	441

F, forward; R, reverse.

RT-PCR

RNA was isolated from staged embryos using Trizol Reagent, and purified and used for reverse transcriptase (RT) reactions as described previously (Jiang et al., 1998). Conditions for semi-quantitative RT-PCR were established empirically by testing cycle number and ensuring that a 2-fold increase of input RNA led to an approximately 2-fold increase in product. 20 μl RT reactions contained either 1 or 2 μg of RNA for one hour at 37°C. PCR reactions used 2 μl of an RT sample in 50 μl. PCR primers are described in Table 1.

Results

Generation of a Gata4 loss-of-function model in zebrafish

It was of interest for us to examine the function of Gata4 in zebrafish development for several reasons. The comparative genetics of Gata factors needs clarification, as fish embryos with a mutation of Gata5 (the *faust* locus) have a cardia bifid phenotype that resembles, at least superficially, that of the mouse Gata4 knockout (Reiter et al., 1999). This has led to speculation that zebrafish Gata5 is the functional ortholog of mammalian Gata4. In addition, the mouse Gata4 knockout is early embryonic lethal because of a requirement for the gene in visceral endoderm, complicating the evaluation of other phenotypes, if they exist. For these reasons, we used morpholino oligomers to target the specific knockdown of Gata4 in zebrafish embryos. The first morpholino (MO) was designed to target a sequence just upstream of the putative initiation codon (ATG). To test the efficacy of the MO, it was convenient to exploit a line of transgenic animals expressing GFP under the control of *gata4* genomic regulatory sequences, including the 5′UTR encompassing the MO target sequence. We chose a transgenic line we had generated that expresses GFP in the heart and also in the notochord (Heicklen-Klein and Evans, 2004). Although notochord does not express Gata4 (presumably the transgene lacks a repressor element), this allowed us to evaluate the effectiveness of the MO throughout the developing embryo and throughout early development. Thus, in this experiment GFP expression serves as a surrogate marker for Gata4, and inhibition of GFP expression should reflect an effective block to translation of endogenous Gata4.

As shown in Fig. 1, transgenic *gata4:GFP* embryos that had been injected at the one-cell stage with the Gata4-specific MO fail to express GFP in the heart or notochord. GFP expression is fully blocked until at least 5 dpf. The analysis indicates that the ATG-targeted morpholino effectively interacts with the *gata4* UTR sequence to inhibit translation. In addition, 100% of the MO-injected embryos develop a consistent and penetrant phenotype by 2-3 dpf (Fig. 1B), although they continue to survive until approximately 6-8 dpf, thereby allowing us to investigate thoroughly the embryonic functions of Gata4. Injection of control morpholinos caused no abnormal phenotypes (data not shown).

Gata4 is required for development of the heart, gut, pancreas, liver and swim bladder

In contrast to Gata5/*faust* mutants [and Gata6 morphants

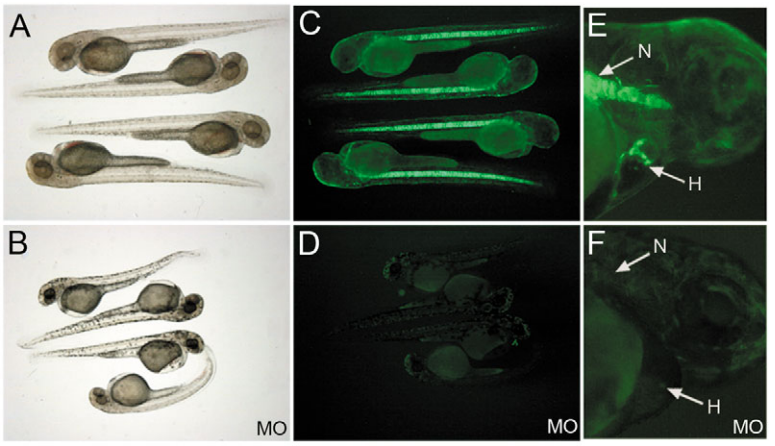

Fig. 1. The Gata4 morpholino effectively blocks GFP translation in the *gata4:GFP* line, and generates a consistent phenotype. (A,C,E) Uninjected control fish; (B,D,F) embryos derived from eggs injected with MO. Living embryos are shown at 2 dpf in brightfield (A,B) or under fluorescence (C-F). (E,F) Control (E) and morphant (F) embryos at 3 dpf (lateral views, dorsal to the top with anterior to the right), demonstrating the complete block to GFP expression in both the notochord (N) and the heart (H).

(Peterkin et al., 2003)], the Gata4 morphant embryos never display a bifid phenotype. However, by 2-3 dpf, every injected embryo is distinguished by a kink in the tail, regression of the yolk stalk extension, and a non-looping but beating heart tube within an edemic cavity (Fig. 2A,B). Development is slightly delayed and the embryos are approximately 25% smaller than controls. To demonstrate that these phenotypes are specific to the loss of Gata4, we designed a distinct morpholino (MO1), to target the splice site at the junction of the first intron and the second exon, upstream of the region encoding the zinc fingers. Injection of embryos with MO1 consistently generates a phenotype that is indistinguishable in all respects from that caused by the ATG-targeted MO (Fig. 2C, and data not shown).

Between 3 and 4 days of development, circulating blood is no longer evident in the morphant embryos (Fig. 2D,E) and they fail to inflate the swim bladder. The heart tube of MO-injected embryos forms, beats, and appears to demarcate chambers, but looping and growth never occurs, leading to cardiac edema and, by 5 dpf, a severe heart-string phenotype (Fig. 2F). The embryos survive for several more days, and this revealed an additional striking phenotype. From 6-8 dpf, a long thin gut tube is evident through the body cavity that extends the length of the embryo (Fig. 2G). Histological analysis at 5 dpf shows that in morphant embryos both the heart and gut tubes form lumens, but subsequent organogenesis fails (Fig.

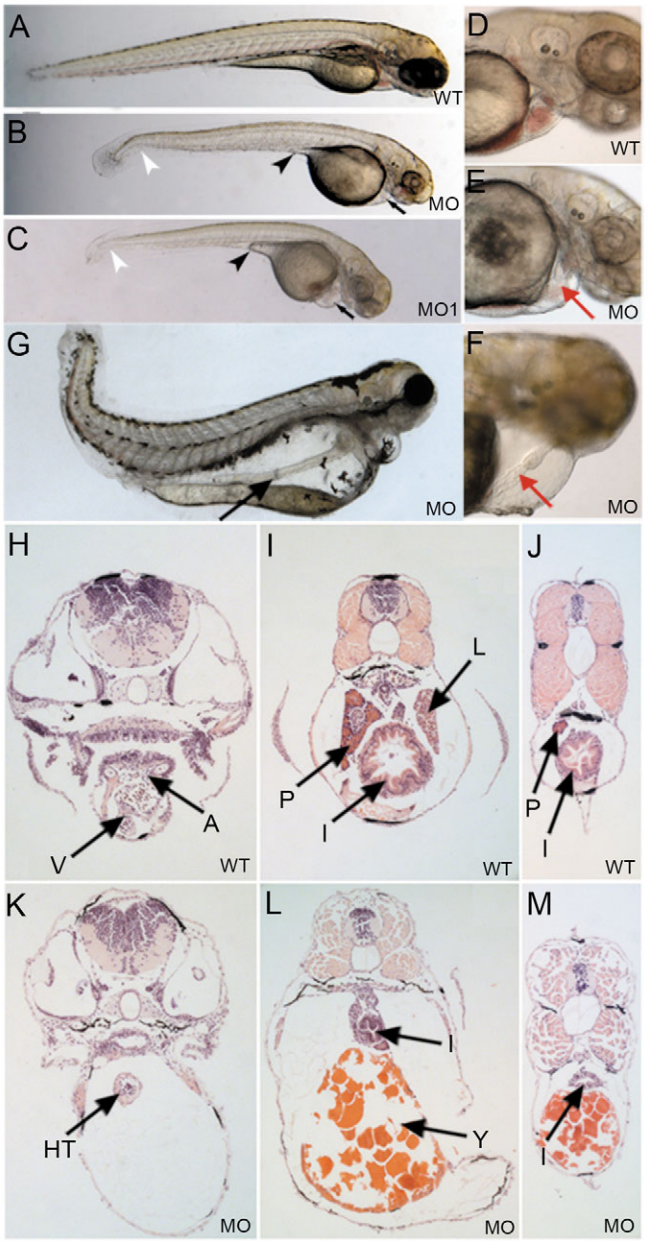

Fig. 2. Gata4 deficient embryos develop cardiac and gut tubes but no derivative organs. (A-C) Shown at 3 dpf are (A) a representative control embryo (WT), and embryos derived from eggs injected with (B) the morpholino targeting the Gata4 5′UTR (MO) or (C) the morpholino targeting an intron/exon boundary (MO1). The morphant fish display a kink in the tail (white arrowheads), a pericardial edema (black arrows), and regression of the yolk stalk extension (black arrowheads). (D,E) Control embryo (D) and a representative morphant (E), showing a relative lack of blood in the linear but constricted heart tube at 3 dpf in the morphant (red arrow). (F) At 5 dpf, the morphant linear heart tube (red arrow) is distended. Views are lateral, dorsal to the top and anterior to the right. (G) At 8 dpf, a linear gut tube is shown (arrow), running the length of the morphant body. (H-M) Representative histological cross sections through control (WT) or morphant (MO) embryos. Equivalent sections are progressively more caudal, at the level of the heart (H,K), mid-pancreas (I,L) or the caudal end of the swim bladder (J,M). A, atrium; V, ventricle; HT, heart tube; P, pancreas; L, liver; I, intestine; Y, yolk.

2H-M). The intestine lacks normal epithelial folds, a phenotype that is more severe in caudal regions of the tube. Liver tissue is either not apparent, or is reduced to a small patch of cells when compared to the control. The exocrine pancreas is also often not apparent, although in some embryos there is evidence of a small islet-like structure. The swim bladder is missing, whereas the large store of maternal yolk material, which has been fully absorbed in control embryos, remains in the large edemic body cavity of Gata4-depleted embryos. By contrast, many other tissues appear grossly normal, including mesonephros, notochord, neural tube and body wall muscle.

Loss of Gata4 disrupts late cardiac morphogenetic movements after initial heart tube formation

We first characterized the cardiomyopathy caused by Gata4 deficiency. Given the hematopoietic defect, it seemed possible that the heart defect and edema could be a secondary consequence of poor fluid exchange. We cultured injected embryos in a solution of 250 mM mannitol, which prevents systemic edema by eliminating the osmotic water gradient (Hill et al., 2004). However, the morphant embryos cultured with or without mannitol developed an identical cardiac edema (not shown), indicating that the morphants have a specific defect in heart function. We evaluated whether the heart tube defect reflected abnormalities in overall vascular development by comparing control and morphant embryos transgenic for the *fli1:GFP* reporter, which labels endothelium. Embryos injected with the Gata4-specific MO develop a normal embryonic trunk vasculature when compared with control embryos at 3 dpf (see Fig. S1 in the supplementary material). Thus the defect is specific to the heart tube.

Molecular markers were analyzed to evaluate the stage of heart development affected by the loss of Gata4. The expression of Nkx2.5, an early marker for cardiogenic progenitors, is normal at 19.5 hours post-fertilization (hpf), indicating that the initial stages of cardiogenesis are intact, including formation of the cardiac cone and the subsequent extension of the heart tube (Fig. 3A,B). Chamber specification is also normal, as Amhc (an atrial marker) and Cmlc2 (an atrial and ventricular marker) are both expressed in the MO-injected embryos (Fig. 3C-F). By evaluating GFP expression in *tie2:GFP* transgenic fish injected with the Gata4 MO, we find that endocardium forms in the morphant embryos, including the establishment of cardiac cushions (see Fig. S2 in the supplementary material). However, the marker analysis reveals that between 2 and 3 dpf the shape of the heart tube becomes disturbed relative to that in control embryos. This late defect was also confirmed by watching morphogenetic changes in the heart tube of transgenic embryos that express GFP in cardiomyocytes (data not shown). Specifically, the more caudal presumptive atrial portion of the tube fails to make the appropriate rostral-anterior jogging movement, or to expand in size relative to the presumptive ventricular region. Therefore, at this stage the heart tube remains straight and uniformly thin, and by 4 dpf forms a distended heart string.

Organogenesis from the primordial gut tube requires Gata4

Similarly, we characterized the progression of endoderm development in morphant embryos compared with controls. Early markers specific to endoderm, including Sox17 and

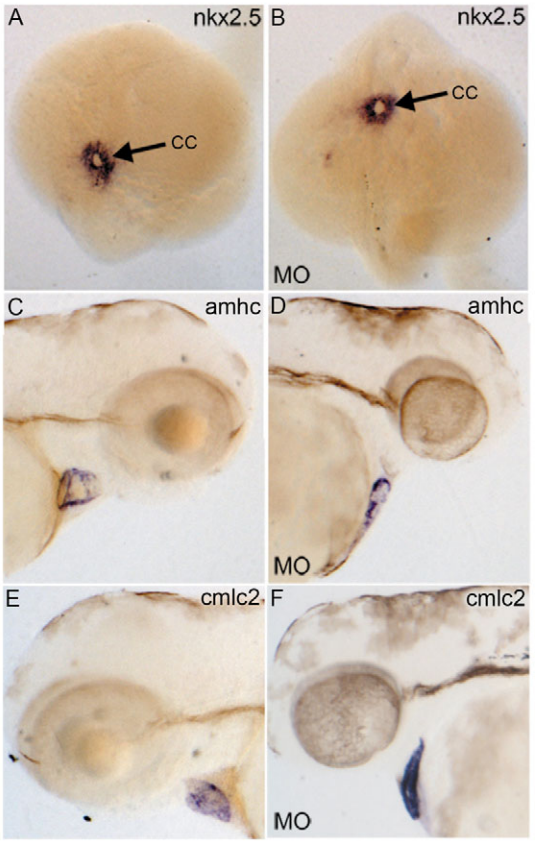

Fig. 3. Heart tube formation is normal, but morphogenesis fails in Gata4 deficient embryos. Control uninjected embryos (A,C,E) are compared with embryos derived from eggs injected with MO (B,D,F). Embryos were processed for in situ hybridization to detect transcripts for Nkx2.5 (A,B), Amhc (C,D) or Cmlc2 (E,F). The morphant embryos show normal formation of the cardiac cone (cc) at the 20-somite stage (B). Likewise, the cardiomyocyte markers Amhc and Cmlc2 are expressed normally, shown here at 3 dpf. (A,B) Dorsal views with anterior to the top; (C,D) lateral views with anterior to the right and dorsal to the top; (E,F) lateral views with anterior to the left and dorsal to the top. Embryos are shown in both orientations to show the lack of looping in the linear heart tube of the morphant embryos.

Foxa2, are expressed equivalently in control and morphant embryos at 28 or 48 hpf (Fig. 4A), indicating that endoderm specification is normal in embryos depleted for Gata4. However, at 4 dpf, expression of organ-specific markers for differentiated intestine (I-fabp), liver (transferrin) or pancreas (elastaseB) are either absent or significantly reduced in the morphant embryos (Fig. 4B). The reproducible low levels of transferrin led us to consider whether some limited tissue had in fact differentiated in the morphant embryos. Therefore, control and morphant embryos were analyzed for transferrin expression by in situ hybridization at 2, 3, 4 and 5 dpf. As shown in representative samples (Fig. 4D,F,H,J), we consistently find a small patch of transferrin-positive cells in morphant embryos at the position of the liver bud by 2 dpf. However, this patch fails to expand at subsequent stages, when compared with the control embryos. This result suggests that, in embryos deficient for Gata4, the liver bud is specified from the gut tube at the appropriate time and place, and that specified

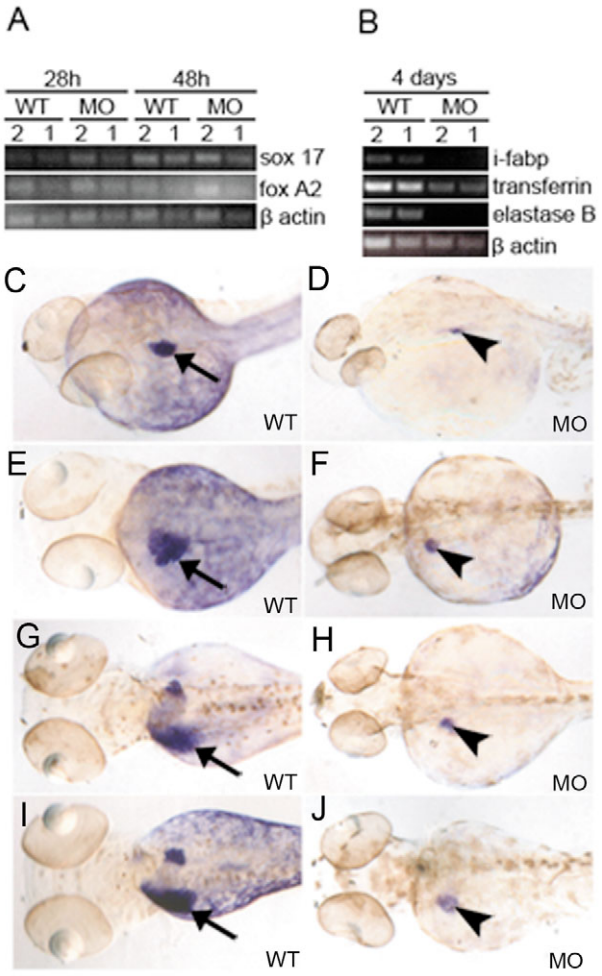

Fig. 4. Gata4 deficient embryos express early endoderm markers but organogenesis fails. (A) Shown are representative semi-quantitative RT-PCR results for the early endoderm markers Sox17 and FoxA2. Assays used either 2 or 1 μg of RNA, isolated for the RT reaction at 28 hpf or 48 hpf, as indicated. We consistently found no significant difference in the expression levels comparing control (WT) and morphant (MO) embryos. (B) RT-PCR assays were also performed using RNA isolated at 4 dpf for markers of differentiated intestine (I-fabp), liver (transferrin), and pancreas (elastaseB). (C-J) Representative embryos are shown following in situ hybridization to detect transferrin RNA, comparing control (WT) and morphant (MO) embryos at 2 dpf (C,D), 3 dpf (E,F), 4 dpf (G,H) and 5 dpf (I,J). In all cases, the morphant embryos develop with a small patch of transferrin-positive cells (arrowheads in MO panels), but this patch fails to grow when compared with controls (arrows in WT panels). Views are dorsal, anterior to the left.

hepatocytes can differentiate, but that the bud fails to grow and expand in size, thus compromising organogenesis.

The stage that gut-derived organ formation fails, and the potential for cross-regulation among Gata factors, was further investigated by evaluating the expression patterns of Gata4, Gata5 and Gata6 in Gata4 morphant embryos (Fig. 5). Early endoderm development has been described in zebrafish (Field et al., 2003; Wallace and Pack, 2003). By 28 hpf, a solid column of cells called the intestinal rod has formed from endoderm at the midline, with two thickened regions that correspond to the future budding points for the liver (rostral on the left) and pancreas (caudal on the right). At this stage (Fig. 5, left panels), Gata4 transcript patterns are not significantly altered by the MO, which is to be expected as the morpholino targets translational arrest and not transcription. The *gata5* gene is expressed at a low level in the developing intestine and heart, and this is increased significantly in the heart tube of morphant embryos. The pattern of Gata5 expression in the

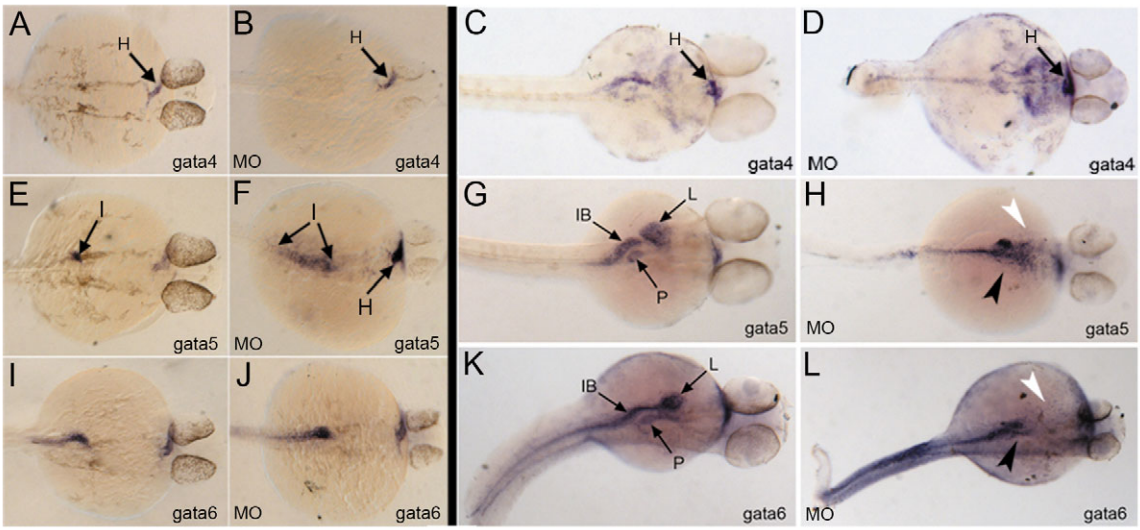

Fig. 5. Gata4 deficient fish are blocked at a relatively late stage of gut tube morphogenesis. Shown are representative examples of control embryos (A,C,E,G,I,K) and corresponding morphant (MO) embryos (B,D,F,H,J,L) following in situ hybridization using probes to detect Gata4 (A-D), Gata5 (E-H) or Gata6 (I-L). Embryos were fixed at 28 hpf (A,B,E,F,I,J) or 48 hpf (C,D,G,H,K,L). Note that, at 28 hpf, Gata5 expression is enhanced in the morphant embryos (arrows in F), whereas at 48 hpf Gata4 transcript levels are clearly increased (D), but Gata5 and Gata6 expression (H,L) is missing in the regions that normally show signal for pancreas (black arrowhead) and liver (white arrowhead). Views are dorsal, with anterior to the right. I, intestinal rod; H, heart; IB, intestinal bulb; L, liver; P, pancreas.

intestinal rod appears relatively diffuse, when compared with controls, suggesting that at these early stages endoderm is present in the morphant embryos but that the developing gut tube is beginning to show some morphological disruption. Expression levels for Gata6 are not significantly altered by the Gata4-specific MO, either in the intestinal rod or in the heart tube. The analysis of Gata6 expression at 28 hpf shows convincingly that, in both control (Fig. 5I) and morphant (Fig. 5J) embryos, the intestinal rod has formed and has made initial budding movements (and in addition that the heart tube has formed and moved appropriately to the left). However, morphological defects are obvious by 2 dpf (Fig. 5, right panels), and are best seen in the expression patterns of Gata5 and Gata6, which at this stage in control embryos clearly define the budding liver, intestinal bulb and pancreas (Fig. 5G,K). Each of these patterns is either not apparent or severely reduced in the morphant embryos (Fig. 5H,L). By this stage, transcript levels for Gata4 are increased appreciably when compared with control embryos (Fig. 5C,D). Therefore, Gata4 negatively regulates transcription of both its own gene and Gata5.

As shown above, Gata6 is an excellent marker for early endoderm and is also expressed in each of the gut-derived organs, including the intestine, liver and pancreas. To evaluate endoderm development closely and rule out the possibility that the phenotype reflects a delay in organ formation, a reporter fish line was generated that expresses GFP under the control of the *gata6* locus. These embryos recapitulate with GFP the normal Gata6 expression pattern, and allow the continued evaluation of the morphant phenotype in living embryos, as development proceeds (Fig. 6). The early activation of the reporter around 24 hpf is equivalent in control and morphant embryos (not shown), confirming that activation of the *gata6* gene is not dependent on Gata4, and that endoderm is specified normally. By 2 dpf, control embryos express GFP strongly in the gut-derived organs, and the budding organ morphology of the intestine, liver and pancreas is easily visualized in the transgenic animals (Fig. 6A). These organ structures grow significantly from 2 to 3 dpf (Fig. 6C). In the morphant embryos, a primordial tube is present at 2 dpf, and the initial stages of organ budding are visualized, including a slight looping of the intestinal bulb to the left, and the initial emergence of the posterior (endocrine) pancreatic bud (Fig. 6B). However, between 2 and 3 dpf there is no further growth of these organs in morphant embryos, and by 4 dpf GFP is difficult to detect, whereas control embryos display bright green intestinal bulb, liver and pancreas.

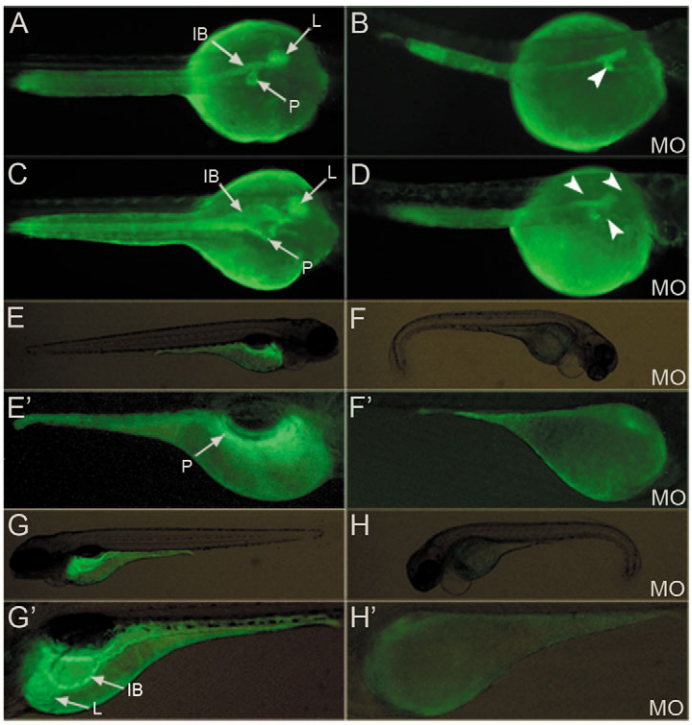

Fig. 6. Gut morphogenesis visualized in *gata6:GFP* transgenic embryos. (Left) Representative control embryos that express GFP regulated by the *gata6* transgene at 2 dpf (A), 3 dpf (C) and 4 dpf (E,G). (Right) Corresponding representative morphants from the same batch of transgenic embryos as controls. Initial organ budding is evident at 2-3 dpf in the morphant, in particular for the posterior (endocrine) pancreatic bud (arrowhead). However, as indicated in D (arrowheads), the intestinal bulb, liver and pancreas fail to grow. (A-D) Dorsal views with anterior to the right; (E,F) lateral views with dorsal to the top and anterior to the right, in order to visualize the pancreas; (G,H) lateral views with dorsal to the top and anterior to the left, in order to visualize the intestinal bulb and liver. E′,F′,G′ and H′ are higher order magnification views of E,F,G and H, respectively. IB, intestinal bulb; L, liver; P, pancreas.

Gata4 and Gata6 are redundant for liver budding, but both are essential and non-redundant for subsequent organ growth

The expression pattern and structural relatedness of Gata6 suggests the potential for functional redundancy with Gata4 in both heart and gut formation. Recently, the function of Gata6 was evaluated in mouse embryos using the tetraploid complementation approach, in which the early requirement for Gata6 in extra-embryonic endoderm is rescued. Mice lacking Gata6 have apparently normal heart development, but show a liver defect that is very similar to the zebrafish Gata4 morphants, with a failure in the expansion of the liver bud (Zhao et al., 2005). An effective morpholino for blocking Gata6 was described recently (Peterkin et al., 2003). Zebrafish embryos blocked for Gata6 expression displayed variable early heart defects, although endoderm-derived organ development was not evaluated. We tested this morpholino sequence and confirmed that it effectively blocks expression of GFP in the *gata6:GFP* transgenic embryos for at least 5 days (data not shown), and also confirmed the variable cardiac defects described by Peterkin et al. (Peterkin et al., 2003). For example, in the background of *cmlc2*:GFP fish, the morpholino results in GFP expression that is often either reduced, misplaced, or present in two non-fusing bifid primitive heart tubes (data not shown).

At the highest morpholino concentration (5 ng), the embryos blocked for Gata6 expression undergo a developmental arrest starting around the 14-somite stage, which precludes analysis of gut development. However, embryos injected with a slightly lower amount (2.5 ng) of the Gata6 morpholino continue to develop beyond the heart tube defect stage. These embryos (because they continue embryonic development) presumably still express low levels of Gata6, although it is likely to be very low, as GFP

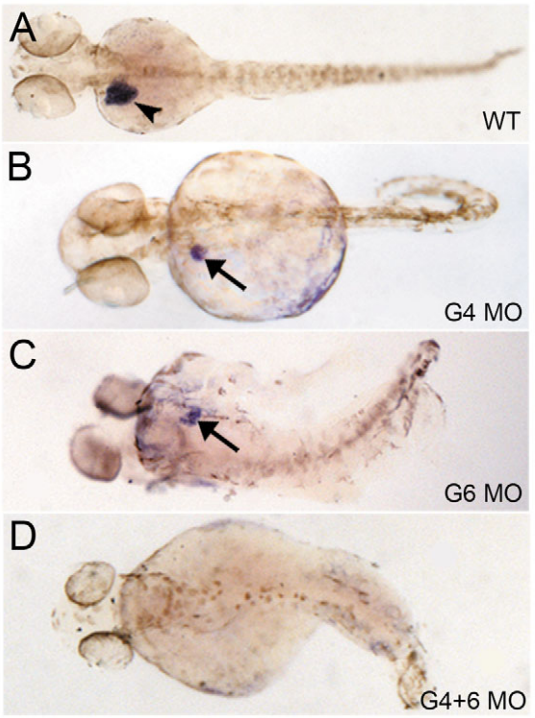

Fig. 7. Gata4 and Gata6 have redundant functions for liver development, but each gene is essential for liver bud growth. Representative control embryos (A, WT), and embryos injected with the morpholinos specific for Gata4 (B, G4 MO), Gata6 (C, G6 MO), or both (D, G4 +6 MO), are shown following in situ hybridization to detect transcript for the liver differentiation marker transferrin. At 3 dpf, the control embryos show a much enhanced liver growth (arrowhead) compared with either the Gata4 or Gata6 morphants (arrows). However, both morphants consistently show small liver buds. By contrast, injection of both morpholinos results in embryos that completely lack transferrin-positive liver tissue. Views are dorsal, anterior to the left.

expression from the *gata6*:GFP transgene is not evident. These embryos are markedly shorter than control embryos and display the same heart tube defects described above. When analyzed at 3 dpf, transferrin expression is variable, but it is always reduced when compared with control embryos, and in many cases it strongly resembles that of the Gata4 morphants (Fig. 7). Typically, transferrin expression marks a small liver bud, similar to that seen in the Gata4 morphants, except that it is sometimes misplaced to the right side, or else shows a more diffuse staining pattern in the region of the intestinal rod. Therefore, zebrafish Gata6, similar to mouse Gata6 and to zebrafish Gata4, is required for liver development. Using these same conditions, we next tested the effect of reducing both Gata4 and Gata6 by co-injection of morpholinos specific to each gene. In this case the results are very clear, in that there is a complete failure in the development of transferrin-positive liver buds (Fig. 7D). To determine whether this block to liver development is caused by an early endoderm defect, embryos injected with morpholinos targeting Gata4, Gata6, or both genes, were isolated at early stages and examined for the expression of early endoderm markers by semi-quantitative RT-PCR. At the shield stage, the Gata4, Gata6 and double morphants all express normal levels of the endoderm markers FoxA2 and Sox17, when compared with controls. Therefore, the complete block to liver development caused by the injection of both morpholinos cannot be explained by an early failure in endoderm specification.

Discussion

The function of Gata4 in heart development is conserved

The zebrafish Gata5/*faust* mutant displays a cardia bifid phenotype, grossly similar to the Gata4 knockout mouse, whereas the Gata5 mouse mutation does not have a cardiac phenotype. Therefore, it has been considered that zebrafish Gata5 is the functional ortholog of mammalian Gata4 (Wallace and Pack, 2003). However, we find that the zebrafish Gata4 morphant shares a remarkably similar phenotype with Gata4 null mouse embryos that are rescued, by tetraploid complementation, for the requirement of Gata4 in extra-embryonic endoderm (Watt et al., 2004). Both sets of embryos show the same defect following normal heart tube formation, with the failure of the atrial (caudal) region to move rostrally, and the failure of chamber expansion. In both cases, the myocardium is specified normally and cardiomyocytes differentiate, as indicated by the beating tube and the expression of both early and later cardiomyocyte markers. Our data therefore indicate that Gata4 gene function is well conserved across vertebrate species, and that its initial essential role in the heart is for a relatively late step of organ morphogenesis. Based on mouse models (Crispino et al., 2001; Tevosian et al., 2000; Watt et al., 2004), this function is probably relevant to the epicardium, although confirmation of this in the fish awaits the development of appropriate markers. Later cell-autonomous functions for Gata4 in the developing myocardium are suggested by conditional null alleles in the mouse (Pu et al., 2004; Zeisberg et al., 2005).

Gata4/Gata5/Gata6 regulates organogenesis from the gut tube

An advantage of the zebrafish model is that embryos can survive a cardiomyopathy, which facilitates the analysis of other phenotypes (Heicklen-Klein et al., 2005). In addition to the heart, we find that Gata4 is required for formation of the intestine, liver, pancreas and swim bladder. Gata4/Gata5/Gata6 gene expression has been previously associated with endoderm-derived organs, and mouse knockout models showed specific functions for Gata4 in the foregut (Kuo et al., 1997; Molkentin et al., 1997) and gastric (Jacobsen et al., 2002) epithelium, and for Gata6 in the visceral endoderm (Morrisey et al., 1998) and lung epithelium (Yang et al., 2002). The zebrafish *gata5* gene is also essential for gut morphogenesis and liver development, based on the analysis of the *faust* mutant (Reiter et al., 1999; Reiter et al., 2001), and depletion of Gata6 in *Xenopus* or zebrafish endoderm appears to disrupt normal intestinal morphogenesis (Peterkin et al., 2003). Therefore, all three of these Gata genes have essential non-redundant functions in regulating organogenesis or differentiation of the gut. However, it is not entirely clear which functions are conserved, and whether certain functions are masked by redundancy, as the experiments have used various model systems.

We find that, in zebrafish, Gata4 is required for both liver

and pancreas development. Gata4 was shown previously to be involved in the establishment of albumin gene expression in murine liver progenitor cells (Bossard and Zaret, 1998; Cirillo et al., 2002; Zaret, 1999), and to be capable of trans-activating the expression of the glucagon gene (Ritz-Laser et al., 2005). The defects seen in the Gata4 morphant embryos do not appear to be related to specification or differentiation. Initial morphogenetic movements occur, including intestinal tube looping to the left, and initial liver and pancreatic budding. However, failure of intestinal bulb, liver and pancreas growth is evident by 3-4 dpf, supported by the decreased levels of differentiation markers. Previous work by Stainier and colleagues demonstrated that vascularization is not required for liver development (Field et al., 2003). This, in addition to the apparently normal trunk vasculature, indicates that the phenotype is not secondary to a vasculature defect. It is interesting that the endocrine pancreas initiates budding, as the specific defect in exocrine pancrease development is consistent with the observation that, in the mouse, Gata4 is expressed in the murine exocrine (but not endocrine) pancreas (Ketola et al., 2004).

Most recently, a requirement for Gata6 in liver development was documented in the mouse, by analyzing embryos rescued for the requirement of Gata6 in extra-embryonic endoderm by tetraploid embryo complementation (Zhao et al., 2005). These mutant embryos fail in liver bud expansion, although hepatic specification is intact and the cells of the ventral hepatic endoderm are capable of differentiation, based on RT-PCR analysis for liver-specific markers. This phenotype is essentially identical to that of zebrafish embryos blocked for expression of Gata4. We cannot definitively rule out that the small transferrin-positive liver buds arise in the Gata4 morphant embryos as a result of leakage of the block, because the embryos are not genetically null. However, this seems unlikely, because when analyzed for transferrin expression by in situ hybrdization, every morphant embryo develops the same sized small bud, over a range of morpholino concentrations that appear to block completely Gata4:GFP expression. Zhao et al. suggest that the mouse Gata6 phenotype might result from a functional redundancy of Gata4 and Gata6 at the earlier stage of hepatic specification. Our data supports this hypothesis, as injection of morpholinos that inhibit the expression of both Gata4 and Gata6 completely eliminates the liver bud and blocks liver-specific gene expression. Furthermore, we show that the function of Gata6 in liver bud growth appears to be conserved with mouse, and that both Gata4 and Gata6 have secondary non-redundant functions for liver bud growth. Such a role for Gata4 in the mouse was considered unlikely (Zhao et al., 2005), as Gata4 expression levels are reduced in hepatoblasts at this stage; however, our results suggest that this should be functionally investigated in the mouse.

Gata factors and early endoderm development

Early endoderm development appears to be normal in both the Gata4 and Gata6 morphants, based on the expression of early markers that are not specific to defined organ systems. A recent study by Patient and colleagues (Afouda et al., 2005) supports a more general role for Gata4/Gata5/Gata6 genes in the specification of early endoderm, in response to TGFβ signaling during germ layer patterning. The specific defects that we describe here were not noted by Afouda et al., when using a morpholino to target *Xenopus* Gata4. It is difficult to compare the experiments, as the *Xenopus* genome encodes two distinct *Gata4* alleles (Jiang and Evans, 1996), which are difficult to target with a single MO (A.H. and T.E., unpublished). Therefore, *Xenopus* Gata4 might have been only partially targeted, or *Xenopus* may be better compensated, with respect to organogenesis, by Gata5 and/or Gata6 than zebrafish. We show that depletion of both Gata4 and Gata6 is not sufficient to eliminate the expression of early endoderm markers. Although our analysis was limited to a few genes, these have been considered to be reliable markers of endoderm specification. Unlike the Gata4 or Gata6 morphants, loss of Gata5 in the *faust* embryo is sufficient to show a significant loss of early endoderm (Reiter et al., 2001). Therefore, if Gata4 and/or Gata6 do regulate endoderm specification, Gata5 is likely to compensate for their loss.

However, our analysis shows that Gata4 and Gata6 have distinct essential functions in both heart and gut development. The cardiac phenotype for each morphant is unique, and the double morphant indicates a lack of functional redundancy and an earlier morphogenetic function for Gata6 than for Gata4. At the highest doses of Gata6 morpholino, presumed to be the most similar to a null allele, there is a developmental arrest that precludes analysis of the gut endoderm, although it does not block the expression of the early endoderm markers. At slightly lower doses, the embryos continue to grow and, although the liver fails to develop, the phenotype is similar but not identical to that of the Gata4 morphant. Much like the heart phenotype, it is variable and the transferrin-expressing cells are often misplaced from the normal site of liver budding. This is in contrast to the Gata4 morphant. However, depletion of both genes demonstrates a functional redundancy with respect to the earliest stages of liver budding and differentiation. In summary, there are likely to be overlapping (redundant) functions for Gata4/Gata5/Gata6 during endoderm specification, and both redundant and non-overlapping (non-redundant) functions with respect to specific organ systems at later stages. There is also an essential and redundant role for Gata factors during mesendoderm specification in *C. elegans* (Maduro et al., 2001; Maduro and Rothman, 2002; Zhu et al., 1997). The fact that Gata4 functions in diverse organ systems, including the heart, liver and pancreas, may be related to the common embryonic origins of heart and gut tubes from mesendoderm. With respect to the early Gata factor network, we find that, in zebrafish, Gata4 is not required for maintaining the expression of its own gene, or that of Gata5 or Gata6, but that it negatively regulates both itself and Gata5.

Novel functions for Gata4 revealed by the fish model

The swim bladder also fails to develop in the Gata4 morphant embryo, and although this could be caused by a number of direct or indirect mechanisms (McCune and Carlson, 2004), in this case it is probably related to a general failure of endoderm-derived organogenesis. In addition to its roles in heart and gut organogenesis, our experiments reveal functions for Gata4 in tail mesoderm and hematopoiesis. Gata4 morphant embryos develop with a kink at a specific position of the caudal tail. Although the significance of this is not known, we note that Gata4 is expressed in the posterior tail bud mesoderm by the 10-somite stage (Griffin et al., 2000; Heicklen-Klein and Evans, 2004). The failure in blood development occurs around

3-4 days of development, coinciding with the anticipated contribution of the definitive 'adult' stage of hematopoiesis, which replaces the transient 'embryonic' or yolk sac population (Davidson and Zon, 2004). This might not have been noticed in the *Gata4* knockout mouse model, as the mutant embryos die before the emergence of the definitive lineage. Because Gata4 is not known to be expressed in hematopoietic cells, it seems likely that the defect in definitive erythropoiesis is secondary to the lack of an appropriate inductive signal. An excellent candidate source is the liver, which fails to develop at this stage in the morphant embryos, and is a possible source of erythropoietin. Indeed, Gata4 regulates the expression of the Epo gene in mouse hepatocytes (Dame et al., 2004). The availability of a zebrafish loss-of-function model for Gata4 will facilitate further investigation of these issues, and should stimulate additional efforts to determine conserved functions in other model systems, including mammals.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/17/4005/DC1

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