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# Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis

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The zinc finger transcription factor GATA4 has been implicated in heart development based on its early expression in precardiogenic splanchnic mesoderm and its ability to activate the expression of a number of cardiac-specific genes. To determine the role of GATA4 in embryogenesis, we generated mice homozygous for a *GATA4* null allele. Homozygous *GATA4* null mice arrested in development between E7.0 and E9.5 because of severe developmental abnormalities. Mutant embryos most notably lacked a primitive heart tube and foregut and developed partially outside the yolk sac. In the mutants, the two bilaterally symmetric promyocardial primordia failed to migrate ventrally but instead remained lateral and generated two independent heart tubes that contained differentiated cardiomyocytes. We show that these deformities resulted from a general loss in lateral to ventral folding throughout the embryo. GATA4 is most highly expressed within the precardiogenic splanchnic mesoderm at the posterior lip of the anterior intestinal portal, corresponding to the region of the embryo that undergoes ventral fusion. We propose that GATA4 is required for the migration or folding morphogenesis of the precardiogenic splanchnic mesodermal cells at the level of the AIP.

[*Kew Words:* GATA4; cardiac morphogenesis; cardia bifida; anterior intestinal portal; foregut; transcription]

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The embryologic events associated with cardiac morphogenesis have been studied for decades, but the underlying molecular mechanisms that control this process are poorly understood (Olson and Srivastava 1996). Early in development, uncommitted splanchnic mesoderm residing on each lateral half of the developing embryo, at about the level of the future head fold, becomes specified to a cardiogenic fate by diffusible factors released from the underlying endoderm (for review, see Nascone and Mercola 1996). Once specified, the bilaterally symmetric cardiogenic precursors organize and migrate along the anterior intestinal portal (AIP), where they converge at the midline of the embryo to form the cardiac crescent. This cardiogenic crescent-shaped structure then folds ventrally resulting in fusion of the cardiac primordia at the midline and formation of the linear heart tube. The linear heart tube then undergoes looping morphogenesis such that the posterior-lying atrial region moves in an anterior and dorsal direction while the future right ventricular region rapidly distends rightward. The looped heart tube then undergoes septation, and the atrial and ventricular chambers become distinct. The basic helix-

loop-helix (bHLH) transcription factors eHAND and dHAND (Srivastava et al. 1995) and the homeobox gene *Nkx2.5* (Lyons et al. 1995) have been implicated in looping morphogenesis of the heart.

Six GATA proteins have been identified in vertebrate species, each of which contains a highly conserved DNA-binding domain consisting of two zinc fingers of the motif Cys-X<sub>2</sub>-Cys-X<sub>17</sub>-Cys-X<sub>2</sub>-Cys (Felsenfeld 1989; Tsai et al. 1989; Yamamoto et al. 1990; Arceci et al. 1993; Kelley et al. 1993; Laverriere et al. 1994; Morrisey et al. 1996, 1997). These two zinc fingers have been shown to direct binding to the DNA sequence element (A/T)GATA(A/G) (Ko and Engel 1993; Merika and Orkin 1993), although just the carboxy-terminal finger is sufficient for site-specific interaction (Martin and Orkin 1990; Yang and Evans 1992; Omichinski et al. 1993; Visvader et al. 1995). Examination of the DNA-binding site specificities of different GATA factors has demonstrated that all six members of the family bind the same consensus sequence, suggesting that they may substitute for one another in lineages in which they are coexpressed (Ko and Engel 1993; Merika and Orkin 1993; Morrisey et al. 1996, 1997). The artificial introduction of GATA4 into *GATA1* null embryonic stem (ES) cells has been shown to partially restore erythropoiesis (Blobel et al. 1995).

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Based on their expression patterns, the GATA proteins have been divided into two subfamilies. GATA1/2/3 are expressed early in hematopoietic progenitors (Evans and Felsenfeld 1989; Tsai et al. 1989; Martin et al. 1990; Wilson et al. 1990; Yamamoto et al. 1990; Lee et al. 1991; Zon et al. 1991; Dorfman et al. 1992), whereas GATA4/5/6 are expressed in the mesodermal precursors that give rise to the heart as well as in the endoderm of the gut epithelium (Arceci et al. 1993; Kelley et al. 1993; Laverriere et al. 1994; Morrisey et al. 1996, 1997). *GATA4* is subsequently expressed in the myocardium throughout embryonic development and into adulthood (Arceci et al. 1993; Laverriere et al. 1994).

*GATA1/2/3* have each been shown to be important regulators of hematopoietic-specific gene expression (for review, see Orkin 1992). *GATA1*, for example, was originally identified as an activator of globin gene expression and was subsequently shown to be involved in the regulation of virtually every erythroid-specific gene examined to date (for review, see Orkin 1992). Targeted disruption of the *GATA1*, *GATA2*, and *GATA3* genes in the mouse has demonstrated that each is an important regulator that establishes the identity of the hematopoietic lineage in which it is expressed (Pevny et al. 1991; Tsai et al. 1994; Pandolfi et al. 1995; Ting et al. 1996). Loss of *GATA1* results in the absence of mature erythroid cells but not megakaryocytes or mast cells (Pevny et al. 1991; Blobel et al. 1995), whereas the loss of *GATA2* results in a deficit in hematopoietic progenitor cells (Tsai et al. 1994). Loss of *GATA3* results in the complete absence of mature T cells as well as minor defects in liver hematopoiesis (Pandolfi et al. 1995; Ting et al. 1996).

*GATA4* has been implicated in cardiac development based both on its early expression pattern and on its ability to regulate a number of cardiac structural genes, such as  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC), cardiac troponin-C (*cTNC*), atrial natriuretic factor (*ANF*), and brain natriuretic peptide (*BNP*) (Grepin et al. 1994; Ip et al. 1994; Molkentin et al. 1994; Thuerauf et al. 1994). Stably transfected P19 embryonic carcinoma cells that express

antisense *GATA4* transcripts have also been shown to be deficient in their ability to differentiate into cardiomyocytes in the presence of DMSO (Grepin et al. 1995), and injection of *GATA4*, *GATA5*, or *GATA6* RNA into *Xenopus* embryos is capable of prematurely activating expression of the  $\alpha$ -cardiac actin and  $\alpha$ -MHC genes (Jiang and Evans 1996).

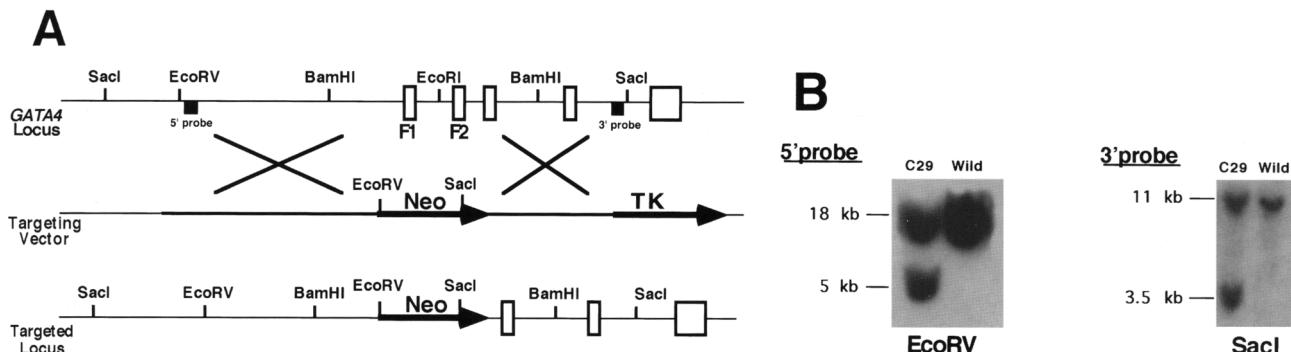
To investigate the function of *GATA4* during embryonic development, we generated mice deficient for this gene product by targeted disruption in ES cells. Although heterozygous mice were normal, homozygous null mice arrested in development between embryonic day 7.0 (E7.0) and E9.5 because of severe morphogenic defects. *GATA4* null embryos lacked a centralized heart tube and foregut, and developed partially outside the yolk sac. These defects resulted from a fundamental failure in lateral to ventral folding early in embryogenesis, suggesting that *GATA4* is essential for the formation of the primitive heart tube, the AIP, and the foregut.

## Results

### *Targeted disruption of GATA4 in ES cells and generation of null mice*

To mutate the *GATA4* locus in ES cells, a replacement targeting vector was designed that would result in deletion of the exons encoding the two DNA-binding zinc finger domains and insertion of the *neomycin* resistance gene (*neo*) by homologous recombination. The *thymidine kinase* (*TK*) gene was included in the targeting vector as a negative selection marker for nonhomologous recombination events (Fig. 1A). The targeting vector was electroporated into ES cells that were selected for the presence of the *neo* gene and the loss of the *TK* gene. Southern blot analysis identified four independent clones that had been properly targeted at the *GATA4* locus.

ES cells heterozygous for the targeted *GATA4* allele were injected into E3.5 blastocysts derived from C57BL/6 mice, and blastocysts were subsequently im-



**Figure 1.** Partial map of the *GATA4* genomic locus and genomic Southern strategy. (A) The *GATA4* genomic locus surrounding the targeted area is shown. Homologous recombination resulted in the replacement of the two zinc finger-encoding exons with the *neo* resistance gene. The positions of the 5' and 3' Southern probes are shown, external to the targeting arms and internal to the restriction sites used to characterize the targeted locus. (B) A Southern blot was performed with genomic DNA that was generated from an ES cell clone (C29) used to generate chimeric mice. The results show that homologous recombination had occurred as predicted based on the additional *EcoRV* and *SacI* sites that were introduced with the *neo* cassette.

planted into pseudopregnant Swiss foster mothers to obtain chimeric mice. Breeding of chimeras into a C57BL/6 background resulted in transmission of the mutation through the germ line and generation of *GATA4* heterozygous mice of the composite C57BL/6 × Sv129 genotype. Heterozygotes for the *GATA4* mutation were intercrossed to generate null mice and to determine the role of *GATA4* in development. As a control, *GATA4* null embryos were analyzed for *GATA4* mRNA expression by RT-PCR and demonstrated that the replacement event resulted in a loss of mature *GATA4* message (data not shown).

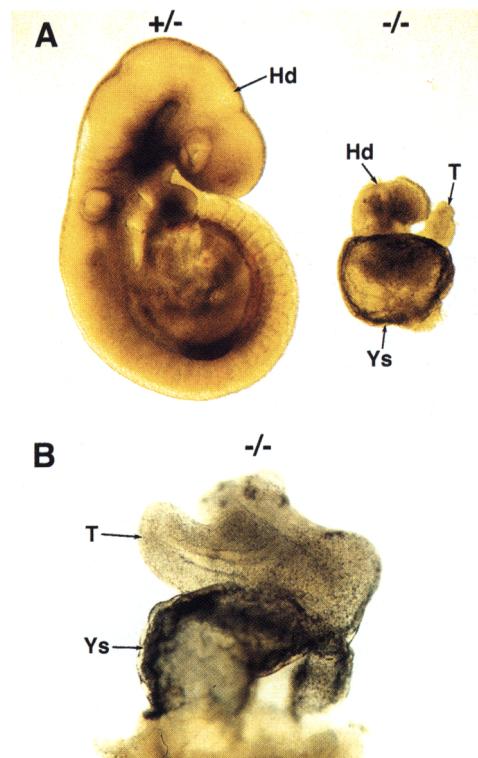
#### Morphological phenotype of *GATA4* $-/-$ embryos and allelic distribution

Although *GATA4* heterozygous mice showed no discernable phenotype, neonates born to *GATA4* heterozygotes revealed no viable null animals, suggesting that the homozygous mutation resulted in embryonic lethality (data not shown). Analysis of a large group of embryos from heterozygote intercrosses taken at E8.5 and E9.5 demonstrated that the allelic distribution was roughly Mendelian, at the predicted 1:2:1 ratio (Table 1). However, no homozygous null embryos were found at E10.5, suggesting that they had been resorbed by that stage.

At E9.5, *GATA4* null embryos exhibited a number of severe defects including a general developmental delay and reduction in size, loss of a centralized heart, and protrusion of the head and tail from the posterior aspect of the yolk sac (Fig. 2A). A day earlier in development, the tail region of the embryo could already be seen developing outside of the yolk sac (Fig. 2B). The majority of the *GATA4* null embryos (65%) at E8.5 or E9.5 were of the phenotype shown in Figure 2; however, a fraction (35%) had arrested at a developmental stage that was morphologically equivalent to the egg cylinder normally seen at ~E7.0 (Table 2). Of the *GATA4* null embryos that developed beyond the egg cylinder stage, none contained a central heart tube or were positioned correctly within the yolk sac (Table 2). This analysis demonstrated that the *GATA4* null phenotype had two varying degrees of severity, either developmental arrest at the egg cylinder stage, presumably before gastrulation, or progression past this stage to ~E9.5.

#### Histological analysis of *GATA4* $-/-$ embryos

To further define the morphologic defects in *GATA4* null embryos, histologic sections of wild-type and mu-



**Figure 2.** Gross morphologic appearance of *GATA4* null embryos. (A) An E9.5 litter from a *GATA4* heterozygote intercross was collected. The *GATA4* mutant embryo on the right shows severe developmental abnormalities, including protrusion outside the yolk sac and the lack of a primitive heart tube. The embryo on the left is heterozygous for the *GATA4* mutation and is normal in appearance. The yolk sac has been removed from this embryo to show normal developmental landmarks at E9.5 for comparison to the null embryo. (B) At E8.5, *GATA4* null embryos were already protruding from their yolk sacs and showed severe developmental defects. The tail region can be seen as the embryo is in the process of turning while outside the yolk sac. (Hd) Head; (T) tail; (Ys) yolk sac.

tant embryos within their decidua were compared (Fig. 3). Parasagittal sections of embryos taken at E7.5 showed that the mutants were already reduced in size and developmentally delayed by half a day compared to wild-type or heterozygous littermates. Mutant embryos at E7.5 also showed a delay in closure of the proamniotic canal and incomplete cavitation of the exocoelomic cavity (Fig. 3B). However, null embryos contained both parietal and visceral endoderm as well as an allantois. A day later, the structural defects were more obvious. Parasagittal sections of *GATA4* null embryos within their decidua showed that regions of the embryo were already developing outside the posterior aspect of the yolk sac (Fig. 3D, arrows). Normally by this time in development, each lateral half of the layer containing the splanchnic mesoderm and underlying endoderm has folded ventrally to place the embryo within the yolk sac at the level of the heart and above. A parasagittal section of an E8.5 unaffected littermate shows that the head and heart region are already free within the yolk sac at this stage (Fig. 3C).

**Table 1.** Genotypes resulting from *GATA4* heterozygote intercrosses

Embryonic day	Allelic distribution		
	+/ <sup>+</sup>	+/-	-/-
8.5	14 (29%)	25 (51%)	10 (20%)
9.5	33 (26%)	68 (54%)	25 (20%)

Numbers of embryos of each genotype and their percentage of the total are shown for each day.

**Table 2.** Prevalence of developmental defects in GATA4 null embryos

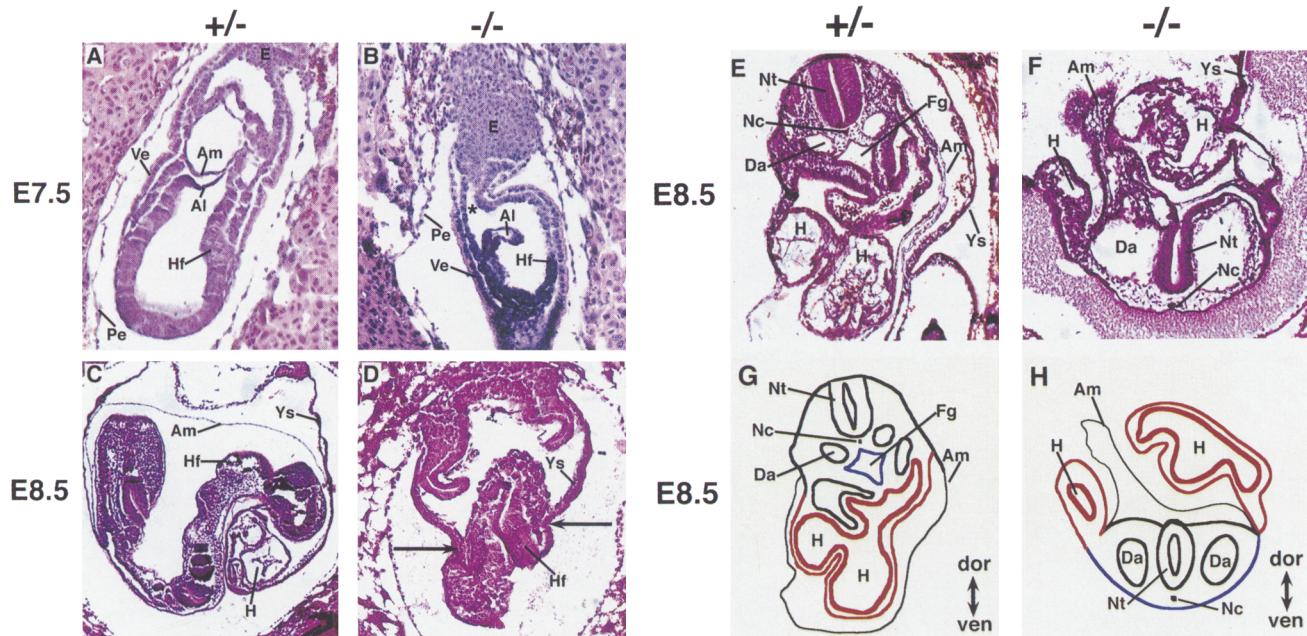
Embryonic day	Arrested before gastrulation	Outside of yolk sac <sup>a</sup>	Lack of a central heart <sup>a</sup>	Structurally unrecognizable <sup>a,b</sup>
8.5	4/10	5/6	5/6	1/6
9.5	8/25	15/17	15/17	2/17

<sup>a</sup>Represents the remaining embryos that progressed through gastrulation.

<sup>b</sup>These embryos were severely affected so that landmarks of development were not obvious but gastrulation had occurred based on marker analysis.

Normally by E8.5, the lateral wings of splanchnic mesoderm with the underlying endoderm have migrated ventrally at the level of the heart to generate the foregut and the centrally located primitive heart tube (Fig. 3E,G). However, transverse sections demonstrated that the *GATA4* null embryos lacked any structures ventral to the notochord (Fig. 3F,H). In *GATA4* null embryos, the lateral wings of endoderm and splanchnic mesoderm remained at the sides and were displaced dorsally as the midline of the embryo continued to grow. This resulted in the lack of

foregut formation and the failure of the paired endocardial tubes to fuse at the ventral midline (Fig. 3F,H). Because the mutant embryos never folded ventrally, the yolk sac was not pulled over and in front of the embryo, but instead was pinched off dorsally, at the anterior and posterior ends of the embryo so that the head and tail came to lie outside the yolk sac (see Fig. 9 and Discussion, below). Also of note, the overlying ectoderm and somatic mesoderm never moved ventrally so that the amnion remained dorsal and did not surround the embryo (Fig. 3F,H).



**Figure 3.** Analysis of *GATA4* null embryos by hematoxylin and eosin staining of decidual sections. Parasagittal section through a *GATA4* heterozygote (A) and a *GATA4* mutant embryo (B) at E7.5. Notice that the mutant embryo is delayed in development and is smaller than the heterozygous embryo. The proamniotic canal, indicated with an asterisk, has not yet closed and the ectoplacental cone region has not cavitated to the same extent in the mutant as in the heterozygous embryo. Parasagittal section through a heterozygous wild-type embryo (C) and a *GATA4* mutant embryo (D) at E8.5 shows that regions of the mutant embryo are developing outside the yolk sac (large arrows) while the normal embryo is becoming surrounded by the yolk sac. A transverse section through an E8.5 heterozygous wild-type embryo (E) and a *GATA4* mutant embryo (F) at the level of the developing heart shows that the normal embryo has already folded resulting in the placement of the foregut and heart ventral to the neural tube and notochord. The *GATA4* null embryo is unable to fold ventrally so that the foregut never forms and the heart-forming region remains lateral and dorsal to the neural tube and notochord. These relationships are shown more clearly in a diagram representing the transverse sections of the wild-type (G) and the mutant (H) embryos. The foregut endoderm is shown in blue, and the heart regions are shown in red. Notice how the most ventral developmental landmark in the *GATA4* mutant embryo is the notochord while the wild-type embryo shows the foregut and heart region ventral to the notochord. (Am) Amnion; (E) ectoplacental cone; (Al) allantois; (Hf) head fold; (H) heart; (Ve) visceral endoderm; (Pe) parietal endoderm; (Ys) yolk sac; (Nt) neural tube; (Nc) notochord; (Fg) foregut; (Da) dorsal aorta.

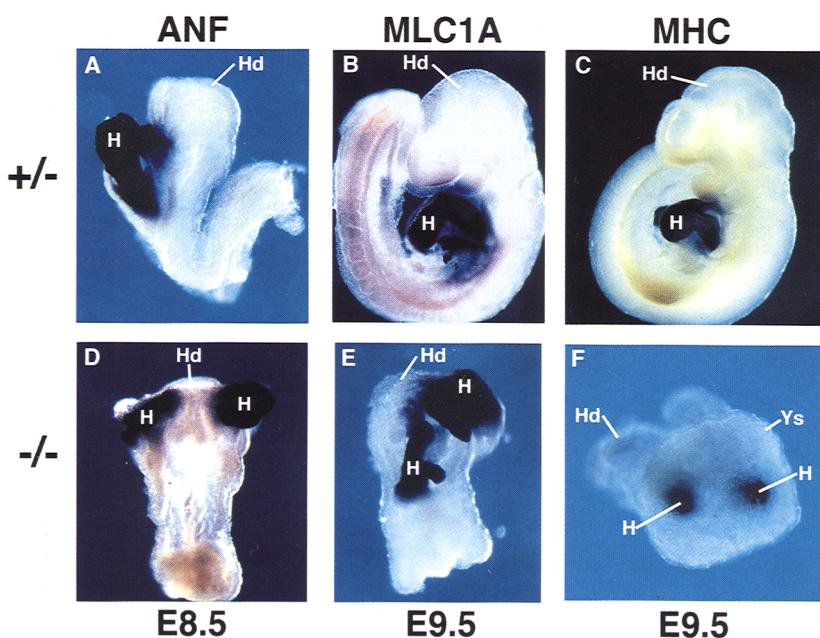
### Analysis of GATA4 null embryos by whole-mount *in situ* hybridization

It was of interest to analyze the expression of a number of cardiac markers, as *GATA4* null embryos lacked a ventrally located heart tube but appeared to contain two independent heart tubes on either lateral edge. Whole-mount hybridization for the cardiac-expressed genes *ANF*, *myosin light chain 1A [MLC1A]*, and  $\alpha$ -*MHC* demonstrated that *GATA4* null embryos contained differentiated cardiomyocytes but that the heart-forming region was split on either side of the embryo, resulting in cardia bifida (Fig. 4D–F). The cardiac-staining regions showed that the cardiogenic primordia remained bilateral in null embryos, whereas in wild-type embryos, they moved ventrally and fused at the midline to generate a single heart tube (Fig. 4A–C). It is also of interest that the bilateral heart tubes in the mutant embryos sometimes looped rightward (Fig. 4E) as normally occurs in the single heart tube of wild-type embryos (Fig. 4A). The cardiac marker genes *MLC2A*, *MLC2V*, *Nkx2.5*, *eHAND*, and *dHAND* appeared to be expressed by whole-mount *in situ* hybridization at roughly comparable levels between the mutant and wild-type hearts (data not shown). Despite expression of a number of differentiation markers, we never observed contractions in the bilateral heart tubes of the mutant embryos, suggesting that the individual cardiomyocytes had not functionally differentiated.

To investigate the relationship between the bilateral heart-forming regions and the dorsal–ventral axis of the embryo, cardiac-stained embryos were sectioned transversely. Sections of embryos stained for *ANF* clearly revealed the dorsolateral positions of the two heart-forming regions in the mutants (Fig. 5B). During normal development, committed splanchnic mesoderm with its associated underlying pharyngeal endoderm at the level

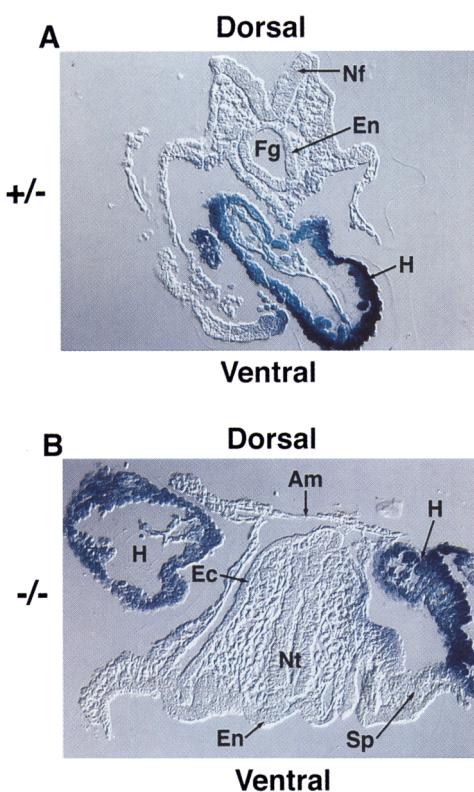
of the heart moves ventrally and folds along the AIP. This lateral to ventral folding encloses the endoderm within the foregut and fuses the two promyocardial regions in front of the neural tube and foregut (Fig. 5A). However, in *GATA4* null embryos, the endoderm and the overlying splanchnic mesoderm were unable to move ventrally, resulting in the formation of two independent heart-forming regions on either side of the embryo and the lack of an enclosed foregut (Fig. 5B). Inspection of the cardiomyocytes from *GATA4* null embryos suggested that they had undergone the characteristic mesenchymal to epithelial transition, despite a lack of ventral midline positioning. Endocardial cells could also be visualized in the split cardiac tubes of the *GATA4* null embryo, suggesting that there was not a defect in the specification of this lineage (Fig. 5B; data not shown). Also of note, the underlying pharyngeal endoderm layer appeared to aggregate and form pouches, suggesting that it continued to grow in place without folding out ventrally (Fig. 5B).

The morphological defects in *GATA4* null embryos were specific to the heart-forming region, as many other major developmental events occurred normally. Null embryos at E8.5 consistently contained between four and eight somites as visualized by hybridization for *Mox1* RNA (Candia et al. 1992) (Fig. 6D). The presence of this number of somites suggested that null embryos could develop to a stage equivalent to E8.5, despite the lack of a centralized heart tube or the proper association of the embryo with the yolk sac. RNA for the forkhead transcription factor hepatocyte nuclear factor 3 $\beta$  (*HNF3 $\beta$* ) was also present in null embryos, suggesting that they developed a relatively normal notochord and neural tube throughout the axis of the embryo (Ang et al. 1993; Sasaki and Hogan 1993) (Fig. 6E). Interestingly, analysis of this marker demonstrated the loss of the AIP and all other ventral structures in *GATA4* null embryos.



**Figure 4.** Whole-mount *in situ* hybridization analysis of cardiac markers. (A) *ANF* was expressed in an E8.5 heterozygous embryo as expected in the primitive heart tube in its proper ventral position. (D) A dorsal view of an E8.5 *GATA4* mutant embryo also demonstrates abundant expression of *ANF*; however, the heart-forming region is split and is positioned laterally and dorsally. Expression of *MLC1A* was also analyzed in an E9.5 heterozygous embryo (B), shown in side view, and a mutant littermate (E) shown in dorsal view. (C) Analysis of  $\alpha$ -*MHC* (*MHC*) mRNA expression demonstrated high levels in both the heterozygous embryo and in the *GATA4* null embryo (F). Part of the yolk sac was left attached to this mutant embryo so its association within the edges of the yolk sac could be visualized. (Hd) Head; (H) heart; (Ys) yolk sac.

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**Figure 5.** Transverse sections through ANF-stained embryos at the level of the heart. Sections of an E8.5 heterozygous embryo (A) show that the primitive heart tube has formed correctly, ventral to the embryo, whereas the heart-forming region in the *GATA4* null embryo (B) has not moved ventrally. The heart-forming region in the mutant remains lateral and differentiates into two independent heart tubes. Because the mutant embryo has not folded ventrally it also lacks a foregut. Notice that the amnion remains dorsal and has not moved ventrally to position itself around the mutant embryo. (Am) Amnion; (Nf) neural fold; (H) heart; (Nt) neural tube; (En) endoderm; (Sp) splanchnic mesoderm; (Ec) ectoderm; and (Fg) foregut.

A ventral view of a wild-type embryo at E8.5 shows the AIP clearly, whereas in the mutant embryo, HNF3 $\beta$  staining is visible along the entire length of the embryo because it is not obscured by the AIP (Fig. 6B,E). When these HNF3 $\beta$ -stained mutant embryos were sectioned transversely, expression could also be demonstrated in the underlying endoderm, suggesting that endoderm was not compromised by the null mutation (data not shown). The homeodomain transcription factor OTX2 is normally expressed in both the neuroectoderm and head mesenchyme within the forebrain and diencephalon (Fig. 6C) (Simeone et al. 1993). In *GATA4* null embryos, OTX2 was expressed in the correct pattern within the developing head (Fig. 6F); however, the head folds of the *GATA4* null embryos were slightly delayed in their development and were not fused. It is likely that this delay in head fold fusion is a secondary effect attributable to the development outside the yolk sac. Markers for lateral mesoderm such as the homeobox gene *Lim1* and the

bHLH genes *eHAND* and *dHAND* were also examined and demonstrated that lateral mesoderm was specified and positioned correctly in *GATA4* null embryos (Barnes et al. 1994; Srivastava et al. 1995; data not shown).

#### GATA4 expression at the level of the anterior intestinal portal

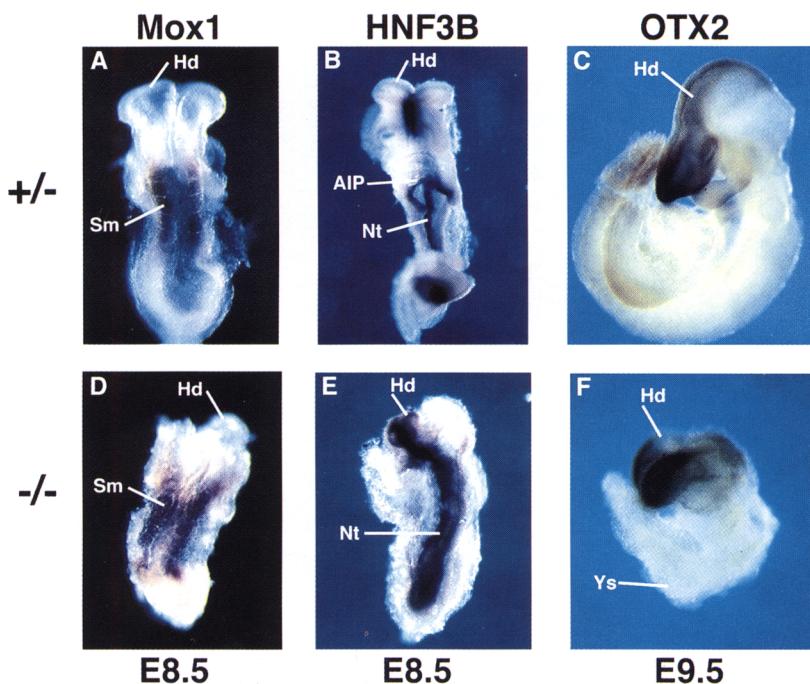
Our analysis of *GATA4* null embryos demonstrated an essential role for *GATA4* in ventral morphogenesis. However, based on previous studies it was unclear how this defect correlated with the endogenous pattern of *GATA4* expression. Prior to E7.5, *GATA4* was reported to be expressed in the visceral endoderm, the allantois, and the anterior aspect of the embryo that is thought to be the precardiac splanchnic mesoderm (Heikinheimo et al. 1994; Laverriere et al. 1994; Morrisey et al. 1996). At E8.5, *GATA4* continues to be expressed in the visceral endoderm, which has fused with extraembryonic mesoderm to form the yolk sac, in the allantois, and in the heart and underlying septum transversum (Heikinheimo et al. 1994; Morrisey et al. 1996).

In *GATA4* null embryos, the first signs of morphogenic abnormality occur at ~E8.0 when ventral folding is occurring and the AIP is prominent. We examined more carefully the normal expression pattern of *GATA4* during this critical time point of ventral closure and found that *GATA4* mRNA was highly localized to the posterior aspect of the AIP, whereas it was expressed more weakly in the fusing heart tubes just anterior to the AIP (Fig. 7). Sectioning of this embryo demonstrated that expression was restricted to the splanchnic mesodermal cell layer (data not shown). Thus, *GATA4* was expressed most intensely within the region of the AIP that was actively migrating and folding in a ventral–medial direction. Together, these results suggest that *GATA4* partly directs the process of lateral to ventral folding at the level of the AIP.

#### Pregastrulation arrest in a small percentage of *GATA4* null embryos

Approximately 40% of the E8.5 embryos and 30% of the E9.5 embryos appeared to be severely delayed or arrested at the egg cylinder stage (Table 2). We examined this affected population further by whole-mount *in situ* analysis of E7.0–E7.5 litters for markers of gastrulation and consistently observed a population of null embryos that failed to express *Brachyury*, *goosecoid*, or *Flkl* (data not shown). Because *GATA4* is expressed in visceral endoderm and a defect in visceral endoderm was also observed in *HNF4* null embryos which arrested at a similar stage (Chen et al. 1994), we also investigated a possible defect in this cell population using the visceral endoderm marker  $\alpha$ -fetoprotein. Mutant embryos in this class, taken at any time point between E7.0 and E9.5, lacked expression of  $\alpha$ -fetoprotein as detected by whole-mount *in situ* hybridization (data not shown).

*GATA4* null embryos that had morphologically progressed past the egg cylinder stage showed expression of



**Figure 6.** Whole-mount *in situ* hybridization analysis for normal developmental landmarks. Analysis of *Mox1* mRNA expression in an E8.5 embryo (*A*) revealed approximately the same number of somites in wild-type and mutant embryos (*D*), shown in dorsal view. Although the mutant embryos generate somites, they are not organized as tightly as the somites from normal embryos. Ventral views of E8.5 wild-type (*B*) and mutant (*E*) embryos hybridized for *HNF3 $\beta$*  mRNA showed expression in the notochord and neural tube floor plate. Notice how the AIP is visible in the normal embryo while a ventrally located AIP structure is not present in the *GATA4* null. A lateral view of an E9.5 heterozygous wild-type embryo (*C*) hybridized for *OTX2* mRNA showed expression in the forebrain and diencephalon. Mutant embryos (*F*) also generate a discernible head structure that showed expression of *OTX2*, although the head region is slightly delayed in development and has not fused completely. A portion of yolk sac was left on this mutant embryo (*F*) while the yolk sacs were completely removed from the rest of the embryos in this panel. (Ys) yolk sac; (Hd) head; (Nt) neural tube; (Sm) Somites; (AIP) anterior intestinal portal.

$\alpha$ -fetoprotein by both whole-mount hybridization and RT-PCR as well as expression of the visceral endoderm markers HNF4 and apolipoprotein E (ApoE) by RT-PCR (data not shown). These data suggest that a percentage of *GATA4* null embryos are rescued by a compensatory mechanism, possibly by functionally similar molecules. Candidate factors that might compensate for the loss of *GATA4* are *GATA5* and *GATA6*. Expression levels of *GATA5* and *GATA6* were tested by semiquantitative RT-PCR and demonstrated that *GATA5* mRNA re-

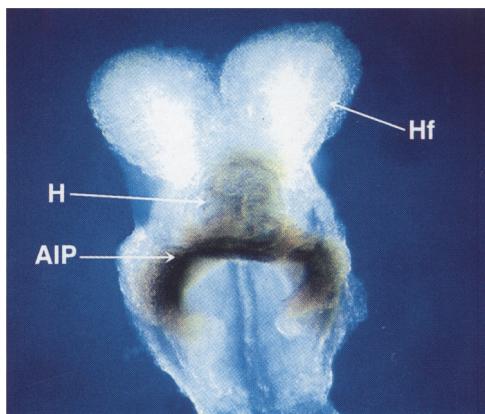
mained constant between wild-type and mutant embryos, as did the ribosomal control marker L7, whereas the *GATA6* message was up-regulated two- to threefold in mutant embryos (Fig. 8). These data suggest that *GATA6* can compensate for the loss of *GATA4* and partially rescue aspects of development.

## Discussion

The phenotype of the *GATA4* null embryo reveals a critical role for *GATA4* in early morphogenetic movements of the mouse embryo. *GATA4* is essential for the formation of the linear cardiac tube and the AIP, as well as for yolk sac development. Despite previous studies that have suggested an essential role for *GATA4* in controlling cardiac gene expression, myocyte differentiation, and visceral endoderm specification, our results show that *GATA4* is not required for these processes.

### Role of *GATA4* in ventral morphogenesis of the embryo

In *GATA4* mutant embryos, the loss of a ventral heart tube and development outside the yolk sac are the result of a failure in lateral to ventral folding. At E7.5, the embryo is in late gastrulation and the region just caudal to the buccopharyngeal membrane (prochordal plate) thickens to form the AIP (DeRuiter et al. 1992), which consists of splanchnic mesoderm and its underlying endoderm (Fig. 9). At the midline of the AIP, a thickening is observed in which the underlying endoderm splits either half of the precardiac splanchnic mesoderm. As development progresses, the bilateral precardiac splanchnic mesoderm with the underlying endoderm fold inward at



**Figure 7.** Whole-mount *in situ* hybridization for *GATA4* expression in a wild-type embryo. Expression of *GATA4* mRNA is shown at the critical developmental time point (E8.0) when the AIP is actively folding and the centrally located heart tube is forming. *GATA4* is expressed most intensely at the posterior lip of the AIP, the region undergoing active ventral morphogenesis. (H) Heart; (Hf) head fold; (AIP) anterior intestinal portal.



**Figure 8.** RT-PCR analysis of GATA5 and GATA6 mRNA levels in *GATA4* null embryos. Wild-type and *GATA4* null embryos were collected at E8.5, and total RNA was extracted and reverse transcribed. The rRNA product L7 was used as a control for quantitation between these two groups (lanes 1,2). The results show identical levels of GATA5 mRNA (lanes 3,4) between wild-type and *GATA4* null embryos while GATA6 mRNA levels (lanes 5,6) were up-regulated in the mutant embryos.

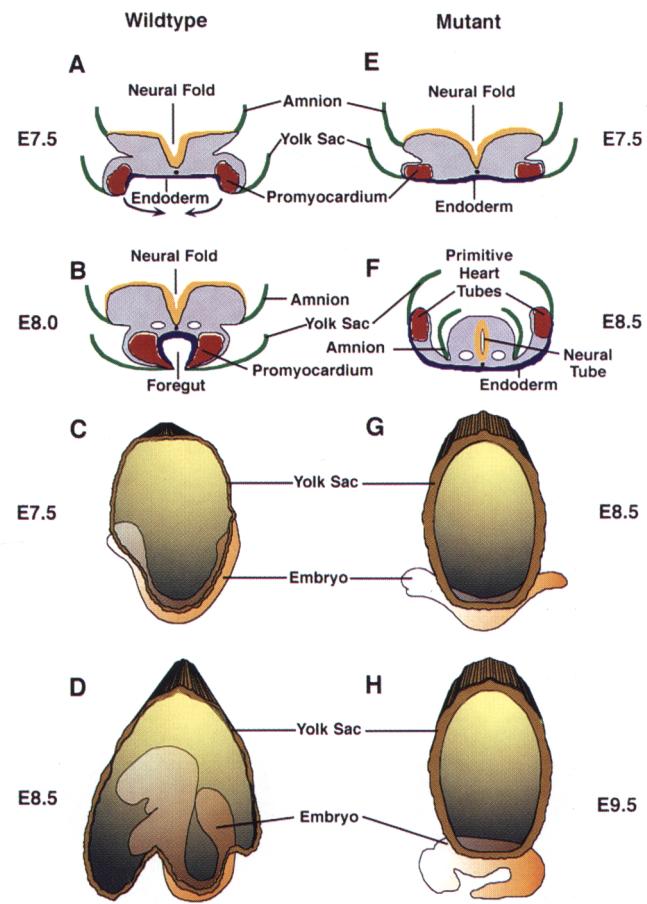
this thickening and converge at the midline of the embryo (Stalsberg and DeHaan 1968; DeRuiter et al. 1992). As this process continues, the two lateral precardiac regions fuse along their lengths and entrap the underlying endoderm, generating the foregut (Fig. 9B). This lateral to ventral fusion progresses in an anterior to posterior direction to "zipper" the embryo together and results in fusion of the lateral edges of yolk sac endoderm in front of the embryo so that the area of the embryo anterior to the AIP becomes free within the yolk sac (Fig. 9C,D).

In *GATA4* null embryos, the AIP does not form and as a result, the bilateral cardiac primordia remain lateral and attached at their edges to the yolk sac (Fig. 9E). Despite this failure in ventral morphogenesis, the bilateral precardiac splanchnic mesoderm continues to develop and differentiates into two independent heart tubes. As the center of the mutant embryo continues to grow, it displaces the lateral halves of yolk sac endoderm dorsal to the embryo proper (Fig. 9F). As this region of endoderm comes together behind the embryo, it pinches off dorsally, resulting in the progressive anterior to posterior zippering of the embryo outside the yolk sac (Fig. 9G,H).

Descriptions of other vertebrate mutants support the conclusion that a fundamental loss in ventral folding is linked to cardia bifida and improper yolk sac development. A naturally occurring mouse mutation called *kinky* has been described that results in duplication or twinning of the embryonic axis (Glueksohn-Schoenheimer 1949). As a result of the lateral axis duplication, *kinky* embryos develop outside the yolk sac and lack a central heart. This mutation likely blocks the ability of these embryos to fold ventrally along their lengths, given that the heart-forming regions and AIP are obscured between the two different axes. Recently, two zebrafish mutations named *miles apart* and *bonnie and clyde* were reported to result in cardia bifida resembling that of *GATA4* mutant mice (Chen et al. 1996; Stainier et al. 1996). It is interesting to speculate that the genes responsible for these mutant phenotypes may act in the same morphogenetic pathway as *GATA4*.

Very little is known about the process that establishes the AIP and the ensuing lateral to ventral folding that generates the foregut and places the heart at the midline. DeHaan (1963) concluded that the active process resulting in lateral to ventral folding at the level of the AIP

likely occurs as a result of the mobility of clusters of precardiac cells. However, more recent evidence suggests that the underlying endoderm may also participate in this process (Suzuki et al. 1995). Thus, it is likely that the process of lateral to ventral folding occurs as a result of an interplay between the overlying splanchnic mesoderm and the underlying endoderm, possibly because of differential growth between these two layers and the embryo proper. *GATA4* is expressed in precardiac splanchnic mesoderm at a time when the active processes of heart tube formation and foregut closure are occurring.



**Figure 9.** Schematic representation of the processes of ventral morphogenesis and heart tube formation in a wild-type and *GATA4* mutant embryo. [A] At E7.5 the precardiogenic splanchnic mesoderm precursors and the associated underlying endoderm begin to migrate ventrally. By E8.0 these cardiogenic cells migrate ventrally to the midline [B] and fuse to generate the primitive heart tube and foregut lined with endoderm. The yolk sac, which is contiguous with the underlying endoderm, is pulled from its lateral position [C] early in development, to a ventral position [D] so that the edges of the yolk sac meet and pinch off to release the embryo anterior to this fusion. In mutant embryos, at E7.5, the precardiogenic splanchnic mesoderm precursors fail to involute or migrate [E] so that by E8.5, they become displaced laterally and dorsally [F]. This dorsal displacement results in the fusion of the yolk sac edges behind the *GATA4* mutant embryo resulting in the progressive release [G,H] of the head and tail outside the yolk sac.

GATA4 expression is strongest at the posterior edge of the AIP, which is the region that actively converges ventrally. Given the phenotype of the *GATA4* null embryo, it is likely that *GATA4*, in part, directs this active process of lateral to ventral folding.

How might GATA4 control the process of lateral to ventral folding? One possibility is that GATA4 regulates the migration of cardiogenic splanchnic mesodermal precursors along the endodermal cell layer. Alternatively, GATA4 might be required for differential proliferation of splanchnic mesodermal precursors versus the underlying endoderm to promote folding.

For a number of reasons, we believe it is unlikely that disruption of the *GATA4* gene affects the underlying endoderm. First, sections of *GATA4* null embryos demonstrated that the underlying pharyngeal endoderm continues to grow so that this layer appeared convoluted and expresses HNF3 $\beta$  (Figs. 5B and 6E), suggesting that there is not a defect in the proliferative ability of this cell layer or its ability to express markers of differentiation. Second, many studies have demonstrated that anterior endoderm is required for the commitment of unspecified splanchnic mesoderm to the cardiac lineage (for review, see Nascone and Mercola 1996). Given that the *GATA4* null embryo contains specified and differentiated cardiomyocytes, it is likely that the underlying endoderm is not defective in its ability to communicate with the splanchnic mesoderm. Third, the endoderm in the *GATA4* null embryo is competent in its ability to fuse and pinch off at the midline, although it occurs dorsally. Thus, it is likely that the molecular defect in the *GATA4* null embryo is not intrinsic to the endodermal cell layer, but is likely intrinsic to the cardiogenic splanchnic mesodermal cells. Together, these data suggest a model of development in which lateral to ventral folding and AIP formation are directed, in part, by the precardiogenic splanchnic mesodermal precursors.

#### *The role of GATA4 in controlling cardiac lineage commitment and differentiation*

GATA4 was first implicated in controlling the cardiac gene program based on its ability to direct the expression of four cardiac structural genes,  $\alpha$ -MHC, *cTNC*, ANF, and BNP (Grepin et al. 1994; Ip et al. 1994; Molkentin et al. 1994; Thuerauf et al. 1994). Furthermore, a recent study reported that P19 embryonic carcinoma cells stably expressing antisense *GATA4* transcript were unable to differentiate into cardiomyocytes in response to DMSO (Grepin et al. 1995). Our analysis demonstrates that *GATA4* null mice have differentiated cardiac tissue and that a number of structural genes previously thought to be GATA4 dependent are expressed normally in mutant embryos. A likely explanation for these differences is that GATA6, which is expressed early in the developing heart, can compensate for some GATA4 functions in cardiomyocytes during embryogenesis. Consistent with this notion, GATA4 and GATA6 are highly conserved within their DNA-binding domains and recognize a GATA consensus element equally (Morrisey et al. 1996,

1997) and GATA6 mRNA is up-regulated in E8.5 mutant embryos (Fig. 8). Furthermore, a recent study reported that GATA6 overexpression in *Xenopus* embryos was capable of delaying cardiomyocyte differentiation resulting in enlarged heart-forming regions, suggesting that GATA6 plays an important role in controlling cardiomyocyte differentiation (Gove et al. 1997).

A greater understanding of the role of GATA4 in cardiac development may require analysis in the background of *GATA5*- or *GATA6*-targeted disruptions. Loss-of-function analysis in the mouse has, in many cases, demonstrated that members of a highly related gene family can compensate for one another's function if they are coexpressed within the same lineage during development. The disruption of the *GATA4* gene in the mouse demonstrates that it has an autonomous function in directing lateral to ventral folding and cardiac tube morphogenesis, yet its role in controlling the identity or differentiation of cardiac myocytes may only be revealed by combinatorial disruption with *GATA5* or *GATA6*. Consistent with this notion is the fact that targeted disruption of the *GATA5* gene in the mouse does not result in embryonic lethality, suggesting that its functions may overlap with that of *GATA4* and *GATA6* in cardiomyocyte development (J. Molkentin and E. Olson, unpubl.).

#### *Loss of gastrulation in a percentage of GATA4 mutant embryos*

Approximately one-third of the *GATA4* null embryos arrested at the egg cylinder stage and failed to gastrulate. Compromised function of either parietal or visceral endoderm can result in arrested development at this stage (for review, see Copp 1995; see also Chen et al. 1994; Healy et al. 1995; Duncan et al. 1997). Visceral and parietal endoderm arise from primitive endoderm by ~E4.5 in the mouse (Gardner 1983; Duncan et al. 1994). Visceral endoderm forms a layer of columnar-shaped cells that surrounds the entire embryo where it secretes important nutrients and filters substances between the maternal and embryonic compartments. *GATA4* is expressed in visceral endoderm, suggesting that it may be required for the proper development of this cell layer. Recently, *GATA4* null ES cells were shown to be deficient in their ability to differentiate in culture into visceral endoderm, suggesting that GATA4 is necessary for the differentiation of this cell lineage (Soudais et al. 1995). Although all *GATA4* mutant embryos contained a layer of morphologically distinct visceral endoderm cells, it is likely that the function of this cell layer is compromised to a variable extent.

The finding that approximately two-thirds of the *GATA4* null embryos progressed through gastrulation suggests that there is a critical point in development in which visceral endoderm function can be rescued. Obvious candidates for the factors that might compensate for the loss of GATA4 are GATA5 and GATA6. Analysis of GATA5 and GATA6 mRNA levels demonstrated that GATA6 is up-regulated two- to threefold in *GATA4* null

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embryos, suggesting that compensation may result in partial developmental rescue (Fig. 8).

Together, these analyses demonstrate that GATA4 plays a critical role in at least two developmental programs within the embryo. GATA4 is likely required for the proper function of the visceral endoderm and is indispensable for establishing a morphogenic cue that results in lateral to medial folding of the embryo at the level of the AIP and the subsequent generation of the centrally located primitive heart tube and foregut. Our analysis of the *GATA4* mutant phenotype suggests that the process of heart formation is linked to the ability of the embryo to fold ventrolaterally, and it suggests that precardiac mesodermal cells actively direct AIP formation.

## Materials and methods

### *GATA4 targeting strategy*

To construct the *GATA4* targeting vector, a genomic clone was isolated from an SV129 λ genomic library and mapped against a panel of restriction enzymes. Because the two exons encoding the zinc finger domain are located ~900 bp apart and are indispensable for GATA4 function, they were chosen for replacement. On the 3' end of the *GATA4* genomic locus, a PCR primer was designed ~500 bp inside of the 3' *SacI* site and another was designed just inside the carboxy-terminal zinc finger exon to generate the 2.0-kb short arm by PCR with a high-fidelity polymerase composite called Expand Long Template (Boehringer Mannheim). The short arm primers contained *Xba*I linkers and were of the sequence 3'-GAGCCGCGCTCGAGGGAGGGTC-TCACCAGCAG, and 5'-CTGGGCGCCTCGAGTGGATGCC-CTGCCCTGCT. The long arm was generated by PCR of a 4.0-kb fragment that was just inside of the intron 5' to the amino-terminal zinc finger exon and ~500 bp 3' of the *EcoRV* site. The long arm primers contained *Cla*I linkers and were of the sequence 5'-GACTCCAGGCCATCGATCGTAGCTGGGCA-CCCAT, and 3'-CGACCGACTCCATCGATGTCTCCGGGAGACTGAT. The long and short arms were then cloned into the *Cla*I and *Xba*I sites of the vector pTK-NEO (Fig. 1).

### *ES cell transfection and chimera production*

Methods for electroporation of AB2.2 ES cells with the linearized targeting vector, growth of ES cells on STO feeder fibroblasts for G418, and FIAU resistance were described previously (Ramirez-Solis et al. 1993). Cell cloning and isolation of genomic DNA for Southern analysis was performed as described previously (Ramirez-Solis et al. 1993). Four targeted clones were obtained from a total of 400, and one of these clones, called C29, was injected into the blastocysts of C57BL/6 mice, which were subsequently implanted into pseudopregnant Swiss foster female mice.

### *Southern blot and PCR analysis of targeting*

ES cell genomic DNA, as well as tail genomic DNA, was analyzed for the correct targeting event by Southern blot analysis with probes made against sequences outside the targeting vector on both the 5' and 3' ends of the *GATA4* locus (Fig. 1A). Genomic DNA was prepared as described previously (Matzuk et al. 1992). The 5' probe was generated from a region just 3' of the *EcoRV* site (Fig. 1A) by PCR of a 320-bp product. Use of this

probe on *EcoRV*-digested genomic DNA resulted in the identification of an 18-kb band on a Southern blot, whereas targeted genomic DNA containing the *neo* gene resulted in the identification of a 5.0-kb band (Fig. 1B). The 3' Southern probe was generated from a region 5' to the 3' *SacI* site by PCR of a 360-bp product. This probe identified an 11.0-kb wild-type band and a 3.5-kb targeted band following *SacI* digestion (Fig. 1B). For PCR genotyping, ~50 ng of genomic tail or yolk sac DNA was used. For substantially smaller embryos lacking a yolk sac, the entire embryo was lysed in *Taq* PCR buffer containing 0.1% Triton X-100 and treated with 0.5 mg/ml of proteinase K for 1 hr at 55°C in a volume of 20 μl. This reaction was heat inactivated at 100°C for 5 min, and half was used directly in a PCR reaction with *Taq* DNA polymerase. The primer pair for the wild-type locus was 5'-ACTCTGGAGGCAGATGGG, and 3'-CTCG-GCATTACGACGCCACAG, which generated a 900-bp band. The primer pair for the targeted locus was 5'-GCCCTGA-ATGAACTGCAGG, and 3'-CACGGGTAGCCAACGCTAT, which generated a 500-bp band. Reaction conditions were as follows: denaturation for 20 sec at 96°C, primer annealing for 25 sec at 62°C, and extension for 60 sec at 72°C for a total of 30 cycles with one unit of *Taq* DNA polymerase in a volume of 50 μl containing 200 μM dNTPs and 50 pmoles of each primer in *Taq* PCR buffer (Perkin Elmer Cetus).

### *Whole-mount in situ analysis*

To detect endogenous RNA by whole-mount *in situ* analysis, a number of specific riboprobes were generated. To visualize the developing somites, notochord and neural tube floor plate, and the developing head region, plasmids containing cDNA inserts for *Mox1*, *HNF3β*, and *OTX2* were used, respectively (Candia et al. 1992; Ruiz i Altaba et al. 1993; Simeone et al. 1993). To visualize the developing heart, a number of cardiac markers were examined, including *ANF*, *MLC1A*, *MLC2A*, *MLC2V*,  $\alpha$ -*MHC*, and *Nkx2.5*. Plasmids for each of these markers were cut, and antisense transcripts were generated with *Xba*I and *T7* (*ANF*), *EcoRI* and *T3* (*MLC1A*), *BamHI* and *T7* (*MLC2A*), *DdeI* and *T7* (*MLC2V*), *HindIII* and *T7* ( $\alpha$ -*MHC*), and *Xba*I and *T7* (*Nkx2.5*). A probe for *GATA4* was used as an *Xba*I digest and *T7* product.

The probes described above were generated as antisense RNA runoff products in the presence of digoxigenin-dUTP. Probe processing and the conditions for embryo hybridization and subsequent immunodetection with AP-conjugated anti-digoxigenin antibody and colorization reaction were described previously (Barth and Ivarie 1994). Embryos for sectioning were processed as described previously (Kaufman 1992).

### *RT-PCR analysis*

Two pools of three and four *GATA4* null embryos and wild-type embryos were collected at E8.5 for total RNA extraction with Trizol reagent (GIBCO BRL). RNA was resuspended in 20 μl of water, and half was reverse transcribed with 5 units of Moloney murine leukemia virus (Mo-MLV) reverse transcriptase in 20 μl with 400 μM dNTPs and 1 μg of random hexamers (GIBCO BRL) at 42°C for 1 hr. The remaining half of RNA was used as a no-reverse transcriptase control. Added to a *Taq* polymerase-directed PCR reaction (1 unit) was 0.5 μl of these reactions with 200 μM dNTPs, 0.1 μCi of [ $\alpha$ -<sup>32</sup>P]dCTP, and 1 μM of each primer in a 50-μl reaction. Cycling conditions were as follows: denaturation at 96°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec for 20 cycles with both L7 primers and *GATA6* primers and 25 cycles with *GATA5* primers. The ribosomal control marker L7 was amplified with primers con-

sisting of 5'-GAAGCTCATCTATGAGAAGGC (sense) and 5'-AAGACGAAGGAGCTGCAGAAC (antisense). GATA5 was amplified with primers consisting of 5'-CGACGTAGCC-CCTTCGTGG (sense) and 5'-GCCACAGTGGTAGACAG (antisense), whereas GATA6 was amplified with primers consisting of 5'-CCCAGCGCAGACCTGTTGGAGGACC (sense) and 5'-TGTGACAGTTGGCACAGGACAG (antisense). Both the GATA5 and GATA6 product spanned at least one intron.

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