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Dev Biol. Author manuscript; available in PMC 2011 October 15.

Published in final edited form as:
Dev Biol. 2010 October 15; 346(2): 346–355. doi:10.1016/j.ydbio.2010.07.032.

Direct transcriptional regulation of *Gata4* during early endoderm specification is controlled by FoxA2 binding to an intronic enhancer

Anabel Rojas[1,*], **William Schachterle**[2], **Shan-Mei Xu**[2], **Franz Martín**[1], and **Brian L. Black**[2,3]

[1] Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER) -CIBERDEM, Avda. Americo Vespucio s/n. 41092, Sevilla, SPAIN [2] Cardiovascular Research Institute and [3] Department of Biochemistry and Biophysics University of California, San Francisco, CA 94143-2240, USA

Abstract

The embryonic endoderm is a multipotent progenitor cell population that gives rise to the epithelia of the digestive and respiratory tracts, the liver and the pancreas. Among the transcription factors that have been shown to be important for endoderm development and gut morphogenesis is GATA4. Despite the important role of GATA4 in endoderm development, its transcriptional regulation is not well understood. In this study, we identified an intronic enhancer from the mouse *Gata4* gene that directs expression to the definitive endoderm in the early embryo. The activity of this enhancer is initially broad in all endodermal progenitors, as demonstrated by fate mapping analysis using the *Cre/loxP* system, but becomes restricted to the dorsal foregut and midgut, and associated organs such as dorsal pancreas and stomach. The function of the intronic *Gata4* enhancer is dependent upon a conserved Forkhead transcription factor-binding site, which is bound by recombinant FoxA2 in vitro. These studies identify *Gata4* as a direct transcriptional target of FoxA2 in the hierarchy of the transcriptional regulatory network that controls the development of the definitive endoderm.

Keywords

GATA4; Forkhead proteins; FoxA2; endoderm progenitors; enhancer; transgenic mouse

Introduction

During gastrulation, the developing embryo undergoes a precise morphogenetic program that leads to the establishment of the basic body plan. Just before gastrulation, the amniote embryo consists of an epithelial layer called the epiblast, which is covered by an extraembryonic layer, known as the visceral endoderm (Lawson et al., 1986; Tam and Loebel, 2007). During gastrulation, cells in the epiblast undergo an epithelial-to-mesenchymal transition, and ingress through the primitive streak (Lawson et al., 1991).

[*]Corresponding author: Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), Avda. Americo Vespucio s/n. 41092, Sevilla, SPAIN, Tel: +34 954 467, 427; Fax: +34 954 461 664, anabel.rojas@cabimer.es.

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Ingressing epiblast cells form the mesoderm and endoderm while the embryonic surface forms the ectoderm. The presumptive definitive endoderm, which gives rise to endodermal organs, displaces the extraembryonic visceral endoderm, which gives rise to extraembryonic structures such as the yolk sac (Kadokawa et al., 1987; Weber et al., 1999). The visceral endoderm shares the expression of many genes with the definitive endoderm (Sherwood et al., 2007).

After gastrulation, a series of morphogenetic movements transform the definitive endoderm into a primitive gut tube surrounded by mesoderm. The anterior and posterior regions of the primitive tube fold ventrally and the tube becomes regionalized along the dorsal-ventral and anterior-posterior axes into foregut, midgut, and hindgut. These subregions are defined by specific genetic programs controlled by cell non-autonomous factors derived from nearby mesoderm and ectodermal tissues and mediated by transcription factors that directly regulate gene expression in different endoderm-derived organs. (Bort et al., 2004; Grapin-Botton and Melton, 2000; Kumar et al., 2003; Molotkov et al., 2005; Serls et al., 2005; Stainier, 2002; Tremblay and Zaret, 2005; Wells and Melton, 2000). The ventral foregut gives rise to the liver, ventral pancreas, and lungs, while dorsal foregut and anterior regions of the midgut contribute to the dorsal pancreas, stomach, esophagus, and duodenum. The midgut and hindgut form the small and large intestines, respectively (Lawson et al., 1986).

Among the transcription factors that have been shown to be crucial for endoderm development is FoxA2, a member of the Forkhead family of transcription factors (Costa et al., 1989; Lai et al., 1990). In the early embryo, FoxA2 is expressed in both visceral and definitive endoderm, the node, the notochord, and the floorplate (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993). Later in development, FoxA2 is also present in endoderm-derived tissues, including liver, lung, stomach, pancreas, small intestine, and colon (Besnard et al., 2004). Targeted disruption of *foxa2* leads to embryonic lethality, and *foxa2*-null embryos display malformation of the node, notochord, and foregut endoderm (Ang et al., 1993; Weinstein et al., 1994). Recent studies, using conditional inactivation approaches, have revealed that FoxA2 is also required at later stages of development in various endoderm-derived organs (Gao et al., 2008; Kaestner, 2005; Lantz et al., 2004; Lee et al., 2005; Wan et al., 2004; Zhang et al., 2005). Mechanistically, FoxA2 functions as a “pioneer factor” by displacing linker histones from compacted chromatin and facilitating the binding of other transcription factors, including the zinc finger transcription factor GATA4 (Gualdi et al., 1996; Zaret, 1999; Zaret, 2008; Zaret et al., 2008).

GATA4 belongs to a family of transcription factors involved in the differentiation of the endoderm in several evolutionarily diverse organisms (Rehorn et al., 1996; Reiter et al., 2001; Zhu et al., 1997). Among the members of the GATA family, GATA4 is the first to be expressed in the visceral and definitive endoderm. GATA4 is also expressed broadly in the mesoderm of the early mouse embryo (Arceci et al., 1993; Molkentin, 2000). Homozygous inactivation of *Gata4* in mice leads to embryonic lethality, and *Gata4*-null embryos display severe defects in the visceral endoderm, malformations in the ventral foregut, and major disruptions in heart morphogenesis (Kuo et al., 1997; Molkentin et al., 1997), establishing a crucial role of this transcription factor in these cell lineages. More recently, conditional inactivation, tetraploid complementation, and mosaic analyses of *Gata4* mutant mouse embryos have also revealed its important role in the differentiation of a variety of endodermal-derived tissues (Battle et al., 2008; Bosse et al., 2006; Dusing et al., 2001; Jacobsen et al., 2002; Watt et al., 2007). Similarly, morpholino knockdown experiments demonstrated a requirement of GATA4 for the proper formation of endodermal organs in the zebrafish (Holtzinger and Evans, 2005). In addition to its role in differentiation of endodermal derivatives, GATA4 also appears to participate in endoderm specification in

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cooperation with FoxA2 (Bossard and Zaret, 1998; Cirillo et al., 2002; Zaret, 1999). However, despite the importance of GATA4 in the development of the early endoderm, relatively little is known about its transcriptional regulation *in vivo*.

In this study, we identified a transcriptional enhancer from the mouse *Gata4* gene, referred to as *Gata4* G4, which is located in the second intron of the mouse *Gata4* gene. Importantly, this novel intronic enhancer directs expression to the definitive endoderm from early stages in mouse development. As development proceeds, the activity of the *Gata4* G4 enhancer becomes restricted to cells of the dorsal foregut and midgut. The cells marked by the activity of this novel *Gata4* enhancer at early stages contribute to all endodermal progenitors, as indicated by Cre-based lineage tracing experiments. Reporter gene expression directed by the *Gata4* G4 enhancer in transgenic embryos *in vivo* matches the expression of transcripts for the Forkhead transcription factor FoxA2, and we show the *Gata4* G4 enhancer contains an essential conserved Forkhead binding site that is efficiently bound by recombinant FoxA2 protein and is required for enhancer activity *in vivo*. Thus, our studies identify *Gata4* as a direct transcriptional target of the Forkhead transcription factor FoxA2 in the early endoderm and establish a *Gata4*-expressing progenitor population that contributes to the entire definitive endoderm.

Materials and Methods

Bioinformatic analyses, cloning, and mutagenesis

Sequence comparisons were performed by using BLAST and VISTA algorithms (Altschul et al., 1990; Mayor et al., 2000). The 1,107-bp G4 fragment of the mouse *Gata4* gene was generated by PCR from mouse genomic DNA using the following primers: 5′-tctgcatggaaccaagcttccag-3′ and 5′-ccctgctaactgcagtcatgg-3′. This fragment was then cloned as a HindIII-PstI fragment into the HindIII-PstI sites of the transgenic reporter plasmid *Hsp68-lacZ* (Kothary et al., 1989). The Forkhead site mutant in *Gata4* G4 was created by using primers with the following plus strand sequence: 5′-attataatcacagactagtcgcagaagtctcaaag-3′. The sequence of the mutant fragment was confirmed by sequencing on both strands. The *Gata4* G4 fragment fused to the *Gata4* minimal promoter containing the mouse *Gata4* sequences from −443 to +52, relative to the transcriptional start site, was cloned into a *Cre* expression plasmid containing the Cre cDNA and the SV40 splice and poly A signal sequences to generate plasmid *Gata4*-G4Gp-Cre. The GenBank Accession number for the sequence of *Gata4* G4 endodermal enhancer is HM212783.

Generation of transgenic mice

Transgenic *lacZ* reporter and Cre constructs were digested from their parental plasmid backbones with SalI, gel purified, and suspended in 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.4, at a concentration of 2 μg/ml for pronuclear injection as described previously (Hogan et al., 1994). Injected embryos were implanted into pseudopregnant FVB females, and embryos were collected at indicated times for F_0 analysis or were allowed to develop to adulthood for the establishment of stable transgenic lines. DNA was extracted from the yolk sac of embryos or from tail biopsies as previously described (Dodou et al., 2003). The presence of the *Gata4*-G4-*lacZ* and *Gata4*-G4Gp-Cre transgenes were detected by Southern blot using specific probes for each allele.

X-gal staining, immunohistochemistry, and *in situ* hybridization

β-galactosidase expression in *lacZ* transgenic embryos or tissues was detected by X-gal staining, which was performed as described previously (Dodou et al., 2003). For the analysis of the *Gata4*-G4Gp-Cre line, transgenic male founders were crossed with female *Rosa26R*

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lacZ reporter mice (Soriano, 1999) and embryos were collected at different stages of development. Transverse and sagittal sections from X-gal stained embryos and tissues were prepared and counterstained with Neutral Fast Red as described previously (Anderson et al., 2004). GATA4 immunohistochemistry was performed as previously described (Rojas et al., 2009). Briefly, dewaxed sections were boiled in antigen retrieval and incubated with 3% H_2O_2 for 15 min prior to block with 3% normal goat serum. Incubation with anti-GATA4 antibody (1:300 dilution) was done overnight at 4ºC. Incubation with biotinylated anti-goat antibody (Vector Laboratories) at a 1:300 dilution was done at room temperature for 1 h. Immunoperoxidase staining was performed using the Vectastain Elite ABC kit (Vector Laboratories) and developed using the peroxidase substrate DAB (Vector Laboratories).

Whole mount *in situ* hybridization was performed as described previously (Rojas et al., 2005). Following staining, embryos were sectioned at a thickness of 7 μm and counterstained with Neutral Fast Red for visualization of embryonic structures. *foxa2* antisense probe was generated from pBluescript-FoxA2 containing the full-length cDNA linearized with BamHI, and transcribed using T3 polymerase.

Electrophoretic mobility shift assay (EMSA)

DNA binding reactions were performed as described previously (Dodou et al., 2003). The *foxa2* cDNA was transcribed and translated using the TNT Coupled Transcription-Translation System (Promega), as described in the manufacturer's directions. FoxA2 protein was generated from pcDNA1-FoxA2 plasmid using T7 polymerase. The sense strand sequence of the mouse *Gata4* G4 Forkhead site used for EMSA was: Fox 5′-gggggttaattataatcacaaataaacgcagaagtctcaaag-3′. The sense strand sequences for the Forkhead mutant site was the same as for the mutagenic primers described above.

Chromatin Immunprecipitation (ChIP)

ChIP assays were performed using the ChIP assay kit from Upstate Pharmaceuticals following the recommendations of the manufacturer. Briefly, a 10 cm plate containing approximately 1×10^6 AR42J rat cells (Rosewicz et al., 1992), grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), were treated with 1% paraformaldehyde at 37°C for 10 min to crosslink protein-DNA complexes. Cells were then lysed and sonicated to shear the DNA into fragments approximately 500 bp in size. The cleared supernatant was divided into two samples. One of the samples was incubated with 4 μg of anti-FoxA2 antibody (Santa Cruz Biotechnology) and the other sample was incubated with 4 μg of anti-rabbit IgG (Santa Cruz Biotechnology) as a nonspecific control overnight at 4°C. The DNA fragments were then precipitated after incubating the lysate and antibody mixture with protein A-agarose beads for 1 h. Reactions were incubated in 200 mM NaCl at 65°C for 4 h to reverse the crosslinks, and DNA was recovered by phenol-chloroform extraction. The following primers were used to amplify a fragment of 290 bp encompassing the *Gata4* G4 Forkhead site: 5′-cagttgaattgtcccaaatcagatc-3′ and 5′-ccaactgtggatgaacttagcttcc-3′.

Results

An intronic *Gata4* endoderm-specific enhancer directs expression to the dorsal foregut and midgut in mouse transgenic embryos

Comparison of the *Gata4* sequences of mouse, human, and opossum identified nine major regions of conserved non-coding sequences, referred to as G1–G9 (Fig. 1). Previously, we demonstrated that two of these conserved regions function as independent, modular transcriptional enhancers: *Gata4* G2 functions as a specific enhancer in lateral mesoderm and a subset of its derivatives in the liver mesenchyme and *Gata4* G8 functions as a late-

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acting endoderm enhancer (Rojas et al., 2005;Rojas et al., 2009). The *Gata4* G8 enhancer directs expression to the foregut and midgut in transgenic mouse embryos, beginning at embryonic day (E) 9.5 (Rojas et al., 2009), but this enhancer is not active at earlier embryonic stages when endogenous *Gata4* transcripts are already robustly expressed (Molkentin, 2000;Nemer and Nemer, 2003). Together, these observations suggested that additional *Gata4* endoderm enhancers must exist and prompted us to test additional conserved sequences from the *Gata4* locus for enhancer activity in early transgenic embryos.

To identify additional *Gata4* endoderm enhancers, we cloned each conserved noncoding region from the *Gata4* locus individually into the transgenic reporter plasmid *Hsp68-lacZ* (Kothary et al., 1989) and tested for enhancer activity in transgenic mouse embryos. These analyses indeed resulted in the identification of an additional endoderm-specific enhancer element, which we refer to as *Gata4* G4, with early activity in the visceral and definitive endoderm (Fig. 1). The *Gata4* G4 enhancer element encompasses a conserved region of 1,107 bp, located between exons 2 and 3 (Fig. 1). To evaluate *Gata4* G4 activity *in vivo*, we generated seven independent transgenic lines harboring the *Gata4*-G4-*lacZ* transgene and analyzed them for β-galactosidase reporter expression during embryonic development. Six of the transgenic lines showed a consistent *lacZ* expression pattern in all the embryonic stages analyzed. We first observed enhancer activity at E7.5 (Fig. 2A). At that stage, *Gata*4-G4-*lacZ* directed expression to the visceral endoderm of the yolk sac, in agreement with the previously described early expression of endogenous *Gata4* in the extra-embryonic endoderm (Kuo et al., 1997; Molkentin et al., 1997; Nemer and Nemer, 2003) (Fig. 2A). Extra-embryonic expression was transient and no longer observed at any developmental stage after E7.5 (Fig. 4A and data not shown). Later, at E8.5, robust *Gata4*-G4-*lacZ* transgene expression was observed in the primitive gut tube formed by the definitive endoderm (Fig. 2B–D). By E9.5, β-galactosidase activity was observed in the ventral and dorsal part of the gut tube, and became clearly restricted to the midgut (Fig. 2E, F, K). In the foregut, lacZ staining was only observed in the most caudal/posterior regions, indicating that enhancer activity did not extend more rostrally than the developing liver (Fig. 2H). The expression of the transgene was completely absent from the mesoderm component of the gut, demonstrating the endodermal specificity of the *Gata4* G4 element *in vivo*.

To characterize the *Gata4* endoderm enhancer in more detail, we compared the expression pattern directed by the enhancer with the expression pattern of the endodermal transcription factor FoxA2 (Ang et al., 1993). The expression patterns of the *Gata4*-G4-*lacZ* transgene and *foxa2* appeared to be nearly identical in the posterior foregut and midgut (Fig. 2, compare panels E, F, H, K to panels G, I, L). The expression of the *Gata4*-G4-*lacZ* transgene also partially overlapped the expression of endogenous GATA4 protein in the posterior foregut and midgut at E9.5 (Fig. 2 compare panels H, K to panels J, M). Thus, these results indicate that G4 enhancer faithfully recapitulates *Gata4* endogenous expression in the early endoderm in transgenic embryos.

Endogenous GATA4 protein expression was broader than *Gata4*-G4-*lacZ* and included other derivatives of the endoderm and mesoderm, such as the entire foregut and gut mesenchyme (Fig. 2J, M), consistent with previously published reports (Arceci et al., 1993). The broader expression of endogenous GATA4 protein relative to *Gata4*-G4-*lacZ* was expected since endogenous GATA4 expression is controlled by multiple independent enhancers with activity in the gut (Rojas et al., 2005; Rojas et al., 2009), and the *Gata4* G4 enhancer controls only a subset of the endogenous pattern of expression. In addition to its expression in the developing gut endoderm, we also observed *Gata4*-G4-*lacZ* expression in the floor plate (Fig. 2H, K) even though endogenous GATA4 protein was not detectable in the floor plate by immunohistochemistry (Fig. 2J, M). This discrepancy might reflect the

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absence of additional regulatory sequences in the *Gata4* G4 enhancer that normally repress endogenous *Gata4* expression in the floor plate.

Gata4 G4 enhancer activity is restricted to the epithelium of the hind-stomach and dorsal pancreatic bud

At E11.5, expression directed by the *Gata4* G4 enhancer was observed only in a subset of endoderm-derived organs, the epithelium of the glandular stomach, and the dorsal pancreatic bud (Fig. 3A–C). By E13.5, transgene activity was restricted to the glandular (hind) stomach, although at lower levels compared to earlier stages of development and it was absent in the anterior stomach (Fig. 3D). These results are in agreement with the previously described expression of endogenous *Gata4* in the developing stomach (Jacobsen et al., 2005). In the dorsal pancreas, only a few cells in the epithelium displayed detectable β-galactosidase activity at E13.5 (Fig. 3E). No transgene expression was observed in the mesenchyme surrounding the stomach and pancreas, indicating the specificity of the G4 enhancer to direct expression in the endodermal component of these organs. At later stages in fetal development, the *Gata4*-G4-*lacZ* transgene was no longer active since no β-galactosidase activity was observed in either the hind stomach epithelium or the pancreas (Fig. 3F). The decreased expression of the transgene coincides with terminal cell differentiation and maturation, suggesting that the *Gata4* G4 enhancer is active mainly at early stages of endoderm development.

Gata4-G4-Cre expressing cells and their descendents contribute to all endodermally-derived organs

The results shown in Figure 2 demonstrated that the *Gata4*-G4-*lacZ* transgene was expressed very early in the definitive endoderm of the mouse embryo but then became restricted to a subset of endoderm derivatives. However, the broad early activity of the *Gata4* G4 enhancer in the endoderm suggested that the cells initially and transiently marked by *Gata4*-G4-*lacZ* might contribute extensively to organs derived from the definitive endoderm. Therefore, to investigate the developmental potential of the progenitors marked by the early activity of the *Gata4* G4 enhancer, we generated transgenic mice in which the expression of Cre recombinase was controlled by the *Gata4* G4 enhancer linked to the minimal promoter region of *Gata4*. We crossed these mice, referred to as *Gata4*-G4Gp-Cre, to *Rosa26R lacZ* reporter mice, which express β-galactosidase constitutively and indelibly in a Cre-dependent manner (Soriano, 1999). We compared the Cre-dependent *lacZ* expression in these embryos to the *lacZ* expression directly under the control of the *Gata4* G4 enhancer at different stages of development. As mentioned above, *Gata4* G4 enhancer activity in the yolk sac was transient, rapidly extinguishing after E7.5 (Figs. 2A, 4A). However, in *Gata4*-G4Gp-Cre$^{Tg/0}$; *Rosa26*$^{lacZ/+}$ embryos, β-galactosidase activity was observed in the yolk sac at all stages of embryonic development (Fig. 4B. and data not shown). These results indicate that the early activation of the *Gata4* G4 enhancer in the visceral endoderm marks all of the progenitors that will give rise to the extra-embryonic endoderm of the yolk sac. As shown in Figure 3, at E11.5, the *Gata4*-G4-*lacZ* transgene was clearly active in the hind stomach and pancreatic epithelium (Fig. 3A-C and 4C, E). In *Gata4*-G4Gp-Cre$^{Tg/0}$; *Rosa26*$^{lacZ/+}$ embryos, β-galactosidase activity was also observed in the stomach epithelium and pancreatic bud, but its expression was broader than the expression of the *Gata4*-G4-*lacZ* transgene and was present in the epithelium of other endoderm derivatives, including the liver, and duodenum (Fig. 4D, F), indicating that these structures are derived from a *Gata4*-G4-*lacZ*-expressing progenitor population.

At E13.5, the Cre-dependent *lacZ* expression of the *Gata4*-G4Gp-Cre$^{Tg/0}$; *Rosa26*$^{lacZ/+}$ embryos was detected throughout the endodermal organs of the gastrointestinal and respiratory tract, including fore- and hind stomach, esophagus, duodenum, branching

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epithelium of the lung and in both dorsal and ventral pancreases (Fig. 4H, J). By comparison, the direct activity of the *Gata4* G4 enhancer (as measured by *Gata4*-G4-*lacZ* expression) was detectable only in a few cells of the dorsal pancreas and hind stomach and was completely absent from other endodermal organs, including the lungs and the liver (Fig. 4G, I, K). The β-galactosidase activity in *Gata4*-G4Gp-Cre$^{Tg/0}$; *Rosa26*$^{lacZ/+}$ embryos remained robust at later stages of fetal development and after birth in all endodermal organs, including the liver and the lungs (Figs. 4L and 5F), indicating that the *Gata4* G4-marked progenitor population stably contributed the majority of cells to all of the endodermal derivatives in the mouse. The specificity of the *Gata4* G4 enhancer for the endoderm was clearly evident based on the complete absence of contribution of any *Gata4*-G4Gp-Cre-marked cells in any organs of mesodermal origin, such as kidney, spleen, and heart (Fig. 5B, D, F). Likewise, Cre activity was completely absent in the mesenchymal compartment of the lung (Fig. 4J). Taken together, these results show that the *Gata4* G4 enhancer is expressed in a progenitor cell population, which gives rise to the majority of cells in endoderm-derived organs.

The Forkhead transcription factor FoxA2 binds to a conserved, consensus Forkhead binding site in the *Gata4* G4 endoderm enhancer

To identify how *Gata4* expression is controlled in the early endoderm via the *Gata4* G4 enhancer, we analyzed the *Gata4* G4 element for evolutionarily conserved sequences that might serve as potential *cis*-regulatory elements. These analyses identified a consensus binding site for Forkhead proteins (Fig. 6A). The presence of a perfect, conserved potential binding site for Forkhead transcription factors, combined with the overlapping expression patterns of *Gata4*-G4-*lacZ* and *foxa2* throughout development (Fig. 2), suggested a potential direct regulatory relationship between FoxA2 and *Gata4* via the *Gata4* G4 enhancer through the conserved Forkhead site in the enhancer. As an initial test of this hypothesis, we examined the ability of recombinant FoxA2 protein to bind to the Forkhead site in the *Gata4* G4 enhancer by Electrophoretic Mobility Shift Assay (EMSA) (Fig. 6B). FoxA2 efficiently bound to the *Gata4* G4 Fox site (Fig. 6B, lane 8). Binding was specific because it was competed by excess unlabeled control Forkhead site site (Fig. 6B, lane 9) and by excess unlabeled *Gata4* G4 Forkhead site (Fig. 6B, lane10), but not by excess unlabeled mutant versions of either Forkhead site (Fig. 6B, lanes 11, 12). To examine the binding of FoxA2 to the *Gata4* G4 enhancer in more detail, we determined the ability of the *Gata4* G4 Forkhead site and the mutant version of this site to compete for FoxA2 binding to a *bona fide* control Fox site. Binding of FoxA2 to the control site was abolished by the addition of unlabeled *Gata4* G4 Forkhead site but was not affected by the addition of an excess of mutant version of the *Gata4* G4 Forkhead site (Fig. 6B, lanes 4, 6).

To investigate whether the *Gata4* G4 Forkhead site is occupied by FoxA2 in cells of endodermal origin, we performed chromatin immunoprecipitation (ChIP) assays in cells of the AR42J pancreatic cell line (Fig. 6C). The AR42J cell line was derived from a chemically-induced rat exocrine pancreatic tumor and expresses numerous endodermal pancreatic transcription factors, including GATA4 and FoxA2 (Aldibbiat et al., 2008;Decker et al., 2006;Rosewicz et al., 1992). Importantly, the DNA fragment encompassing the Forkhead site in the endogenous *Gata4* G4 enhancer was precipitated and amplified by PCR when anti-FoxA2 antibody was added to the reaction but not when non-specific IgG was used for ChIP (Fig. 6C). These results demonstrate that endogenous FoxA2 binds to the Forkhead site in the endogenous *Gata4* G4 enhancer in its normal chromatin context in endodermally-derived cells. Taken together with the results of the EMSA shown in Fig. 6B, these results establish the Forkhead site in the *Gata4* G4 enhancer as a FoxA2 binding site, supporting the notion that *Gata4* is a direct transcriptional target of FoxA2 via the *Gata4* G4

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enhancer. Direct regulation of *Gata4* by FoxA2 would be consistent with the overlapping expression of *Gata4*-G4-*lacZ* and *foxa2* (Fig. 2).

Gata4 G4 endoderm enhancer activity is dependent on Forkhead-binding site *in vivo*

To determine the requirement for Forkhead transcription factor binding to the *Gata4* G4 enhancer for function *in vivo*, we introduced mutations identical to those used in EMSA to completely abolish FoxA2 binding to the site *in vitro* (Fig. 6B). We then generated transgenic mice harboring the *Gata4*-G4-*lacZ* mutant version and determined the effect of the Fox site mutation on enhancer activity compared to the wild type *Gata4*-G4-*lacZ* construct at E8.5 (Fig. 7). As in the results shown in Fig. 2, robust expression of the wild type *Gata4*-G4-*lacZ* transgene was observed in the definitive endoderm (Fig. 7A, C). In sharp contrast, mutation of the Forkhead binding site in the *Gata4* G4 enhancer completely eliminated transgene expression in all three independently generated transgenic lines examined (Fig. 7B, D), indicating a critical role for Forkhead transcription factor binding, likely FoxA2, in the activation of the enhancer. Taken together, the coexpression data showing near precise overlap of the expression of *foxa2* and *Gata4*-G4-*lacZ* (Fig. 2), the strong binding of FoxA2 protein to the perfect consensus Forkhead site in the enhancer (Fig. 6), and the absolute requirement of the Fox site for enhancer function *in vivo*, strongly support a pathway in which FoxA2 is a direct transcriptional activator of *Gata4* in the early endoderm via the enhancer identified and described in these studies.

Discussion

Dynamic expression and modular regulation of *Gata4* during endoderm development

The *Gata4* G4 enhancer described here directs robust expression to the endoderm in the early developing mouse embryo. The activity of this enhancer becomes restricted at midgestation to a small subset of endodermal derivatives in the dorsal foregut and midgut that will form the dorsal pancreas and stomach. Our Cre-based fate mapping experiments show that the total temporal and spatial activity of the *Gata4 G4* enhancer from its initial activation broadly in the endoderm at early times to its more restricted activity later results in the marking of all definitive endodermal derivatives from both ventral and dorsal foregut, midgut, and hindgut. When this broad contribution of Cre-marked cells is compared to the very restricted expression of the *Gata4-G4-lacZ* transgene at later stages of development, the results suggest that that the *Gata4*-G4 positive cells represent a pool of early multipotent endodermal progenitors, and while the activity of the enhancer itself is extinguished, the cells marked by the enhancer at early stages ultimately contribute to essentially all endodermally-derived cells in the embryo. However, we cannot formally exclude a very transient expression of the *Gata4*-G4 enhancer in other gut domains at later stages that may account for the further contribution of *Gata4*-G4 positive cells to all endodermal derivatives.

We have recently described another *Gata4* endoderm enhancer, referred to as *Gata4* G8 (Rojas et al., 2009). Interestingly, the *Gata4* G8 enhancer directs expression to the visceral and definitive endoderm at later stages of development than the *Gata4* G4 enhancer described in the present studies. The previously described *Gata4* G8 enhancer is active in the primitive gut tube and yolk sac beginning at E9.5. In contrast to the *Gata4* G4 enhancer described here, the previously described *Gata4* G8 enhancer remains active at later stages of development, although its activity also becomes restricted to pancreas, stomach and duodenum and yolk sac (Rojas et al., 2009).

The existence of two endoderm-specific enhancers in the *Gata4* gene might be related to the multiple roles of this transcription factor in endoderm development and, more specifically, in the development of pancreas and stomach. Alternatively, the two enhancers might

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represent temporally discrete transcriptional units with the early *Gata4* G4 enhancer serving as an initiation element and the later *Gata4* G8 enhancer serving as a maintenance unit. This type of model would be consistent with previously described examples of transcriptional control in the endoderm and other lineages where multiple enhancers differentially regulate initial activation versus maintenance of gene expression (Chen and Goldhamer, 2004; Meredith et al., 2009; Sasaki and Hogan, 1996; Teboul et al., 2002). It is interesting that the activities of *Gata4* G4 and *Gata4* G8 enhancers overlap at E9.5 in the dorsal foregut and midgut, a key period in endoderm development when organ buds start to form. The selective advantage of the modularity is not well understood, but perhaps it serves to fine tune the spatiotemporal and quantitative control of *Gata4* expression and the subsequent transcriptional programs downstream of this key transcription factor gene.

The rapid and progressive restriction of the *Gata4* G4 enhancer activity from E7.5 to E11.5 suggests that the activators of the enhancer might also become restricted during development. Alternatively, the enhancer may contain specific sites for repressors that inhibit enhancer activation in other tissues. One of the most intriguing aspects of the expression pattern directed by the *Gata4* G4 enhancer is the exclusive localization to the caudal foregut and midgut region of the gut tube at later times in its activity. It is well known that the developing endoderm displays clear anterior-posterior patterning influenced by signals from the adjacent germ layers that act in a graded manner (Kumar et al., 2003). These signals are critical for the proper development of the gut tube. The specific expression pattern directed by the *Gata4* G4 enhancer suggests that it may respond to different thresholds of mesodermal signals that restrict its activity in the precise anterior-posterior pattern observed in the present studies.

A transcriptional regulatory network for endoderm development by GATA4 and FoxA2

GATA and Forkhead transcription factors are involved in the specification and differentiation of different endodermal cell types in different organisms, including the fly, the nematode, the mouse, and the fish (Dufort et al., 1998; Fukushige et al., 1998; Mango et al., 1994; Narita et al., 1997; Reiter et al., 2001; Weigel et al., 1989; Zhu et al., 1997). GATA4 and FoxA2 are among the earliest transcription factors to be expressed in the embryonic endoderm in the mouse, and they synergistically activate the expression of endoderm-specific genes (Cirillo et al., 2002; Denson et al., 2000). In this study, we demonstrate that the *Gata4* G4 endoderm enhancer requires a conserved Forkhead binding site for function *in vivo*. Based on the overlapping expression pattern of *foxa2* and *Gata4*-G4-*lacZ* transgene and the efficient binding of FoxA2 to the Forkhead site in the *Gata4* G4 enhancer in vitro and in vivo (Figs. 2, 5), our studies strongly support a role for FoxA2 as a direct transcriptional regulator of *Gata4* in the early endoderm.

It would be interesting to determine if the direct regulatory relationship between GATA4 and FoxA2 is reciprocal, since it has been shown in other organisms that GATA factors are likely direct regulators of *Forkhead* genes. For example, the *Drosophila* GATA4 ortholog Serpent influences the expression of *forkhead* in the midgut (Casanova, 1990; Reuter, 1994). Similarly, the promoter of *PHA-4*, the *C. elegans* FoxA2 ortholog, is bound and transactivated by two different GATA factors, ELT-1 and ELT-2, in the worm (Azzaria et al., 1996; Horner et al., 1998; Kalb et al., 1998). The reciprocal activation of *Gata4* and *FoxA2* could serve to reinforce in a feed-forward fashion a regulatory circuit designated to amplify a transcriptional response for specification and differentiation of different endodermal organs.

A mechanism by which FoxA2 and GATA4 might function together in multipotent endoderm cells has been described previously. GATA4 and FoxA2 serve as pioneer factors for the induction of endoderm gene expression, and they were shown to bind to the

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chromatin of the silent *Alb* gene in the endoderm prior to the hepatic induction (Bossard and Zaret, 1998; Cirillo et al., 1998; Zaret, 1999). Upon inductive and permissive signals, the two transcription factors relax the compacted chromatin, allowing the binding of other transcription factors to activate the transcription of *Alb* and other genes to initiate the liver program (Bossard and Zaret, 1998; Cirillo et al., 2002). Our studies, described here, support a feed forward model for endoderm specification in which FoxA2 directly activates the expression of its own transcriptional partner GATA4. It will be interesting to determine whether this mechanism involving GATA4 and FoxA2 represents a general mechanism by which endoderm competence is predermined. Genome-wide analyses of FoxA2 and GATA4 bound to their targets in multipotent endodermal cells will provide new insights into how cell specification is achieved along the gut tube.

Acknowledgments

We thank Dr. Benoit Gauthier and Dr. Karim Hmadcha for helpful comments on the manuscript. We also thank Jillian Jarrett and Antonio Cárdenas for assistance with these studies. This work was supported by funds from the Instituto Salud Carlos III (CP07/00120, PI080018), Consejería Salud Junta de Andalucía (PI0008) and CIBERDEM to AR and by NIH grants HL099707, HL064658, and DE019118 to BLB.

References

Aldibbiat A, Marriott CE, Scougall KT, Campbell SC, Huang GC, Macfarlane WM, Shaw JA. Inability to process and store proinsulin in transdifferentiated pancreatic acinar cells lacking the regulated secretory pathway. J Endocrinol. 2008; 196:33–43. [PubMed: 18180315]

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215:403–10. [PubMed: 2231712]

Anderson JP, Dodou E, Heidt AB, De Val SJ, Jaehnig EJ, Greene SB, Olson EN, Black BL. HRC is a direct transcriptional target of MEF2 during cardiac, skeletal, and arterial smooth muscle development in vivo. Mol Cell Biol. 2004; 24:3757–68. [PubMed: 15082771]

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development. 1993; 119:1301–15. [PubMed: 8306889]

Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol. 1993; 13:2235–46. [PubMed: 8455608]

Azzaria M, Goszczynski B, Chung MA, Kalb JM, McGhee JD. A fork head/HNF-3 homolog expressed in the pharynx and intestine of the Caenorhabditis elegans embryo. Dev Biol. 1996; 178:289–303. [PubMed: 8812130]

Battle MA, Bondow BJ, Iverson MA, Adams SJ, Jandacek RJ, Tso P, Duncan SA. GATA4 is essential for jejunal function in mice. Gastroenterology. 2008; 135:1676–1686. e1. [PubMed: 18812176]

Besnard V, Wert SE, Hull WM, Whitsett JA. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expr Patterns. 2004; 5:193–208. [PubMed: 15567715]

Bort R, Martinez-Barbera JP, Beddington RS, Zaret KS. Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. Development. 2004; 131:797–806. [PubMed: 14736744]

Bossard P, Zaret KS. GATA transcription factors as potentiators of gut endoderm differentiation. Development. 1998; 125:4909–17. [PubMed: 9811575]

Bosse T, Piaseckyj CM, Burghard E, Fialkovich JJ, Rajagopal S, Pu WT, Krasinski SD. Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. Mol Cell Biol. 2006; 26:9060–70. [PubMed: 16940177]

Casanova J. Pattern formation under the control of the terminal system in the Drosophila embryo. Development. 1990; 110:621–8. [PubMed: 2133557]

Chen JC, Goldhamer DJ. The core enhancer is essential for proper timing of MyoD activation in limb buds and branchial arches. Dev Biol. 2004; 265:502–12. [PubMed: 14732408]

NIH-PA Author Manuscript

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Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol Cell. 2002; 9:279–89. [PubMed: 11864602]

Cirillo LA, McPherson CE, Bossard P, Stevens K, Cherian S, Shim EY, Clark KL, Burley SK, Zaret KS. Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. EMBO J. 1998; 17:244–54. [PubMed: 9427758]

Costa RH, Grayson DR, Darnell JE Jr. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. Mol Cell Biol. 1989; 9:1415–25. [PubMed: 2786140]

Decker K, Goldman DC, Grasch CL, Sussel L. Gata6 is an important regulator of mouse pancreas development. Dev Biol. 2006; 298:415–29. [PubMed: 16887115]

Denson LA, McClure MH, Bogue CW, Karpen SJ, Jacobs HC. HNF3beta and GATA-4 transactivate the liver-enriched homeobox gene, Hex. Gene. 2000; 246:311–20. [PubMed: 10767553]

Dodou E, Xu SM, Black BL. mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. Mech Dev. 2003; 120:1021–1032. [PubMed: 14550531]

Dufort D, Schwartz L, Harpal K, Rossant J. The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development. 1998; 125:3015–25. [PubMed: 9671576]

Dusing MR, Florence EA, Wiginton DA. Pdx-1 is required for activation in vivo from a duodenum-specific enhancer. J Biol Chem. 2001; 276:14434–42. [PubMed: 11278481]

Fukushige T, Hawkins MG, McGhee JD. The GATA-factor elt-2 is essential for formation of the Caenorhabditis elegans intestine. Dev Biol. 1998; 198:286–302. [PubMed: 9659934]

Gao N, LeLay J, Vatamaniuk MZ, Rieck S, Friedman JR, Kaestner KH. Dynamic regulation of Pdx1 enhancers by Foxa1 and Foxa2 is essential for pancreas development. Genes Dev. 2008; 22:3435–48. [PubMed: 19141476]

Grapin-Botton A, Melton DA. Endoderm development: from patterning to organogenesis. Trends Genet. 2000; 16:124–30. [PubMed: 10689353]

Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev. 1996; 10:1670–82. [PubMed: 8682297]

Hogan, B.; Beddington, R.; Costantini, F.; Lacy, E. Manipulating the mouse embryo. Cold Spring Harbor Laboratory Press; Plainview, NY: 1994.

Holtzinger A, Evans T. Gata4 regulates the formation of multiple organs. Development. 2005; 132:4005–14. [PubMed: 16079152]

Horner MA, Quintin S, Domeier ME, Kimble J, Labouesse M, Mango SE. pha-4, an HNF-3 homolog, specifies pharyngeal organ identity in Caenorhabditis elegans. Genes Dev. 1998; 12:1947–52. [PubMed: 9649499]

Jacobsen CM, Mannisto S, Porter-Tinge S, Genova E, Parviainen H, Heikinheimo M, Adameyko II, Tevosian SG, Wilson DB. GATA-4:FOG interactions regulate gastric epithelial development in the mouse. Dev Dyn. 2005; 234:355–62. [PubMed: 16127717]

Jacobsen CM, Narita N, Bielinska M, Syder AJ, Gordon JI, Wilson DB. Genetic mosaic analysis reveals that GATA-4 is required for proper differentiation of mouse gastric epithelium. Dev Biol. 2002; 241:34–46. [PubMed: 11784093]

Kadokawa Y, Kato Y, Eguchi G. Cell lineage analysis of the primitive and visceral endoderm of mouse embryos cultured in vitro. Cell Differ. 1987; 21:69–76. [PubMed: 3607886]

Kaestner KH. The making of the liver: developmental competence in foregut endoderm and induction of the hepatogenic program. Cell Cycle. 2005; 4:1146–8. [PubMed: 16123587]

Kalb JM, Lau KK, Goszczynski B, Fukushige T, Moons D, Okkema PG, McGhee JD. pha-4 is Ce-fkh-1, a fork head/HNF-3alpha, beta, gamma homolog that functions in organogenesis of the C. elegans pharynx. Development. 1998; 125:2171–80. [PubMed: 9584117]

Kothary R, Clapoff S, Darling S, Perry MD, Moran LA, Rossant J. Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. Development. 1989; 105:707–14. [PubMed: 2557196]

NIH-PA Author Manuscript

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NIH-PA Author Manuscript

Kumar M, Jordan N, Melton D, Grapin-Botton A. Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. Dev Biol. 2003; 259:109–22. [PubMed: 12812792]

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 1997; 11:1048–60. [PubMed: 9136932]

Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE Jr. HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. 1990; 4:1427–36. [PubMed: 2227418]

Lantz KA, Vatamaniuk MZ, Brestelli JE, Friedman JR, Matschinsky FM, Kaestner KH. Foxa2 regulates multiple pathways of insulin secretion. J Clin Invest. 2004; 114:512–20. [PubMed: 15314688]

Lawson KA, Meneses JJ, Pedersen RA. Cell fate and cell lineage in the endoderm of the presomite mouse embryo, studied with an intracellular tracer. Dev Biol. 1986; 115:325–39. [PubMed: 3709966]

Lawson KA, Meneses JJ, Pedersen RA. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development. 1991; 113:891–911. [PubMed: 1821858]

Lee CS, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. Nature. 2005; 435:944–7. [PubMed: 15959514]

Mango SE, Lambie EJ, Kimble J. The pha-4 gene is required to generate the pharyngeal primordium of Caenorhabditis elegans. Development. 1994; 120:3019–31. [PubMed: 7607089]

Mayor C, Brudno M, Schwartz JR, Poliakov A, Rubin EM, Frazer KA, Pachter LS, Dubchak I. VISTA: visualizing global DNA sequence alignments of arbitrary length. Bioinformatics. 2000; 16:1046–7. [PubMed: 11159318]

Meredith DM, Masui T, Swift GH, MacDonald RJ, Johnson JE. Multiple transcriptional mechanisms control Ptf1a levels during neural development including autoregulation by the PTF1-J complex. J Neurosci. 2009; 29:11139–48. [PubMed: 19741120]

Molkentin JD. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J Biol Chem. 2000; 275:38949–52. [PubMed: 11042222]

Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 1997; 11:1061–72. [PubMed: 9136933]

Molotkov A, Molotkova N, Duester G. Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn. 2005; 232:950–7. [PubMed: 15739227]

Monaghan AP, Kaestner KH, Grau E, Schutz G. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. Development. 1993; 119:567–78. [PubMed: 8187630]

Narita N, Bielinska M, Wilson DB. Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. Development. 1997; 124:3755–64. [PubMed: 9367431]

Nemer G, Nemer M. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. Dev Biol. 2003; 254:131–48. [PubMed: 12606287]

Rehorn KP, Thelen H, Michelson AM, Reuter R. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. Development. 1996; 122:4023–31. [PubMed: 9012522]

Reiter JF, Kikuchi Y, Stainier DY. Multiple roles for Gata5 in zebrafish endoderm formation. Development. 2001; 128:125–35. [PubMed: 11092818]

Reuter R. The gene serpent has homeotic properties and specifies endoderm versus ectoderm within the Drosophila gut. Development. 1994; 120:1123–35. [PubMed: 7913013]

Rojas A, De Val S, Heidt AB, Xu SM, Bristow J, Black BL. Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. Development. 2005; 132:3405–17. [PubMed: 15987774]

NIH-PA Author Manuscript

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Rojas A, Schachterle W, Xu SM, Black BL. An endoderm-specific transcriptional enhancer from the mouse Gata4 gene requires GATA and homeodomain protein-binding sites for function in vivo. Dev Dyn. 2009; 238:2588–98. [PubMed: 19777593]

Rosewicz S, Vogt D, Harth N, Grund C, Franke WW, Ruppert S, Schweitzer E, Riecken EO, Wiedenmann B. An amphicrine pancreatic cell line: AR42J cells combine exocrine and neuroendocrine properties. Eur J Cell Biol. 1992; 59:80–91. [PubMed: 1361433]

Ruiz i Altaba A, Prezioso VR, Darnell JE, Jessell TM. Sequential expression of HNF-3 beta and HNF-3 alpha by embryonic organizing centers: the dorsal lip/node, notochord and floor plate. Mech Dev. 1993; 44:91–108. [PubMed: 8155584]

Sasaki H, Hogan BL. Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development. 1993; 118:47–59. [PubMed: 8375339]

Sasaki H, Hogan BL. Enhancer analysis of the mouse HNF-3 beta gene: regulatory elements for node/notochord and floor plate are independent and consist of multiple sub-elements. Genes Cells. 1996; 1:59–72. [PubMed: 9078367]

Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development. 2005; 132:35–47. [PubMed: 15576401]

Sherwood RI, Jitianu C, Cleaver O, Shaywitz DA, Lamenzo JO, Chen AE, Golub TR, Melton DA. Prospective isolation and global gene expression analysis of definitive and visceral endoderm. Dev Biol. 2007; 304:541–55. [PubMed: 17328885]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–1. [PubMed: 9916792]

Stainier DY. A glimpse into the molecular entrails of endoderm formation. Genes Dev. 2002; 16:893–907. [PubMed: 11959838]

Tam PP, Loebel DA. Gene function in mouse embryogenesis: get set for gastrulation. Nat Rev Genet. 2007; 8:368–81. [PubMed: 17387317]

Teboul L, Hadchouel J, Daubas P, Summerbell D, Buckingham M, Rigby PW. The early epaxial enhancer is essential for the initial expression of the skeletal muscle determination gene Myf5 but not for subsequent, multiple phases of somitic myogenesis. Development. 2002; 129:4571–80. [PubMed: 12223413]

Tremblay KD, Zaret KS. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Dev Biol. 2005; 280:87–99. [PubMed: 15766750]

Wan H, Xu Y, Ikegami M, Stahlman MT, Kaestner KH, Ang SL, Whitsett JA. Foxa2 is required for transition to air breathing at birth. Proc Natl Acad Sci U S A. 2004; 101:14449–54. [PubMed: 15452354]

Watt AJ, Zhao R, Li J, Duncan SA. Development of the mammalian liver and ventral pancreas is dependent on GATA4. BMC Dev Biol. 2007; 7:37. [PubMed: 17451603]

Weber RJ, Pedersen RA, Wianny F, Evans MJ, Zernicka-Goetz M. Polarity of the mouse embryo is anticipated before implantation. Development. 1999; 126:5591–8. [PubMed: 10572036]

Weigel D, Jurgens G, Kuttner F, Seifert E, Jackle H. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell. 1989; 57:645–58. [PubMed: 2566386]

Weinstein DC, Ruiz i Altaba A, Chen WS, Hoodless P, Prezioso VR, Jessell TM, Darnell JE Jr. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell. 1994; 78:575–88. [PubMed: 8069910]

Wells JM, Melton DA. Early mouse endoderm is patterned by soluble factors from adjacent germ layers. Development. 2000; 127:1563–72. [PubMed: 10725233]

Zaret K. Developmental competence of the gut endoderm: genetic potentiation by GATA and HNF3/fork head proteins. Dev Biol. 1999; 209:1–10. [PubMed: 10208738]

Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nat Rev Genet. 2008; 9:329–40. [PubMed: 18398419]

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Zaret KS, Watts J, Xu J, Wandzioch E, Smale ST, Sekiya T. Pioneer factors, genetic competence, and inductive signaling: programming liver and pancreas progenitors from the endoderm. Cold Spring Harb Symp Quant Biol. 2008; 73:119–26. [PubMed: 19028990]

Zhang L, Rubins NE, Ahima RS, Greenbaum LE, Kaestner KH. Foxa2 integrates the transcriptional response of the hepatocyte to fasting. Cell Metab. 2005; 2:141–8. [PubMed: 16098831]

Zhu J, Hill RJ, Heid PJ, Fukuyama M, Sugimoto A, Priess JR, Rothman JH. end-1 encodes an apparent GATA factor that specifies the endoderm precursor in Caenorhabditis elegans embryos. Genes Dev. 1997; 11:2883–96. [PubMed: 9353257]

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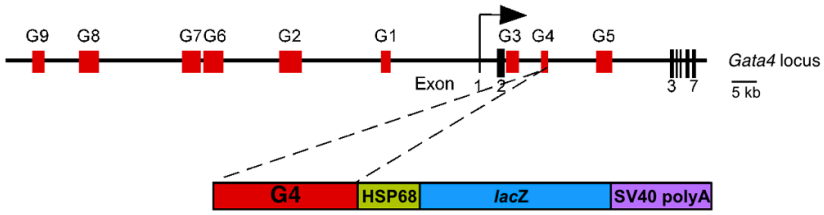

Fig. 1.

A schematic representation of the mouse *Gata4* locus and the *Gata4*-G4-*lacZ* transgene. The mouse *Gata4* locus, including the seven *Gata4* exons (vertical lines) and the transcriptional start site (bent arrow) is depicted in the top schematic. Conserved non-coding regions among the human, mouse and opossum *Gata4* sequences are represented as red boxes (G1–G9). The lower schematic depicts the *Gata4* G4 intronic sequences cloned into the reporter vector HSP68-*lacZ*.

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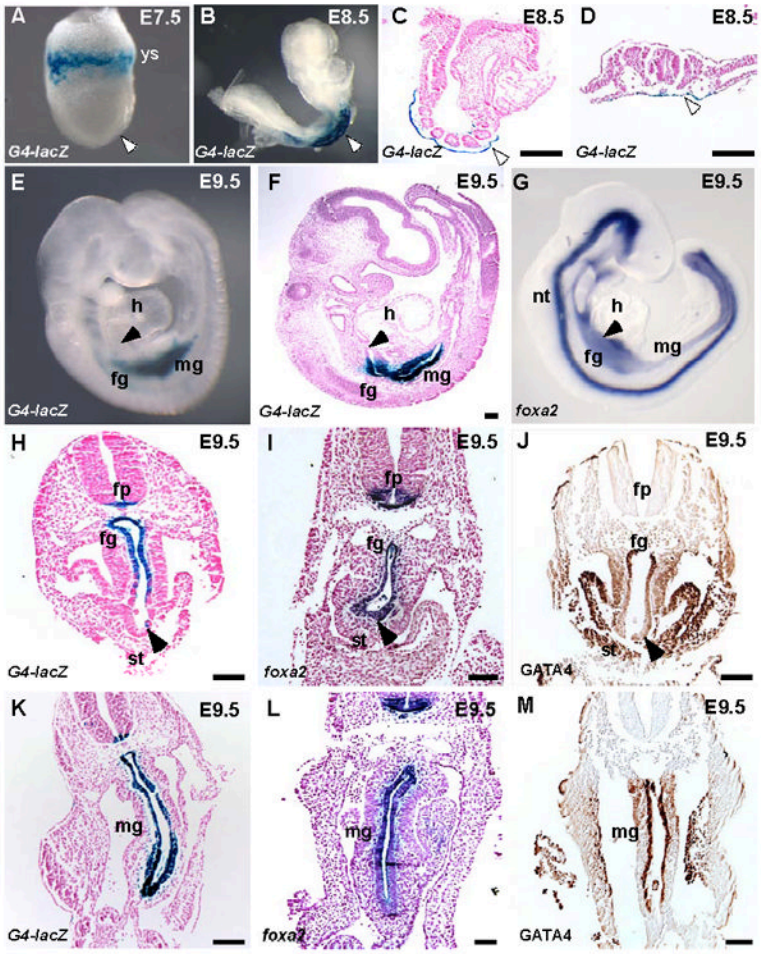

Fig. 2.

The *Gata4*-G4-*lacZ* transgene is expressed in the visceral and definitive endoderm, dorsal foregut, midgut, and derivatives of the definitive endoderm in the developing stomach and pancreas during mouse embryonic development. Whole mount (A, B, E), sagittal (C, F) and transverse (D, H, K) sections of X-gal stained *Gata4*-G4-*lacZ* transgenic embryos are shown. For comparison, whole mount (G) and transverse (I, L) sections of *foxa2 in situ* hybridization and transverse sections (J, M) of GATA4 immunohistochemistry are shown. White arrowheads mark the definitive endoderm. Transgene expression was only detected transiently in the visceral endoderm of the yolk sac (ys) at E7.5 (A). At slightly later stages, *Gata4*-G4-*lacZ* expression appeared in the definitive endoderm (B–D). By E9.5, *lacZ* expression became restricted to the caudal foregut (fg) and midgut (mg) where expression mirrored the expression of the endodermal marker *foxa2* (G, I, L) and endogenous GATA4 (J, M) but was absent in the anterior foregut (black arrows). Bars in all panels=100μm. h, heart; st, septum transversum.

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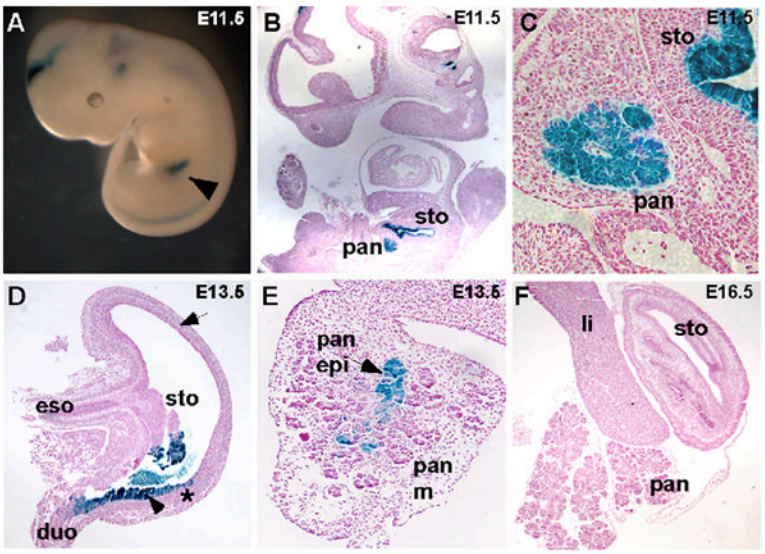

Fig. 3.

Expression directed by the *Gata4*-G4-*lacZ* transgene becomes restricted to the stomach epithelium and dorsal pancreatic bud. Whole mount (A) and sagittal sections (B–F) of X-gal-stained *Gata4*-G4-*lacZ* transgenic embryos are shown. At E11.5, X-gal staining was observed in the nascent epithelium of the hind stomach and in the pancreatic epithelium (A–C). By E13.5, transgene activity began to diminish in both the hind stomach (arrowhead in D) and the pancreas epithelium (pan epi) (E). No transgene expression was observed at E16.5 (F). Arrow marks the anterior stomach and asterik indicates the stomach mesenchyme in D. (sto) stomach; (eso) esophagus; (duo) duodenum; (pan) pancreas; (pan m) pancreas mesenchyme (li), liver.

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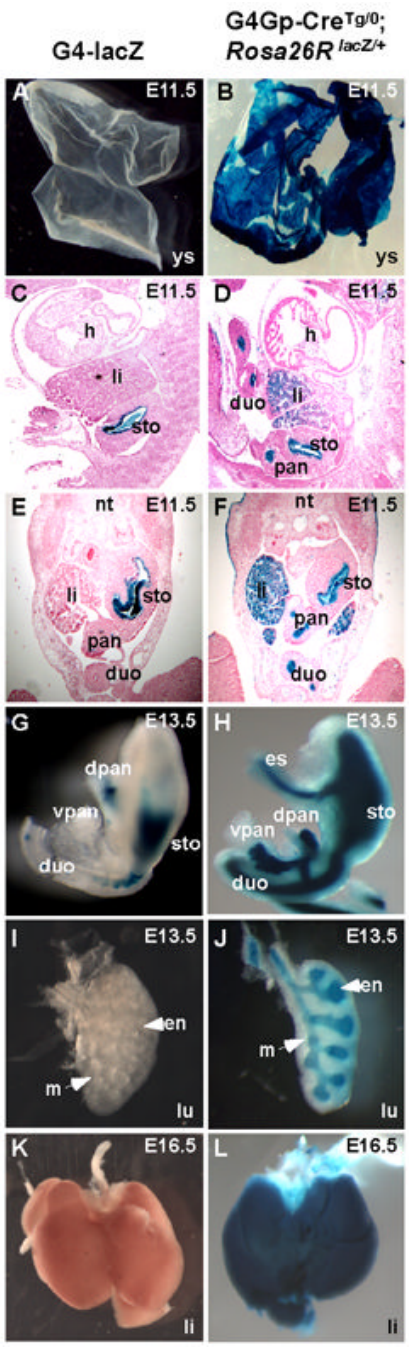

Fig. 4.

The *Gata4* G4 enhancer is active in all endodermal progenitors in the mouse embryo. Yolk sac (A, B), sagittal (C, D) and transverse (E, F) sections and dissected organs (G–L) collected from either *Gata4*-G4-*lacZ* (A, C, E, G, I, K) or *Gata4*-G4Gp-Cre$^{Tg/0}$, *Rosa26* $^{lacZ/+}$ (B, D, F, H, J, L) transgenic embryos were stained for β-galactosidase activity with X-gal. At E11.5, *Gata4*-G4-*lacZ* expression was absent in the yolk sac and detected only in the epithelium of the hind stomach and pancreas (A, C, E). *Gata4*-G4Gp-Cre$^{Tg/0}$, *Rosa26* $^{lacZ/+}$ embryos exhibited a similar pattern of *lacZ* expression in these tissues, but expression was also apparent in other endoderm-derived including the yolk sac and organs, such as liver, duodenum(B, D, F), indicating that the *Gata4* G4 enhancer was once active in

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the progenitors of these organs at earlier stages. By E13.5, the activity of *Gata4*-G4-*lacZ* transgene activity became weaker and staining was only observed in the dorsal pancreas and hind-stomach (E). No staining was observed in the lungs of *Gata4*-G4-*lacZ* transgenic embryos (I) at E13.5 or in the liver at E16.5 (K). By contrast, *Gata4*-G4Gp-Cre$^{Tg/0}$, *Rosa26* $^{lacZ/+}$ embryos exhibited X-gal staining in both dorsal and ventral pancreases, fore- and hind stomach, duodenum, esophagus, liver, and lungs (H, J, L), indicating that these structures were derived from an earlier *Gata4*-G4Gp-Cre-expressing population of cells. Note the specific X-gal staining of *Gata4*-G4Gp-Cre$^{Tg/0}$, *Rosa26* $^{lacZ/+}$ embryos in the endoderm compartment of the lung (en) but not in the mesenchymal (m) component (H). (sto), stomach; (pan), pancreas; (dpan), dorsal pancreas; (vpan), ventral pancreas; (h), heart; (nt), neural tube; (li), liver; (lu), lungs; (duo), duodenum.

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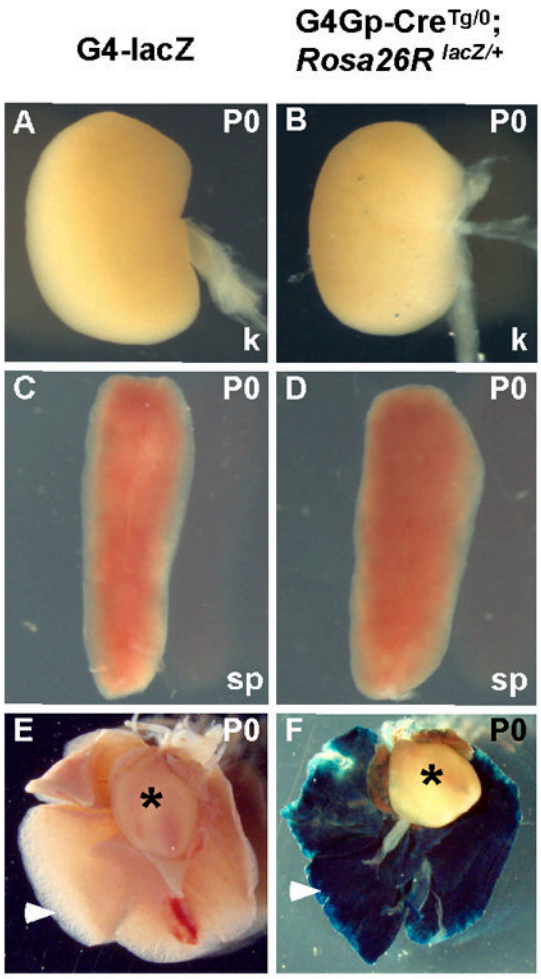

Fig. 5.

The *Gata4* G4 enhancer is not active in the mesoderm or its derivatives at any stage in development. Representative organs dissected from *Gata4*-G4-*lacZ* (A, C, E) and *Gata4*-G4Gp-Cre$^{Tg/0}$, *Rosa26* $^{lacZ/+}$ (B, D, F) newborn mice (P0) were assayed for β-galactosidase activity. *Gata4*-G4Gp-Cre indelibly labeled cells in the lung epithelium of newborn mice (arrowhead in F), consistent with its activity in the endoderm. At that stage, no direct expression of the *Gata4*-G4-*lacZ* transgene was observed in the lungs (arrowhead E). No mesodermal organs, including kidney (k) (A, B), spleen (sp) (C, D), or heart (asterisks in E, F) were labeled in either *Gata4*-G4-*lacZ* or *Gata4*-G4Gp-Cre$^{Tg/0}$; *Rosa26R*$^{lacZ/+}$ mice.

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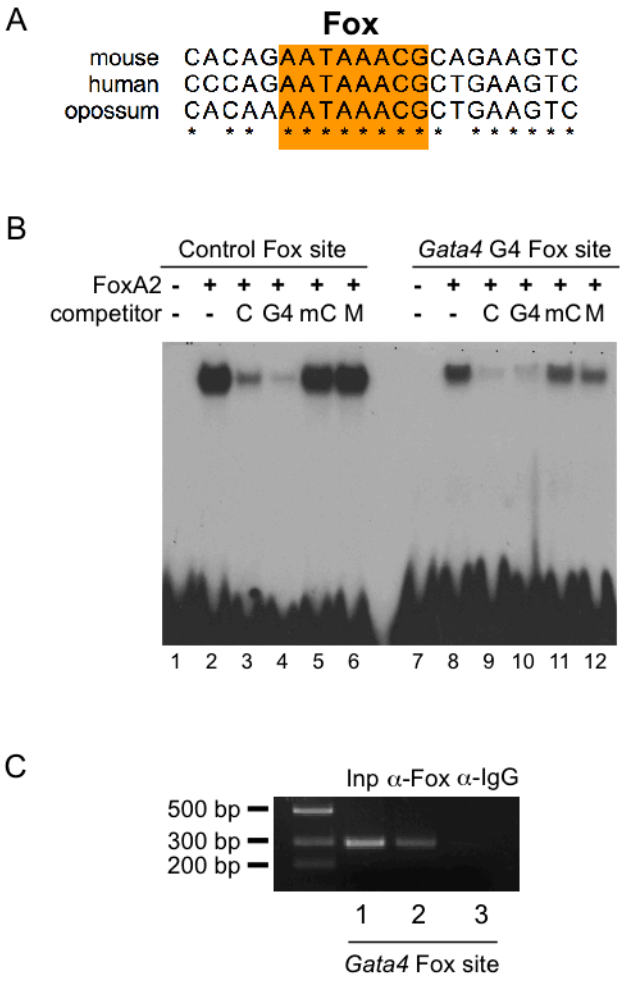

Fig. 6.

The Gata4-G4-*lacZ* transgene contains an evolutionary conserved Forkhead binding site that is bound by FoxA2 protein *in vitro* and *in vivo*. (A) ClustalW analysis of *Gata4* G4 sequences from mouse, human, and opossums revealed a highly conserved, perfect consensus Forkhead binding site (orange box). Asterisks denote nucleotides that have been perfectly conserved among the three species. (**B**) Recombinant FoxA2 protein was used in EMSA with radiolabeled double-stranded oligonucleotide probe representing a canonical Forkhead binding site from the *Gata4* G2 lateral mesoderm enhancer (Rojas et al., 2005) (lanes 1–6) or the *Gata4* G4 Forkhead site (lanes 7–12). Lanes 1 and 7 contain reticulocyte lysate without recombinant FoxA2 protein. FoxA2 efficiently bound to the Forkhead control site and to the *Gata4* G4 Forkhead site (lanes 2, 8). In both cases, binding was competed by excess, unlabeled control Forkhead site (C, lanes 3 and 9) or unlabeled *Gata4* G4 Forkhead site (G4, lanes 4 and 10), but not by excess unlabeled mutant control (mC, lanes 5 and 11) or an unlabeled mutant version of the *Gata4* G4 (M, lanes 6 and 12) Forkhead sites. (**C**) FoxA2 binds to the endogenous *Gata4* G4 enhancer *in vivo*. AR42J pancreatic cells were subjected to ChIP to detect endogenous FoxA2 bound to the *Gata4* G4 enhancer using anti-FoxA2 antibody. Following ChIP, the *Gata4* G4 Forkhead site was detected using specific primers flanking the *Gata4* G4 Fox binding site. PCR products were analyzed by agarose gel electrophoresis. Lane 2 contains PCR product obtained following ChIP using anti-FoxA2 antibody (α-Fox). Lane 3 contains PCR product obtained following ChIP using a non-

specific anti-IgG (α-IgG). Lane 1 contains PCR product from input (Inp) amplified prior to immunoprecipitation. Sizes in bp are shown at the left.

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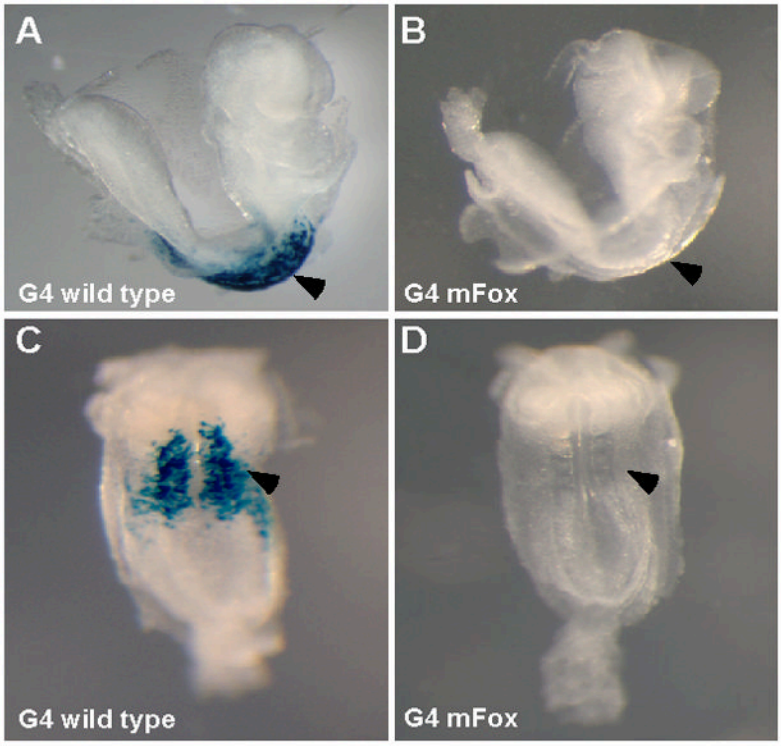

Fig. 7.

The *Gata4* G4 early endoderm enhancer is dependent on its conserved Forkhead (Fox) site for activity *in vivo*. Wild type and mutant versions of the *Gata4*-G4-*lacZ* transgene were used to generate transgenic embryos. Lateral (A, B) and frontal view (C, D) of X-gal stained transgenic embryos at E8.5 are shown. The wild type construct reproducibly directed expression to the definitive endoderm (A, C). Mutation of the *Gata4* G4 Forkhead site completely abolished transgene expression in each of three independently-generated F_0 transgenic embryos analyzed (B, D). Arrowheads mark the definitive endoderm in all four panels.