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An endoderm-specific transcriptional enhancer from the mouse *Gata4* gene requires GATA and homeodomain protein binding sites for function *in vivo*

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Abstract

Several transcription factors function in the specification and differentiation of the endoderm, including the zinc finger transcription factor GATA4. Despite its essential r ole in endoderm development, the transcriptional control of the *Gata4* gene in the developing endoderm and its derivatives remains incompletely understood. Here, we identify a distal enhancer from the *Gata4* gene, which directs expression exclusively to the visceral and definitive endoderm of transgenic mouse embryos. The activity of this enhancer is initially broad within the definitive endoderm but later restricts to developing endoderm-derived tissues, including pancreas, glandular stomach, and duodenum. The activity of this enhancer *in vivo* is dependent on evolutionarily-conserved HOX and GATA binding sites, which are bound by PDX-1 and GATA4, respectively. These studies establish *Gata4* as a direct transcriptional target of homeodomain and GATA transcription factors in the endoderm and support a model in which GATA4 functions in the transcriptional network for pancreas formation.

INTRODUCTION

The embryonic body of bilaterian animals is composed of three germ layers: ectoderm, mesoderm and endoderm (Beddington and Smith, 1993). The endoderm gives rise to the epithelia of the major part of the gut, which develops via a highly orchestrated process involving numerous morphogenetic events, reciprocal interactions with the adjacent mesoderm and ectoderm, and cell determination and differentiation (Bienz, 1997; Grapin-Botton and Melton, 2000; Wells and Melton, 2000; Stainier, 2002; Kumar et al., 2003). The result of these patterning events is a gut tube in which defined regions are predetermined to contribute to certain organs (Rosenquist, 1971; Keller, 1976; Lawson et al., 1986). At approximately embryonic day (E) 9.5 in the mouse, many organs of the respiratory and gastrointestinal tracts, including pancreas and stomach, begin to form as buds along the gut tube. These buds subsequently expand and differentiate to form mature organs (Grapin-Botton and Melton, 2000). In the developing bud epithelia of the pancreas, growth and morphogenesis result in the formation of small ductules, which contain the precursor cells of the acini, ducts, and islets of Langerhans (Jonsson et al., 1995). The developing gut tube itself becomes regionalized along the anterior-posterior axis, as cells adopt functions specific for their eventual roles in digestion (Grapin-Botton and Melton, 2000). Although some of the embryological processes involved

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in the development of endoderm-derived organs are well understood, many of the molecular events and transcriptional pathways controlling endoderm-derived organ development remain undefined.

The homedomain transcription factor PDX-1 is one of the first markers of the developing pancreas (Wright et al., 1989; Leonard et al., 1993; Ohlsson et al., 1993; Kim and MacDonald, 2002). PDX-1 is first observed in the mouse around E8.5 in the dorsal endoderm while the gut is still an open tube. At E9.5, PDX-1 expression marks the dorsal and ventral pancreas buds and the endoderm-derived component of the duodenum (Miller et al., 1994; Guz et al., 1995). By E11.5, PDX-1 expression has expanded from the pancreas and duodenum to also include the posterior part of the stomach (Stoffers et al., 1999). At later stages of development, when the exocrine pancreas appears and hormone-producing cells are clustered in the islets, PDX-1 expression is restricted to the endocrine compartment where it plays a critical role in the transcriptional regulation of the insulin gene (Ohlsson et al., 1993; Guz et al., 1995). In the adult, PDX-1 expression is maintained in the duodenal epithelium and in insulin-secreting β-cells in the islets (Hui and Perfetti, 2002). Mice lacking *Pdx-1* display pancreatic agenesis and have defects throughout the posterior foregut region, including a distorted gastro-duodenal junction, loss of Brunner's gland and deficiency of enteroendocrine differentiation in the stomach and duodenum (Larsson et al., 1996; Offield et al., 1996; Jepeal et al., 2005). Loss of PDX-1 function also results in pancreatic agenesis in human (Stoffers et al., 1997).

The GATA family of zinc finger transcription factors is also involved in the specification and differentiation of the endoderm (Rehorn et al., 1996; Zhu et al., 1998; Reiter et al., 2001; Patient and McGhee, 2002; Afouda et al., 2005; Watt et al., 2007). In vertebrates, three members of this family, GATA4, 5, and 6 directly bind and activate endoderm genes, including *gastric* H^+/K^+*-ATPase*, *albumin*, and *HNF4* (Mushiake et al., 1994; Nishi et al., 1997; Morrisey et al., 1998; Cirillo et al., 2002). *Gata4* is expressed in the primitive endoderm, heart, liver, small intestine, gastric epithelium and gonads (Arceci et al., 1993; Molkentin, 2000). *Gata4* is also expressed at the onset of mouse pancreas development and restricts to the exocrine pancreas as development proceeds (Ketola et al., 2004; Decker et al., 2006). *Gata4* expression is observed in the duodenum and jejunum but is undetectable in the ileum; this gradient of GATA4 along the anterior-posterior axis is important for the maintenance of regional identities along the gastrointestinal tract of adult mice (Bosse et al., 2006). In the adult duodenum, GATA4 directly activates the *adenosine deaminase* gene via a duodenum-specific enhancer, which is also activated by the homedomain transcription factor PDX-1 (Dusing et al., 2001; Dusing et al., 2003). In the adult stomach, *Gata4* is expressed in the glandular portion, which comprises the distal two-third of the stomach (Jacobsen et al., 2002). In this region, known as the hind stomach, thousands of mucosal invaginations produce mucus and digestive enzymes (Nomura et al., 1998; Larsson, 2000). *Gata4* deficiency has been associated with impaired differentiation into glandular epithelium in the developing stomach and intestine (Jacobsen et al., 2002; Jacobsen et al., 2005).

Inactivation of *Gata4* in the germline of mice leads to embryonic lethality around E10 as a result of severe defects in extraembryonic endoderm, and *Gata4*-null mice display defects in heart and foregut morphogenesis (Kuo et al., 1997; Molkentin et al., 1997). The use of tetraploid complementation to overcome the early lethality in *Gata4*$^{-/-}$ embryos revealed additional roles for GATA4 in mouse endoderm development (Watt et al., 2007). In *Gata4*$^{-/-}$ ES cell-derived embryos, the liver bud fails to expand and the ventral pancreas is absent (Watt et al., 2007). Similarly, an earlier study conducted using morpholino knockdown of *Gata4* in the zebrafish demonstrated an absolute requirement of *Gata4* for the formation of multiple endoderm-derived organs, including the liver and exocrine pancreas (Holtzinger and Evans, 2005). In addition to its requirement for endoderm-derived organ development, GATA4 also plays a later role in the function of the endoderm-derived small intestine by regulating jejunal

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morphology and expression of genes involved in lipid metabolism in adult mice (Battle et al., 2008)

Despite the importance of GATA4 in endoderm-derived organ development and gene regulation, the transcriptional regulation of the *Gata4* gene itself in the endoderm and its derivative organs has not been defined. In the work described here, we identified an enhancer from the mouse *Gata4* gene that directs expression to the foregut in transgenic embryos. As development proceeds, this novel *Gata4* endoderm enhancer directs robust expression in the regions of the gut that will give rise to the stomach and to the dorsal and ventral pancreases. Later in development, the activity of this endoderm-specific regulatory element becomes restricted to the glandular stomach, pancreatic exocrine acinar cells, and duodenal crypts. We show that the *Gata4* endoderm enhancer contains a conserved, tandem homeodomain transcription factor (HOX) binding site and a perfectly conserved consensus GATA site. We show that these *cis*-acting elements are bound efficiently by PDX-1 and GATA4, respectively. Importantly, we show that the PDX/HOX and GATA binding sites are each independently required for enhancer activity in transgenic embryos. Thus, these studies identify *Gata4* as a direct transcriptional target of homedomain and GATA transcription factors *in vivo* and support a feed-forward transcriptional model for *Gata4* regulation in the endoderm and its derivatives in the pancreas, stomach, and duodenum.

RESULTS

A novel *Gata4* endoderm-specific enhancer directs expression to the foregut and midgut *in vivo*

To identify regulatory elements that control *Gata4* expression *in vivo*, we compared the sequences of the mouse, human, and opossum *Gata4* loci using BLAST and VISTA analyses (Altschul et al., 1990; Mayor et al., 2000). The comparison showed at least nine major regions of conservation in non-coding sequences, referred to as G1–G9, within the *Gata4* locus (Fig. 1). Because these sequences have been conserved since placental and marsupial mammals diverged approximately 150 million years ago (Graves, 1996), we hypothesized that one or more of these sequences might be important regulatory modules. To test this notion, we cloned each of these regions into the transgenic reporter plasmid *Hsp68-lacZ* (Kothary et al., 1989) and tested for enhancer activity in transgenic mouse embryos. These studies identified several unique and highly specific transcriptional enhancers, including a previously described lateral mesoderm enhancer regulated by Forkhead and GATA transcription factors (Rojas et al., 2005).

We also identified a unique endoderm enhancer from the mouse *Gata4* locus, which we refer to as *Gata4* G8. The *Gata4* G8 genomic fragment spans 3541 bp and is located approximately 80 kb upstream of the *Gata4* transcriptional start site (Fig. 1). At E9.5, *Gata4* G8 directed *lacZ* expression to the ventral and dorsal foregut, midgut (Fig. 2A–C, E), and yolk sac (data not shown). β-galactosidase expression was also observed in the foregut-derived ventral pancreatic bud entering the septum transversum just below the developing heart (Fig. 2B, arrowhead). The expression of endogenous GATA4 protein expression completely overlapped the expression of the *Gata4-G8-lacZ* transgene in the foregut and midgut at E9.5, although the expression of endogenous GATA4 was broader than transgene expression (Fig. 2, compare panels C, E to panels D, F). The broader expression of endogenous GATA4 likely reflects control of the *Gata4* gene by additional modular transcriptional enhancers, which control expression in distinct subsets of the developing gut mesoderm and endoderm.

By E11.5, the broad pattern of *Gata4* G8 enhancer activity became restricted to the epithelium of the stomach, with the strongest expression in the hind stomach, and to the nascent pancreatic epithelium (Fig. 2G, I). The epithelia of the hind stomach and the pancreas are marked by the

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expression of PDX-1 at E11.5 (Ashizawa et al., 2004), and β-galactosidase expression directed by *Gata4* G8 overlapped PDX-1 positive cells almost perfectly at that stage (Fig. 2I, J). Importantly, no *Gata4-G8-lac*Z transgene expression was observed in the mesenchyme of these developing organs, suggesting that the activity of the enhancer is endoderm-specific. By E13.5, expression directed by the *Gata4* G8 endoderm-specific enhancer appeared only in the glandular (hind) stomach and was absent in the anterior portion of the stomach, (Fig. 2K), which corresponded almost precisely to endogenous GATA4 protein expression in the stomach (Fig. 2L). At this stage, *Gata4* G8-directed β-galactosidase expression was also very robust in the branching epithelial tree of the dorsal and ventral pancreases (Fig. 2H, M), and comparison of immediately adjacent sections stained for expression of endogenous GATA4 and PDX-1 proteins showed that the pattern of β-galactosidase expression overlapped with endogenous GATA4 almost perfectly (Fig. 2N). β-galactosidase expression also overlapped the expression PDX-1 protein (Fig. 2O), which is important since PDX-1 is a marker of endoderm-derived pancreatic epithelium at this stage (Ashizawa et al., 2004).

Gata4 G8 endoderm enhancer activity restricts to the gastric epithelium, duodenal crypts, and pancreatic acinar cells

At E18.5, the activity of the *Gata4* endoderm enhancer remained robust in the epithelium of the glandular stomach, which again, tightly mirrored endogenous GATA4 expression (Fig. 3A, B). Indeed, expression of both *Gata4-G8-lacZ* and endogenous GATA4 could be seen throughout the epithelial invaginations in the hind stomach but not in the underlying gastric mesenchyme (Fig. 3A, B). Similarly, transgene-directed β-galactosidase expression in the duodenum was restricted to the endoderm component and tightly overlapped endogenous GATA4 expression in this intestinal compartment (Fig. 3C, D).

By later stage in fetal development, the pancreas has developed clearly distinct endocrine and exocrine compartments (Kim and MacDonald, 2002). The exocrine pancreas is marked by the expression of acinar cell digestive enzymes, such as amylase. In contrast, differentiation of the endocrine pancreas results in a large increase in the number of hormone-producing cells (Kim and MacDonald, 2002). PDX-1 becomes restricted to a subset of exocrine acinar cells and endocrine cells during fetal development and its expression becomes progressively more restricted to insulin-producing β-cells (Ashizawa et al., 2004). By E18.5, strong expression of PDX-1 can be seen in β-cells, while only low levels are detectable in the nuclei of acinar cells (Ashizawa et al., 2004). Therefore, we examined the overlap of *Gata4* G8-directed expression in the pancreas with endogenous GATA4 and with markers of the endocrine and exocrine pancreases. Endogenous GATA4 protein and *Gata4-G8-lacZ* transgene expression within the pancreas was confined to the exocrine acinar cells, marked by the expression of amylase (Fig. 3E, G). By contrast, neither GATA4 nor β-galactosidase protein was detected in insulin-producing cells (Fig. 3E, F; arrowheads). Importantly, this expression pattern essentially mirrored the expression of endogenous GATA4 protein within the pancreas at this stage (Fig. 3F). PDX-1 protein expression began to become restricted to islets within the pancreas at E18.5 (Fig. 3H, arrowheads), although some expression of PDX-1 was still observed within acinar cells (Fig. 3H, arrows). The reduced expression of PDX-1 in acinar cells coincided with the weaker expression of *Gata4-G8-lacZ* compared to earlier stages in development, hinting at a possible regulatory relationship between PDX-1 and *Gata4*.

Importantly, the overlap of the *Gata4-G8-lacZ* transgene expression with endogenous GATA4 protein expression throughout the development of the gut and pancreas strongly supports the notion that the *Gata4* G8 enhancer is a *bona fide* regulatory element controlling expression of *Gata4*. In addition, the overlap of *Gata4* G8 enhancer activity with PDX-1 expression, suggested the possibility that *Gata4* might be regulated in a common transcriptional pathway

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with the homeodomain protein PDX-1 and that this molecular circuitry might function *via* the G8 enhancer.

The *Gata4* endoderm enhancer contains deeply conserved HOX and GATA binding sites

As a first step toward defining a transcriptional pathway upstream of *Gata4* in the endoderm, we determined the minimal region of the G8 enhancer sufficient to direct endoderm-specific expression. The G8 enhancer contains two regions that are conserved between human, mouse, and opossum, denoted CR1 and CR2 (Fig. 4A). We deleted the majority of the 5′ end of the G8 enhancer, including the CR1 conserved region, to generate a smaller fragment, referred to as G8[890], which contains 890 bp from the 3′ end of G8, extending from nucleotides 2652 to 3541. We examined the ability of G8[890], which includes the entire CR2 conserved region, to direct expression in transgenic embryos. At E9.5, this smaller construct directed robust *lacZ* expression to the ventral and dorsal foregut, as well as the midgut, in a pattern identical to full-length G8 (Fig. 4B, C). At E11.5, *Gata4-G8[890]-lacZ* expression was restricted to the stomach and pancreas (data not shown), which was also consistent with the expression of the larger *Gata4-G8-lacZ* transgene. A fragment lacking the CR2 region, but encompassing all of CR1 showed no activity in transgenic embryos (data not shown). These results demonstrate that G8[890] is sufficient to direct expression to the developing gut at E9.5 and to the stomach, ventral and dorsal pancreases, and duodenum at E11.5. These observations suggest that the minimal, essential *cis*-regulatory elements of this *Gata4* endoderm enhancer reside within CR2.

We examined CR2 for candidate transcription factor binding sites and for cross-species conservation. Interestingly, CR2 contains two overlapping candidate HOX binding sites (2x HOX) and one candidate GATA transcription factor binding site (Fig. 4D). Because the expression pattern of *Gata4-G8-lacZ* largely overlapped the expression of PDX-1 from the onset of foregut formation through pancreas, stomach, and duodenum development, we reasoned that PDX-1 might bind to the *Gata4* enhancer via the overlapping HOX sites contained within G8 CR2. To test this possibility, we examined the ability of PDX-1 protein to bind to the HOX sites in the *Gata4* endoderm enhancer by EMSA (Fig. 5A). PDX-1 bound efficiently to a probe encompassing the two overlapping HOX sites in *Gata4* G8 (Fig. 5A, lane 8). Binding was specific because it was competed by excess unlabeled control HOX site (Wang et al., 2004) and by excess unlabeled G8 2x HOX self-probe (Fig 5A, lanes 9, 11). By contrast, binding of PDX-1 to the *Gata4* G8 2x HOX site was not competed by unlabeled mutant versions of the PDX-1 control site or the *Gata4* HOX sites (Fig. 5A, lanes 10, 12). In addition, the *Gata4* G8 2x HOX site efficiently competed for PDX-1 binding to a control PDX site from the *lactase* gene promoter (Wang et al., 2004; Fig. 5A, lane 5), further confirming the ability of the *Gata4* G8 2x HOX site to be bound efficiently by PDX-1.

In addition to PDX-1 binding to the 2x HOX site in the enhancer, GATA4 bound to the G8 enhancer via the conserved GATA site (Fig. 5B, lane 8). Binding of GATA4 to the GATA site in the *Gata4* endoderm enhancer was specific since binding was efficiently competed by excess unlabeled GATA control probe (Rojas et al., 2005;Fig. 5B, lane 9) and by excess unlabeled self-probe (Fig. 5B, lane 11) but not by mutant versions of the control or *Gata4* CR2 GATA probes (Fig. 5B, lanes 10, 12). The *Gata4* G8 GATA site also efficiently competed for GATA4 binding to the *bona fide* GATA binding site from the *Gata4* G2 lateral mesoderm enhancer (Rojas et al., 2005; Fig. 5B, lane 5) but a mutant version of the G8 GATA site did not compete for binding to the control GATA site (Fig. 5B, lane 6). Thus, the results presented in Fig. 5 demonstrate that the HOX and GATA sites in the *Gata4* G8 endoderm enhancer are efficiently and specifically bound *in vitro* by PDX-1 and GATA4, respectively.

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Gata4 endoderm enhancer function is dependent on HOX and GATA binding sites *in vivo*

To test the requirement of the HOX and GATA sites in G8 for enhancer activity *in vivo*, we generated transgenic embryos harboring *Gata4-G8-lacZ* transgenes with mutations in either the 2x HOX or the GATA sites in the context of the full-length G8 fragment (Fig. 6). The mutations were identical to those used in the EMSA analyses, where they mutations abolished PDX-1 and GATA4 binding (Fig. 5). At E11.5, the wild-type *Gata4-G8-lacZ* transgene directed robust expression to the epithelium of the stomach and pancreas (Fig.6A, B). In contrast, mutation of the two tandem HOX sites in the enhancer resulted in a complete disruption of enhancer activity (Fig. 6C, D). Similarly, mutation of the GATA site abolished enhancer activity (Fig. 6E, F). These data demonstrate that the HOX and GATA sites in CR2 are required for *Gata4* G8 endoderm enhancer function *in vivo*. Taken together with the data from the EMSA in Fig. 5, these observations suggest that *Gata4* is a transcriptional target of PDX-1 and GATA factors via direct binding to the *Gata4* G8 endoderm enhancer.

DISCUSSION

A model for pancreas development involving FoxA2, GATA4, and PDX-1

The formation of organ primordia along the length of the developing gut tube requires sub-specification of the early definitive endoderm and the proper activation of several overlapping but discrete transcriptional programs in the developing embryo (Kumar and Melton, 2003; Servitja and Ferrer, 2004). Precisely how this is achieved remains unclear, but several key regulators have been identified as playing important roles in endoderm development. Among these, *Gata4* is one of the earliest markers of the endoderm and regulates the proliferation and differentiation of many endoderm-derived cell types (Arceci et al., 1993; Heikinheimo et al., 1994; Maduro and Rothman, 2002; Nemer and Nemer, 2003; Heicklen-Klein and Evans, 2004; Murakami et al., 2005; Zorn and Wells, 2007). In the mouse, inactivation of *Gata4* causes cardia bifida due to severe defects in endoderm differentiation and migration (Kuo et al., 1997; Molkentin et al., 1997), and GATA4 has been implicated in the development of several endoderm-derived organs (Rehorn et al., 1996; Zhu et al., 1998; Reiter et al., 2001). Similarly, the Forkhead transcription factor FoxA2 is an essential regulator of endoderm development through its role as a pioneer factor capable of reconfiguring chromatin to an active state (Cirillo et al., 1998). Interestingly, FoxA2 functions cooperatively with GATA4 to define the competency of the endoderm to adopt a hepatic fate (Bossard and Zaret, 1998; Zaret, 1999; Cirillo et al., 2002). However, *Gata4* and *Foxa2* are co-expressed broadly throughout the endoderm, suggesting that other factors may be important to restrict or refine the activity of FoxA2 and GATA4 to give rise to different organ primordia (Zaret, 2008). In this regard, PDX-1 appears to be the critical factor for promoting a pancreatic fate in the early definitive endoderm (Miller et al., 1994; Guz et al., 1995; Larsson et al., 1996; Offield et al., 1996; Stoffers et al., 1997). An attractive model for pancreatic development would involve FoxA2 and GATA4 functioning cooperatively to reposition nucleosomes in the *Pdx1* locus only in regions of the developing gut that also receive additional spatially-restricted signals. This would allow GATA4 and FoxA2 to promote pancreatic gene expression, followed by amplification of the program via PDX-1 activation of *Gata4* and possibly *Foxa2* as well.

Our EMSA data (Fig. 5) clearly demonstrate that GATA4 and PDX-1 bind to their respective sites in the *Gata4* G8 enhancer, and our mutagenesis data show that the GATA and HOX (PDX) sites in the *Gata4* G8 enhancer are absolutely required for enhancer function *in vivo* (Fig. 6). Together, these observations support our model for PDX-1/GATA-dependenent regulation of *Gata4* in the endoderm and its derivatives. The very strong overlap in the expression directed by the *Gata4* G8 enhancer and PDX-1 and GATA4 further support this notion. However, it remains possible that other transcription factors, in addition to or in lieu of, GATA4 and PDX-1 may regulate the *Gata4* G8 enhancer *in vivo* via the essential *cis*-acting elements described in

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these studies. Taken together, however, the data presented here suggests that *Gata4* expression is activated and maintained by the combined activities of PDX-1 and GATA4 via essential binding sites within the *Gata4* G8 enhancer and are consistent with a feed-forward model for endoderm differentiation. Alternatively, *Gata4* expression may be activated by other GATA factors, such as GATA6, which is also expressed in the developing pancreas at early stages (Decker et al., 2006). It will be interesting to determine whether GATA4 and other GATA factors may also contribute to pancreas specification and differentiation via direct activation of *Pdx1* and other endoderm- and pancreas-restricted transcription factor genes.

Modular regulation of *Gata4* by multiple, independent enhancers

During embryonic development, *Gata4* is expressed in multiple tissues with dynamic and distinct patterns of expression in different cell lineages (Arceci et al., 1993; Molkentin, 2000). The *Gata4* endoderm enhancer described here directs expression throughout the foregut and midgut at early stages of development but becomes restricted to the epithelia of pancreas, stomach, and duodenum as development proceeds (Fig. 2, Fig 3). This type of progressive restriction from an initially broad pattern to a narrow pattern may be a common theme in the regulation of enhancers present in the *Gata4* gene, as a similar phenomenon was observed in the expression directed by the previously identified *Gata4* G2 mesoderm enhancer (Rojas et al., 2005). The rapid restriction of enhancer activity suggests that the activators of the G8 enhancer may also become restricted as cells within the endoderm differentiate and adopt a more restricted fate. Alternatively, the restriction of *Gata4* enhancer activity might be explained by the action of repressors that impair the activation of the enhancer in some cell types.

The activity of the *Gata4* G8 enhancer is restricted to a subset of the endogenous *Gata4* expression pattern, suggesting that other *Gata4* enhancers direct expression to other endoderm and mesoderm derivatives where the *Gata4* gene is normally expressed. Our previous identification of a discrete *Gata4* enhancer that recapitulates endogenous GATA4 protein expression in the lateral mesoderm and its derivates in the liver mesenchyme supports this notion (Rojas et al., 2005). It is likely that additional modular enhancers from the *Gata4* locus will regulate *Gata4* expression in the mesoderm and endoderm. Indeed, we have recently identified an additional endoderm enhancer from *Gata4* with distinct spatiotemporal activity compared to the G8 enhancer described here (AR and BLB, unpublished observations). Understanding how the activity of these different modular regulatory elements are integrated and controlled during development will be an important area for future studies to define the precise regulation of the *Gata4* gene *in vivo*.

EXPERIMENTAL PROCEDURES

Cloning and mutagenesis

The 3541-bp G8 fragment of the mouse *Gata4* gene was generated by PCR using the following two primers: 5′-caggcaccactcgagttctggaaac-3′ and 5′-gtttctctttcccgggacctctggaag-3′. This fragment was then cloned as an XhoI-XmaI fragment into the transgenic reporter plasmid *Hsp68-lacZ* (Kothary et al., 1989) to create plasmid *Gata4-G8-Hsp68-lacZ*. The following mutant sequences were created in the context of the G8 transgene: m-HOX, 5′-gttcacaggacacccactagtcaaacagttccc-3′; m-GATA 5′-ctttggtctcagcttaagaaactgccctg-3′. The sequence of each mutant fragment was confirmed by sequencing on both strands. The GenBank accession number for the sequence of the mouse *Gata4* G8 endoderm enhancer described in this manuscript is currently pending.

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Generation of transgenic mice

Transgenic reporter constructs were digested from the *Gata4-G8-Hsp68-lacZ* plasmid backbone with SalI to create *Gata4*-G8-*lac*Z and with SphI and SalI to create *Gata4-G8 [890]-lac*Z, gel purified, and suspended in 5 mM Tris-HCl, 0.2 mM EDTA, pH7.4, at a concentration of 2 μg/ml for pronuclear injection, as described previously (Dodou et al., 2003). Injected embryos were implanted into pseudopregnant CD-1 females, and embryos were collected at indicated developmental stages for transient analyses 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 from mice by digestion in tail lysis buffer (100 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate, 10 mM Tris-Cl, 200 μg/ml proteinase K, pH 8.0) at 56°C overnight. Digested samples were extracted once with phenol-chloroform and ethanol precipitated. The presence of the *lacZ* transgene was detected by Southern blot. All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

X-gal staining, H&E and immunohistochemistry

β-galactosidase activity in *lacZ* transgenic embryos or tissues was detected by X-gal staining, which was performed as described previously (Dodou et al., 2003). 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). For immunohistochemistry, sections were dewaxed, incubated in PBS for 5 min, boiled in antigen retrieval solution (Biogenex), and blocked in 3% normal goat serum for 1 h. Incubation with the following primary antibodies was done overnight at 4°C in a humid chamber at a 1:300 dilution: rabbit anti β-galactosidase (ICN), rabbit anti-amylase (Sigma), goat anti-GATA4 (Santa Cruz Biotechnologies), guinea pig anti-insulin (Linco), and rabbit anti-PDX-1 (kind gift of M. German, UCSF). Following incubation with primary antibodies, sections were washed three times with PBS and incubated with appropriate secondary antibodies, including Alexa Fluor 488 donkey anti-goat (Invitrogen), Alexa Fluor 594 donkey anti-rabbit (Invitrogen), Oregon Green 488 goat anti-rabbit (Invitrogen), andCyanine 5 (Cy5)-conjugated anti-guinea pig (Invitrogen) at 1:300 dilutions in 3% normal goat serum at room temperature for 1 h. Slides were then washed three times in PBS, mounted using SlowFade Light antifade (Molecular Probes) and photographed on a fluorescence microscope. Immunoperoxidase staining was performed using the Vectastain Elite ABC kit and developed using the peroxidase substrate DAB (Vector Laboratories).

Electrophoretic mobility shift assay (EMSA)

DNA binding reactions were performed as described previously (Dodou et al., 2003). Briefly, double-stranded oligonucleotides were labeled with [^{32}P]-dCTP, using Klenow to fill in the overhanging 5′ ends, and then purified on a nondenaturing polyacrylamide-TBE gel. Binding reactions were pre-incubated at room temperature in 1X binding buffer (40mM KCl, 15mM HEPES [pH 7.9], 1mM EDTA, 0.5 mM DTT, 5% glycerol) containing recombinant protein, 1.5 μg of poly-dI:dC and competitor DNA for 10 min prior to probe addition. Reactions were incubated for an additional 20 min at room temperature after probe addition and were then electrophoresed on a 6% nondenaturing polyacrylamide gel.

The *Gata4* and *Pdx-1* cDNAs were transcribed and translated using the TNT Coupled Transcription-Translation System (Promega), as described in the manufacturer's directions. GATA4 protein was generated from pCITE-GATA4 plasmid, which has been described previously (Dodou et al., 2004). PDX-1 protein was generated from pBat-PDX-1 plasmid (kindly provided by M. German, UCSF) using T7 polymerase. The sense strand sequences of the mouse *Gata4* G8 GATA and HOX sites used for EMSA were: GATA: 5′-ggctttggtctcagcttatcaaactgccctg-3′; HOX: 5′-ggcaggttcacaggacacccattaatcaaacagttccc-3′. The sense strand sequences of the GATA and HOX mutant sites were the same as for the mutagenic

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primers described above. The GATA control site used here was the gata I site from the *Gata4* G2 lateral mesoderm enhancer, which has been described previously (Rojas et al., 2005). The PDX control site from the rat *lactase* gene has also been described previously (Wang et al., 2004).

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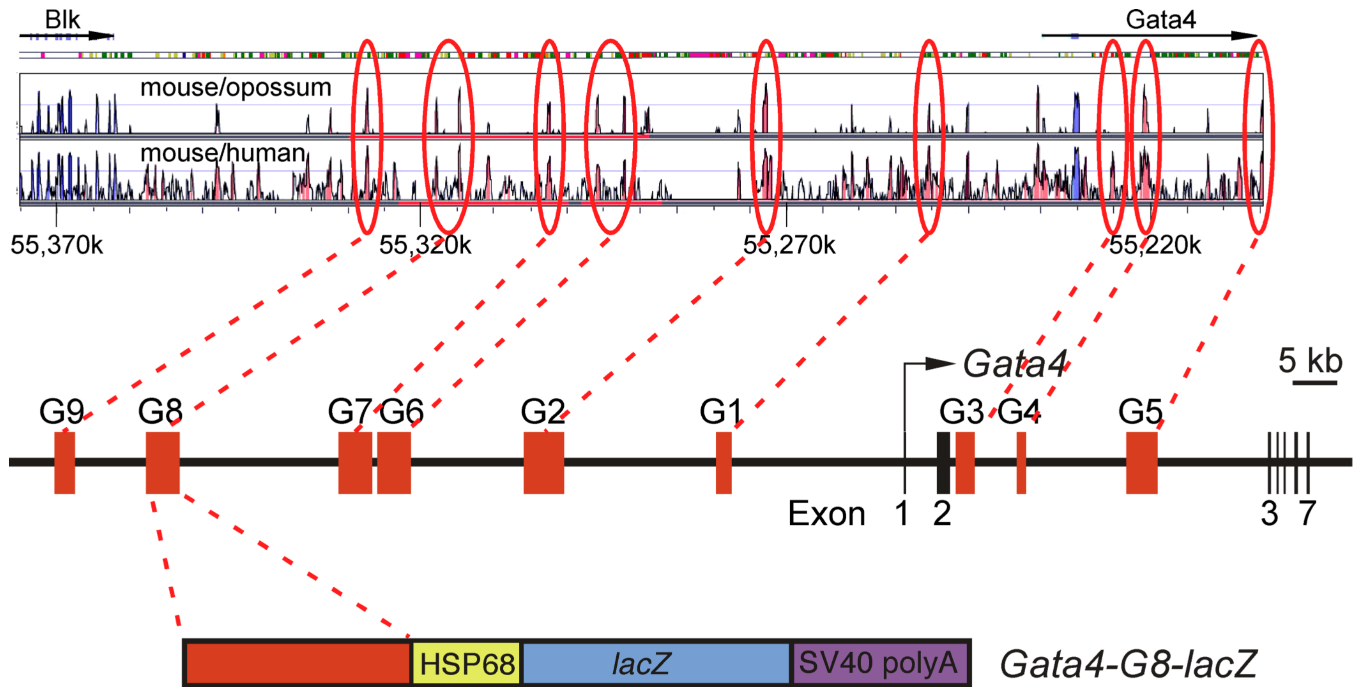

Fig.1.

VISTA plot and schematic representation of the mouse *Gata4* locus and the *Gata4-G8-lac*Z transgene. The top of the figure shows VISTA plots (Mayor et al., 2000) comparing the sequence conservation between the mouse and opossum and the mouse and human *Gata4* loci. The red circled regions represent regions of the mouse *Gata4* locus that were tested for enhancer activity in transgenic embryos, including the G8 region, which is the foucus of the present study. The horizontal line in the middle of the figure represents the mouse *Gata4* locus. Exons are depicted as vertical black lines. The bent arrow represents the transcriptional start. The red boxes (G1–G9) represent nine regions of non-coding sequence, which are highly conserved between the human, mouse, and opossum *Gata4* sequences. The lower line depicts the *Gata4-G8-lac*Z transgene.

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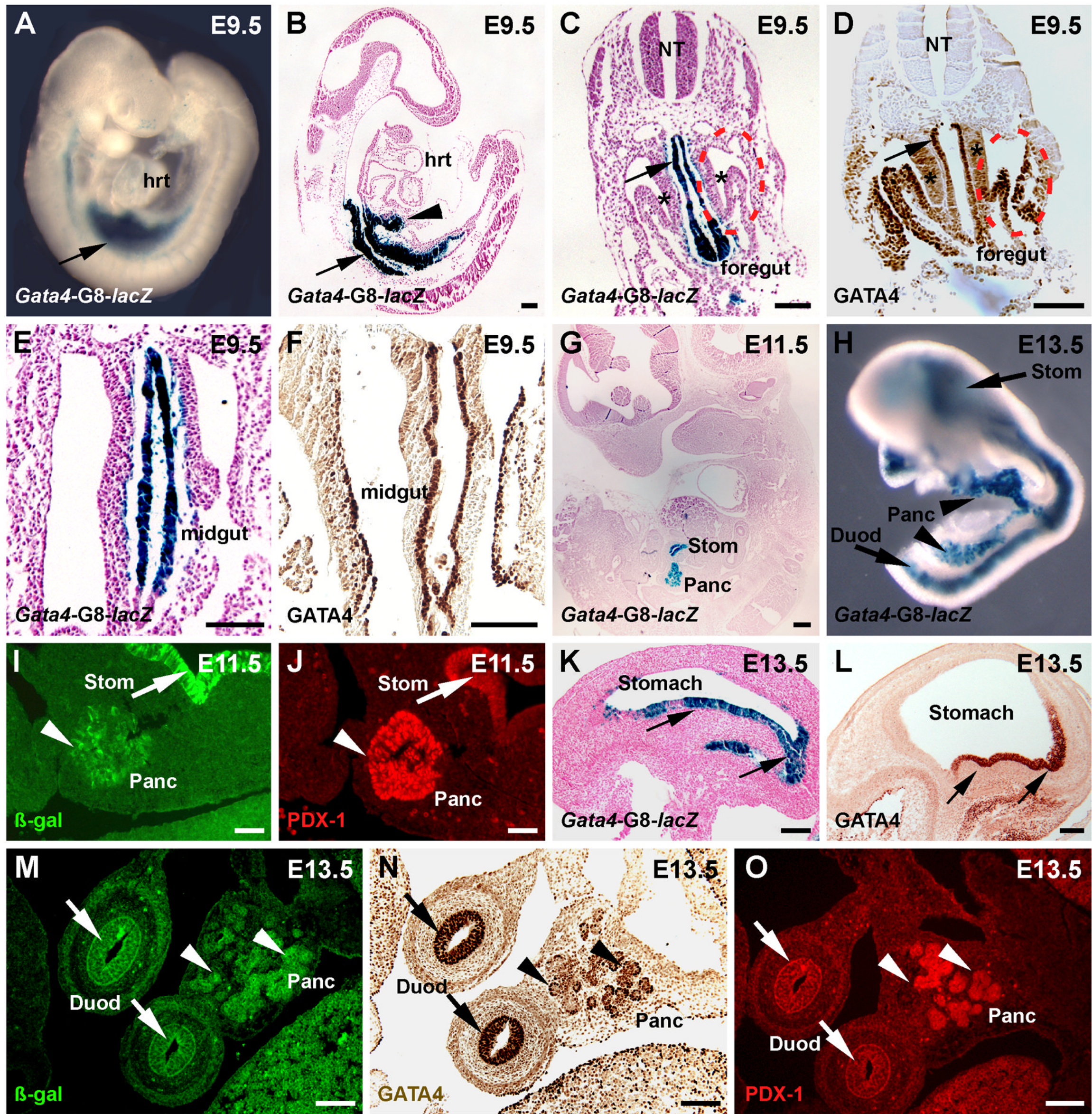

Fig.2.

The *Gata4-G8-lacZ* transgene is expressed in the foregut and midgut and its derivatives in the developing stomach, pancreas, and duodenum during mouse embryonic development. Whole mount (A, H), sagittal (B, G, K) and transverse (C, E) sections of X-gal-stained *Gata4-G8-lac*Z transgenic embryos or dissected organs from transgenic embryos are shown. For comparison, transverse (D, F, M, N, O) and sagittal (I, J, L) sections stained with α–β-galactosidase, α–GATA4, and α–PDX-1 antibodies are shown. Arrows mark the developing gut. The dashed lines and asterisks in (C, D) show regions of the gut mesoderm marked by endogenous GATA4 expression (D) but not by the activity of the modular *Gata4* G8 enhancer (C) in the corresponding region of the mesoderm. Arrowheads mark the nascent pancreatic epithelium. Expression of *Gata4-G8-lacZ* was confirmed at each stage in four independent transgenic lines, which showed nearly identical patterns of expression at all stages. Bars in all panels are equal to 100 μm. Expression of Duod, duodenum; hrt, heart; Panc, pancreas; Stom, stomach.

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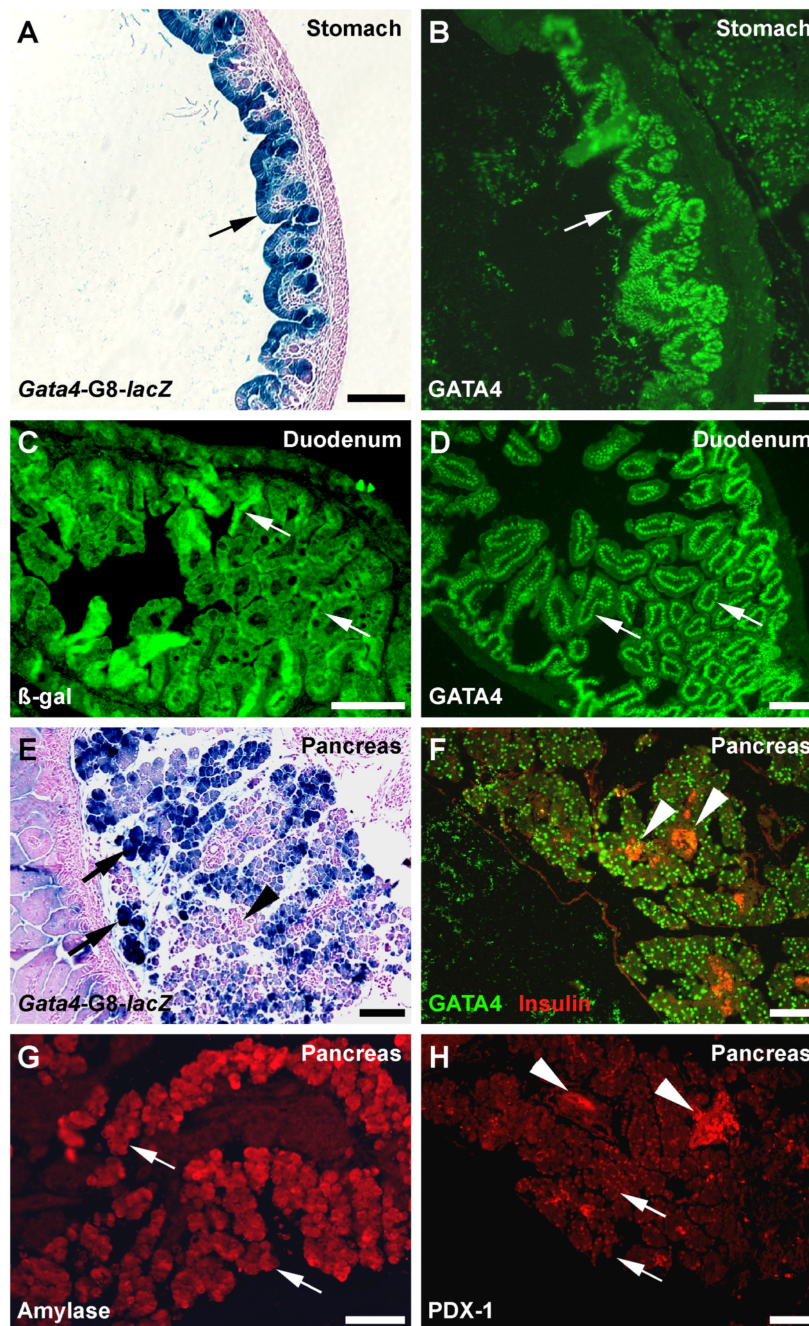

Fig.3.

Gata4 G8 endoderm enhancer activity is restricted to the glandular stomach, crypts of the duodenum, and pancreatic acinar cells at E18.5. Transverse sections of stomach (A, B), duodenum (C, D) and pancreas (E–H) were stained with X-gal (A, E), α–β-galactosidase (C), α–GATA4 antibody (B, D, F), α-Insulin antibody (F), α–amylase antibody (G), or α–PDX-1 antibody (H). *Gata4-G8-lacZ* transgene expression was apparent in the glandular stomach and crypts of the duodenum (arrows in A and C), which overlaps with the expression of the endogenous GATA4 (arrows in B and D). In the pancreas, expression was restricted to acinar cells (arrows in E, G, H), marked by amylase positive cells (G). Islets (arrowheads in E, F, H), marked by Insulin expression (F) and PDX-1 expression (H) were negative for β-galactosidase

expression (E). *Gata4-G8-lacZ* expression was nearly identical to the expression of endogenous GATA4, which was also restricted to acinar cells (green staining in F) and was excluded from the Insulin-producing cells and PDX-1-positive cells (arrowheads in F and H, respectively). Expression of *Gata4-G8-lacZ* was confirmed at this stage in four independent transgenic lines, which showed nearly identical patterns of expression. Bars in all panels are equal to 100 μm.

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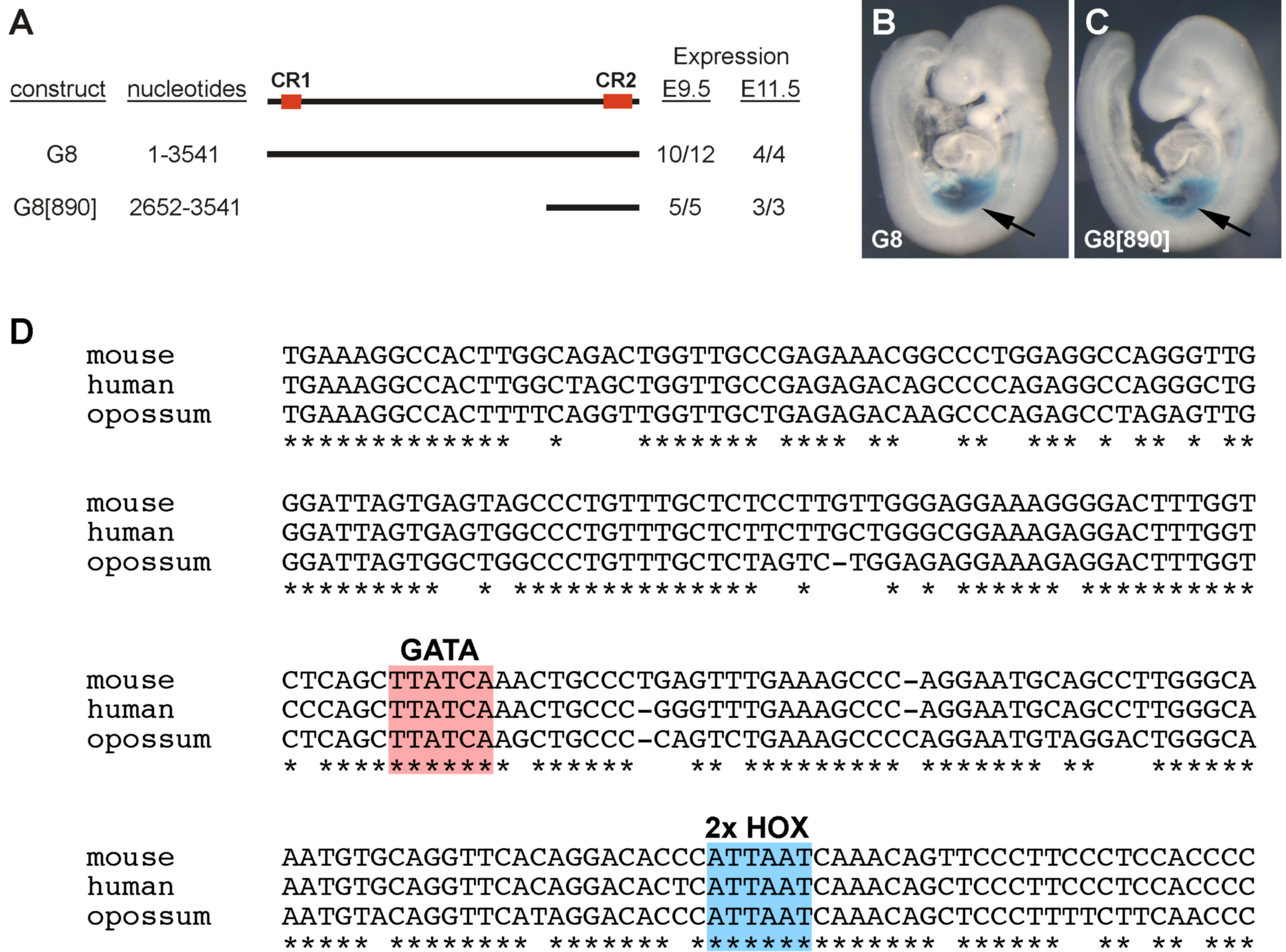

Fig.4.

The *Gata4* G8 endoderm enhancer contains a highly conserved region that is sufficient for enhancer activity *in vivo*. (A) The top line represents the genomic organization of the G8 enhancer. Red boxes represent two regions of high conservation between the human, mouse, and opossum *Gata4* sequences, denoted CR1 and CR2. Schematic diagram of the G8 enhancer and a deletion construct G8[890]. The nucleotide positions of the G8 and G8[890] are denoted to the left. The column on the right indicates the number of independent transgenic lines or F0 embryos that expressed *lacZ* in the primitive gut at E9.5 and in the stomach, pancreas, and duodenum at E11.5 as a fraction of the total number of transgene-positive embryos. (B, C) Representative, X-gal-stained G8 and G8[890] transgenic embryos. The G8 and G8[890] constructs directed robust expression in the developing gut (arrows). (D) ClustalW analysis of the *Gata4* G8 CR2 sequence, comparing the mouse, human, and opossum sequences. CR2 contains two conserved, overlapping candidate HOX binding sites (2x HOX, blue box) and one conserved, candidate GATA binding site (red box). Asterisks denote nucleotides that have been perfectly conserved among the three species.

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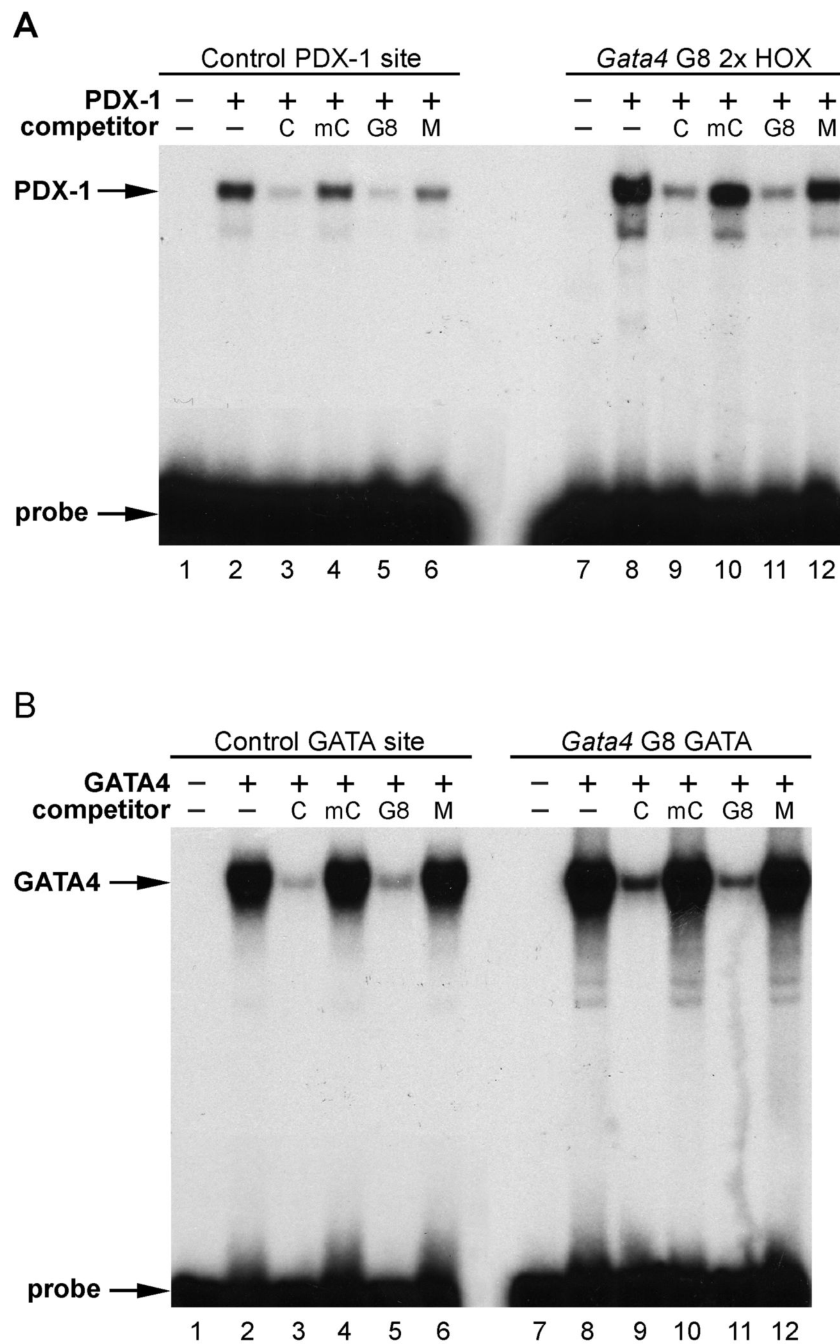

Fig.5.

The *Gata4* G8 endoderm enhancer is bound by PDX-1 and GATA4. (A) Recombinant PDX-1 protein was used in EMSA with a radiolabeled, double-stranded oligonucleotide probe encompassing a canonical PDX-1 binding site from the promoter of the *lactase* gene (lanes 1–6) or the *Gata4* G8 2x HOX site (lanes 7–12). Lanes 1 and 7 contain reticulocyte lysate without recombinant PDX-1 (represented by a minus sign). PDX-1 efficiently bound to the control PDX site and to the *Gata4* G8 2x HOX site (lanes 2, 8). Binding was competed by excess unlabeled control site (C, lanes 3 and 9) or unlabeled *Gata4* 2x HOX site (G8, lanes 5 and 11), but not by an excess of unlabeled mutant control site (mC, lanes 4 and 10) or mutant G8 2x HOX site (M, lanes 6 and 12). (B) Recombinant GATA4 protein was used in EMSA with a

radiolabeled double-stranded oligonucleotide probe representing a canonical GATA binding site from the *Gata4* G2 lateral mesoderm enhancer (lanes 1–6) or the *Gata4* G8 GATA site (lanes 7–12). Lanes 1 and 7 contain reticulocyte lysate without recombinant GATA4 protein. GATA4 efficiently bound to the GATA control site and to the *Gata4* G8 GATA site (lanes 2, 8). In both cases, binding was competed by excess, unlabeled control GATA site (C, lanes 3 and 9) or unlabeled *Gata4* G8 GATA site (G8, lanes 5 and 11), but not by excess unlabeled mutant control GATA site (mC, lanes 4 and 10) or an unlabeled mutant version of the *Gata4* G8 GATA site (M, lanes 6 and 12).

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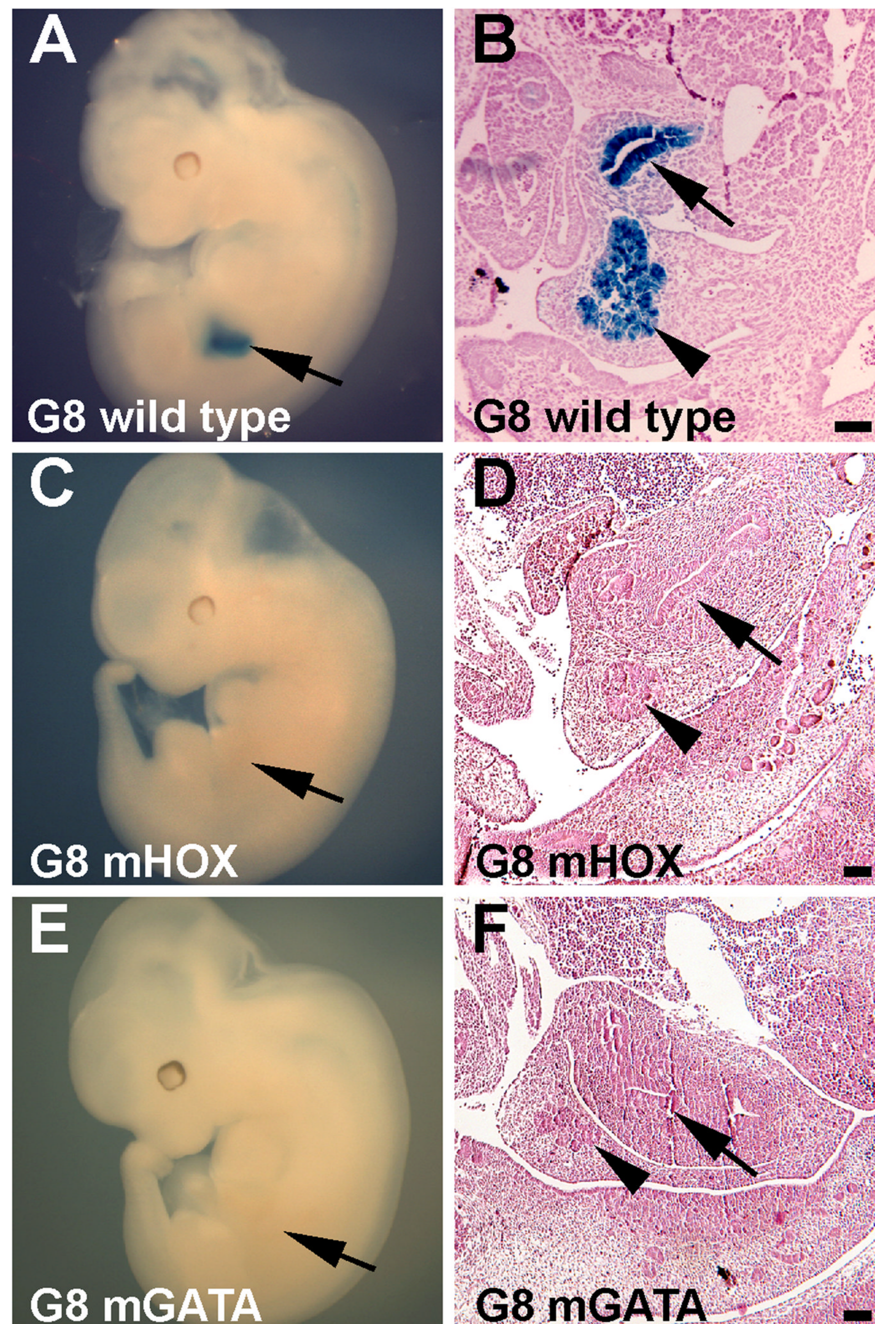

Fig.6.

The *Gata4* G8 endoderm enhancer is dependent on conserved HOX and GATA sites for activity *in vivo*. Wild-type and mutant versions of the *Gata4-G8-lacZ* transgenes were used to generate transgenic embryos. Representative X-gal-stained whole mount (A, C, E) or sagittally sectioned (B, D, F) transgenic embryos at E11.5 are shown. The wild-type construct directed strong expression in the stomach (A, B, arrow) and in the nascent pancreatic epithelium (B, arrowhead) in four independent transgenic lines. Mutation of the 2x HOX site in the *Gata4* G8 enhancer (mHOX) abolished the expression in all five independently generated embryonic transgenic lines analyzed (C, D). Similarly, mutation of the *Gata4* G8 GATA site completely

eliminated transgene expression in all four independently-generated embryonic transgenic lines analyzed (E, F). The bars in panels B, D, and F are equal to 100 μm.

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