Cdx4 is required in the endoderm to localize the pancreas and limit β-cell number

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Cdx transcription factors have crucial roles in anteroposterior patterning of the nervous system and mesoderm. Here we focus on the role of cdx4 in patterning the endoderm in zebrafish. We show that cdx4 has roles in determining pancreatic β-cell number, directing midline convergence of β-cells during early pancreatic islet formation, and specifying the anteroposterior location of foregut organs. Embryos deficient in cdx4 have a posteriorly shifted pancreas, liver and small intestine. The phenotype is more severe with knockdown of an additional Cdx factor, cdx1a. We show that cdx4 functions within the endoderm to localize the pancreas. Morpholino knockdown of cdx4 specifically in the endoderm recapitulates the posteriorly shifted pancreas observed in cdx4 mutants. Conversely, overexpression of cdx4 specifically in the endoderm is sufficient to shift the pancreas anteriorly. Together, these results suggest a model in which cdx4 confers posterior identity to the endoderm. Cdx4 might function to block pancreatic identity by preventing retinoic acid (RA) signal transduction in posterior endoderm. In support of this, we demonstrate that in cdx4-deficient embryos treated with RA, ectopic β-cells are located well posterior to the normal pancreatic domain.

KEY WORDS: Cdx4, Cdx1a, Retinoic acid, Pancreas, β-cell, AP patterning

INTRODUCTION

In recent years, many molecular signals have been identified that are crucial for the early steps of pancreas development. We currently understand pancreatogenesis as a process in which mesodermal signals elicit a program of differentiation in the adjacent endoderm. Known signals include FGFs, BMPs, retinoic acid (RA), activins and Sonic hedgehog (Shh) (Cano et al., 2007). A variety of studies has shown that the timing and location of these signals must be tightly controlled for correct pancreas development along the anteroposterior (AP) axis. For example, in zebrafish, treatment with exogenous RA produces anterior ectopic β-cell differentiation (Stafford and Prince, 2002), and disinhibition of BMP signaling enlarges the pancreatic domain, whereas Bmp2b deficiency reduces the pancreatic domain (Tiso et al., 2002). In mice, pancreas development requires inhibition of Shh signals from the region of the notochord that overlies the endodermal domain of pancreatic precursors (Hebrok, 2003). Studies such as these have yielded important insights into how the pancreatic endoderm is induced. However, our current understanding of endodermal patterning is limited, relative to our more detailed knowledge of patterning in the other germ layers.

There is intense interest in Cdx transcription factors because of their function as modulators of AP patterning in all three germ layers. In a variety of contexts, Cdx factors have been shown to function downstream of RA, FGFs and Wnts, and, in turn, to convey these signals by directly regulating Hox gene expression (Lohnes, 2003). All vertebrates have three Cdx genes: Cdx1, Cdx2 and Cdx4 in tetrapods, and cdx1a, cdx1b and cdx4 in zebrafish (Mulley et al., 2006). In the endoderm, Cdx factors are crucial for patterning the intestine along the AP axis as well as along the crypt-villus axis. Loss of mouse Cdx2 leads to development of gastric epithelium in more-posterior intestinal domains and, conversely, overexpression of Cdx2 or Cdx1 transforms gastric epithelium to an intestinal fate (Beck et al., 1999; Mutoh et al., 2004; Mutoh et al., 2005; Silberg et al., 2001). In light of their ability to transform epithelia, it is not surprising that both Cdx1 and Cdx2 have been studied intensively for their roles in gastric and intestinal cancers (Guo et al., 2004). By contrast, little is known about the role of a third Cdx family member, Cdx4, in patterning the endoderm. However, recent studies in zebrafish have begun to reveal crucial roles for Cdx4 in patterning the ectoderm and mesoderm (Davidson et al., 2003; Shimizu et al., 2005; Shimizu et al., 2006; Skromne et al., 2007).

In the zebrafish endoderm, cdx4 is required to establish the boundary between hindbrain and spinal cord territories (Shimizu et al., 2006; Skromne et al., 2007). A second Cdx factor, Cdx1a, exhibits partial functional redundancy with Cdx4 such that embryos deficient in both factors have enhanced phenotypes (Shimizu et al., 2006; Skromne et al., 2007). Loss of Cdx4 results in posterior expansion of segmented hindbrain at the expense of spinal cord. Conversely, overexpressing cdx4 has a posteriorizing effect (Skromne et al., 2007). Loss of zebrafish Cdx4 also disrupts mesoderm patterning; for example, the anterior limits of expression of kidney markers are shifted posteriorly, and hematopoiesis is disrupted (Davidson et al., 2003; Wingert et al., 2007). Again, this phenotype is exacerbated with additional removal of Cdx1a function (Davidson and Zon, 2006). Thus, cdx4 and cdx1a are crucial for patterning both the ectoderm and mesoderm.

Here, we study zebrafish cdx4 function in the endoderm and show that cdx4 has multiple roles in patterning the foregut. In Cdx4 loss-of-function embryos, we find that pancreatic β-cells are mislocated toward the posterior, and this is indicative of a more general AP patterning defect in which the entire foregut is shifted posteriorly. Using targeted cell transplantations, we show that cdx4 functions directly within the endoderm to localize the pancreas. Morpholino knockdown of cdx4 specifically in the endoderm is sufficient to shift the pancreas posteriorly. Conversely, endoderm-specific overexpression of cdx4 shifts the pancreas anteriorly. In addition to these general AP regionalization defects, we also find that kugelig (kgg; cdx4) mutants exhibit delayed midline convergence of β-cells and an increase in β-cell number during early pancreatogenesis. Knockdown of a second Cdx gene, cdx1a, in cdx4-deficient embryos also results in posterior pancreas localization, suggesting that Cdx4 acts downstream of cdx1a.

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embryos results in a more severe phenotype. Thus cdx4, together with cdx1a, is important for localizing the pancreas and modulating the size of the islet.

**MATERIALS AND METHODS**

**Embryo collection and genotyping**
Zebrafish were maintained following standard procedures (Westerfield, 1995) and staged as described (Kimmel et al., 1995). Embryos were collected from the wild-type AB line and from a locally obtained pet-store line, kgg (Sun et al., 2004). Tg(insulin:GFP) (Huang et al., 2001) and Tg(gut:GFP) (Ober et al., 2003). Genotyping of kgg and Sox32 sibling siblings was performed using primers: mutant forward, 5′-TGAATCGAGGTCCTTGGGAGGGTCCCTC; wild-type forward, 5′-CGTAATTCCATCACGTGGATGAC; and shared reverse, 5′-CCAGTCACCT-GACCTCAACCCCT.

**Microinjections**
Capped mRNAs encoding zebrafish Cdx4 and Sox32 were synthesized using mMachine mMessage (Ambion), following the manufacturer’s protocol. sox32 mRNA was injected at 40 ng/μl and cdx4 mRNA was injected at 84 ng/μl. Antisense morpholinos (Gene Tools) for Cdx4 (Davidson et al., 2003), Cdx1a (Shimizu et al., 2005; Skromne et al., 2007) and Sox32 (Stafford et al., 2006) were used as previously described.

**Cell transplantation**
Transplantation was performed as previously described (Ho and Kane, 1990; Stafford et al., 2006). For transplants in which Sox32 was used to target regents to the endoderm, donor embryos were co-injected at the one-cell stage with sox32 mRNA, cdx4 mRNA or Cdx4 MO, and 40 kDa lysinated fluorescein dextran (Molecular Probes). Hosts were injected at the one-cell stage with Sox32-MO. At 4hpf, ~25-40 cells from donor embryos were transplanted along the blastoderm margin of stage-matched host embryos. The resulting chimeras were raised to 24hpf and fixed in 4% paraformaldehyde.

**In situ hybridization, immunohistochemistry and imaging**
In situ probes were used as previously described (Prince et al., 1998), using probes for cdx4 (Joly et al., 1992), cebpa (Lyons et al., 2001), glucagon and somatostatin 2 (Argenton et al., 1999), somatostatin 1 (Devos et al., 2002), insulin (Milewski et al., 1998), islet1 (Inoue et al., 1994), trypsin (Biemar et al., 2001), pdx1 (Lin et al., 2004) and krox20 (also known as egr2 – ZFIN) (Oxtoby and Jowett, 1993). In situ for pax9 were performed with modifications as previously described (Jackman et al., 2004). For sections, embryos were embedded in Durcupan (Fluka) and cut at 5.5 μm and using a Sorvall MT-2 ultramicrotome. Immunohistochemistry was performed following standard protocols. Somites were labeled using mouse monoclonal anti-myosin antibody (1:100) (Developmental Studies Hybridoma Bank), followed by an AlexaFluor 488-conjugated secondary antibody (1:2000) (Molecular Probes), or followed by the Vectastain Universal ABC Kit (Vector Labs) using the secondary antibody at 1:500. ABC labeling was followed by either tyramide labeling (Perkin Elmer) using the manufacturer’s protocol, or by labeling with an avidin-conjugated AlexaFluor 546 at 1:1000 (Molecular Probes). To analyze the AP location of endodermal expression domains, embryos were deyolked, flat-mounted and photographed under brightfield and fluorescence using a Zeiss Axioskop. To analyze the location of endodermal cdx4, paraxial and lateral plate mesoderm was trimmed off, and embryos were mounted laterally and photographed under brightfield and fluorescence, as above. Images were merged and analyzed using Adobe Photoshop.

**Histology**
Larvae were fixed in 10% neutral buffered formalin, embedded in 1% low melting temperature agarose, and processed for paraffin embedding. Sections were cut at 4 μm and stained with Hematoxylin and Eosin following standard protocols.

**BrdU treatment**
Mutant and sibling embryos at 19hpf and 24hpf were manually dechorionated, then incubated with 10 mM BrdU (5-Bromo-2′-Deoxyuridine, Sigma) in embryo medium and 15% DMSO for 1 hour at 28.5°C. Following treatment, embryos were washed three times with embryo medium and fixed in 4% paraformaldehyde. Antibody labeling was performed using a standard protocol with the addition of a 1-hour incubation in 2N HCl following enzymatic digestion. The BrdU antibody was used at 1:100 (G3G4, Developmental Studies Hybridoma Bank), AlexaFluor 546 secondary at 1:2000 (Molecular Probes), insulin antibody at 1:1000 (Dako) and AlexaFluor 488 at 1:2000 (Molecular Probes). For BrdU treatment and antibody labeling, mutants and siblings were processed in the same tubes.

**Retinoic acid treatment**
Embryos were incubated at 28.5°C in the dark in embryo medium containing 10^{-6} M RA (Sigma) for 1 hour. The RA was removed by repeated washing with embryo medium. Embryos were grown to 24hpf and fixed in 4% paraformaldehyde.

**RESULTS**

**cdx4 and pdx1 are expressed in overlapping gradients**
Vertebrates express Cdx4 in all three germ layers during development (Davidson et al., 2003; Gamer and Wright, 1993; Joly et al., 1992). In zebrafish, the presumptive endoderm begins to express cdx4 during late epiboly (Joly et al., 1992), but details of its subsequent endodermal expression pattern are lacking. We therefore investigated whether zebrafish cdx4 is expressed in the endoderm during early pancreas development. In situ hybridizations on embryos from tailbud stage [10 hours post-fertilization (hpf)] to the 20-somite stage (19hpf) revealed cdx4 expression in all three germ layers (Fig. 1 and data not shown). Sectional analysis revealed robust expression of cdx4 within the endoderm (Fig. 1A′,B′). We found that endodermal expression is in a gradient from high posteriorly to low anteriorly, and extends further anteriorly with time, approaching the foregut (Fig. 1G′-J′). We compared the location of cdx4 to that of pdx1, a foregut marker of pancreatic and anterior intestinal precursors (Fig. 1C′-J′). pdx1 expression was localized exclusively to the endoderm, in a gradient that is high anteriorly and low posteriorly. These reciprocal gradients of pdx1 and cdx4 expression were non-overlapping until ~16hpf (Fig. 1C′-J′ and data not shown). At this stage, a small number of scattered cdx4-positive cells were found within the domain of pdx1-positive cells and this number increased at 18hpf.

**Cdx4 functions in endodermal AP patterning**
The cdx4 gene plays an important role in regionalization of neural ectoderm and mesoderm. We hypothesized that this gene might similarly be important for endodermal regionalization. As cdx4 expression is localized to the posterior part of the embryo (Joly et al., 1992), we postulated that it might play a role in setting the boundary between the posteriorly located intestine and the foregut. To explore this possibility, we compared various foregut markers in wild-type embryos and in kgg (cdx4) mutants (Figs 2, 3). In situ hybridization analysis of markers for pancreatic β-cells (insulin), α-cells (glucagon) and δ-cells (somatostatin 1), as well as a general marker of endocrine pancreas cells (islet1), demonstrated that the entire endocrine pancreas is shifted posteriorly in kgg mutants (Fig. 2A-D, Fig. 3 and data not shown). Interestingly, mutants did not express the δ-cell marker somatostatin 2 (sst2) at 48hpf (data not shown), although by 72hpf we detected two embryos (of 17) with five and eight sst2-positive cells, respectively, versus ~20 cells in wild types (data not shown). Markers for the exocrine pancreas (trypsin) and the liver (cebpa) were also shifted posteriorly in kgg mutants, as was expression of pdx1 (Fig. 2E-L). In addition to showing a posterior shift in expression, the mutant pdx1 domain was expanded anteroposteriorly, typically lying adjacent to somites 1-7.
at 19hpf. By contrast, the wild-type pdx1 domain at 19hpf typically extended from just anterior to the first somite, to somite 4 (Fig. 2, compare G-J). In 50% (17/34) of mutants, the pax9 domain was bifurcated posteriorly (Fig. 2H).

In contrast to the posterior shift of foregut gene markers, the expression domain of pharyngeal endoderm marker pax9 was unaltered in kgg mutants, indicating that this more-anterior region of the endoderm is unaffected (see Fig. S1 in the supplementary material). We hypothesized that the expanded mutant endoderm between the pax9-positive pharynx and the intestine would be esophageal; histology of the pax9-positive pharynx and the intestine would be between the material). We hypothesized that the expanded mutant endoderm of the endoderm is unaffected (see Fig. S1 in the supplementary material). We further found that most siblings of kgg homozygous mutant embryos express insulin in an intermediate location relative to wild types and homozygous mutants from 19hpf onwards. PCR genotyping of these siblings confirmed that kgg heterozygous embryos exhibit a gene dosage effect (Fig. 3F-H and data not shown). However, at 16hpf, the gene dosage effect is not yet apparent; at this stage, we could not detect any consistent difference in AP location of insulin expression between wild type and siblings (P=0.3075, χ² test for trend). In summary, we conclude that cdx4 is required to correctly localize β-cells and functions in a dosage-dependent manner.

Cdx4 is required for proper β-cell localization

Next, we examined the β-cell location defect in more detail. We used insulin as a marker of differentiated β-cells, and began our analysis at 16hpf, shortly after the onset of expression (Biemar et al., 2001). In wild-type embryos, we found that insulin was initially expressed in bilateral domains in the anterior trunk, as previously reported (Biemar et al., 2001; Kim et al., 2005). Using anti-myosin antibody to visualize somites, we found that β-cells are typically located adjacent to somites 0-2 at 16hpf (Fig. 3A,A’). At subsequent stages, the bilateral insulin domains resolved into a single, midline domain that shifts posteriorly with time (Fig. 3B-D), such that by 48hpf the wild-type insulin domain was located adjacent to somites 4-5. We visualized the posterior movement and convergence of individual β-cells in live insulin:GFP transgenic embryos (Huang et al., 2001) using time-lapse imaging (data not shown). This analysis confirmed that the β-cells undergo movement [as previously described (Kim et al., 2005)].

In kgg mutants, the insulin expression domain was located more posteriorly than in wild types at each stage (Fig. 3I-L), such that by 48hpf the mutant expression domain was adjacent to somites 6-7. This domain is shifted relative to the wild-type insulin domain by two somite widths. As with wild types, time-lapse imaging of kgg mutants transgenic for insulin:GFP confirmed that the posterior shift of insulin expression is caused by β-cell movement (data not shown). We further found that most siblings of kgg homozygous mutant embryos express insulin in an intermediate location relative to wild types and homozygous mutants from 19hpf onwards. PCR genotyping of these siblings confirmed that kgg heterozygous embryos exhibit a gene dosage effect (Fig. 3F-H and data not shown). However, at 16hpf, the gene dosage effect is not yet apparent; at this stage, we could not detect any consistent difference in AP location of insulin expression between wild type and siblings (P=0.3075, χ² test for trend). By contrast, the posterior shift in the homozygous mutant insulin domain was detectable as early as 16hpf. Statistical analysis revealed a significant difference in the AP location of the insulin domain, with kgg mutant β-cells showing a trend for being clustered more posteriorly compared with both wild types (P=0.0039) and siblings (P=0.0006, χ² test for trend). In summary, we conclude that cdx4 is required to correctly localize β-cells and functions in a dosage-dependent manner.

Cdx4 modulates β-cell number and midline convergence

In addition to determining the location of the pancreas along the AP axis, we also quantitated the size of the insulin expression domain. At 16hpf, the size and location of the insulin expression domain are more variable than at earlier timepoints. We could not detect a statistically significant difference in domain size between kgg mutants and wild types and could not designate a modal domain
mutants, only 33% of embryos showed a single cluster of β-cells by 19hpf (Fig. 3J, Fig. 4C), and bilateral domains of insulin were still evident at 24hpf (29%). We conclude that cdx4 is also required for the normal temporal and spatial pattern of β-cell convergence.

Cdx1a functions redundantly with Cdx4 to establish the pancreatic domain

Studies on cdx1a function in the ectoderm and mesoderm have established that deficiency in both Cdx1a and Cdx4 results in a more severe phenotype than loss of Cdx4 alone (Davidson and Zon, 2006; Shimizu et al., 2005; Shimizu et al., 2006). We therefore tested whether cdx4 cooperates with cdx1a during pancreas development. Morpholino (MO) knockdown of cdx1a had no overt effect on endoderm patterning (data not shown), similar to findings in other germ layers. We next asked whether Cdx1a deficiency in a kgg background results in a more severe pancreas phenotype than loss of Cdx4 alone.

We used Cdx1a-MO to knockdown cdx1a expression in kgg mutants and siblings. We found that in double-deficient embryos at 19hpf and 24hpf, the pdx1 domain is further expanded, and is more posteriorly located, than in embryos deficient in Cdx4 only (summarized in Fig. 4D and compare Fig. 5B,D with Fig. 2H,J). cdx1a knockdown had a similar, though less dramatic, effect on kgg siblings (compare Fig. 5A,C with Fig. 2G,I). Next, we examined the effect of Cdx double-deficiency on β-cell development, using insulin expression to determine AP location of β-cells. Interestingly, cdx1a knockdown in kgg mutants caused the insulin domain to expand anteriorly, but not posteriorly, compared with kgg mutants at 19 and 24hpf (Fig. 5F,H and compare Fig. 4A,D). The anterior expansion of insulin expression in Cdx double-deficient embryos suggests that β-cells differentiate adjacent to anterior somites as in wild-type embryos, but are delayed in moving posteriorly. As with pdx1 expression, there was a similar, but less dramatic, effect on insulin expression in Cdx1a-deficient kgg siblings (Fig. 5E,G and Fig. 4A,D).

Because the pdx1 domain was further expanded in the AP axis in response to Cdx double-deficiency, we counted insulin-positive cells to determine whether there was a corresponding increase in β-cell number. We saw no significant increase in β-cell number at 19hpf, for either kgg siblings or mutants with cdx1a knockdown, relative to β-cell number in embryos deficient in Cdx4 only (Fig. 4, compare B with E). However, by 24hpf, there was a dramatic increase in β-cell number for Cdx1a-deficient kgg mutants (Fig. 4E). Cell counts and statistical analysis (Table 1) revealed that embryos deficient in both Cdx4 and Cdx1a have a greater increase in β-cell number than embryos lacking Cdx4 alone. Additionally, the data show the importance of Cdx dosage from 24hpf, with β-cell number increasing as more Cdx expression is lost or knocked down. Finally, cdx1a knockdown resulted in a further delay in convergence of β-cells to form the islet (Fig. 4F). In summary, Cdx1a and Cdx4 function in concert to control the position and size of the pancreas.

Altered cell proliferation is not the primary cause of the kgg pancreatic phenotype

Next we attempted to address the mechanism by which Cdx4 modulates β-cell number. Experiments shown in Fig. 2G-J demonstrated that the pdx1-positive field is expanded in kgg mutants during early pancreas development. As pdx1-positive cells include the pancreas progenitors, this suggests that β-cells might be more numerous in mutants either due to increased specification or to increased proliferation of the precursors. Additionally, β-cells might also be expanded owing to increased proliferation of the β-cells themselves (Brennand et al., 2007). To begin to distinguish between
these possibilities, we labeled proliferative endodermal cells with BrdU, and labeled β-cells with an anti-insulin antibody. Our double-labeling strategy revealed that mature β-cells, detected by the insulin antibody at 24hpf, were not co-labeled by BrdU (n = 13; data not shown), suggesting that proliferation of β-cells at this developmental stage is absent or rare. The insulin antibody did not label β-cells at 19hpf, indicating that the translated product is below detection level. In general, our BrdU labeling revealed extremely limited proliferation of cells within the pre-pancreatic field, with no obvious differences between wild-type and mutant embryos (data not shown). We conclude that although proliferation may be subject to Cdx4 regulation, this cannot be the primary mode through which the cdx4 gene limits β-cell number.

**Cdx4 functions within the endoderm to localize the pancreas**

As we found that cdx4 is expressed in the endoderm during timepoints critical for pancreas specification and development, we asked whether Cdx4 functions in the endoderm to localize the pancreas. These experiments relied on a cell transplantation approach to target (or exclude) reagents specifically to (or from) the endoderm (Stafford et al., 2006). This approach utilizes Sox32, which is necessary and sufficient to specify endoderm (Kikuchi et al., 2001; Sakaguchi et al., 2001). We previously demonstrated that cell transplantations from Sox32-expressing donors can restore the endoderm, including insulin expression, of hosts injected with Sox32 morpholino (Sox32-MO) (Stafford et al., 2006). Here we performed additional control experiments to test whether endodermal cell transplantation restores insulin expression in the correct AP location. We blocked endoderm development in wild-type hosts using Sox32-MO, transplanted endodermal precursors from sox32 mRNA-expressing donors, and allowed the chimeras to develop to 24hpf. Then we probed for insulin expression and determined the AP location of the domain. Similar to unmanipulated embryos, the insulin domain was located either adjacent to somite 2-4 (n=3) or somite 3-4 (n=2), indicating a normal β-cell location (data not shown). However, development might be slightly delayed, as suggested by the embryos that expressed insulin adjacent to somite 2-4, which is consistent with a more immature islet.

To test whether Cdx4 functions in the endoderm, host embryos deficient in Sox32 received endoderm from donors injected with a fluorescein-dextran lineage tracer, Cdx4 MO and sox32 mRNA (schematized in Fig. 6A). These chimeric embryos develop with normal gross morphology, and combine host-derived wild-type mesoderm and ectoderm with donor-derived FITC-labeled Cdx4-deficient endoderm. Chimeric embryos were raised to 24hpf and
probed for insulin expression. We found that when only the endoderm is Cdx4-deficient, β-cells are localized in unusually posterior positions (n=6/9; e.g. Fig. 6B). Our criteria were that the insulin domain spanned at least three somite widths and extended posteriorly to somite 5 or further, consistent with the insulin pattern observed in kgg mutant embryos lacking Cdx4 in all three germ layers. Three chimeric embryos had insulin expression adjacent to somites 4-5, and we considered these pancreases to be in a ‘wild-type’ location, as wild-type H9252 cells can occasionally be found adjacent to somites 4-5 at 24hpf (data not shown). However, it should be noted that cdx4 heterozygotes typically express insulin adjacent to somites 4-5 at 24hpf.

We also employed an alternative technique to target Cdx4 MO to the endoderm. We injected one cell of a wild-type embryo at the 32-cell stage with FITC, Cdx4 MO and sox32 mRNA, and grew the embryos to 24hpf. In those specimens in which the FITC label was confined to the endoderm, we found that most embryos had insulin expression that resembled the mutant pattern (n=17/23), consistent with our cell transplantation data. Embryos typically expressed insulin at somites 3-6 or 4-6, although some expressed insulin in much larger domains, for example from somites 1-6. Four embryos had H9252 cells adjacent to somites 4-5, and we conservatively scored these as ‘wild-type’, as above.

Fig. 4. Cdx4 has roles in determining β-cell localization, cell number and in regulating β-cell convergence to the midline during early pancreatogenesis. (A) Summary of Fig. 3 data showing average (modal) insulin domain locations for post-16hpf timepoints relative to somite number (shown as numbered boxes). mut, kgg mutants (cdx4−/−); sib, siblings of kgg mutants, including both heterozygotic and wild-type (wt) zebras. (B) The number of β-cells increases more rapidly in cdx4−/− embryos than in siblings (mean±s.d.). (C) Midline convergence of β-cells is delayed in cdx4−/− embryos. y-axis indicates percentage of embryos in which β-cells have converged to the midline. (D) Summary of Fig. 5 data showing modal insulin and pdx1 domain locations for Cdx1a-MO-injected kgg mutants and siblings. (E) β-cell number increases further in Cdx1a-MO-injected kgg siblings and mutants (mean±s.d.). (F) Midline convergence is further delayed in Cdx1a-MO-injected kgg siblings and mutants. y-axis indicates percentage of embryos in which β-cells have converged to the midline. Note than in E and F, mutant and sibling data from B and C are included for ease of comparison. Sample sizes for A-C were as follows. Wild type (wt): 16hpf, n=38; 19hpf, n=36; 24hpf, n=60; 48hpf, n=17. Siblings (sib): 16hpf, n=38; 19hpf, n=82 for A and C, n=45 for B; 24hpf, n=80 for A and C, n=40 for B; 48hpf, n=33. Mutant (mut): 16hpf, n=23; 19hpf, n=30; 24hpf, n=24; 48hpf, n=23. Sample sizes for D-F were as follows. insulin data, siblings+Cdx1a-MO (sib+MO): 19hpf, n=22; 24hpf, n=9. Siblings+Cdx1a-MO (sib+MO): 19hpf, n=15; 24hpf, n=17. Mutant+Cdx1a-MO (mut+MO): 19hpf, n=16; 24hpf, n=20.
Cdx4 and pancreas development

Reciprocal experiments were performed in which host embryos deficient in both Cdx4 and Sox32 received Cdx4-positive donor endoderm (schematized in Fig. 6C). These embryos show typical kgg mutant gross morphology, and combine host-derived Cdx4-deficient mesoderm and ectoderm, with donor-derived FITC-labeled Cdx4-positive endoderm. We found that in these specimens, in which only the endodermal cells are Cdx4-positive, β-cells are correctly localized (n=4/4; e.g. Fig. 6D).

To further test whether cdx4 functions only in the endoderm to localize the pancreas, we performed transplantation experiments to manipulate Cdx4 expression in paraxial mesoderm. In these experiments, cells were transplanted to the paraxial mesoderm by placing donor-derived cells close to the host blastoderm margin, again as previously described (Stafford et al., 2006). Transplanted cells contributed to at least five somites in the anterior trunk on one side of the chimeric embryos. In chimeras combining wild-type host cells with donor-derived Cdx4-deficient paraxial mesoderm, the β-cells showed a wild-type location at 24hpf (n=16/16, data not shown). Similarly, in chimeric embryos combining Cdx4-deficient host cells with donor-derived wild-type paraxial mesoderm, the β-cells showed a kgg mutant location at 24hpf (n=4/4, data not shown). Taken together, these transplantation experiments suggest that Cdx4 functions directly within the endoderm to localize the pancreas.

Finally, to test whether Cdx4 has the capacity to confer posterior fate directly to the endoderm, we generated embryos overexpressing cdx4 mRNA throughout this germ layer. Our expectation was that overexpression of Cdx4 would posteriorize more-anterior endodermal structures. We transplanted FITC-labeled endodermal cells from embryos previously injected with cdx4 mRNA to endoderm-deficient hosts (schematized in Fig. 6E). In the resultant chimeras, Cdx4 is expressed in anterior endoderm, where cdx4 transcripts are not normally detected. As predicted, we found that in these embryos insulin expression was shifted anteriorly by 2 to 3 somites at 24hpf (n=6/9; Fig. 6F). Of the three chimeras that expressed insulin in the wild-type location, adjacent to somites 3-4, two expressed insulin in anterior trunk locations as well, indicating a partial shift. In a single chimeric specimen raised to 48hpf, imaging of the gut:gfp transgene revealed that the entire pancreas (endocrine and exocrine), as well as the intestinal bulb and liver bud, was shifted anteriorly (data not shown). We conclude that endodermal cdx4 functions similarly to Cdx genes in other germ layers: namely, that overexpression in anterior domains causes posteriorization of fates.

**Cdx4 maintains posterior endodermal identity**

Previous work has demonstrated that RA is necessary and sufficient to specify the pancreas in vertebrates (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002). When wild-type zebrasfish embryos are treated with RA, endodermal cells express insulin throughout the anterior endoderm, ectopic to the normal expression domain (Stafford and Prince, 2002). Additionally, expressing a dominant active RA receptor throughout the endoderm also produces anterior ectopic insulin expression (Stafford et al., 2006). Interestingly, neither of these manipulations is sufficient to induce posterior ectopic insulin. We therefore hypothesized that posterior cdx4-positive endoderm is not competent to respond to RA signaling. To test this, we combined cdx4 knockdown with RA treatments. Uninjected and Cdx4 MO-injected specimens were treated with RA for 1-hour intervals during gastrulation stages, and assayed for insulin expression at 24hpf. Uninjected RA-treated embryos expressed ectopic insulin only in anterior domains, as previously reported (Fig. 7A) (Stafford and Prince, 2002). By contrast, in Cdx4-deficient RA-treated embryos we observed a small number of ectopic insulin-expressing cells well posterior to the expected location of the pancreas, in addition to ectopic anterior insulin (Fig. 7B,C). We observed more ectopic β-cells, located further towards the posterior, in those embryos treated at earlier stages (Fig. 7D). We conclude that the normal posterior expression of Cdx4 functions to prevent posterior endodermal precursors from responding to RA signaling.

**DISCUSSION**

**cdx4 is required to correctly localize foregut organs**

Cdx genes play a role in AP patterning of all three germ layers. Loss-and gain-of-function studies have demonstrated that disruption of Cdx1 and/or Cdx2 expression in mice results in homeotic transformations along the axial skeleton (Subramanian et al., 1995; van den Akker et al., 2002). Similarly, Cdx1 and Cdx2 overexpression...
produces intestinal homeosis in mice (Beck et al., 1999; Chawengsaksophak et al., 1997; Mutoh et al., 2004; Silberg et al., 2002). Recently, studies in zebrafish have shown that deficiency of cdx4 results in a posteriorly shifted hindbrain/spinal cord boundary (Shimizu et al., 2006; Skromne et al., 2007), as well as a posterior shift in the boundary between anterior angioblasts and posterior hematopoietic progenitors (Davidson et al., 2003). Here, we have shown that cdx4 has a role in establishing the AP location of the foregut. In loss-of-function studies, we show that cdx4-deficient embryos have posteriorly shifted foregut organs, including the endocrine and exocrine pancreatic buds, the liver and the intestine.

Examination of β-cell location during the first 48 hours of pancreas development revealed that there is a gene dosage effect on islet location by 19hpf. Specifically, at 48hpf, the wild-type islet is located adjacent to somites 4-5; with loss of one cdx4 copy the islet shifts posteriorly by one somite, and with loss of two copies the islet shifts posteriorly by two somites so that it lies adjacent to somites 6-7 at 48hpf. The intermediate phenotype in heterozygotes is consistent with previous studies of mouse Cdx genes that showed dosage effects for axial skeletal patterning and for intestinal patterning (Beck et al., 1999; Chawengsaksophak et al., 1997; Mutoh et al., 2004; Subramanian et al., 1995; van den Akker et al., 2002). A gene dosage effect for cdx4 has not been previously reported in zebrafish.

We observed a posterior shift for all foregut organ markers tested, including somatostatin 1, glucagon, islet1, trypsin, cebpa and pdx1, indicating that cdx4 has a role in setting the posterior boundary of the foregut. Interestingly, a similar 2-somite posterior shift of ectoderm marker gene expression, including neuronal markers and Hox genes, was recently reported in cdx4-deficient embryos (Skromne et al., 2007). Disruptions in Cdx1 and Cdx2 in mice are known to cause shifts in Hox domains in the mesoderm, with concomitant homeotic transformations of the vertebralae (Subramanian et al., 1995; van den Akker et al., 2002). In zebrafish, loss of cdx4 again produces a 2-somite posterior shift in Hox expression domains in the mesoderm (I. Skromne, personal communication). It is likely that endodermal Hox domains are also shifted posteriorly when cdx4 is lost. Future studies will determine the timing and location of specific Hox expression in zebrafish endoderm.

cdx4 is required to limit β-cell number and has a role in midline convergence

Examination of insulin expression in cdx4 mutant embryos revealed that at 19hpf, shortly after the onset of expression, the insulin domain is abnormally expanded along the AP axis. This expansion is characterized by an increase in the number of insulin-positive β-cells in cdx4 mutant embryos compared with wild type. As our cell proliferation analysis did not reveal any obvious differences in proliferation rates between wild-type and kgg mutant embryos, we suggest that the primary role for cdx4 is in limiting the specification of β-cells. Interestingly, at 19hpf, the size of the pdx1 expression domain, which labels pancreatic and intestinal precursors, is also significantly expanded in mutants, suggesting an expanded progenitor pool. The early excess of β-cells might be at the expense of δ-cells, as we could not detect somatostatin 2 expression in mutants until 72hpf, at which time a small number of positive cells were detected in a few embryos. In the pancreas, subsets of δ-cells have been reported, with some cells expressing both somatostatins and others expressing only one (Devos et al., 2002). However, because the number of somatostatin 1-positive δ-cells is increased, as are numbers of other differentiated endocrine pancreas derivatives, it is more likely that the field of endocrine precursors is generally expanded in the absence of Cdx4 function.

The excess β-cell number in kgg mutants at 19 and 24hpf is associated with a delay in midline convergence of these cells. This is consistent with a similar report of a midline convergence delay for angioblasts in kgg mutants (Davidson et al., 2003). Both cell number and midline convergence may be modulated by Cdx4, which is a direct Wnt target (Pilon et al., 2006). Wnts are involved in both cell proliferation and convergence movements (Clevers, 2006) (reviewed by Torban et al., 2004), and Wnt signaling is implicated in midline convergence of foregut precursors (Kim et al., 2005; Matsui et al., 2005).

cdx4 functions within the endoderm during pancreas development

We have used cell transplantation experiments to demonstrate that cdx4 functions within the endoderm to localize the pancreas. Additionally, we showed that overexpression of cdx4 throughout the endoderm shifts the pancreas anteriorly. This is consistent with Cdx4 function in other vertebrates. Previous studies in chick showed that FGF treatment resulted in an anterior shift in Cdx-B (chick Cdx4), which in turn resulted in anterior shifts in downstream endoderm gene expression (Dessimoz et al., 2006). Similarly, overexpressing Cdx4 in mice resulted in anteriorly shifted Hoxb8 expression in the neural tube and somites (Charite et al., 1998). We found that overexpression of cdx4 in endoderm shifted the pancreas anteriorly, such that insulin was expressed anterior to the somites or adjacent to the first somite at 24hpf. This anteriorly localized insulin...
expression closely resembles the wild-type location of newly differentiated β-cells at 16hpf. This suggests that in the cdx4 overexpression chimeras, β-cells are specified normally but fail to move posteriorly owing to their location in an environment of already high Cdx4 expression. Our expression analysis showed that cdx4 is expressed in posterior endoderm and excluded from anterior foregut during the earliest stages of pancreas development, prior to the onset of insulin expression. Subsequently, cdx4 is expressed at low levels more anteriorly, in a salt-and-pepper pattern, throughout much of the pdx1-positive (and insulin-positive) domain. We suggest that in cdx4 overexpression chimeras, the newly differentiated β-cells fail to move posteriorly because they interpret their position as already being in the posterior of the foregut.

**Cdx4 and Cdx1a function redundantly during pancreas development**

Our studies on Cdx1a/Cdx4-deficient embryos revealed that Cdx genes function redundantly to localize the pancreas and modulate β-cell number. At 24hpf, a Cdx dosage effect on β-cell number was observed, such that cell number increased as Cdx dosage decreased, suggesting that these two genes exhibit partial functional redundancy. A similar Cdx dosage effect was reported for mesoderm-derived blood cell precursors in the intermediate cell mass (Davidson and Zon, 2006). Whereas Cdx1a-deficient kgg mutants have a pdx1-positive domain that is expanded posteriorly compared with kgg mutants, the β-cell location expands anteriorly rather than posteriorly. As reducing Cdx1a activity did not allow the pancreatic islet to shift further posterior than in the cdx4/kgg mutant, we suggest that additional mechanisms operate in the posterior of the embryo to control the location of the pancreas. Although a third Cdx gene, cdx1b (Mulley et al., 2006), has been described for zebrafish, our preliminary data show that this gene is not expressed during the first 48 hours of development (M.D.K., M.R.A. and V.E.P., unpublished), making it an unlikely candidate to modulate pancreas position. We therefore suggest that Cdx-independent mechanisms also play a role in establishing the posterior limit of the pancreas.

During gastrulation, cdx1a is expressed in marginal cells and becomes restricted to the posterior tailbud during somitogenesis, but expression in the endoderm earlier than 48hpf has not been reported (Davidson and Zon, 2006). We were unable to detect endodermal transcripts between the 5-somite stage and 24hpf (our unpublished results), stages when critical steps in AP patterning of the endoderm are taking place. Thus, whereas the endoderm expresses cdx4, and our cell transplantations demonstrate that cdx4 functions within the endoderm to localize the pancreas, cdx1a is likely to function cell-non-autonomously within adjacent mesoderm. Interestingly, Cdx1a-deficient kgg mutants show an anterior expansion of β-cells compared with kgg mutants. This suggests that in addition to the endodermal role of Cdx4, Cdx1a and Cdx4 might function together within the mesoderm to further refine β-cell number and location. The mesoderm is a source of various signals that pattern the endoderm; the expanded domain of pdx1-positive precursors and insulin-positive cells in cdx1a/cdx4-deficient embryos is consistent with a model in which Cdx deficiency alters expression of key mesodermal signals.

**cdx4 prevents insulin expression in posterior endoderm**

We have established that RA signaling from anterior paraxial mesoderm is required for pancreas specification, which requires precise control of the RA signals generated in the mesoderm and received by the endoderm (Stafford et al., 2006). However, the RA synthesis enzyme Raldh2 (also known as Aldh1a2 – ZFIN) is expressed along the trunk mesoderm in domains that extend posterior to the pancreatic domain (Begemann et al., 2001; Grandel et al., 2002), and RA receptors are expressed throughout the posterior endoderm (Waxman and Yelon, 2007). It is thus likely that the RA-degrading Cyp26 enzymes are also important for modulating RA signals during pancreas development. Both Raldh2 and Cyp26a1 are regulated by Cdx factors, and their expression domains are shifted posteriorly in response to Cdx1a/Cdx4 deficiency during early somitogenesis (Shimizu et al., 2006). Such shifts might underlie the subsequent shift in foregut expression markers that we have observed.

Additionally, we have shown that Cdx4 has a role in preventing insulin expression in posterior endoderm. cdx4 morphant embryos treated with RA responded by expressing insulin throughout the AP extent of the endoderm, including regions posterior to the trunk and notochord. These results are consistent with a model in which high levels of cdx4 expression in the posterior renders endodermal cells unable to respond to RA signals, thus maintaining a posterior identity. Interestingly, early-stage RA treatments proved most effective at producing posterior insulin-expressing cells, perhaps suggesting that at later stages additional mechanisms block RA signaling in the posterior. Our RA-treatment experiments do not distinguish between models in which RA acts directly on posterior to the pancreatic domain (Begemann et al., 2001; Grandel et al., 2002), and RA receptors are expressed throughout the posterior endoderm (Waxman and Yelon, 2007). It is thus likely that the RA-degrading Cyp26 enzymes are also important for modulating RA signals during pancreas development. Both Raldh2 and Cyp26a1 are regulated by Cdx factors, and their expression domains are shifted posteriorly in response to Cdx1a/Cdx4 deficiency during early somitogenesis (Shimizu et al., 2006). Such shifts might underlie the subsequent shift in foregut expression markers that we have observed.

Although cdx4 prevents insulin expression in posterior endoderm, our cdx4-mRNA overexpression experiments demonstrated that anteriorly expressed cdx4 is nevertheless compatible with β-cell differentiation in the anterior-most region of the trunk. This can be attributed to the fact that cdx4 is normally expressed in a posterior-
to-anterior gradient, with a low expression level in the foregut/pancreatic domain of wild-type embryos. In the overexpression assay, the level of ectopic cdx4 expression in the anterior-most trunk is likely to be consistent with the low expression level observed in the wild-type foregut, and thus is compatible with β-cell differentiation in this context.

We have shown that Cdx4 is a crucial factor in localizing the pancreas and in limiting its size. Although the mesoderm expresses Cdx4, it is endodermal Cdx4 that is required for localizing the pancreas. By contrast, Cdx1a functions in mesendoderm to influence adjacent endoderm. Our work reveals that Cdx genes are important regulators of AP patterning in all three germ layers.

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Supplementary material
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