Zebrafish cdx1b regulates expression of downstream factors of Nodal signaling during early endoderm formation

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We identified a zebrafish caudal-related homeobox (cdx1b) gene, which shares syntenic conservation with both human and mouse Cdx1. Zebrafish cdx1b transcripts are maternally deposited. cdx1b is uniformly expressed in both epiblast and hypoblast cells from late gastrulation to the 1-2s stages and can be identified in the retinas, brain and somites during 18-22 hpf stages. After 28 hours of development, cdx1b is exclusively expressed in the developing intestine. Both antisense morpholino oligonucleotide-mediated knockdown and overexpression experiments were conducted to analyze cdx1b function. Hypoplastic development of the liver and pancreas and intestinal abnormalities were observed in 96 hpf cdx1b morphants. In 85% epiboly cdx1b morphants, twofold decreases in the respective numbers of gata5, cas-, foxa2- and sox17-expressing endodermal precursors were identified. Furthermore, ectopic cdx1b expression caused substantial increases in the respective numbers of gata5, cas-, foxa2- and sox17-expressing endodermal precursors and altered their distribution patterns in 85% epiboly injected embryos. Conserved Cdx1-binding motifs were identified in both gata5 and foxa2 genes by interspecific sequence comparisons. Cdx1b can bind to the Cdx1-binding motif located in intron 1 of the foxa2 gene based on an electrophoretic mobility shift assay. Co-injection of either zebrafish or mouse foxa2 mRNA with the cdx1b MO rescued the expression domains of ceruloplasmin in the liver of 53 hpf injected embryos. These results indicate that zebrafish cdx1b regulates foxa2 expression and may also modulate gata5 expression, thus affecting early endoderm formation. This study underscores a novel role of zebrafish cdx1b in the development of different digestive organs compared with its mammalian homologs.

KEY WORDS: Nodal signaling, cdx1b, Digestive organ development, Zebrafish

INTRODUCTION

The digestive system is important for the maintenance of vertebrate physiology. After the endoderm progenitors are specified, they differentiate into epithelial cells of the embryonic gut. In amniotes such as mice, the endodermal layer of the mouse embryo begins to form a gut tube at embryonic day 8.5 (E8.5) by folding at the anterior end, resulting in the anterior intestinal portal (AIP), followed by creation of the caudal intestinal portal (CIP) at the posterior end (reviewed by Wells and Melton, 1999; Grapin-Botton and Melton, 2000). Next, a fully extended gut tube forms by extension and fusion of the AIP and CIP. Organ buds form during the E10.5-14.5 stages. Eventually, the esophagus, stomach, thyroid, lungs, pancreas and liver are derived from the foregut region.

In amniotic vertebrates, various paracrine and transcription factors have been shown to be essential for specifying endoderm progenitors, patterning and morphogenesis during gastrointestinal tract development (Harmon et al., 2002; de Santa Barbara et al., 2003; Schier, 2003; Mutoh et al., 2004). Among these, Nodal paracrine factors play crucial roles in mesendoderm induction in vertebrates (reviewed by Schier, 2003). In the absence of Nodal signaling, no endoderm- or mesoderm-derived organs or tissues can develop in zebrafish embryos (Feldman et al., 1998). The molecular pathway leading to early endoderm development in several vertebrates has been established (Alexander and Stainier, 1999; Shivdasani, 2002; Stainier, 2002; Tam et al., 2003). In zebrafish embryos, two Nodal factors (Squint and Cyclops; also known as Nodal-related 1 and 2, respectively – ZFIN) interact with the TGFβ-related type I receptor; Taram-a (Tar; Acvr1b – ZFIN), and the One-eyed pinhead (Oep) EGF-CFC co-receptor. Nodal signaling can be transduced either by association of the phosphorylated Smad2-Smad4 complex with Bonnie and clyde (Bon) or with Gata5 to activate the HMG domain transcription factor, Casanova (Cas; also known as Sox32 – ZFIN). Alternatively, Cas may function in parallel with Gata5/Bon. Subsequent cooperation between Cas and the POU domain protein, Spg (Pou5f1 – ZFIN), activates the HM domain transcription factor, Sox17, leading to endoderm formation (Alexander and Stainier, 1999; Alexander et al., 1999; Kikuchi et al., 2001; Aoki et al., 2002; Kikuchi et al., 2001; Aoki et al., 2002; Lunde et al., 2004; Reim et al., 2004).

Successive patterning and morphogenesis of the gut tube are regulated by coordinated transcriptional activity (reviewed by Well and Melton, 1999). Both mouse Cdx1 and Cdx2 are expressed in the embryonic and adult intestine and colon. In the adult intestine and colon, Cdx1 expression increases along the anteroposterior axis, with the highest expression in the distal colon, whereas Cdx2 expression increases progressively from the duodenum to the distal intestine, with the highest level observed in the proximal colon (Silberg et al., 2000) (reviewed by Guo et al., 2004). Conversion of the gastric mucosa to intestinal metaplasia was detected in either Cdx1- or Cdx2-expressing transgenic mice (Mutoh et al., 2002; Mutoh et al., 2004). In heterozygote Cdx2+/− mutant mice and Cdx2-null
mutant chimeric mice, polyps with stomach heteroplasia were found in the midgut (Chawengsaksophak et al., 1997; Beck et al., 2003). However, Cdx1b mice do not show any intestinal abnormalities (Beck, 2004). Taken together, those studies indicate that Cdx2 functions in controlling intestinal development and homeostasis.

Recently, zebrafish (Danio rerio) have become a new model organism for studying endoderm development, owing to the availability of both their forward and reverse genetics, which can be used to dissect the molecular mechanisms responsible for digestive tract morphogenesis. Several studies have shown that the digestive organs of zebrafish and amniotes form differently (Field et al., 2003a; Field et al., 2003b; Ober et al., 2003; Wallace and Pack, 2003). The zebrafish digestive tract system contains no stomach (Pack et al., 1996). In contrast to the mouse, endothelial cells are needed for neither development of the pancreas nor budding of the liver in developing zebrafish embryos (Lammert et al., 2001; Matsumoto et al., 2001; Field et al., 2003a; Field et al., 2003b).

In addition, disparities in regulatory mechanisms have also been observed in zebrafish. For example, inhibition of shh expression in the gut endoderm is necessary for the induction of the pancreas in amniotic embryos, whereas in zebrafish embryos, Shh secreted from the notochord induces development of the pancreas (Kim and Hebrok, 2001; Roy et al., 2001).

In this study, we report our findings on a zebrafish caudal-related homeodomain protein, Cdx1b, which exerts its novel function during gastrointestinal tract development compared to its mammalian homolog. Antisense morpholino oligonucleotide-mediated knockdown and overexpression analyses revealed that zebrafish cdx1b regulates expression of several downstream factors of Nodal signaling involved in early endoderm development and is therefore essential for the normal development of different digestive organs.

MATERIALS AND METHODS

Zebrafish maintenance and staging

Adult zebrafish were maintained in 20 l aquariums supplied with filtered fresh water and aeration under a 14 hour light and 10 hour dark photoperiod. Different developmental stages were determined according to morphological criteria defined by Kimmel et al. (Kimmel et al., 1995). A homozygote mutant phenotype was identified by a dissecting microscope by the presence of eye fusion and the number of eyes present in oepws3, cycld2 and squint embryos.

Cloning of zebrafish cdx1b, expression vector construction and phylogenetic and syntenic comparison analyses

A 435 bp amplified DNA fragment was obtained using degenerate primers (see Fig. S1 in the supplementary material) in a reverse-transcription PCR (RT-PCR), and this was used as a probe to screen a zebrafish cDNA library (Clontech). In order to obtain the exon containing the start codon, an FIXII zebrafish genomic DNA library (Stratagene) was screened using the same probe. An RT-PCR was conducted to verify the sequence of the full-length coding region. DNA and the deduced amino acid sequences were analyzed using Lasergene software (DNASTAR) and are deposited in GenBank under Accession no. AV761094. For construction of the expression vector, the cdx1b coding region was PCR-amplified with Pfu DNA polymerase (Stratagene). The PCR products were respectively cloned into a T7TS vector for capped cdx1b mRNA synthesis and into a pcDNA3-Myc-His (Invitrogen) vector for in vitro Cdx1b protein synthesis.

Phylogenetic analyses were performed using PHYLIP 3.6 (Felsenstein, 2000). A neighbor-joining (NJ) analysis was performed after genetic distances were calculated based on the Dayhoff PAM model. The robustness of the NJ phylogenies was assessed by 1000 bootstrap replicates using the SEQBOOT and CONSENSE options. The BioMart data-mining program from the Ensembl Genome Browser was used to conduct syntenic analyses among zebrafish linkage group 7, human and mouse genomes.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on embryos treated with 0.003% phenylthiocarbamide using digoxigenin-labeled antisense RNA probes and alkaline phosphatase-conjugated anti-digoxigenin antibodies as described (Peng et al., 2002). Double in situ hybridization was conducted based on procedures described in Jowett (Jowett, 2001). Various templates were linearized, and antisense RNA probes were generated as follows: bon (Ncol/SP6), cas (Ncol/SP6), cdx1b (HindIII/T3), ceruloplasmin (Notll/T7), cyclophil (EcoR I/T7), fgg3 (Ncol/SP6), foxa2 (SpeI/T3), gata5 (SacII/SP6), gsc (EcoRI/T7), ifap (Ncol/SP6), insulin (Ncol/SP6), ifap (SalI/T7), myod (XbaI/T7), ntl (XhoI/T7), oep (Ncol/SP6), rxl (SalI/T7), ssh (BamHI/T7), sox17 (EcoRI/T7), squint (Notl/T7) and trypsin (Notl/T7).

Histologic methods and photography

Cryostat sectioning of whole-mount in situ embryos was conducted according to Westerfield (Westerfield, 1995). Paraffin sectioning and Hematoxylin (Vector) and Eosin (Muto Pure Chemical) staining were performed according to standard procedures. Images of embryos from in situ hybridization, cryostat and paraffin sectioning as well as GFP images from live 27 hours post-fertilization (hpf) embryos were taken using an RT color digital camera (SPOT) on a Zeiss Axioplan 2 microscope or using a Coolpix 5000 digital camera (Nikon) on a Leica MZFLIII stereomicroscope. Two sides of lateral-view images from cas, sox17 and foxa2 in situ hybridization and the dorsal-view image of gata5 in situ hybridization were photographed, and the Image-Pro Plus program (Media Cybernetics) was used to count endocardial cell numbers.

Morpholino, cdx1b1 RNA, foxa2 RNA and Cdx1b protein injections

Respective morpholino oligonucleotides (MOs; Gene Tools) were dissolved in Danieu solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2 and 5 mM Hepes; pH 7.6) at a 1 mM stock concentration. Diluted MOs (0.4 mM) were respectively microinjected (2.3 nl; to a final concentration of 7.5 ng or 0.92 pmole) into the cytoplasm of 1-2-cell zygotes using a Nanoscript II automatic injector (Drummond). The morpholino sequences were as follows: cdx1b MO comprising sequences complementary to the AUG translational start site and the 21 bases in the 5’ UTR region: CAAATTTTCTGTGGCTTCCAGTGCC; and cdx1b-4nm MO containing the same nucleotide sequences as cdx1b MO except for four mismatched sequences: CAAATTTTCTGTGGCTTCCAGTGCC. Respective capped cdx1b1 and lacZ mRNAs were synthesized using a T7 or a SP6 mMESSAGE mMACHINE Kit (Ambion). To ectopically express cdx1b1, cdx1b1 mRNA (50-60 pg) was injected into the cytoplasm of 1-2-cell zygotes. lacZ mRNA (81 pg) was injected into the cytoplasm of 1-2-cell zygotes as the control. To rescue cdx1b morphants, either 10-30 pg of cdx1b1 mRNA or Cdx1b protein (the TNT reaction mixture was diluted 2- to 40-fold) was co-injected with 7.5 ng of the cdx1b MO with 7.5 ng of the cdx1b1 MO except for four mismatched sequences: CAAATTTTCTGTGGCTTCCAGTGCC. Cdx1b protein was synthesized using the TNT-coupled transcription/translation system (Promega) with the pcDNA3-cdx1b1-Myc-His plasmid, and the GFP was synthesized with the pcDNA3-GFP plasmid. foxa2 mRNA rescue experiments were conducted by co-injecting 7.5 ng of the cdx1b MO with either zebrafish foxa2 mRNA (75-100 pg) or mouse Foxa2 mRNA (200 pg) respectively synthesized using the T7 mMESSAGE mMACHINE kit into the cytoplasm of 1- to 2-cell zygotes.

Electrophoretic mobility shift assay (EMSA) and the preparation of nuclear extracts

For sequences of the wild-type Cdx1b-binding motif in the intron 1 of foxa2 and mutant oligonucleotides see Fig. S1 in the supplementary material. Oligonucleotides were 5’-end-labeled with biotin, and the subsequent EMSA was performed according to procedures described in a Lightshift Chemiluminescent EMSA Kit (Pierce). COS-1 cells (5x105) were plated onto a 10 cm Petri dish and cultured for 16 hours. After respective transfection with the pcDNA3-cdx1b1-Myc-His and pcDNA3-Myc-His plasmids, COS-1 cells were harvested at 48 hours post-transfection, and nuclear extracts of transfected cells were prepared as described in Deryckere and Gannon (Deryckere and Gannon, 1994).
**RESULTS**

**Cloning of the zebrafish cdx1b gene, and phylogenetic tree and syntenic analyses**

Full-length cdx1b cDNA was obtained by respectively screening a zebrafish cDNA and a genomic DNA library using a 435 bp DNA fragment of the RT-PCR product as a probe. The deduced amino acid sequence showed that cdx1b encodes a 255 amino acid-long polypeptide that contains a C-terminal homeodomain and an N-terminal caudal-type activation domain. The cdx1b gene containing three exons was found to be located on chromosome 7 by an Ensemble genome (Zv6) search. A global amino acid sequence comparison showed that it shared high (69%) amino acid sequence similarity with that of cad2 from *Xenopus tropicalis*, while it shared 53-56% sequence similarities with *Cdx1* and *Cdx2* from human and mouse. Phylogenetic tree analyses revealed that zebrafish cdx1b was branched with *X. tropicalis cad2* with a high bootstrap value, and they were closely grouped with both human and mouse *Cdx1* and *Cdx2*. By contrast, zebrafish cdx1a was branched with *X. tropicalis cad1*, and zebrafish cdx4 was clustered together with mammalian *Cdx4* and *X. tropicalis cad3* (see Fig. S2 in the supplementary material). Syntenic analyses were conducted to clarify the orthologous relationships among zebrafish cdx1b and mammalian *Cdx1* and *Cdx2* genes. At least 14 genes, including cdx1b from zebrafish linkage group (LG) 7, shared ≥50% amino acid sequence identities with those in the respective human chromosome 5 and mouse chromosome 18 where *Cdx1* resides (see Fig. S3 in the supplementary material). However, there was no syntenic conservation among zebrafish LG7, human chromosome 13 or mouse chromosome 18.
Developmental expression patterns of cdx1b

Zebrafish cdx1b mRNA is a maternal mRNA, as shown by the hybridization signal in one-cell zygotes (Fig. 1A). cdx1b mRNA in blastula embryos was distributed close to the blastoderm margin (Fig. 1D). In shield embryos, cdx1b mRNA was expressed mainly in hypoblasts (Fig. 1E,F). From 80% epiboly to the 1-2-somite stage, cdx1b expression was uniformly detected in both epiblast and hypoblast cells of whole embryos (Fig. 1H-J,L,M). During the late segmentation period (18-20 hpf), cdx1b was expressed in the retinas, brain and somites, and expression in the anus was briefly detected around 20 hpf (Fig. 1O,R-T). Expression of cdx1b in the retinas, diencephalon, midbrain and somites was further confirmed by double in situ hybridization using rx1, shh and myoD as probes (Fig. 1O-Q). From 28 hours of development, cdx1b was exclusively expressed in the developing foregut region and extended to the hindgut region before 48 hpf (Fig. 1V-X). Increased cdx1b expression was observed as the intestines developed, and a high level of expression was detected at 96 hpf (Fig. 1Y,Z,b). Transverse cryostat sectioning showed that cdx1b mRNA was localized in both microvilli and basal nuclear sides of the intestinal epithelium (Fig. 1a).

Semiquantitative RT-PCR further showed that high cdx1b expression was detected in adult fish by RT-PCR (data not shown). These results indicate that cdx1b is an intestine-specific gene during gut tube formation.

Antisense MO-mediated knockdown of cdx1b expression

To explore the function of cdx1b during zebrafish embryonic development, we performed antisense MO-mediated knockdown experiments. We designed a cdx1b-specific MO that corresponds to the sequence from nucleotides –21 to +3 covering the ATG start codon. Four mismatches in this cdx1b MO were introduced and used as a control in this study. Notable morphological changes, such as reductions in ventral neuroectodermal structures including the hypothalamus and basal plate midbrain, a closer distance between the eyes (103±17 μm compared with 133±22 μm in cdx1b-4mm-MO-injected embryos), and pericardial edema, were detected after 24 hours of development in embryos that had been injected with 7.5 ng of the cdx1b MO (Fig. 2A-F). Neurectodermal structure reduction was further confirmed by observations of reduced and altered shh expression domains in the hypothalamus, zona limitans intrathalamica and basal plate midbrain in 28 hpf cdx1b morphants when compared with wild-type embryos (Fig. 2U-X). Roughly half of the 24 hpf cdx1b morphants displayed a curled-down body axis (Fig. 2A-C). We also tested different doses for the cdx1b MO injection and detected the same morphant phenotypes that appeared but with different morphant rates when calculated at 24 hpf (Table 1).

Overall, an increasing morphant rate was detected when an increasing dose of the cdx1b MO was injected. As injecting 7.5 ng of the cdx1b MO gave the highest morphant rate, we decided to use this dose in further experiments. As injecting 7.5 ng of the cdx1b MO also resulted in pericardial edema, we determined that the cdx1b MO could induce these phenotypes.

Table 1. Morphant phenotype characterization based on different doses of cdx1b MO injection

<table>
<thead>
<tr>
<th>Injected (ng)</th>
<th>Wild type (%)</th>
<th>Morphant (%)</th>
<th>Deformities (%)</th>
<th>Death (%)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9</td>
<td>61.9±2.6</td>
<td>26.2±2.4</td>
<td>1.5±0.7</td>
<td>10.4±1.7</td>
<td>336</td>
</tr>
<tr>
<td>4.7</td>
<td>25.1±2.3</td>
<td>61.4±2.6</td>
<td>4.1±1.1</td>
<td>9.4±1.6</td>
<td>342</td>
</tr>
<tr>
<td>7.5</td>
<td>4.6±1.2</td>
<td>83.8±2.0</td>
<td>2.1±0.8</td>
<td>9.5±1.6</td>
<td>328</td>
</tr>
</tbody>
</table>

Data are presented as the mean±standard error (Kuzuma and Bohnenblust, 2004).

mouse chromosomes 5 where Cdx2 resides. Therefore, we named this caudal-related homebox gene cdx1b, which is a newly identified zebrafish cdx1 paralog with significant difference from previously identified cdx1a and cdx4 genes.
Fig. 3. Inhibition of zebrafish cdx1b function affects expression of some digestive organ marker genes and causes hypoplastic growth of the liver, pancreas and intestines. Seventy-two hpf (A, D) and 54 hpf (G) wild type and 72 hpf (B, C, E, F) and 54 hpf (H) morphants were respectively labeled with ifabp/lfabp (A-C), trypsin (D-F) and insulin (G, H) probes. Real-time quantitative PCR (I) indicated a reduction in expression levels of different marker genes in 72 hpf morphants. Histological analyses of paraffin transverse (J-L) and sagittal (S-U) sections of 96 hpf wild-type and transverse (M-R) and sagittal (V-X) sections of morphants are shown. The inset in K indicates the sectioning planes on digestive tracts shown in J-R. Mid-intestine and posterior-intestine regions of wild-type (T, U) and morphant (W, X) at a higher magnification are shown. Scale bars: 100 μm. a, anus; es, esophagus; ep, exocrine pancreas; i, intestine; l, liver; wt, wild type.
to use this dose for subsequent experiments in this study. As a control, 7.5 ng of the cdx1b-4mm MO was injected into one-cell zygotes, and they exhibited a normal morphology when compared with the wild-type ones at 24 hours of development (Fig. 2G-I). Pronounced pericardial edema accompanied by a heart-looping defect was observed in 48 hpf cdx1b morphants when compared with either wild type or embryos that had been injected with the cdx1b-4mm MO (Fig. 2M-R). In order to demonstrate the specificity of the cdx1b MO, we fused the cdx1b MO sequence in front of the GFP start codon. We detected no green fluorescence in 27 hpf embryos that had been co-injected with 7.5 ng of the cdx1b MO and 57.5 pg of the CMV-cdx1b-mo-GFP expression plasmid (Fig. 2T). By contrast, bright GFP fluorescence was detected in embryos that had been co-injected with the same amount of the cdx1b-4mm MO and CMV-cdx1b-mo-GFP plasmids (Fig. 2S). These results demonstrate the specificity of the cdx1b MO used in this study.

**Development of several digestive organs was impaired in cdx1b morphants**

Development of the liver, intestines and endocrine and exocrine pancreases was examined by analyzing expression levels and patterns of two fatty acid-binding proteins (lfabp and ifabp; also known as fabp1a and fabp2 – ZFIN), insulin and trypsin. A substantial reduction in both lfabp and ifabp expression levels and domains were observed in 72 hpf cdx1b morphants (88%, n=50).
when compared with those of the wild-type embryos (Fig. 3A-C). In addition, 20% of morphants (n=50) showed smaller amounts of liver \textit{lfabp} and intestinal \textit{ifabp} expression on the R-axis (Fig. 3C). A decrease in the area of \textit{trypsin} expression was detected in 77% of 72 hpf \textit{cdx1b} morphants (n=104) compared with wild-type embryos (Fig. 3D-F). Similarly, \textit{trypsin} mRNA was localized on the R-axis in 6% of 72 hpf \textit{cdx1b} morphants (n=104) (Fig. 3F). The expression domain of \textit{insulin} was also decreased in 54 hpf \textit{cdx1b} morphants (55.3%, n=64) compared with wild-type embryos (Fig. 3G,H). A real-time quantitative PCR further confirmed the relatively decreased expression levels of \textit{lfabp}, \textit{ifabp}, \textit{trypsin} and \textit{insulin} in 72 hpf \textit{cdx1b} morphants (Fig. 3I). Moreover, histological analyses revealed different degrees of hypoplastic development of the liver, intestines and exocrine pancreas in 96 hpf \textit{cdx1b} morphants (Fig. 3M-R). In 96 hpf wild-type embryos, the liver surrounded the esophagus (Fig. 3J). The epithelial cells adopted a columnar shape and folded in on the intestinal bulb region with the microvilli facing the lumen (Fig. 3K,L,S-U). By contrast, epithelial cells appeared cuboidal with pleomorphic nuclei located at random positions with respect to the apical-basal axis, and no epithelial folding was detected in the intestinal bulb, mid-intestine or posterior-intestine regions with the microvilli facing the lumen (Fig. 3K,L,S-U). Overall, these results indicate that zebrafish \textit{cdx1b} is required for the normal morphogenesis of the liver, intestines and pancreas as well as their left-right asymmetrical distribution in the embryo.

\textit{cdx1b} regulates endoderm development

\textbf{Fig. 5.} Zebrafish \textit{cdx1b} epiboly morphant showed no ectoderm or mesoderm defects. Expression of \textit{fgf3} in wild type (A) and bud morphants (B), \textit{gsc} expression in wild type (C) and shield morphants (D), \textit{ntl} expression in 80% morphants (F) and wild-type (E) embryos. fb, forebrain; n, notochord; pp, prechordal plate; r4, rhombomere 4; wt, wild type. Scale bars: 100 \mu m.

\textbf{Fig. 6.} Effects of ectopic \textit{cdx1b} expression on respective \textit{gata5}-, \textit{cas}-, \textit{sox17}- and \textit{foxa2}-expressing endodermal cell numbers in zebrafish. Increases in the numbers of \textit{gata5}-(C,D), \textit{cas}-(G,H), \textit{sox17}-(K,L) and \textit{foxa2}-expressing (O,P) endodermal cells were detected in 85% epiboly embryos ectopically expressing \textit{cdx1b} when compared with \textit{lacz} (A,B,E,F,I,J,M,N) ectopically expressing epiboly embryos. Scale bars: 100 \mu m. Dfc, dorsal forerunner cell.
cdx1b regulates respective numbers of gata5-, cas-, foxa2- and sox17-expressing endodermal precursor cells in epiboly embryos

As cdx1b is present as maternal transcripts and is uniformly expressed in both epiblast and hypoblast cells in 80% epiboly embryos, and because defects were detected in the development of several digestive organs in cdx1b morphants, we expected that inhibition of Cdx1b protein synthesis would affect early endoderm development. Thus, we examined the expression levels of several protein components of the Nodal signaling pathway that are involved in early endoderm formation. First, we investigated the relationships among cdx1b, Nodal factors and oep by analyzing cdx1b expression levels in 48 hpf squint<sup>c35</sup>, 48-hpf cyc<sup>+/−</sup>, and 4-6-s oep<sup>wt</sup>/oep<sup>−/−</sup> mutant embryos, respectively. There were no differences in cdx1b expression levels in mutant embryos from these three mutant fish lines when compared with their respective wild-type siblings. Likewise, similar expression levels of cyclops, oep and sqt were detected in both 85% and 30% epiboly cdx1b morphants and their wild-type siblings, respectively. In addition, there was no difference in bon expression levels when comparing 40% epiboly cdx1b morphants with wild-type embryos (see Fig. S4 in the supplementary material).

We then analyzed the expression levels of downstream factors of Nodal signaling in 85% epiboly cdx1b morphants. Substantial reductions in the numbers of gata5-expressing endodermal precursors and reduced expression levels in ventral-lateral mesodermal cells were observed in the majority of epiboly morphants when compared with either cdx1b-4mm-MO-injected or wild-type embryos (Fig. 4A-C,Q,R, and data not shown); whereas injection of either the Cdx1b protein or cdx1b mRNA restored the gata5-expressing endodermal cell number to a level comparable to that of wild-type in embryos co-injected with cdx1b MO (Fig. 4D,T). A higher percentage of rescued embryos was obtained by co-injection of the Cdx1b protein when compared with co-injection of cdx1b mRNA, which may be attributed to the translational efficiency of exogenous cdx1b mRNA (Fig. 4S). Approximately 54% decreases in the respective numbers of cas- and sox17-expressing endodermal precursors were also identified in epiboly morphants compared with cdx1b-4mm-MO-injected embryos, whereas expression levels in dorsal forerunner cells were not altered (Fig. 4E-L,Q,R). About a 47% reduction in the number of foxa2-expressing endodermal precursors was observed in epiboly morphants, and the foxa2 expression area in the prechordal plate was also affected (Fig. 4O,Q,R). Similarly, injection of either the Cdx1b protein or cdx1b mRNA restored the foxa2-expressing endodermal cell number to a level comparable to that of wild type in embryos co-injected with the cdx1b MO, thus demonstrating the specificity of the cdx1b MO used in this study (Fig. 4P,S,T). In order to clarify whether the reduced number of endodermal precursor cells in epiboly cdx1b morphants is a primary defect or not, we examined development of the forebrain, hindbrain, prechordal plate and notochord in epiboly morphants. As shown in Fig. 5, similar expression levels and patterns of fgf3, gsc and ntl were detected in both epiboly morphants and wild-type embryos, indicating that a primary endoderm defect occurred in epiboly cdx1b morphants. Taken together, zebrafish cdx1b is required for the occurrence of normal numbers of gata5-, cas-, foxa2- and sox17-expressing endodermal precursors but is not required for the normal expression levels of cyclops, sqt, oep or bon.

Ectopic cdx1b expression increases the respective numbers of gata5-, cas-, foxa2- and sox17-expressing endodermal precursor cells in epiboly embryos

In order to further confirm the decreases in the respective numbers of gata5-, cas-, foxa2- and sox17-expressing endodermal precursors detected in epiboly cdx1b morphants, we also overexpressed cdx1b by injecting cdx1b mRNA into one-cell zygotes. Substantial increases in the respective numbers of gata5-, cas-, foxa2- and sox17-expressing endodermal precursors and altered distribution patterns were detected in 85-90% epiboly embryos that had been injected with cdx1b mRNA compared with either embryos that had

Table 2. Respective percentages of embryos showing increases in the numbers of gata5-, cas-, sox17- and foxa2-expressing endodermal cells in cdx1b-overexpressing epiboly embryos

<table>
<thead>
<tr>
<th>Embryos containing increased endodermal cell numbers</th>
<th>gata5</th>
<th>cas</th>
<th>sox17</th>
<th>foxa2</th>
</tr>
</thead>
<tbody>
<tr>
<td>58.5±2.4</td>
<td>72.7±3.0</td>
<td>70.9±3.2</td>
<td>67.6±3.4</td>
<td></td>
</tr>
<tr>
<td>41.5±2.0</td>
<td>27.3±1.9</td>
<td>29.1±2.0</td>
<td>32.4±3.4</td>
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</tr>
</tbody>
</table>

Data are presented as the mean±standard error (Kuzuma and Bohnenblust, 2004).
been injected with lacZ mRNA or wild-type embryos (Fig. 6, Table 2). When epiboly embryos ectopically expressing cdx1b were examined in the animal view, we could easily detect the distribution of extra gata5-, cas-, foxa2- and sox17-expressing endodermal precursors in the animal pole where no endodermal precursors normally exist (Fig. 6D,H,L,P). In addition, we also detected the appearance of extra foxa2-expressing mesodermal cells in the animal pole, and expansion/distortion of the foxa2-expressing prechordal plate and notochord was also detected in epiboly embryos that had been injected with cdx1b mRNA (Fig. 6O,P, data not shown). Overall, these results demonstrate that ectopic cdx1b expression can extensively increase the respective numbers of gata5-, cas-, sox17- and foxa2-expressing endodermal precursors and alter their distribution patterns in injected 85-90% epiboly embryos.

**Regulation of the foxa2 gene by cdx1b**

In order to investigate possible target genes of cdx1b, we conducted a comparative sequence analysis to identify conserved Cdx1-binding motifs in the gata5, cas, sox17 and foxa2 genes. We identified a conserved Cdx1-binding motif (TTTATA) located in intron 1 of foxa2 genes from human, mouse and zebrafish and two conserved Cdx1-binding motifs (TTTATG) located at 22 120 bp upstream of exon 1 of zebrafish gata5 and its 3' UTR region when comparing the gata5 gene sequences from the zebrafish and stickleback (see Fig. S1 in the supplementary material).

Subsequently, we conducted EMSA experiments to investigate whether the Cdx1b protein specifically binds to the CDX1-binding motif located in intron 1 of the foxa2 gene. Nuclear extracts prepared from cdx1b-overexpressing COS-1 cells were incubated with biotin-labeled double-stranded oligonucleotides containing a potential Cdx1-binding motif. These oligonucleotides produced shifted bands and can be competed by an excess amount of competitor oligonucleotides, but failed to be competed by oligonucleotides containing the mutated Cdx1-binding motif (Fig. 7). This result indicates that Cdx1b can bind to the CDX1-binding motif located in intron 1 of the foxa2 gene.

In addition, we conducted rescue experiments by co-injecting either zebrafish or mouse foxa2 mRNA with the cdx1b MO into one-cell zygotes. Significant reductions in the ceruloplasmin expression domain and level were readily detected in 53 hpf cdx1b morphants (Fig. 3I, Fig. 8B,C). However, injection of either zebrafish or mouse foxa2 mRNA restored the expression domain of ceruloplasmin in the liver of cdx1b MO (Fig. 8D,E) to a size comparable to that of wild-type embryos (Fig. 8A). While slightly increased expression domains of ceruloplasmin in the liver of embryos that had been injected with respective zebrafish or mouse foxa2 mRNA alone were detected (Fig. 8F,G), approximately 32-34% of the cdx1b morphants could be rescued and showed normal expression domains of ceruloplasmin in the liver of embryos that had been co-injected with either zebrafish or mouse foxa2 mRNA (Fig. 8H). By contrast, injection of either zebrafish or mouse foxa2 mRNA could not rescue early endoderm deficiencies in epiboly embryos co-injected with the cdx1b MO when assayed by sox17 expression (data not shown). These results indicate that Cdx1b directly regulates foxa2 expression and may modulate gata5 expression to affect endoderm formation and subsequent development of different digestive organs.

**DISCUSSION**

We identified cdx1b, a caudal-related homeodomain gene, in zebrafish embryos. Our loss-of-function, overexpression, EMSA and rescue studies demonstrated that zebrafish cdx1b controls the morphogenesis of different digestive organs through regulating expressions of foxa2 and gata5, downstream factors of Nodal signaling that are required for early endoderm formation.

**Comparison of expression patterns of cdx1b with zebrafish cdx1a and cdx4 and mouse Cdx1**

Three Cdx genes (Cdx1, Cdx2 and Cdx4) have been identified in mammals (Lohnes, 2003). Previously, two caudal-related homeobox genes, cdx1a and cdx4, were characterized in zebrafish (Davidson et al., 2003; Davidson and Zon, 2006; Shimizu et al., 2006). Results from the sequence comparison, phylogenetic analyses, and expression patterns all indicated that zebrafish cdx1b differs from the previously identified cdx1a and cdx4 (see Figs S2, S3 in the supplementary material; Fig. 1). On the whole, cdx1b is a maternal transcript and is ubiquitously expressed in both epiblast and hypoblast cells during the late gastrulation stage, while both cdx1a and cdx4 are expressed in these two cell types near the margin.

**Fig. 8. Injection of either zebrafish or mouse Foxa2 mRNA restored the expression domain of ceruloplasmin in the liver of cdx1b morphants.** Expression of ceruloplasmin in a 53 hpf wild type (A), respective morphants (B, C), an embryo co-injected with zebrafish foxa2 mRNA and cdx1b MO (D), an embryo co-injected with mouse Foxa2 mRNA and cdx1b MO (E), and respective embryos injected with either zebrafish foxa2 (F) or mouse Foxa2 (G) mRNA alone. (H) Comparison of the percentages of embryos showing reduced levels of the ceruloplasmin expression domain in respective embryos injected with either the cdx1b MO, zebrafish foxa2 mRNA and cdx1b MO, or mouse Foxa2 mRNA and cdx1b MO. Scale bar: 100 μm. wt, wild type.
but are excluded from the dorsal midline. During late segmentation stages, the cdx1b transcript was detected in the brain, retina and somites, whereas almost no cdx1a expression was detected in 22s embryos, and cdx4 mRNA was detected in the posterior spinal cord, notochord, hypochond, ventral mesenchyme and tailbud.

Although the syntenic analyses suggested that zebrafish cdx1b is an ortholog of the mammalian Cdx1 gene, variations between their expression patterns were identified. Zebrafish cdx1b is maternally deposited; however, mouse Cdx1 is not (Fig. 1) (Meyer and Gruss, 1993; Freund et al., 1998; Lohnes, 2003). During late gastrulation, zebrafish cdx1b is uniformly expressed in both epithelium and hypoblast cells, whereas mouse Cdx1 begins to be expressed in the ectoderm and nascent mesoderm of the primitive streak in mouse E7.5 embryos. During somitogenesis, zebrafish cdx1b was found to be expressed in the notochord, midbrain, hindbrain and somites, whereas mouse Cdx1 is expressed in developing somites and neural tubes with an anterior expression boundary that corresponds to the proctodaeum in mouse E8.5 embryos. Taken together, in contrast to mouse Cdx1, zebrafish cdx1b exhibits early expression in endodermal cells during gastrulation and in the anterior neuroectoderm, including the forebrain, midbrain and retinas, during the segmentation stage. This expression difference may contribute to the novel role of zebrafish cdx1b in regulating early endoderm formation reported in this study.

cdx1b regulates expression of downstream factors of Nodal signaling

Nodal signaling is central to early endoderm development. In zebrafish embryos, Squint and Cyclops Nodal factors interact with the Taram-a (Tar) receptor and the One-eyed pinhead EGF-CFC co-receptor. Nodal signaling is transmitted through Bon, Gata5 and Cas, the Taram-a (Tar) receptor and the One-eyed pinhead EGF-CFC co-receptor. Nodal signaling is thus important for early endoderm formation. In 54 hpf embryo, the expression of genes important for liver and pancreas development, including hnfl, hnf1B, hnf4a, pdx1 and α-amylase (Cockell et al., 1995; Levinson-Dushnik and Benvenisty, 1997; Duncan et al., 1998; Gerrish et al., 2000; Lee et al., 2002). Mouse Pdx1 is expressed in the developing foregut, which invaginates with the dorsal and ventral buds of the pancreas (Edlund, 2002). A recent study showing that the homozgyous deletion of a conserved enhancer region containing binding sites for several transcription factors, including Foxa2 from the pdx1 gene, revealed no ventral pancreatic bud specification or dorsal bud hypoplasia (Fujitani et al., 2006). Their results indicated that different levels of Pdx1 protein activity are required for specifying several organs of the posterior foregut, pancreas and gut enterodendroc cell differentiation.

In faul/gata5 mutant embryos, reductions in the respective sox17- and foxa2-expressing endodermal cell numbers were detected, whereas overexpression of gata5 caused increased numbers of foxa2- and sox17-expressing endodermal cells (Reiter et al., 2001). Therefore, perturbations of sox17-expressing endodermal cell numbers in both epiboly cdx1b morphants and embryos ectopically expressing cdx1b are probably indirectly caused by regulation of gata5 expression by cdx1b (Figs 4, 6). Weaker cas expression in endodermal cells was detected in faul/gata5 epiboly mutants, but overexpression of gata5 did not activate cas expression in nonmarginal cells (Kimura et al., 2001). Thus, the suggested regulation of gata5 expression by cdx1b cannot completely account for the alterations of cas-expressing endodermal cell numbers observed in both epiboly cdx1b morphants and embryos ectopically expressing cdx1b. The possibility that cdx1b regulates cas expression exists and remains to be investigated. Altogether, our study adds an extra regulatory path to Nodal signaling in addition to the roles of Eomes and Spg, and cdx1b may participate in early endoderm formation by regulating the expressions of foxa2 and gata5 during gastrulation.

Regulation of foxa2 expression by cdx1b may affect development of the liver and pancreas

A chimeric mouse embryo study showed that Foxa2 (also known as Hnf3β) is required for the formation of the foregut and midgut endoderm (Dufort et al., 1998). A recent study that engineered an endoderm-specific deletion of foxa2 using the Cre/lloxP recombination system demonstrated that foxa1 and foxa2 are required for the establishment of competence within the foregut endoderm and the onset of hepatogenesis (Lee et al., 2005). In vivo footprinting studies have shown that binding of Foxa2 onto the albumin enhancer controls hepatic specification of the gut endoderm, and co-binding of Foxa2 and Gata4 on the albumin enhancer cEF and eG sites, respectively, is essential for albumin enhancer activity (Gualdi et al., 1996; Bossard and Zaret, 1998). Therefore, Foxa family proteins, including Foxa2, are pioneer factors that bind to promoters and enhancers to permit chromatin access for other tissue-specific transcription factors (Friedman and Kaestner, 2006). Additionally, Foxa2 regulates expressions of genes important for liver and pancreas development, including hnf1α, hnf1β, hnf4a, pdx1 and α-amylase (Cockell et al., 1995; Levinson-Dushnik and Benvenisty, 1997; Duncan et al., 1998; Gerrish et al., 2000; Lee et al., 2002). Mouse Pdx1 is expressed in the developing foregut, which invaginates with the dorsal and ventral buds of the pancreas (Edlund, 2002). A recent study showing that the homozygous deletion of a conserved enhancer region containing binding sites for several transcription factors, including Foxa2 from the pdx1 gene, revealed no ventral pancreatic bud specification or dorsal bud hypoplasia (Fujitani et al., 2006). Their results indicated that different levels of Pdx1 protein activity are required for specifying several organs of the posterior foregut, pancreas and gut enterodendroc cell differentiation.

In 54 hpf cdx1b morphants, defects in the growth of the liver and pancreatic buds and abnormal intestinal morphogenesis were readily detected when using gata5, gata6 and hnf4a, respectively, as probes (see Fig. S5 in the supplementary material). In addition, a decreased pdx1 expression level was observed in 72 hpf cdx1b morphants (Fig. 3). As a result, hypoplastic development of the liver and pancreas was detected in 96 hpf cdx1b morphants (Fig. 3). Results of functional analyses, the presence of conserved Cdx1-binding motifs in the gata5 gene, EMSA and Foxa2 mRNA rescue experiments suggest that Cdx1b regulates Foxa2 and may modulate gata5 expression (Figs 4, 6–8). Zebrafish gata5 is thought to be a
functional ortholog of mammalian and avian Gata4, and foxa/gata5 mutant embryos exhibit defects in several endodermal organs, including the liver and pancreas (Reiter et al., 2001; Wallace and Pack, 2003). Zebrafish pdx1 morphants have been shown to display defects in pancreas development (Yee et al., 2001). Judging from the role of Foxa2 as a pioneer transcription factor that displaces linker histones from compacted chromatin and the synergistic interactive effect on liver-specific albumin gene expression with Gata4 and other transcription factors in mouse embryos, decreases in the numbers of gata5- and foxa2-expressing endodermal precursor cells in epiboly cdx1b morphants can cause deficient gene activation in the development of the liver and pancreas, thus resulting in deformities of these two digestive organs.

In conclusion, we have identified a caudal-related homeobox gene, cdx1b, in zebrafish embryos. Results from the antisense MO-mediated knockdown, overexpression, conserved Cdx1-binding motif search, EMSA and rescue experiments demonstrated that cdx1b regulates foxa2 expression and may modulate the expression of gata5, thus resulting in subsequent hypoplastic growth of the liver and pancreas as well as intestinal abnormalities.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/941/DC1

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