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.

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; Murtaugh et al., 2003). 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 HMG domain transcription factor, Sox17, leading to endoderm formation (Alexander and Stainier, 1999; Alexander et al., 1999; Kikuchi et al., 2000; 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 level 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

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mutant chimeric mice, polyps with stomach heteroplasia were found in the midgut (Chawengsaksophak et al., 1997; Beck et al., 2003). However, *Cdx1*^{−/−} 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 under a dissecting microscope by the presence of eye fusion and the number of eyes present in *oep*[*m134*], *cyc*[*b16*] and *squint*[*cz35*] 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 λgt10 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. AY761094. 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 PHYLIP3.6 (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* (*Nco*I/SP6), *cas* (*Nco*I/SP6), *cdx1b* (*Hin*dIII/T3), *ceruloplasmin* (*Not*I/T7), *cyclops* (*Eco*R I/T7), *fgf3* (*Nco*I/SP6), *foxa2* (*Spe*I/T3), *gata5* (*Sac*II/SP6), *gsc* (*Eco*RI/T7), *ifabp* (*Nco*I/SP6), *insulin* (*Nco*I/SP6), *lfabp* (*Sal*I/T7), *myoD* (*Xba*I/T7), *ntl* (*Xho*I/T7), *oep* (*Nco*I/SP6), *rx1* (*Sal*I/T7), *shh* (*Bam*HI/T7), *sox17* (*Eco*RI/T7), *squint* (*Not*I/T7) and *trypsin* (*Not*I/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 endodermal cell numbers.

Morpholino, *cdx1b* RNA, *foxa2* RNA and Cdx1b protein injections
Respective morpholino oligonucleotides (MOs; Gene Tools) were dissolved in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$ 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 Nanoject 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: <u>CA</u>TTTTTTCTGGTGGCTCCAGTGC; and *cdx1b*-4mm MO containing the same nucleotide sequences as *cdx1b* MO except for four mismatched sequences: CAATTTTTGTGGTGCCTCCACTGC. Respective capped *cdx1b* and *lacZ* mRNAs were synthesized using a T7 or a SP6 mMESSAGE mMACHINE Kit (Ambion). To ectopically express *cdx1b*, *cdx1b* mRNA (50-60 pg) was injected into the cytoplasm of 1-2-cell zygotes. *lacZ* mRNA (≥60 pg) was injected into the cytoplasm of 1-2-cell zygotes as the control. To rescue *cdx1b* morphants, either 10-30 pg of *cdx1b* 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 (a total of 2.3 nl) into 1-2-cell zygotes. As a control, a similar amount of green fluorescent protein (GFP) was co-injected with the *cdx1b* MO into 1-2-cell zygotes. The Cdx1b protein was synthesized using the TNT-coupled transcription/translation system (Promega) with the pcDNA3-cdx1b-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 (5×10^6) were plated onto a 10 cm Petri dish and cultured for 16 hours. After respective transfection with the pcDNA3-cdx1b-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).
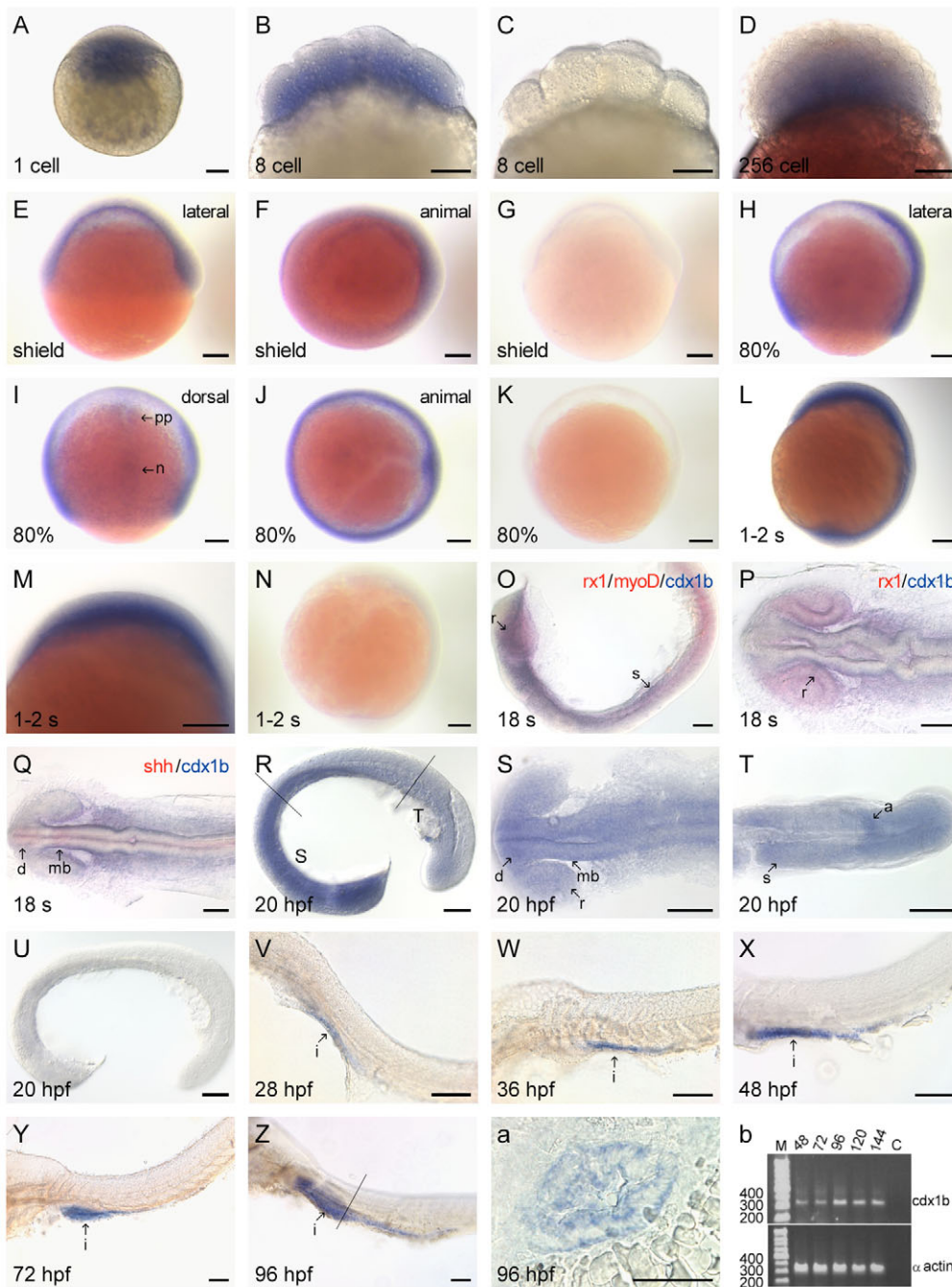
Fig. 1. Developmental mRNA expression pattern of the zebrafish *cdx1b* gene. *cdx1b* mRNA was detected in one-cell zygote (**A**), cleavage (**B**), blastula (**D**), shield (**E,F**), 80% epiboly (**H-J**), 1-2s (**L,M**), 18s (**O-Q**), 20 hpf (**R-T**), 28 hpf (**V**), 36 hpf (**W**), 48 hpf (**X**), 72 hpf (**Y**) and 96 hpf (**Z**) stages. (**C,G,K,N,U**) Embryos hybridized with the sense RNA probe. Double-labeled in situ hybridization showed the expression of *cdx1b* compared with either *rx1*, *myoD* (O,P) or *shh* (Q). The two lines in R show different boundaries of a 20 hpf embryo viewed at higher magnification in S and T. (**a**) Cryostat transverse section along the plane of the line shown in Z. (**b**) Semiquantitative RT-PCR revealed *cdx1b* expression levels in embryos from different developmental stages. a, anus; d, diencephalon; i, intestine; mb, midbrain; n, notochord; pp, prechordal plate; r, retina; s, somite. Scale bars: 100 μm.

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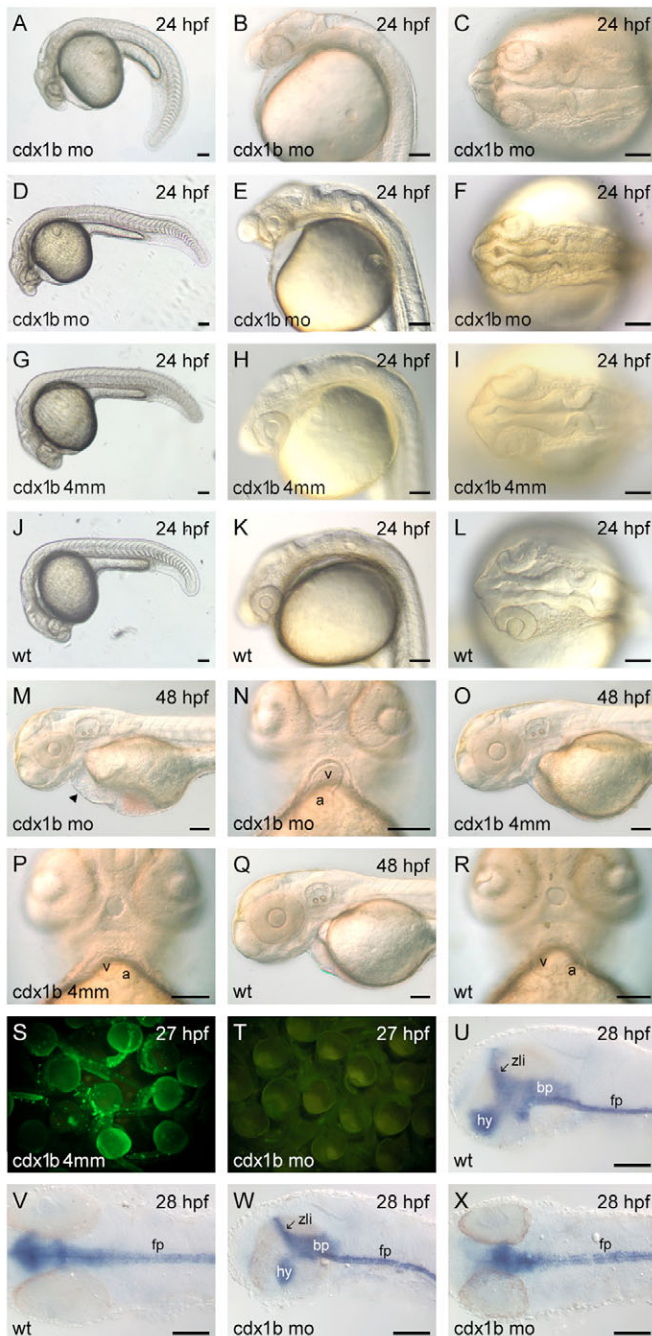
Table 1. Morphant phenotype characterization based on different doses of *cdx1b* MO injection

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

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

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 levels were maintained in 144 hpf embryos (Fig. 1b). Furthermore, *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). Neuroectodermal 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

Fig. 2. *cdx1b* antisense MO knockdown analyses. *cdx1b* 24 hpf (**A-F**) and 48 hpf (**M,N**) zebrafish morphants; *cdx1b*-4mm-MO-injected 24 hpf (**G-I**) and 48 hpf (**O,P**) embryos; wild-type 24 hpf (**J-L**) and 48 hpf (**Q,R**) embryos. Green fluorescence was not detected in *cdx1b* MO and CMV-*cdx1b*-mo-*GFP* co-injected (**T**) 27 hpf embryos, whereas bright green fluorescence was detected in *cdx1b*-4mm MO and CMV-*cdx1b*-mo-*GFP* co-injected embryos (**S**). The expression patterns of *shh* were compared in 28 hpf wild type (**U,V**) and morphants (**W,X**). Scale bars: 100 µm. a, atrium; bp, basal plate midbrain; fp, floor plate; hy, hypothalamus; v, ventricle; wt, wild type; zli, zona limitans intrathalamica.

mouse chromosome 5 where *Cdx2* resides. Therefore, we named this *caudal*-related homeobox gene *cdx1b*, which is a newly identified zebrafish *cdx1* paralog with significant difference from previously identified *cdx1a* and *cdx4* genes.
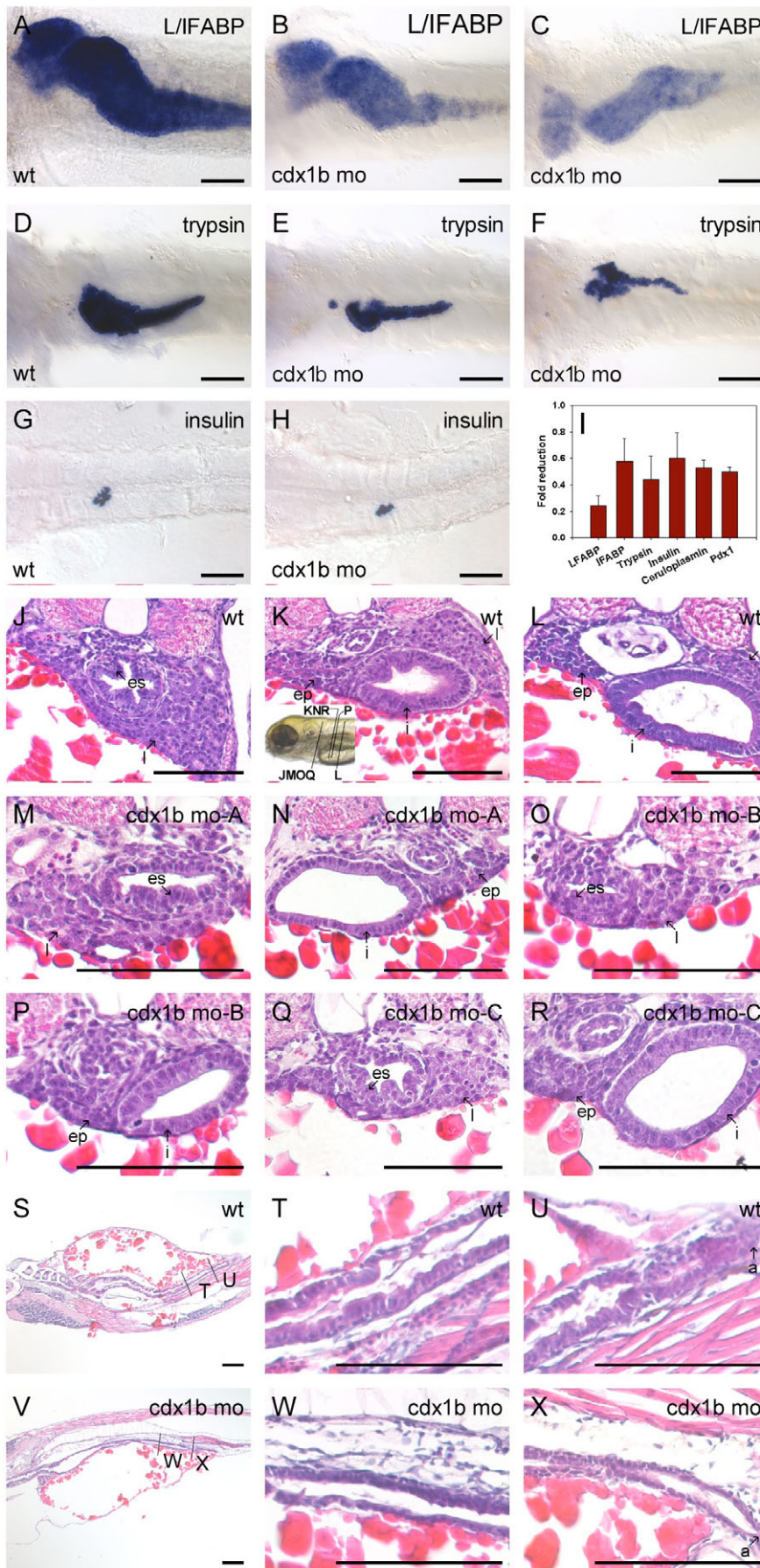
Fig. 3. Inhibition of zebrafish *cdx1b* function affects expression of some digestive organ marker genes and causes hypoplastic growth of the liver, pancreas and intestines. Seventy-two hpf (**A,D**) and 54 hpf (**G**) wild type and 72 hpf (**B,C,E,F**) and 54 hpf (**H**) morphants were respectively labeled with *lfabp/ifabp* (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.
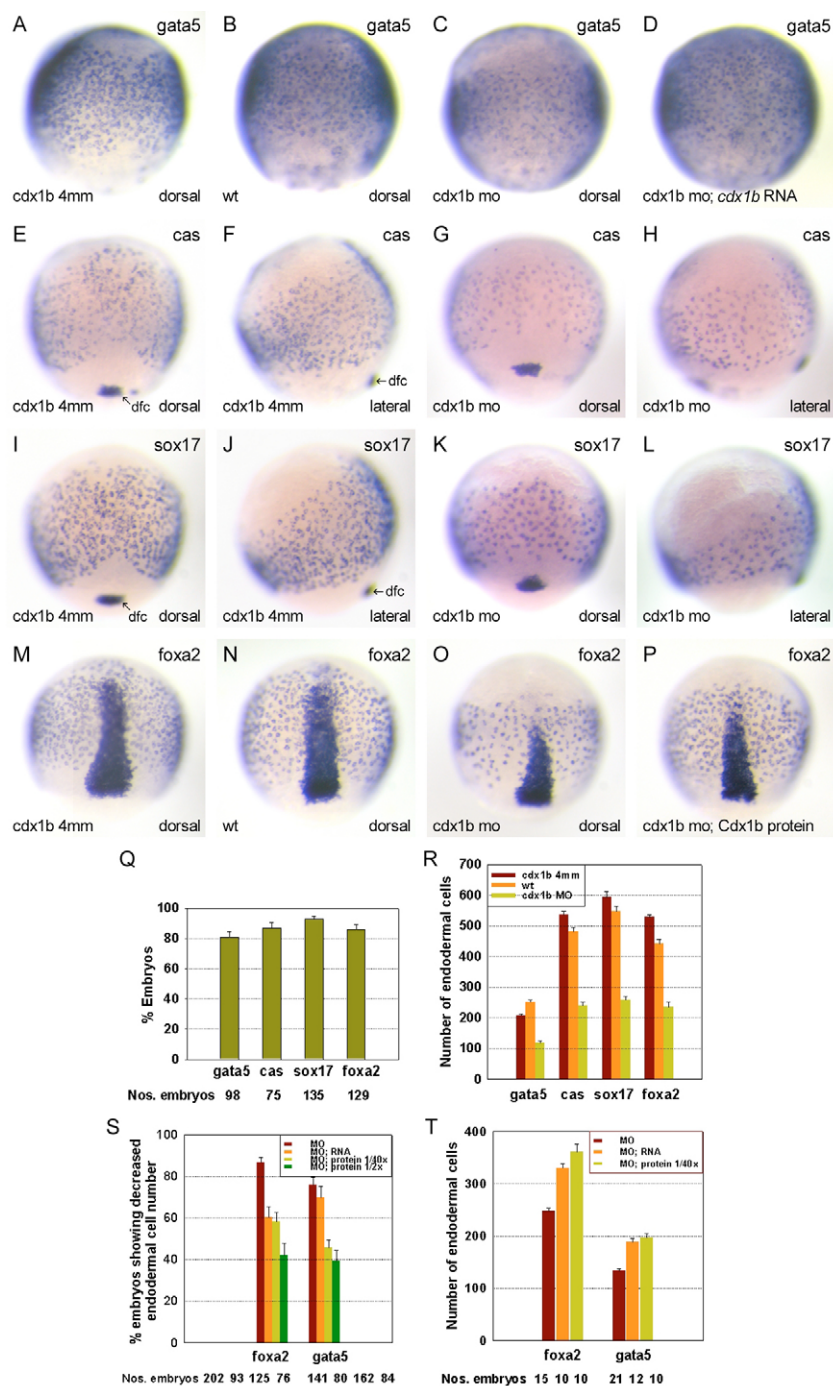
Fig. 4. Analyses of relationships between *cdx1b* and downstream factors of Nodal signaling in zebrafish. Reductions in respective *gata5*-, *cas*-, *sox17*- and *foxa2*-expressing endodermal cell numbers were observed in 85% epiboly morphants (**C,G,H,K,L,O**) compared with respective *cdx1b*-4mm-MO-injected (**A,E,F,I,J,M**) and wild-type (**B,N**) embryos. Embryos co-injected with either *cdx1b* mRNA or protein showed restoration of respective *gata5*- (**D**) and *foxa2*-expressing (**P**) endodermal cell numbers. Percentages of morphants showing decreases in the respective numbers of *gata5*-, *cas*-, *sox17*- and *foxa2*-expressing endodermal cells (**Q**). Comparison of the respective *gata5*-, *cas*-, *sox17*- and *foxa2*-expressing endodermal cell numbers in morphants (yellow bars), and wild type (orange bars) and *cdx1b*-4mm-MO-injected (brown bars) embryos (*n*=10 each) (**R**). Comparison of percentages of embryos showing decreased *foxa2*- or *gata5*-expressing endodermal cell numbers in respective embryos injected with either *cdx1b* MO (brown bars), *cdx1b* MO and *cdx1b* mRNA (orange bars), or *cdx1b* MO and different amounts of Cdx1b protein (1/40×, yellow bars; 1/2×, green bars) (**S**). Comparison of the respective *foxa2*- and *gata5*-expressing endodermal cell numbers in morphants (brown bars) and embryos co-injected with *cdx1b* MO and either *cdx1b* mRNA (orange bars) or protein (yellow bars) (**T**). Error bars represent standard error. Dfc, dorsal forerunner cell; 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)
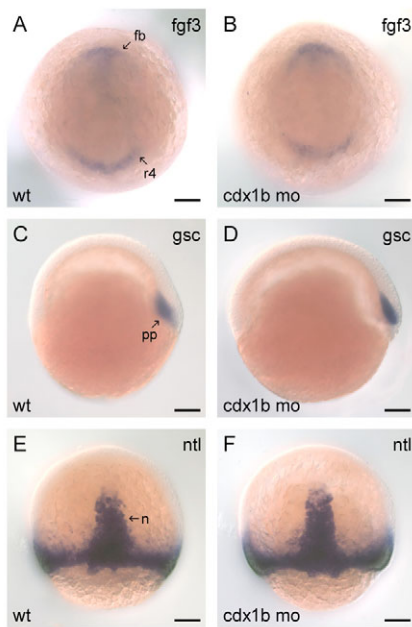
Fig. 5. Zebrafish *cdx1b* epiboly morphant showed no ectoderm or mesoderm defects. Expression of *fgf3* in wild type (**A**) and bud morphants (**B**). *gsc* expression in wild type (**C**) and shield morphants (**D**). *ntl* expression in 80% morphants (**F**) and wild-type (**E**) embryos. fb, forebrain; n, notochord; pp, prechordal plate; r4, rhombomere 4; wt, wild type. Scale bars: 100 μm.

cdx1b morphants (55 %, *n*=64) compared with wild-type embryos (Fig. 3G,H). A real-time quantitative PCR further confirmed the relatively decreased expression levels of *lfabp*, *ifabp*, *trypsin* and *insulin* in 72 hpf *cdx1b* morphants (Fig. 3I). Moreover, histological analyses revealed different degrees of hypoplastic development of the liver, intestines and exocrine pancreas in 96 hpf *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 in 96 hpf *cdx1b* morphants (Fig. 3N,P,R; *n*=11, V-X; *n*=5). Reductions in the sizes of the liver and exocrine pancreas were observed in 96 hpf *cdx1b* morphants (Fig. 3M-R), and these two organs were also detected in the reversed L-R axis (Fig. 3M,N; 5 of 11). Together, these results indicate that zebrafish *cdx1b* is required for the normal morphogenesis of the liver, intestines and pancreas as well as their left-right asymmetrical distribution in the embryo.
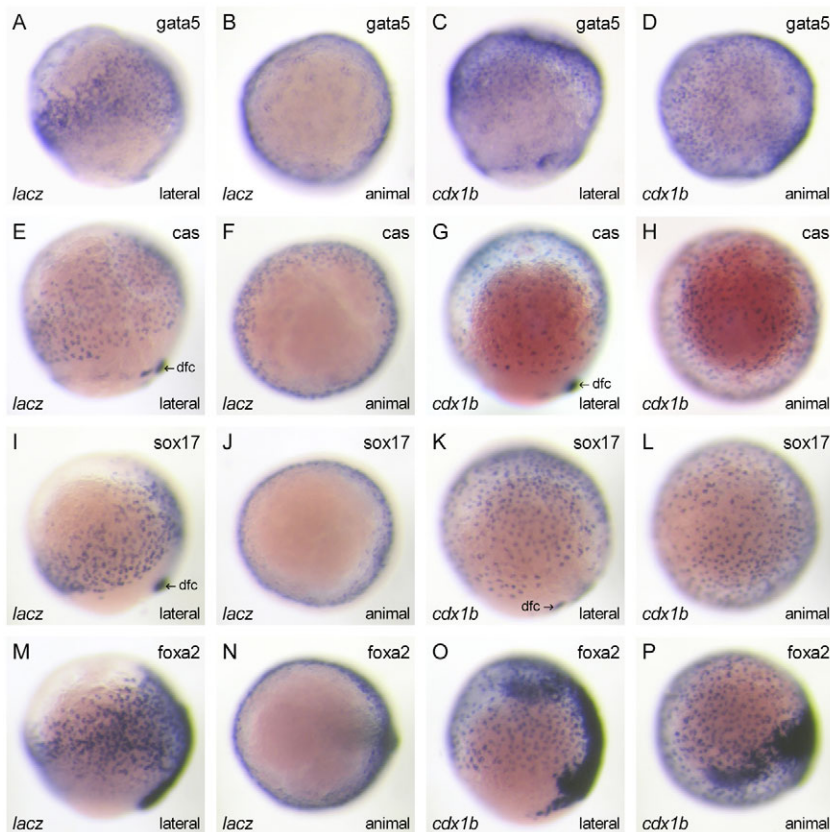
when compared with those of the wild-type embryos (Fig. 3A-C). In addition, 20% of morphants (*n*=50) showed smaller amounts of liver *lfabp* and intestinal *ifabp* expression on the R-axis (Fig. 3C). A decrease in the area of *trypsin* expression was detected in 77% of 72 hpf *cdx1b* morphants (*n*=104) compared with wild-type embryos (Fig. 3D-F). Similarly, *trypsin* mRNA was localized on the R-axis in 6% of 72 hpf *cdx1b* morphants (*n*=104) (Fig. 3F). The expression domain of *insulin* was also decreased in 54 hpf

Fig. 6. Effects of ectopic *cdx1b* expression on respective *gata5*-, *cas*-, *sox17*- and *foxa2*-expressing endodermal cell numbers in zebrafish. Increases in the numbers of *gata5*- (**C**,**D**), *cas*- (**G**,**H**), *sox17*- (**K**,**L**) and *foxa2*-expressing (**O**,**P**) endodermal cells were detected in 85% epiboly embryos ectopically expressing *cdx1b* when compared with *lacZ* (**A**,**B**,**E**,**F**,**I**,**J**,**M**,**N**) ectopically expressing epiboly embryos. Scale bars: 100 μm. Dfc, dorsal forerunner cell.
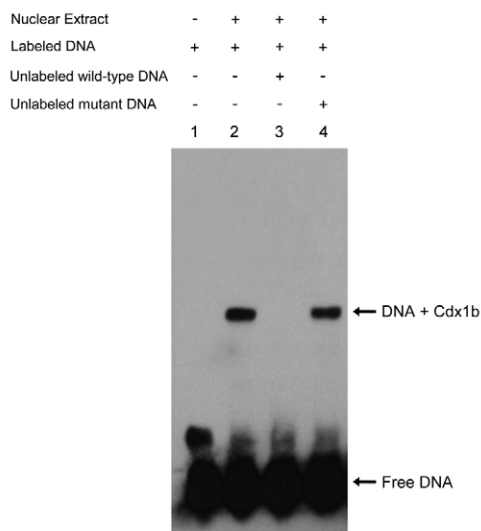
Table 2. Respective percentages of embryos showing increases in the numbers of *gata5*-, *cas*-, *sox17*- and *foxa2*-expressing endodermal cells in *cdx1b*-overexpressing epiboly embryos

	Percentage			
	gata5	*cas*	*sox17*	*foxa2*
Embryos containing increased endodermal cell numbers	58.5±2.4	72.7±3.0	70.9±3.2	67.6±3.4
Embryos containing endodermal cell numbers similar to wild-type or *lacZ*-overexpressing embryos	41.5±2.0	27.3±1.9	29.1±2.0	32.4±3.4
Total number of embryos	412	216	203	185

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

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*^{cz35}, 48-hpf *cyc*^{b16} and 4-6-s *oep*^{m134} 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).

Fig. 7. Electrophoretic mobility shift assay of the Cdx1b protein. The biotin-labeled wild-type oligonucleotide was mixed with buffer (lane 1) or 10 μg of nuclear extract prepared from COS-1 cells transfected with pcDNA3-cdx1b-Myc-His plasmid (lanes 2-4). Binding was completely abolished by the addition of an unlabeled wild-type oligonucleotide competitor in a 50-fold molar excess (lane 3), whereas specific binding was maintained when the same amount of the excess mutant oligonucleotide competitor was added (lane 4).

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
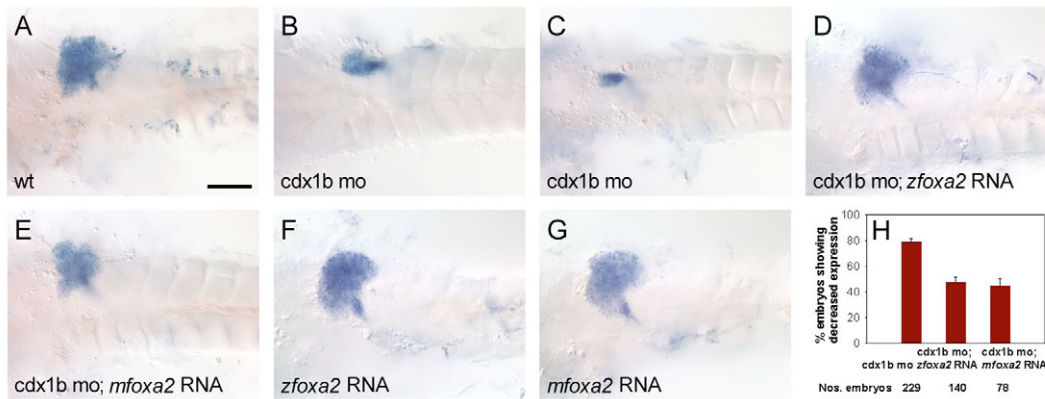
Fig. 8. Injection of either zebrafish or mouse *Foxa2* mRNA restored the expression domain of *ceruloplasmin* in the liver of *cdx1b* morphants. Expression of *ceruloplasmin* in a 53 hpf wild type (**A**), respective morphants (**B,C**), an embryo co-injected with zebrafish *foxa2* mRNA and *cdx1b* MO (**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.

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-biding 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 embryos co-injected with the *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 *cerulopasmin* 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

but are excluded from the dorsal midline. During late segmentation stages, the *cdx1b* transcript was detected in the brain, retinas and somites, whereas almost no *cdx1a* expression was detected in 22s embryos, and *cdx4* mRNA was detected in the posterior spinal cord, notochord, hypochord, 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 epiblast 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 retinas, forebrain, midbrain, hindbrain and somites, whereas mouse *Cdx1* is expressed in developing somites and neural tubes with an anterior expression boundary that corresponds to the preotic sulcus 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, following subsequent activations of *sox17* and several fork-head factors including *foxa2*, leading to endoderm formation (reviewed by Schier, 2003). Nodal signaling for endoderm development became more complex when two maternally deposited transcripts (*spg* and *eomes*) were shown to interact with downstream factors of Nodal signaling and thus participate in early endoderm formation (Lunde et al., 2004; Reim et al., 2004; Bjornson et al., 2005). Eomes can interact with both Bon and Gata5 to induce *cas* expression in the late blastula stage, whereas Spg interacts with Cas to commit mesendodermal precursors to an endodermal fate and activate the expressions of *sox17* and *foxa2* during gastrulation.

Antisense MO-mediated knockdown of *cdx1b* and ectopic *cdx1b* expression experiments demonstrated that *cdx1b* modulates the respective numbers of *gata5*-, *cas*-, *foxa2*- and *sox17*-expressing endodermal cells during gastrulation (Figs 4, 6). Overlapping expressions of *cdx1b*, *gata5*, *cas*, *foxa2* and *sox17* exist during gastrulation, which provides additional support for the potential interactions between *cdx1b* and these genes (Fig. 1) (Alexander and Stainier, 1999; Kikuchi et al., 2001; Reiter et al., 2001). An interspecific sequence comparison revealed conserved Cdx1-binding motifs (A/CTTTATA/G) in intron 1 of the *foxa2* gene as well as in the 5′ upstream and 3′ UTR regions of the *gata5* gene, suggesting potential regulation by Cdx1b of the expression of these two genes (Brown et al., 2005). Direct binding of the Cdx1-binding motif of *foxa2* by Cdx1b was then demonstrated by EMSA experiments (Fig. 7). Furthermore, injection of *foxa2* mRNA rescued the expression domain of *ceruloplasmin* in the liver of 53 hpf embryos that had been co-injected with the *cdx1b* MO (Fig. 8). These results demonstrate that Cdx1b regulates *foxa2* expression.

In *fau/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 *fau/gata5* epiboly mutants, but overexpression of *gata5* did not activate *cas* expression in nonmarginal cells (Kikuchi 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/*loxP* 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 eF 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*, *hnf4α*, *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 analog (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 enterendocrine 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 *hnf4α*, 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. 3I). 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 *fau/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.

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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