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# Endodermal expression of Nkx6 genes depends differentially on Pdx1

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**Abstract**

Nkx family members are essential for normal development of many different tissues such as the heart, lungs, thyroid, prostate, and CNS. Here, we describe the endodermal expression pattern of three Nkx6 family genes of which two shows conserved expression in the early pancreatic epithelium. In chicken, Nkx6.1 expression is not restricted to the presumptive pancreatic area but is more broadly expressed in the endoderm. In mice, expression of Nkx6.1 is restricted to the pancreatic epithelium. In both mice and chicken, Nkx6.2 and Pdx1 are expressed in very similar domains, identifying Nkx6.2 as a novel marker of pancreas endoderm. Additionally, our results show that Nkx6.3 is expressed transiently in pancreatic endoderm in chicken but not mouse embryos. At later stages, Nkx6.3 is found in the caudal stomach and rostral duodenum in both species. Finally, we demonstrate that Pdx1 is required for Nkx6.1 but not Nkx6.2 expression in mice and that ectopic Pdx1 can induce Nkx6.1 but not Nkx6.2 or Nkx6.3 expression in anterior chicken endoderm. These results demonstrate that Nkx6.1 lies downstream of Pdx1 in a genetic pathway and that Pdx1 is required and sufficient for Nkx6.1 expression in the early foregut endoderm.


*Keywords:* Nkx6.1; Nkx6.2; Nkx6.3; Pdx1; Nkx2.2; Chicken; Mouse; Pancreas; Endoderm; Patterning; Development

## Introduction

The Nkx family of proteins constitutes a large group of homeodomain transcription factors with at least 18 members. In addition to the DNA binding homeodomain, the Nkx proteins all contain a conserved engrailed homology domain (eh1) repressor motif (also called the TN domain or the NK decapeptide) (Bodmer, 1995; Lints et al., 1993) that mediates the recruitment of Gro/Tle co-repressors (Muhr et al., 2001). Members of the Nkx family have been shown to be essential for normal development of many different tissues including the heart, lungs, thyroid, prostate, and the CNS (Bhatia-Gaur et al., 1999; Jamali et al., 2001; Sander et al., 2000a; Small et al., 2000). In mice and rats, Nkx6.1 has been detected in the early pancreatic epithelium at E10.5 (Oster et al., 1998b; Sander et al., 2000b). Later, Nkx6.1 expression in the pancreas becomes restricted to insulin-producing β-cells of the islets of Langerhans (Jensen et al., 1996; Oster et al., 1998b; Sander et al., 2000b). Nkx6.1-deficient mice display a severe reduction in β-cell mass after E13 but do not show any defects in pancreas development prior to this time point (Sander et al., 2000b). Nkx6.2 (also called Gtx) mRNA has been detected in the developing rat pancreas by RT-PCR as early as E13.5 (Oster et al., 1998b), suggesting a role for Nkx6.2 in pancreas formation. The transcription factors Nkx6.1 and Nkx6.2 are very similar with almost identical homeodomains (Jorgensen et al., 1999), the major difference being that Nkx6.1 contains poly-alanine and poly-serine tracts (Fig. 1). This sequence similarity suggests that the Nkx6 genes can provide redundant functions (Vallstedt et al., 2001). Nkx6.1 and Nkx6.2 are both expressed in the developing CNS where they are involved in specifying neuronal progenitor identity. Nkx6.1 is essential for normal motor neuron generation, and, in this process, Nkx6.2 can partially

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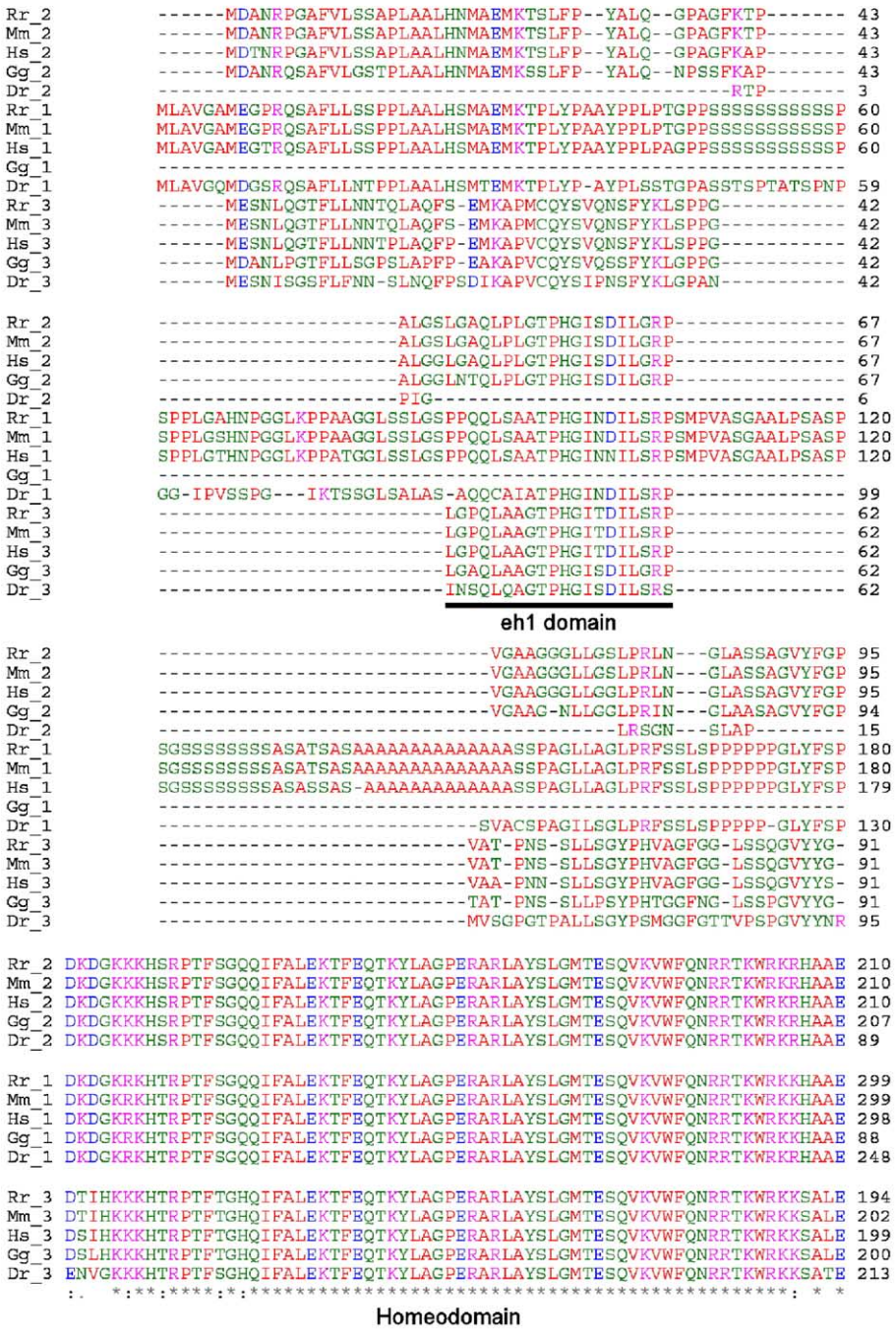
compensate for Nkx6.1 deficiency through ectopic expression in the Nkx6.1 domain of the spinal cord (Sander et al., 2000a; Vallstedt et al., 2001).

Orthologs of the Nkx6.1 and Nkx6.2 genes have been identified in rat, mouse, human, and chicken (Cai et al., 1999; Inoue et al., 1997; Jorgensen et al., 1999; Komuro et al., 1993; Lee et al., 2001; Oster et al., 1998b; Qiu et al., 1998; Watada et al., 2000). Recently, whole genome and EST sequencing projects have revealed the presence of a third Nkx6 family member, Nkx6.3, in mice and chicken. Nkx6.3 lacks the poly-alanine and poly-serine stretches found in Nkx6.1, and phylogenetic analysis reveals that Nkx6.1 and Nkx6.2 are more closely related to each other than to Nkx6.3 (Fig. 1). Apart from Nkx6.1, expression of the Nkx6 family genes has not been well documented outside the CNS.

In this study, we describe the expression patterns of Nkx6 family genes in chicken and mouse endoderm. We identify Nkx6.2 as a novel specific marker of pancreas endoderm up to approximately the 25 somite stage. Later, Nkx6.2 expression expands rostrally and caudally into the posterior stomach and duodenum, respectively. In contrast, expression of the new Nkx6 family member, Nkx6.3, is excluded from mouse pancreas and only transiently expressed in very early chicken pancreas but overlaps significantly with Nkx6.2 in duodenum

A



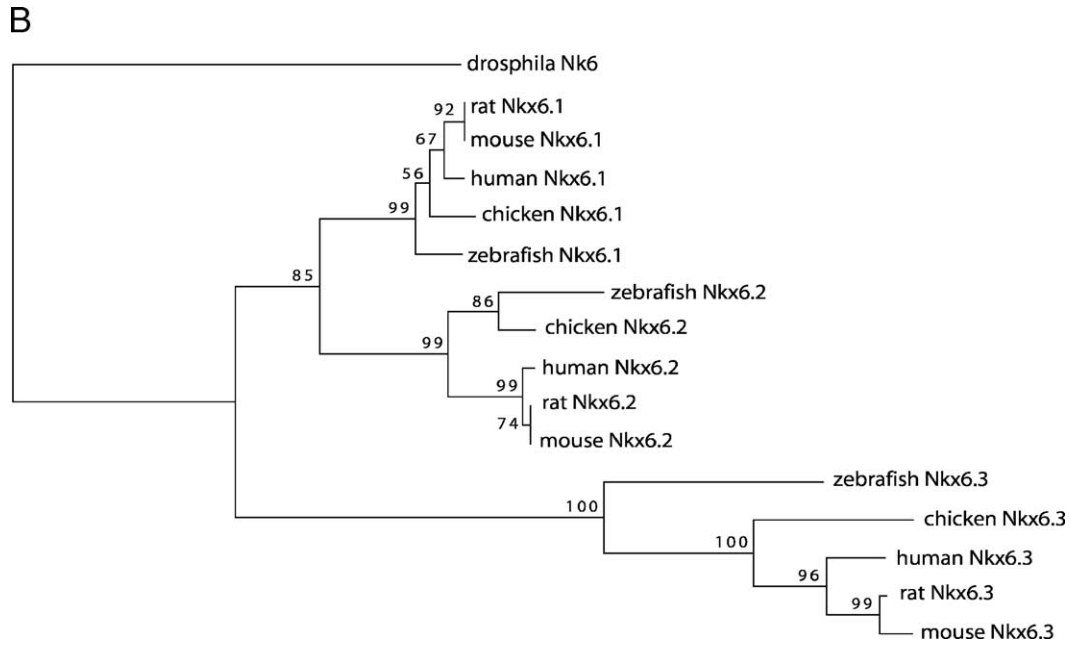



Fig. 1. Alignment and phylogenetic analysis of *Drosophila*, chick, rat, mouse, and human Nkx6 family genes. (A) ClustalW alignment of the N-termini and homeodomains of zebrafish (Dr), chicken (Gg), mouse (Mm), rat (Rr), and human (Hs) Nkx6.1, Nkx6.2, and Nkx6.3. The following sequences were aligned (GenBank accession numbers or ENSEMBL database locations are given in parentheses): *Drosophila* Nk6 (NM_144357), chick Nkx6.1 (AF102991), chick Nkx6.2 (AF189767), chick Nkx6.3 (Ensembl: Contig190.12 and Contig21189.1), mouse Nkx6.1 (NM_144955), mouse Nkx6.2 (L08074), mouse Nkx6.3 (AK018683), rat Nkx6.1 (NM_031737), rat Nkx6.2 (XM_219447), rat Nkx6.3 (XM_224991), human Nkx6.1 (NM_006168), human Nkx6.2 (NM_177400), and human Nkx6.3 (Ensembl: ENSP00000328512 and ENSP00000297748), zebrafish Nkx6.1 (X000991.8.1-170966), zebrafish Nkx6.2 (Zv4_scaffold1370.1), and zebrafish Nkx6.3 (Zv4_scaffold883.12). (B) Phylogentic tree indicating the evolutionary distances between the Nkx6 family genes in *Drosophila*, zebrafish, chicken, mouse, rat, and human. The neighbor-joining (NJ) tree was constructed using ClustalW amino acid alignment and Poisson correction. Bootstrap support values (out of 250 replicates) for the NJ tree are shown above the nodes.

and caudal stomach at later stages. The dynamic expression of Nkx6.2 is similar to that seen for Pdx1, a homeobox gene essential for pancreas development (Guz et al., 1995; Miller et al., 1994; Offield et al., 1996; Ohlsson et al., 1993). Finally, we demonstrate that Pdx1 is required and sufficient for Nkx6.1 expression, while Pdx1 does not appear to be involved in the regulation of Nkx6.2 and Nkx6.3.

## Materials and methods

### *Database searches*

The full-length amino acid sequences of mouse Nkx6.1 (acc: NP_659204), Nkx6.2 (acc: S35304), and Nkx6.3 (acc: AK018683) were used for BLASTP searches on the NCBI server (www.ncbi.nlm.nih.gov/BLAST/) and for TBLASTN searches on the Ensembl Genome Browser (www.ensembl.org). Retrieved sequences were aligned using ClustalW version 1.82 at EBI (www.ebi.ac.uk/clustalw/index.html).

### *Phylogenetic tree*

Amino acid sequences were aligned using ClustalW (Thompson et al., 1994) in the Alignment Explorer in MEGA version 3.0 (Kumar et al., 2004). The phylogenetic tree was constructed using neighbor-joining analysis with Poisson correction and complete deletion of gaps. Results were verified using the Dayhoff Matrix and JTT models.

### *Animals*

Pdx1 (CC4) mice and Nkx6.2$^{tlz/+}$ mice were genotyped by PCR (Offield et al., 1996; Vallstedt et al., 2001). The following primers were used: Pdx1 forward primers: 5′-GAGCTGGAGAAGGAATTCTTA-3′ and 5′-CTACCCGTGATATTGCTGAAGAGCTT-3′; Pdx1 reverse primer (neomycin): 5′-GTTCCTGCGGTCGCAGTGGC-3′; Nkx6.2$^{tlz/+}$ LacZ forward primer 5′-GCGTGTACCACAGCGGATGGTTCGG-3′; LacZ reverse primer 5′-GTCAATCCGCCGTTTGTTCCCACGG-3′.

Chicken embryos (White Leghorn, SPAFAS) were incubated and staged according to Hamburger and Hamilton (1951). To obtain mouse embryos at the desired stages, timed matings were set up, and the morning of the day the vaginal plug was noticed was set to embryonic day 0.5 (E0.5).

### *Fixation and embedding*

Embryos were fixed in 4% PFA overnight at 4°C, equilibrated in 30% sucrose in 0.1 M phosphate buffer, pH 7.5, and embedded in Tissue-Tek (Sakura). Mouse embryos for whole-mount X-gal stainings were fixed as described (Mombaerts et al., 1996).

### *In situ hybridization*

Whole-mount in situ hybridization and in situ hybridization on sections were performed as previously described (Gradwohl et al., 2000; Hecksher-Sorensen et al., 1998; Jensen et al., 2000). The probes used were chicken Nkx6.1 (cNkx6.1, a kind gift from Mengsheng Qiu), cNkx6.2 and cNkx2.2 (kind gifts from Johan Ericson), cPdx1 (a kind gift from Seung Kim and Mattias Hebrok), and cNkx6.3 (BBSRC ChickEST Database (www.chick.umist.ac.uk): dbEST Id: 13564315). Mouse Nkx6.3 was a kind gift from Johan Ericson. A rat Nkx6.1 probe (Jorgensen et al., 1999) was used on mouse embryos. All probes were used in the concentration 1 μg/ml. The probes were digoxygenin-UTP-labeled and developed with NBT/BCIP (Fluka) or Fast Red (Roche). X-gal stainings were performed as described (Mombaerts et al., 1996). Whole-mount X-gal-stained embryos were sequentially dehydrated for 1 h in 25%, 50%, and 75% methanol in PBS followed by 100% methanol overnight. Embryos were cleared in BABB (1:2 benzylalkohol:benzylbenzo-

ate). All stainings were performed on at least three embryos for each stage. When in situ hybridizations were performed on electroporated embryos, we always included a negative control consisting of GFP electroporated embryos and in no case did we observe expression of the assayed gene outside its normal domain.

### *Immunohistochemistry*

Ten-micrometer frozen sections were cut on a cryostat (Leica). Immunohistochemistry was performed according to standard procedures. Briefly, sections were blocked in 5% donkey serum and 1% BSA in PBS for 1 h. The following primary antibodies were used: rabbit anti-Pdx1 raised against the N-terminus of mouse Pdx1 (C.V.E.W 1:5000), rabbit anti-Nkx6.1 ((Jensen et al., 1996) 1:3000 to 1:10,000), mouse anti-Nkx6.1 (antibody core of the BCBC 1:200), guinea pig anti-Nkx6.2 ((Vallstedt et al., 2001) 1:2000 to 1:3000), goat anti-HNF3β (Santa Cruz sc6554 1:200), mouse anti-glucagon (GLU 001, Novo-Nordisk, Bagsværd, Denmark 1:500) and mouse anti-glucagon (Sigma 1:10.000), insulin anti-guinea pig (ABCAM ab7842 1:200), Human Somatostatin anti-rabbit (DAKO A566 1:1000), and amylase (Sigma A 8273 1:1600). Primary antibodies were diluted in 1% BSA in PBS and were incubated overnight at room temperature. The following secondary antibodies were used: Cy2 rabbit anti-goat (1:500), Cy3 donkey anti-guinea pig (1:500 to 1:2000), Cy3 goat anti-rabbit (1.500 to 1:2000), Cy3 donkey anti-mouse (1:500), Cy5 donkey anti-mouse (1:500), and Cy5 donkey anti-rabbit (1.500) all from Jackson ImmunoResearch, Alexa 488 goat anti-mouse (1:2000), Alexa 488 donkey anti-rabbit (1:500), and Alexa 594 donkey anti-mouse (1:500), all from Molecular Probes. Secondary antibodies were diluted in 1% BSA in PBS and were incubated for 1 to 2 h.

Images were collected with the following equipment: Olympus BX51 microscope or a Olympus SZX9 stereomicroscope equipped with a cooled Hamamatsu C5810 CCD camera, a Zeiss AxioCam, and a Zeiss LSM 510 META confocal microscope. Images were further processed in Adobe Photoshop.

### *In ovo electroporation*

In ovo electroporation was performed as previously described (Grapin-Botton et al., 2001). Embryos were electroporated at HH stages 11–13 with CMV promoter-driven rat Pdx1 cDNA together with pCMV-Ds-Red or pCMV-GFP. Control embryos were electroporated with pCMV-Ds-Red or pCMV-GFP alone. Plasmids were diluted to a final concentration of 2 μg/μl. Three 50 ms, 17 V square pulses were applied to the embryo using a BTX T820 square wave electroporator. After electroporation, embryos were incubated 48 h at 38°C in a humidified incubator.

## Results

### *Identification of Nkx6 family members*

Orthologs of the Nkx6.1 and Nkx6.2 genes have previously been identified in rat, mouse, human, and chicken (Cai et al., 1999; Inoue et al., 1997; Jorgensen et al., 1999; Komuro et al., 1993; Lee et al., 2001; Oster et al., 1998b; Qiu et al., 1998; Watada et al., 2000). An Nkx6-related sequence (acc: AK018683) different from Nkx6.1 and Nkx6.2 has previously been noted (Vallstedt et al., 2001) which we here name mouse Nkx6.3. Using the three mouse Nkx6 family amino acid sequences as queries in database searches, we identified complete and partial sequences that represented human, rat, mouse, chick, and zebrafish orthologs of all three genes. Notably, the presence of zebrafish orthologs of all three Nkx6 family genes indicates that the three Nkx6 family genes had arisen prior to the evolutionary segregation of fish and amniotes. The homeodomains of all Nkx6 family members are highly conserved, although the homeodomains of Nkx6.3 orthologs differ from Nkx6.1 and Nkx6.2 homeodomains at positions 2 and 3. At these positions, Nkx6.3 proteins are characterized by the presence of a polar and a hydrophobic amino acid instead of the invariant lysine/aspartic acid found in Nkx6.1 and Nkx6.2 (Fig. 1A). Nkx6.3 from all five species contain an eh1 domain (Engrailed homology 1 domain) which is also found in Nkx6.1 and Nkx6.2 and interacts with the co-repressor Groucho (Jimenez et al., 1997; Muhr et al., 2001) (Fig. 1A). Similar to Nkx6.2, Nkx6.3 lacks the poly-alanine and poly-serine stretches found in Nkx6.1 (Fig. 1A), but phylogenetic analysis revealed that Nkx6.1 and Nkx6.2 are more closely related to each other than to Nkx6.3 (Fig. 1B) and that Nkx6.3 is the closest relative to *Drosophila* Nk6, suggesting that Nkx6.3 represents the ancestral gene in vertebrates and that Nkx6.1 and Nkx6.2 arose by two sequential gene duplications.

### *Nkx6 gene expression in the endoderm in HH stage 11 chicken embryos*

The earliest time point of Nkx6.1 expression detection in the chicken endodermal epithelium is at Hamburger and Hamilton (HH) stage 10+ (11 somites, Hamburger and Hamilton, 1951) (Figs. 2A, G, H). At this stage, Nkx6.1 expression is found in two separate domains of the endoderm along the anterior–posterior (AP) axis: one near the anterior intestinal portal (AIP) and one at the posterior end underlying the sinus rhomboidalis (Fig. 2A). The anterior expression domain appears as two bilateral bands extending along the AP axis from approximately the 3rd to the 8th somite pair, a region of the endoderm that previous fate mapping studies have shown corresponds to prospective pancreatic endoderm (Kumar et al., 2003; Matsushita, 1996). Sectioning of the embryos reveals that the Nkx6.1-expressing endoderm is located beneath and in contact with the endothelium of the paired dorsal aortas, whereas the endodermal epithelium underlying the notochord does not express Nkx6.1 at this stage (Fig. 2G). Posteriorly, the Nkx6.1 expression is evenly distributed along the left–right (LR) axis of the endodermal epithelium (Fig. 2H). At HH stage 11, we detect endodermal Nkx6.1 expression from the level of the AIP and extending caudally along the AP axis to the tail bud (Fig. 2B). Sectioning of the embryos reveals that from the 3rd to the 8th somite level Nkx6.1 expression is present along the entire LR axis at this stage (Fig. 2I). The two bilateral domains first seen at HH stage 10+ display the strongest signal at HH stage 11. This difference in expression level has been confirmed by reducing the time period of the in situ hybridization development, resulting in appearance of the signal only in the area underlying the dorsal aortas from the 3rd to the 8th somite pair as well as in the most posterior part of the endoderm similar to the observations at HH stage 10+. Longer development of the in situ hybridization leads to visualization of the broader Nkx6.1 expression area extending from the level of the AIP to the tail bud and along the entire LR axis. Furthermore, immunohistochemical detection of

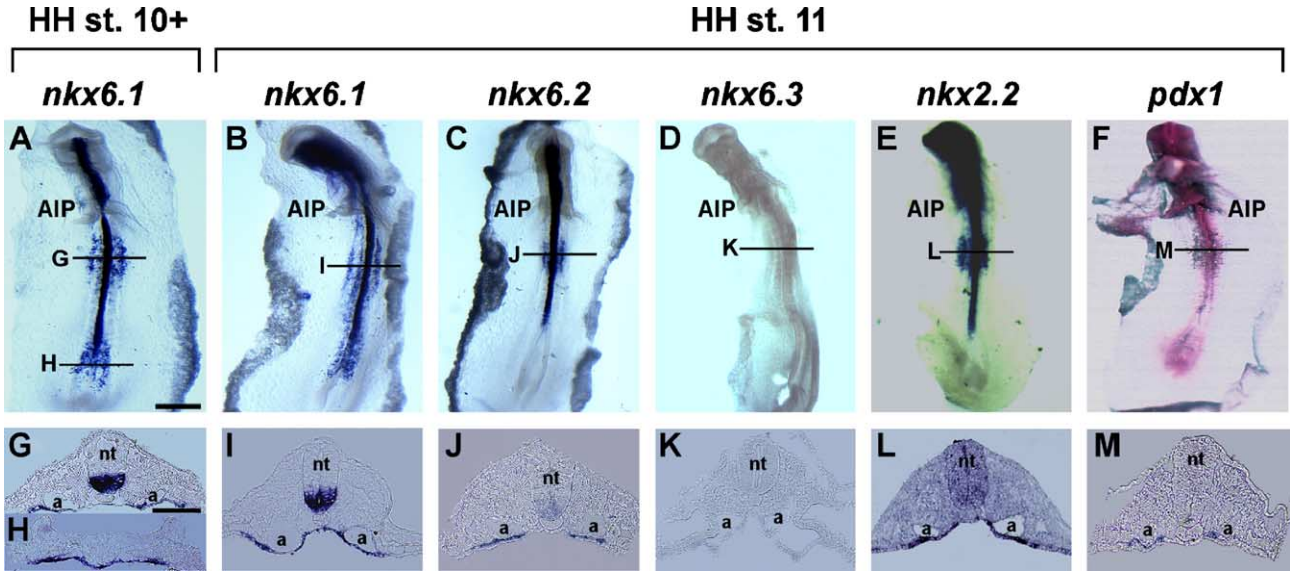



Fig. 2. The Nkx6 genes are expressed in the prospective pancreatic endoderm in chicken embryos. Whole-mount in situ hybridizations of HH stage 10+ (A, G, H) and 11 (B–F, I–M) chicken embryos with antisense probes recognizing Nkx6.1 (A, B), Nkx6.2 (C), Nkx6.3 (D), Nkx2.2 (E), and Pdx1 (F). (A–F) Ventral views of whole embryos with the anterior at the top. (G–M) Cross-sections of representative embryos at the anterior–posterior level indicated in the corresponding panels shown in the top row (dorsal at the top). With the exception of Nkx6.3, all genes are expressed in the prospective pancreatic endoderm. AIP: anterior intestinal portal, a: aorta, nt: neural tube. Scale bars are 1 mm (A–F) and 100 μm (G–M).

Nkx6.1 protein expression reveals that the protein is present in the same (broad) areas as the mRNA at HH stage 11 (data not shown), suggesting that the low mRNA expression level detected by in situ hybridization development may have biological significance.

Nkx6.2 is first detected in chicken endoderm at HH stage 11 (Fig. 2C). In contrast to Nkx6.1 expression, Nkx6.2 expression is exclusively localized to the endoderm underlying the dorsal aortas from the 3rd to the 8th somite pair along the AP axis (Fig. 2J). This expression domain is identical to the anterior Nkx6.1 expression domain observed at HH stage 10+. We cannot detect Nkx6.1 or Nkx6.2 expression in the endoderm of HH stages 8 and 9 embryos, although expression in the spinal cord is clearly present at these stages (data not shown). Expression of Nkx6.3 cannot be detected at HH stage 11+ or earlier (Figs. 2D, K).

Due to the fate mapping of the Nkx6.2 expression domain to prospective pancreatic endoderm (Kumar et al., 2003; Matsushita, 1996), we correlated expression of the Nkx6 genes with expression of known pancreatic markers. Therefore, we examined the expression of the two homeobox genes, Nkx2.2 and Pdx1. We can detect Nkx2.2 and Pdx1 expression from HH stage 10 to 11, respectively. As seen for Nkx6.2, both Pdx1 and Nkx2.2 are expressed in two bilateral domains of the endoderm, extending approximately from somite pairs 3 to 8, (Figs. 2E, L, F, and M).

### *Nkx6 gene expression in the endoderm in HH stages 15 and 17 chicken embryos*

In the chicken, pancreatic morphogenesis initiates at HH stage 15 (25 somites), where the dorsal and the two ventral pancreatic buds emerge as three local thickenings of the endoderm (Romanoff, 1960). At this stage, we find that the anterior border of Nkx6.1 expression is at the level of the pancreas primordium and extends caudally along the AP axis to the tail bud. Anteriorly, the Nkx6.1 expression is divided in a dorsal domain and two ventro-lateral domains. A dorsal domain of strong Nkx6.1 expression corresponds to the thickening of dorsal pancreatic epithelium. In the prospective duodenal region and more posteriorly, a gradual decrease in Nkx6.1 expression is observed. Ventrally, Nkx6.1 expression is restricted to the two ventral pancreatic thickenings of the endoderm. Nkx6.1 expression is also detected in the neural tube and in the mesenchyme surrounding the stomach- and esophageal epithelium (Fig. 3A). Expression of Nkx6.1 protein as detected by immunohistochemical stainings is identical to the mRNA hybridization signals (data not shown).

At HH stage 15, the expression of Nkx6.2 (Fig. 3C), Nkx6.3 (Fig. 3E), Nkx2.2 (Fig. 3G), and Pdx1 (Fig. 3I) continues to be very similar and more restricted than Nkx6.1. Expression of Nkx6.2, Nkx6.3, Nkx2.2, and Pdx1 all marks the pancreatic primordia specifically in one dorsal and two ventro-lateral domains similar to the strong Nkx6.1 expression domain in pancreatic endoderm (Figs. 3C, E, G, and I).

At HH stage 17 (31 somites), the pancreatic buds are evident morphologically compared to the relatively inconspicuous epithelial thickenings of the endoderm at HH stage 15. Accordingly, expression of Nkx6.1, Nkx6.2, Nkx6.3, Nkx2.2, and Pdx1 appears more distinct at HH stage 17 but is otherwise very similar to the HH stage 15 expression (Figs. 3B, D, F, H, and J). Sections of HH stage 15 embryos subjected to whole-mount in situ hybridization demonstrate that expression of Nkx6.1, Nkx6.2, and Nkx6.3 in the pancreatic area is separated into one dorsal domain and two ventro-lateral domains corresponding to the developing dorsal

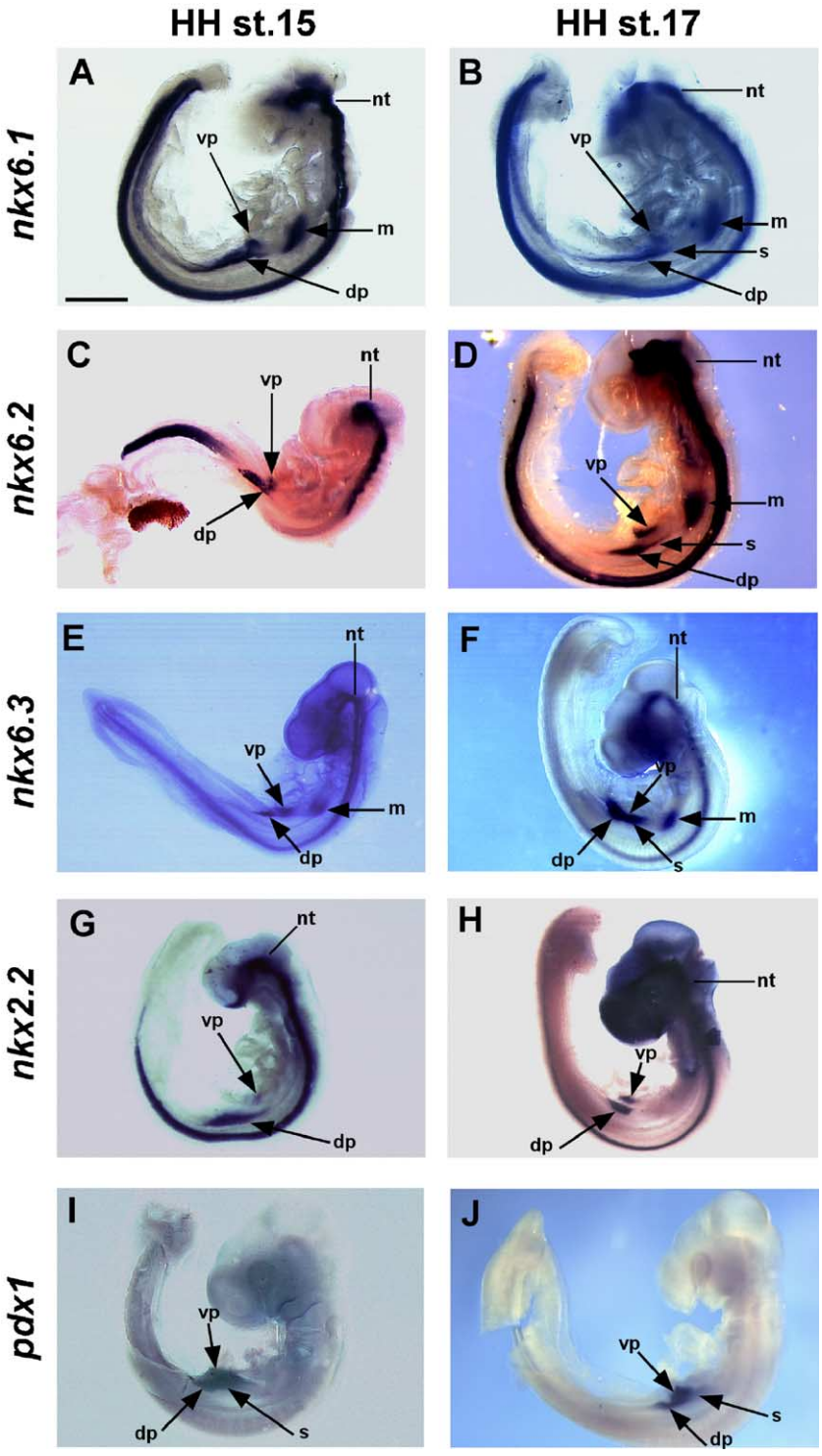



Fig. 3. The Nkx6 genes are expressed in the pancreatic endoderm of HH stages 15 and 17 chicken embryos. Lateral views of whole-mount in situ hybridizations of HH stage 15 (A, C, E, G, and I) and 17 (B, D, F, H, and J) chicken embryos with antisense probes that recognize Nkx6.1 (A, B), Nkx6.2 (C, D), Nkx6.3 (E, F), Nkx2.2 (G, H), and Pdx1 (I, J). dp: dorsal pancreas primordium, vp: ventral pancreas primordium, nt: neural tube, m: stomach mesenchyme, s: posterior stomach epithelium. Scale bar is 1 mm (A–J).

and ventral pancreas buds (Figs. 4A–C′). At HH stage 17, sectioning of whole-mount in situ hybridized embryos revealed that Nkx6.1 and Nkx6.2 are expressed in the chicken pancreas at this stage, while expression of Nkx6.3 is present in the duodenum but absent from the pancreas epithelium (Figs. 4D–F). At HH stage 15, no expression of the Nkx6 genes can be detected in the posterior stomach epithelium (Figs. 4G–I). However, expression of Nkx6.1 and Nkx6.3 can be observed in two dorso-lateral bands in the mesenchyme surrounding the stomach (Figs. 4G, I). In contrast, sectioning of HH stage 17 embryos reveals that expression of all Nkx6 genes can be detected in the caudal part of the stomach

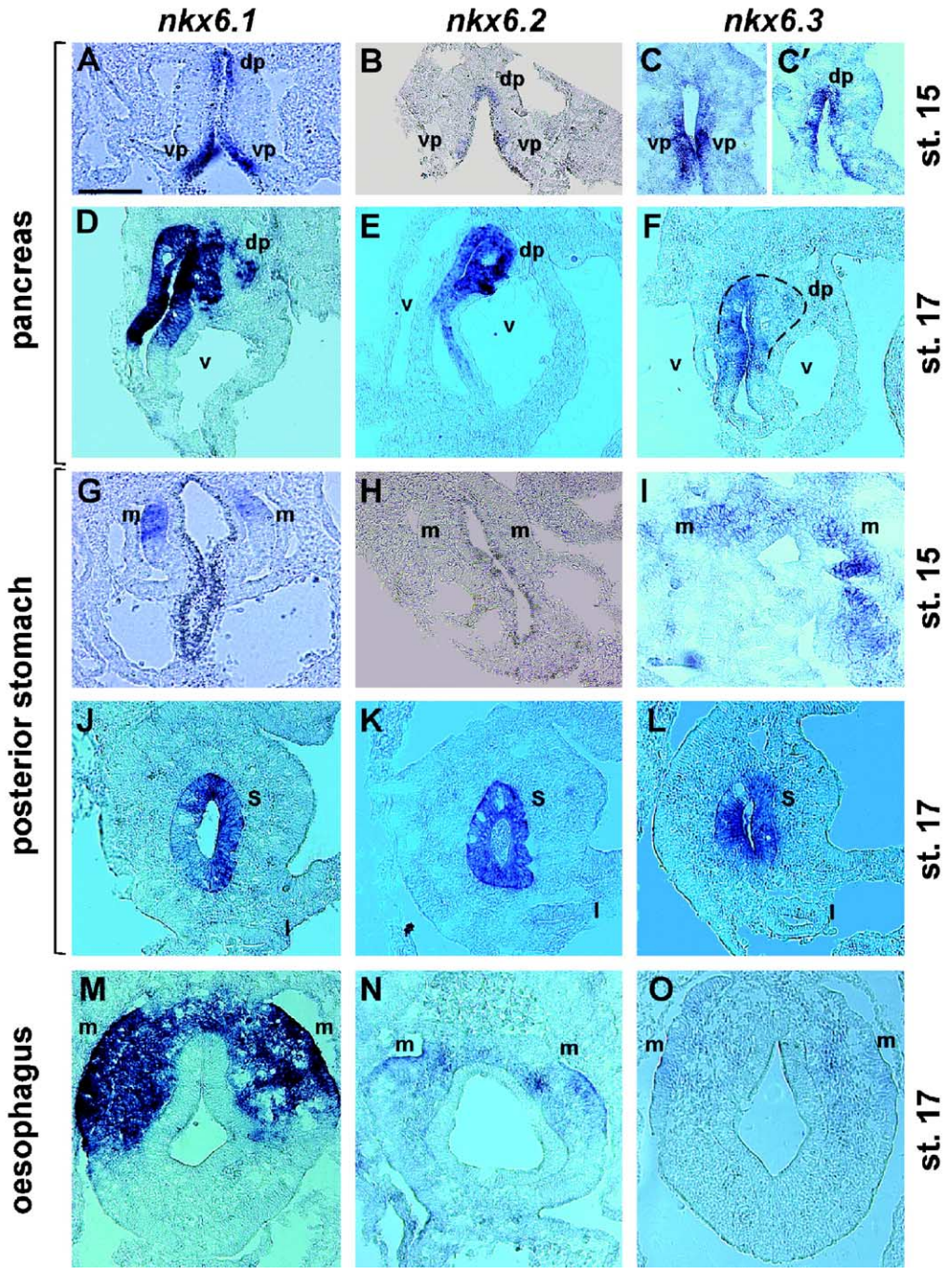



Fig. 4. Expression of the Nkx6 genes marks the domain of pancreatic bud morphogenesis in HH stages 15 and 17 chicken embryos. Sections of embryos previously subjected to whole-mount in situ hybridization as shown in Fig. 3 (dorsal at the top). (A–F) Sections at the pancreatic level of HH stages 15 (A–C′) and 17 (D–F) chicken embryos. (G–L) Sections at the level of posterior stomach of HH stages 15 (G–I) and 17 (J–L) embryos. (M–O) Sections at the level of the esophagus of HH stage 17 embryos. (A–C′) At stage 15, expression of the Nkx6 genes marks the onset of pancreas bud morphogenesis. dp: dorsal pancreas bud, vp: ventral pancreas bud, v: vein, m: mesenchyme, s: posterior stomach epithelium, l: liver. Scale bar is 200 μm.

epithelium at this stage (Figs. 4J–L). Furthermore, at HH stage 17, all the Nkx6 genes are expressed in two lateral domains of the mesenchyme surrounding the anterior stomach and the esophagus (Figs. 4M–O). Additionally, expression of Nkx6.1 and Nkx6.2 can be detected in two small lateral regions of the pharyngeal epithelium, and expression of Nkx6.2 is also observed in the dorsal part of the pharyngeal endoderm (data not shown).

*Nkx6 gene expression in the endoderm of E4 chicken embryos*

To determine Nkx6 gene expression at a later stage, we performed in situ hybridization on sections of embryonic day 4 (E4) chicken embryos. We find that Nkx6.1 expression is present in the pancreatic endoderm and a small area in the hindgut (Fig. 5). Specifically, Nkx6.1 is expressed in the majority of the pancreatic epithelial cells and in the dorsal

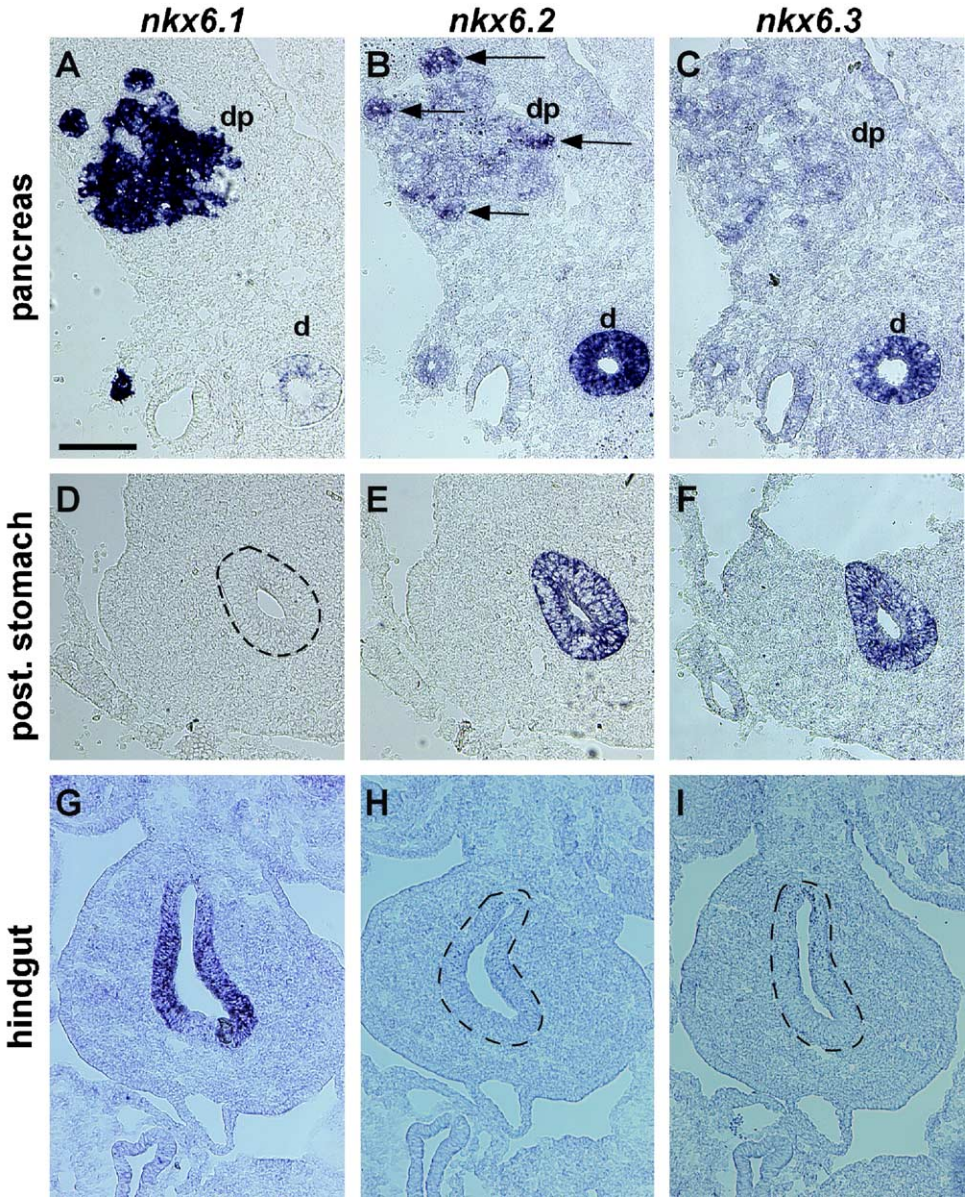



Fig. 5. Expression of Nkx6 genes in endoderm of E4 chicken embryos. In situ hybridization on adjacent sections at different anterior–posterior levels of embryonic day 4 (E4) chicken embryos (dorsal at the top). (A–C) Nkx6.1 is strongly expressed in dorsal pancreas but only weakly in duodenum (A). Nkx6.2 is variably expressed in pancreatic cells and strongly in the duodenum (B). Nkx6.3 is strongly expressed in the duodenum but not above background in the pancreas (C). (D–F) Nkx6.2 and Nkx6.3 but not Nkx6.1 is expressed in posterior stomach. (G–H) Nkx6.1 but not Nkx6.2 and Nkx6.3 is expressed in the hindgut. dp: dorsal pancreas, d: duodenum. Scale bar is 100 μm (A–I).

duodenum (Fig. 5A), while we cannot detect Nkx6.1 expression in the stomach (Fig. 5D). In addition to the pancreas and duodenum, a small area in the hindgut region displays prominent Nkx6.1 expression (Fig. 5G). Identical results were obtained when assaying Nkx6.1 protein expression by immunohistochemistry (data not shown). Next, we analyzed Nkx6.2 expression and find that this gene is expressed in most of the pancreatic epithelium but with varying intensity. Clusters of cells with strong Nkx6.2 expression are interspersed with areas of weaker expression (Fig. 5B). We also find Nkx6.2 expression in the epithelium of the caudal stomach and duodenum (Figs. 5B, E), and, in contrast to HH stage 17, the expression level appears higher here than in the pancreatic epithelium (Figs. 5B, E). Lastly, we analyzed Nkx6.3 expression and detect expression of this gene in the epithelium of the duodenum and caudal stomach (Figs. 5C, F). In the pancreatic epithelium at this stage, Nkx6.3 expression cannot be distinguished from the background signal (Fig. 5C). No expression of Nkx6.2 and Nkx6.3 could be detected in the hindgut area as observed for Nkx6.1 (Figs. 5G–I).

### *Chicken Nkx6.1 and Nkx6.2 is expressed in both endocrine and exocrine cell types*

In order to identify which pancreatic cell types exhibit Nkx6.1 and Nkx6.2 expression, we performed colocalization

studies at HH stage 17, E4, and E9, either by double immunohistochemistry or by combined in situ hybridization and immunohistochemistry. At HH stage 17 and E4, we find that Nkx6.1 colocalizes with insulin-expressing cells, but not with the glucagon- or somatostatin-expressing cells (Figs. 6A, D and data not shown). A similar expression pattern is observed for Nkx6.2. Clusters of cells with strong Nkx6.2 expression are interspersed within cells with weaker Nkx6.2 expression (Fig. 6B). To determine the identity of the various types of Nkx6.2-expressing cells, we performed co-labeling

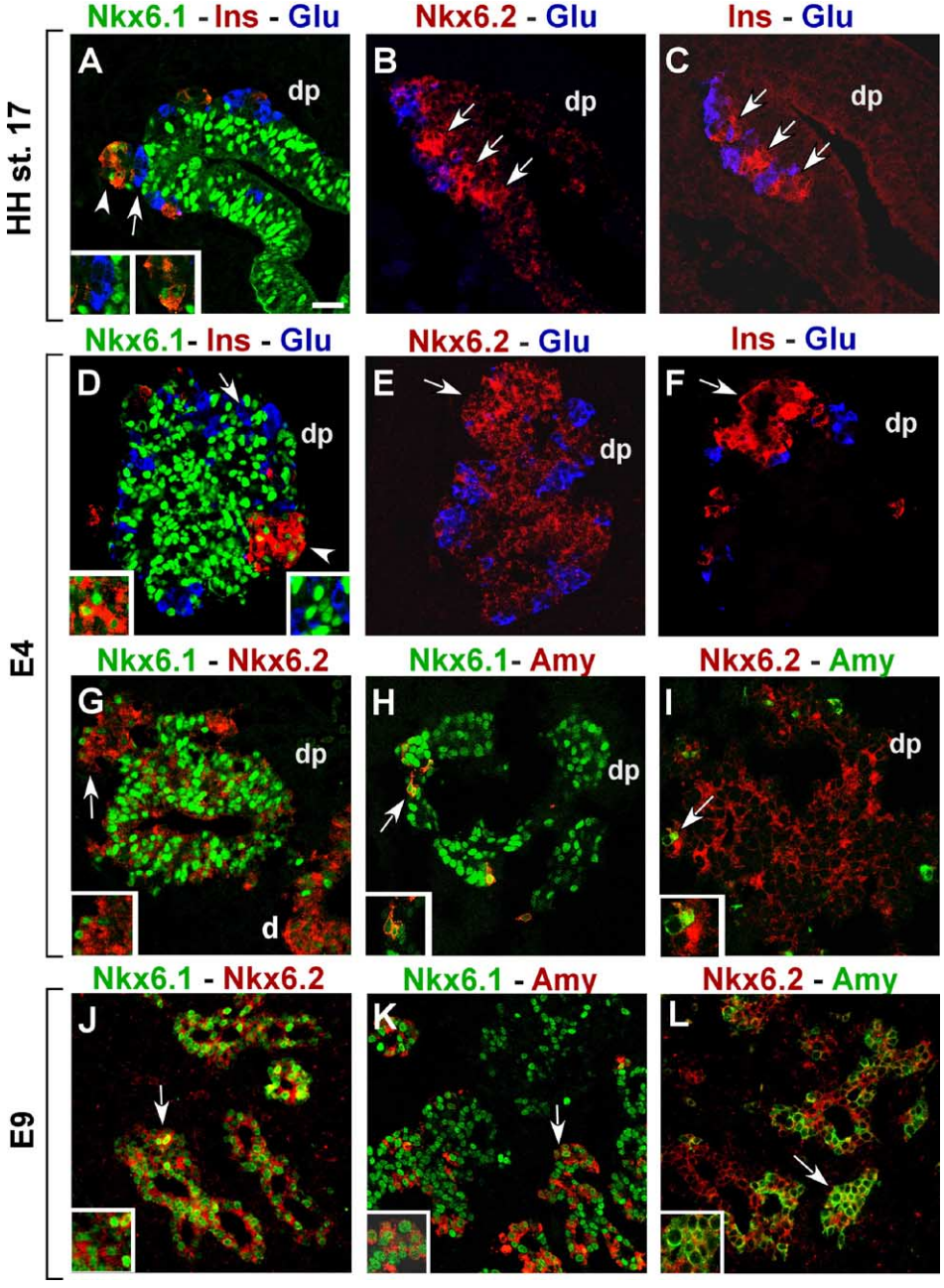



Fig. 6. Distinct expression of Nkx6.1 and Nkx6.2 in pancreatic cell types of E4 chicken embryos. (A, D) Triple immunohistochemical (IHC) localization of Nkx6.1 (green), insulin (red), and glucagon (blue) reveals that Nkx6.1 is expressed in undifferentiated epithelial cells, insulin-producing cells (arrowhead), but not in glucagon-producing cells (arrow) at HH stage 17 (A) and E4 (D). (B, E) Sections of HH stage 17 (B) and E4 (E) pancreas subjected to in situ hybridization to detect Nkx6.2 (red) followed by IHC localization of glucagon (blue). (C, F) Sections of HH stage 17 (C) and E4 (F) chicken pancreas subjected to double IHC localization of insulin (red) and glucagon (blue). The sections in panels C and F are adjacent to the sections shown in panels B and E, respectively. Note that cell clusters expressing high levels of Nkx6.2 in panels B and E (arrows) correspond to insulin-producing cell clusters in panels C and F (arrows). (G, J) Sections of E4 (G) and E9 (J) chicken pancreas subjected to in situ hybridization to detect Nkx6.2 (red) followed by IHC localization of Nkx6.1 (green). Note the extensive overlap between Nkx6.1 and Nkx6.2 in undifferentiated epithelial cells and the presence of Nkx6.1 in strongly Nkx6.2 positive cells (arrows). (H, K) Sections of E4 (H) and E9 (K) chicken pancreas subjected to double IHC detection of Nkx6.1 (green) and amylase (red). Note that amylase positive cells co-express Nkx6.1 (arrows). (I, L) Sections of E4 (I) and E9 (L) chicken pancreas subjected to in situ hybridization to detect Nkx6.2 (red) followed by IHC localization of amylase (green). Note that amylase positive cells co-express Nkx6.2 (arrows). Inserts show high magnification images to help visualize co-expression or the absence of co-expression. dp: dorsal pancreas, d: duodenum. Scale bar is 20 μm.

studies. On adjacent sections, we performed Nkx6.2–glucagon and insulin–glucagon co-labeling. Glucagon was found in cells expressing Nkx6.2 weakly, while cells strongly positive for Nkx6.2 appeared to represent insulin-producing cells (Figs. 6B, C). Additionally, undifferentiated epithelial cells were positive for Nkx6.1 and Nkx6.2 at HH stage 17, E4, and E9 (Figs. 6A, B, G, and J).

Around E4, the first amylase-producing cells appear, and these are co-expressing both Nkx6.1 and Nkx6.2 (Figs. 6H, I). At E9 where many more amylase positive cells are found, a similar pattern is observed (Figs. 6K, L).

### *Nkx6 gene expression in mice*

In order to determine whether the expression patterns that we observe in chicken are conserved in mammals, we analyzed expression of the Nkx6 family in mice. In E9 (20–22 somites) mouse embryos, we do not detect Nkx6.1 expression in any region of the endoderm, although we do detect expression in the CNS and in two lateral regions of the stomach and esophageal mesenchyme (Figs. 7A–C). At E9.5 and E10 (25–30 somites), we detect a few Nkx6.1-expressing cells in the dorsal pancreatic epithelium but none in the ventral pancreas primordia (Figs. 7D, E and data not shown). Nkx6.1 expression is also detected in the mesenchyme around the stomach (Fig. 7F). Slightly later, at E10.5 (>30 somites), we detect Nkx6.1 expression in most of the epithelial cells of the developing dorsal and ventral pancreas (data not shown) which is in agreement with previous work (Sander et al., 2000b).

To analyze Nkx6.2 expression in mice, we took advantage of a mouse strain carrying a LacZ insertion in the Nkx6.2 locus (Vallstedt et al., 2001). We detect β-galactosidase activity in the endoderm as early as E8.5. At this stage, Nkx6.2 is expressed in two lateral regions located at the border between primitive and definitive endoderm, whereas the definitive dorsal endoderm including the prospective dorsal pancreas is Nkx6.2 negative (Figs. 7G, H). The Nkx6.2 expression domain in the ventral endoderm includes the ventro-lateral part of the definitive endoderm as well as the visceral endoderm. At E8.5, we also find Nkx6.2 expression in the CNS (Figs. 7G, H). At E9.5, Nkx6.2 expression is clearly detected in the evaginating dorsal and ventral pancreatic buds. Both the dorsal pancreatic epithelium and the two ventral buds express Nkx6.2 (Figs. 7I, J), whereas the stomach epithelium is Nkx6.2 negative (Fig. 7K) One day later, at E10.5, we still detect expression of Nkx6.2 in the dorsal and ventral pancreatic epithelium but similar to what we observe in chicken embryos, we find that Nkx6.2 expression has expanded into the posterior part of the stomach (Fig. 7N) and duodenal endoderm (Figs. 7L, M). We do not detect sharp borders of expression but rather a gradual decline in expression similar to that observed for Pdx1 (Offield et al., 1996).

Expression of the third Nkx6 family member, Nkx6.3, can be detected at E10.5 mouse embryos in the prospective pyloric sphincter and the duodenum (Fig. 7O). However, in contrast to Nkx6.1 and Nkx6.2, expression of Nkx6.3 could not be detected by in situ hybridization in the pancreatic region (Figs. 7P, Q). We also analyzed Nkx6.3 expression of E12.5 and E14.5 mouse embryos but did not detect pancreatic expression of Nkx6.3 at these stages. However, expression of Nkx6.3 could still be detected in the pyloric sphincter region at these stages (Figs. 7R, S).

### *Nkx6.1 and Nkx6.2 expression is differentially dependent on Pdx1 in the mouse*

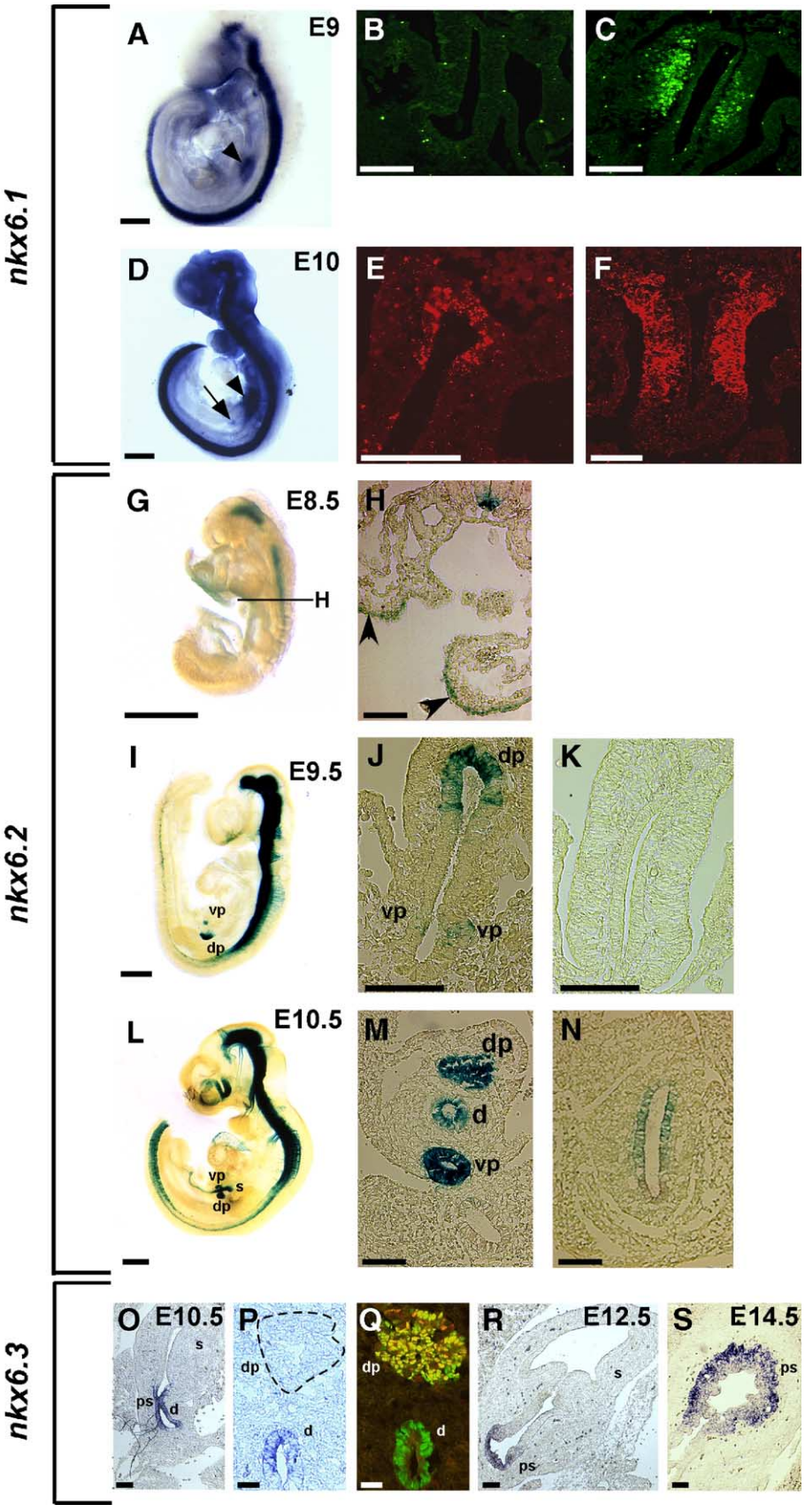
In mice as well as in chicken, the expression patterns of the Nkx6 transcription factors overlap with the expression pattern of Pdx1 in the epithelium of the early pancreas, and, in mice, the onset of Pdx1 expression precedes Nkx6.1 expression. Pdx1 is well known as an activator of gene expression, and Nkx6.1 has been suggested as a target gene (Iype et al., 2004; Watada et al., 2000). We therefore investigated whether the induction and/or maintenance of Nkx6 expression in early pancreas is dependent on Pdx1 expression in mice.

In E10.5 wild-type mice, we find that most of the undifferentiated epithelial cells of both the ventral and the dorsal pancreatic buds express Pdx1, Nkx6.1, and Nkx6.2 (Figs. 8A–C and data not shown). At this stage, some of the early glucagon-expressing cells in the dorsal bud are Nkx6.1 positive (Fig. 8B) and also a few Pdx1 positive glucagon cells can be detected (Fig. 8A) as previously shown (Guz et al., 1995). Nkx6.2 does not colocalize with glucagon (Fig. 8C).

In E10.5 Pdx1-deficient mice, the ventral pancreas bud is absent, and the dorsal pancreas bud is smaller than in wild-type littermates (Fig. 8D and Ahlgren et al., 1996; Offield et al., 1996). However, in the remaining dorsal epithelium, Nkx6.1 expression is absent from the undifferentiated epithelial cells, while a few Nkx6.1 positive nuclei can still be detected in the glucagon-expressing cells (Fig. 8E). The expression pattern of Nkx6.2 in the dorsal pancreas bud is unchanged in the Pdx1-deficient embryos compared to wild-type littermates (Fig. 8F).

Thus, Pdx1 deficiency results in the loss of Nkx6.1 expression in the dorsal pancreas epithelium but has no effect

Fig. 7. Nkx6 gene expression patterns in early developing mouse embryos. (A, D) Whole-mount in situ hybridization of E9 (A) and E10 (D) Nkx6.1 expression with antisense probes that recognize Nkx6.1. Note Nkx6.1 expression in the stomach mesenchyme at both stages (arrowheads) and in the dorsal pancreas at E10 (arrow in D). (B, C) IHC detection of Nkx6.1 on sections at the level of the pancreas (B) and anterior stomach (C). (E, F) In situ hybridizations on sections of E10 mouse embryos using Nkx6.1 antisense probe at the level of the pancreas (E) and anterior stomach (F). (G–N) Whole-mount enzymatic detection of β-galactosidase expression in E8.5 (G), E9.5 (I), and E10.5 (L) Nkx6.2$^{+/tlz}$ mice followed by sectioning to visualize expression of β-galactosidase in pancreatic tissue at E8.5 (H), E9.5 (J), and E10.5 (M) and in posterior stomach tissue at E9.5 (K) and E10.5 (N). (O, P) Nkx6.3 in situ hybridizations on sections of E10.5 mouse embryos. Note expression in the endoderm of the prospective pyloric sphincter (O) and in duodenal, but not in dorsal pancreatic epithelium (P). (Q) Section adjacent to the section shown in panel P subjected to IHC to detect Pdx1 (green) and Nkx6.1 (red). Note co-expression of both factors in dorsal pancreas epithelium. (R, S) Nkx6.3 in situ hybridizations on sections of E12.5 (R) and E14.5 (S) show expression in the pyloric sphincter region. d: duodenum, vp: ventral pancreas bud, dp: dorsal pancreas bud, s: stomach epithelium, ps: pyloric sphincter region. Scale bars are 500 μm on whole-mount specimens and 100 μm on sections.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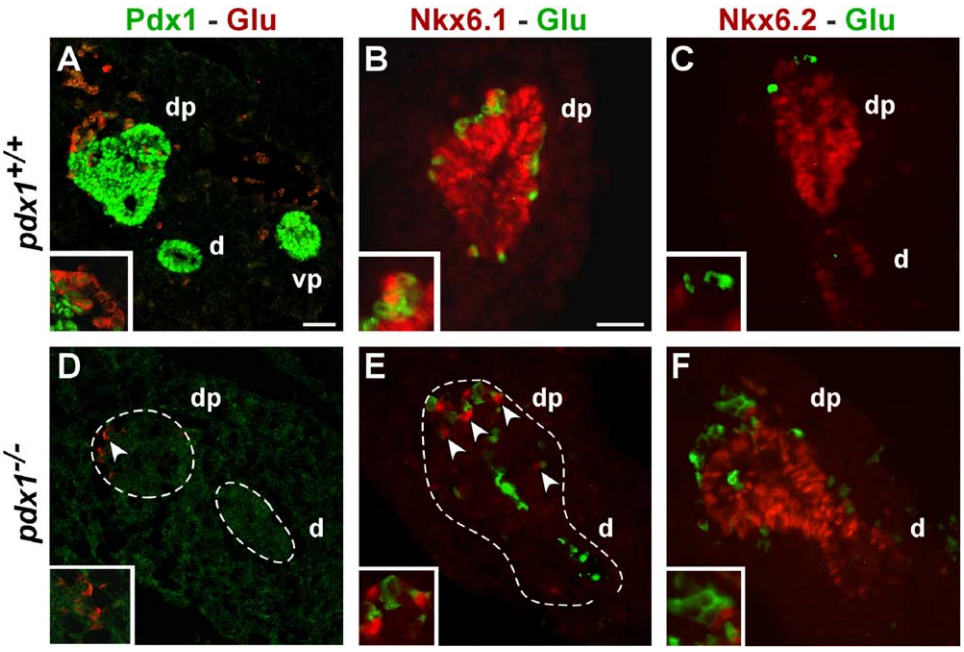



Fig. 8. Nkx6.1 and Nkx6.2 expression is differentially dependent on Pdx1 in the mouse. (A–F) Double IHC detection of Pdx1 (green) and glucagon (red) (A, D), Nkx6.1 (red) and glucagon (green) (B, E), and Nkx6.2 (red) and glucagon (green) (C, F) in E10.5 wild-type (A–C) and *pdx1* mutant (D–F) embryos. Note the absence of Nkx6.1 in the undifferentiated epithelium of the *pdx1* mutant embryo and the continued presence of glucagon-expressing cells in these embryos (arrowheads). Inserts show high magnification images to help visualize co-expression or the absence of co-expression. dp: dorsal pancreas bud, vp: ventral pancreas buds, d: duodenum. Scale bars are 50 μm.

on Nkx6.2 expression. This demonstrates that Nkx6.1 but not Nkx6.2 lies downstream of Pdx1 in the genetic pathway that governs early pancreas development. Notably, the expression of Nkx6.1 in the early glucagon cells seems to be independent of Pdx1.

### *Pdx1 is sufficient to induce cNkx6.1 but not cNkx6.2 or cNkx6.3 expression*

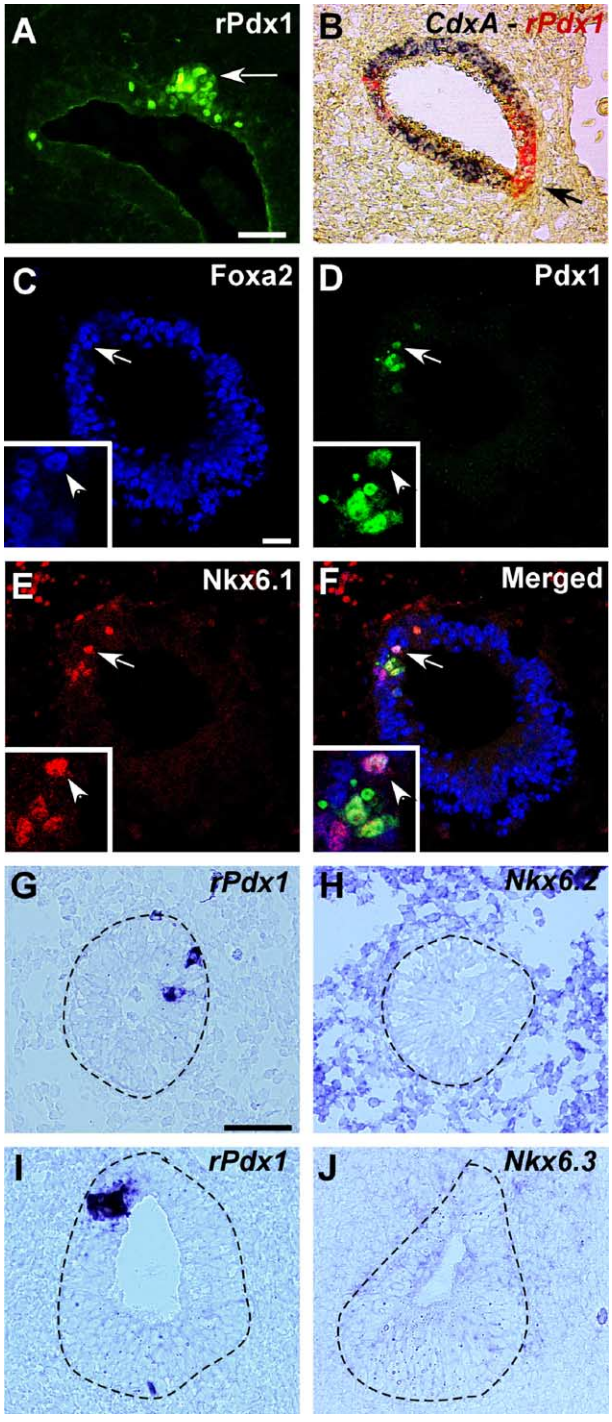
The absence of Nkx6.1 expression in the epithelium of Pdx1-deficient embryos shows that Pdx1 is required for Nkx6.1 expression. To determine if Pdx1 is sufficient for Nkx6.1 expression, we introduced a rat Pdx1 expression vector into the chicken endoderm by in ovo electroporation. We could confirm previous work demonstrating that ectopic expression of Pdx1 leads to formation of ectopic pancreatic bud structures in the intestine and that Pdx1 is able to repress the intestinal marker CdxA (Figs. 9A, B) (Grapin-Botton et al., 2001). The normal presence of Nkx6.1 in the duodenum precluded analysis of whether this gene can be induced by Pdx1 in this area, however, when Pdx1 was ectopically expressed in the yolk sac endoderm, we could not find evidence to suggest that Nkx6.1, Nkx6.2, or Nkx6.3 was induced (data not shown). We next tested whether ectopic expression of Pdx1 in the endoderm of the anterior stomach and posterior esophagus can lead to an induction of Nkx6 gene expression. We find that cells with exogenous expression of Pdx1 in the most anterior stomach and in the esophagus display ectopic cNkx6.1 expression. To validate that the cNkx6.1 expression is localized to endodermal cells and not in the adjacent mesenchyme where endogenous cNkx6.1 expression is observed, we performed triple immunohistochemistry with antibodies against the endodermal marker Foxa2 (HNF3β) and find that the Pdx1–Nkx6.1 double positive cells are indeed also expressing Foxa2 (Figs. 9C–F). In control embryos electroporated with a GFP expression vector, we do not detect Pdx1 or Nkx6.1 expression in the epithelium of the anterior stomach and posterior esophagus (data not shown). We also analyzed sections for Nkx6.2 and Nkx6.3 expression in the most anterior stomach and the esophagus. We cannot detect any cNkx6.2 or cNkx6.3 expression in sections adjacent to sections with exogenous Pdx1 expression (Figs. 9G–J). Control sections containing the pancreas and duodenum from the same embryos showed clear endogenous cNkx6.2 and cNkx6.3 expression (data not shown). We conclude that forced expression of Pdx1 is sufficient to induce expression of cNkx6.1 in the anterior stomach and esophagus without affecting cNkx6.2 or cNkx6.3 expression.

## Discussion

### *Nkx6 gene expression marks prospective pancreatic endoderm in chicken and mouse embryos*

We show here that Nkx6 family genes display conserved expression in midgut endoderm. Additionally, Nkx6.1 and Nkx6.2 display prominent expression in the developing pancreas, suggesting that these genes play important roles during early pancreas development. In chicken, we find that early and strong expression of Nkx6.1 marks the prospective pancreatic domain of the endoderm. However, Nkx6.1 expression is not restricted to the presumptive pancreatic area but

appears to be much more broadly expressed in the early chicken endoderm, albeit the expression levels are weaker outside the pancreatic region. The observation that Pdx1, Nkx2.2, and Nkx6.2 expression in HH stage 11 chicken embryos is confined to two bilateral domains beneath the paired dorsal aortas and in contact with the endothelium suggests that endothelial cells in the chicken might also provide inductive or permissive signals to the pancreatic endoderm, as recently demonstrated in mice and *Xenopus* (Lammert et al., 2003; Yoshitomi and Zaret, 2004).




Later in development, expression of chicken Nkx6.1 becomes narrowed to the pancreas epithelium, dorsal duodenum, and a small area in the hindgut. Notably, onset of Nkx6.1 expression occurs much later in mice and is found to be restricted to the epithelium of the pancreatic buds. In contrast, we find that the onset of Nkx6.2 expression in mouse endoderm is around E8.5 which is approximately the time of onset of Pdx1 and Hb9 (Li et al., 1999). In mice, endodermal Nkx6.2 expression thus resembles the situation in chicken, where Nkx6.2 expression is detected at a similar stage. In addition, the Nkx6.2 and Pdx1 expression in the midgut region of mice and chicken endoderm appears to be in similar domains, particularly at later stages. Also, Nkx6.3 displays a similar expression pattern in mice and chicken, with weak and transient expression in the epithelium of the chicken pancreas appearing to be the exception. Thus, overall, the expression patterns of Nkx6 family genes are conserved in mice and chicken.

The conserved expression of Nkx6 genes suggests that these might serve important functions during early pancreas development. However, Nkx6.1 deficiency does not appear to alter early pancreas development (Sander et al., 2000b), suggesting that different Nkx6 family members might serve redundant functions. This notion was recently confirmed by one of us as shown by the severe reduction in endocrine development in Nkx6.1/Nkx6.2 double mutant mice compared to mice deficient for only one of these genes (Henseleit et al., 2005; Sander et al., 2000b). Redundancy between Nkx6.1 and Nkx6.2 has previously been demonstrated as Nkx6.2 has been shown to be able to partially rescue defects in motoneuron formation in Nkx6.1-deficient embryos due to ventral expansion of Nkx6.2 expression into the domain normally expressing Nkx6.1 (Vallstedt et al., 2001). The fact that Nkx6.2 expression expands into the Nkx6.1 expression domain in the neural tube demonstrates that Nkx6.1 is acting as a repressor of Nkx6.2 expression. It is thus notable that we find expression of both Nkx6.1 and Nkx6.2 in pancreatic endoderm. However, the repressive action of Nkx6.1 upon Nkx6.2 gene activity may be context-dependent. For example, in the hindbrain, Nkx6.1 and Nkx6.2 are co-expressed in branchial motor neurons (Muller et al., 2003). It is therefore possible that presence of Nkx6.1 is not sufficient to fully repress Nkx6.2 expression in the developing

Fig. 9. Exogenous Pdx1 expression induces Nkx6.1 but not Nkx6.2/6.3 expression in anterior endoderm. (A) Exogenous Pdx1 expression visualized by IHC induces formation of bud-like structures (arrow). (B) Double in situ hybridization to visualize ectopic Pdx1 expression (red) and endogenous CdxA expression in chicken intestine. Note that CdxA expression is extinguished in Pdx1 expressing cells (arrow). (C–F) Triple IHC to detect the endodermal marker Foxa2 (blue, C), ectopic Pdx1 (green, D), and Nkx6.1 (red, E) and the corresponding overlay (F). Note that ectopic Nkx6.1 expression is induced in Foxa2 positive endodermal cells that express exogenous Pdx1 (arrow in low magnification panels and arrowhead in high magnification inserts point to the same cell). (G–J) In situ hybridization visualizes exogenous Pdx1 expression (G, I), Nkx6.2 (H), and Nkx6.3 expression (J) in anterior chicken endoderm. Panels H and J represent sections adjacent to the sections shown in panels G and I, respectively. Note that Nkx6.2 and Nkx6.3 expression is not induced in cells expressing exogenous Pdx1. Scale bars are 100 μm (A, B), 20 μm (C–F), and 50 μm (G–J).

pancreas. However, loss of Nkx6.1 does enhance Nkx6.2 expression in the pancreas (Henseleit et al., 2005). One notable difference between mice and chicken is the expression of Nkx6.2 in insulin-producing cells in chicken but not in mice. This is likely due to species-specific differences in the Nkx6.2 promoter as also neural tube expression of chicken Nkx6.2 overlaps with Nkx6.1 (Briscoe et al., 2000; Sander et al., 2000a). Later in chicken embryonic development, we found that Nkx6.1 expression is restricted to the undifferentiated epithelium of the pancreas as well as to amylase- and insulin-producing cells. The Nkx6.1 expression in acinar cells demonstrates another notable difference between mice and chicken. In mice, Nkx6.1 expression is lost when epithelial cells differentiate into acinar cells (Jensen et al., 2000; Sander et al., 2000b). We cannot presently say whether this acinar expression of Nkx6.1 will disappear at later stages. The strong expression in the pancreatic epithelium and the β-cells resembles the expression pattern observed for Nkx6.1 in the mice, indicating that chicken Nkx6.1 might play a role in the differentiation of islet precursor cells into mature insulin-producing β-cells as it does in mice (Sander et al., 2000b).

### *Nkx6.1 and Nkx6.2 gene expression is differentially dependent on Pdx1 activity in the mice*

The finding that induction and/or maintenance of Nkx6.1 (but not Nkx6.2) expression in the undifferentiated dorsal pancreas epithelium is dependent on the presence of a functional Pdx1 allele suggests that expression of Nkx6.1 and Nkx6.2 in the early dorsal pancreas bud is initiated by different mechanisms. Our observations are consistent with previous work showing that Pdx1 is necessary for the maintenance of Nkx6.1 expression in mature β-cells and for expression of Nkx6.1 in endocrine cells in the antropyloric mucosa of newborn mice (Ahlgren et al., 1998; Oster et al., 1998a). However, the phenotype observed in both of these studies can also be explained by the loss of the Nkx6.1-expressing cell type. Here, we clearly demonstrate that the early pancreas epithelium is still present and marked by Nkx6.2 expression in Pdx1 mutant mice, but expression of Nkx6.1 is selectively lost. The fact that Nkx6.1 expression can still be detected in some of the early glucagon-expressing cells in the dorsal pancreas bud of Pdx1-deficient mice indicates that the expression of Nkx6.1 in these early glucagon cells is independent of prior Nkx6.1 expression in the undifferentiated epithelium. This observation could be explained by the presence of more than one Nkx6.1 promoter. It has been demonstrated that Nkx2.2 uses one promoter for expression in undifferentiated pancreatic epithelial cells and another for directing expression to mature islet cells (Watada et al., 2003).

In vitro studies have shown that both Pdx1 and Nkx2.2 can bind to the promoter of Nkx6.1, and it has been suggested that Nkx2.2, like Pdx1, could also play a role in the induction of Nkx6.1 in the early pancreas (Iype et al., 2004; Watada et al., 2000). However, Nkx2.2 is not required for expression of Nkx6.1 in the early pancreatic epithelium (Sussel et al., 1998). But, Nkx2.2 is necessary for Nkx6.1 expression in β-cells (Sander et al., 2000b; Sussel et al., 1998), implying that Nkx6.1 gene activity might be controlled by more than one mechanism.

### *Pdx1 is sufficient for expression of chicken Nkx6.1 when introduced to chicken endoderm*

Since we find that Pdx1 is required for Nkx6.1 expression, we investigated whether Pdx1 induced effects could be mediated through induction of Nkx6 genes. We observed that exogenous expression of Pdx1 does indeed induce the expression of Nkx6.1 but not Nkx6.2 or Nkx6.3 in the anterior chicken endoderm. This demonstrates that Pdx1 is sufficient for Nkx6.1 expression in this region. In contrast, yolk sac endoderm did not respond to exogenous Pdx1. It was recently shown that anterior chicken endoderm can switch to a more posterior fate when cultured together with posterior mesoderm while posterior endoderm could not be respecified when cultured together with more anterior mesoderm (Kumar et al., 2003). Hence, it could be speculated that endoderm anterior to the pancreas is more amenable to induction of Nkx6 expression by Pdx1. Lastly, the chicken Nkx6.1 expression domain is clearly broader than the chicken Pdx1 expression domain. Hence, additional mechanisms must be involved in regulating cNkx6.1 in this area.

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