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The Cdk4-E2f1 pathway regulates early pancreas development by targeting Pdx1$^+$ progenitors and Ngn3$^+$ endocrine precursors

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SUMMARY

Cell division and cell differentiation are intricately regulated processes vital to organ development. Cyclin-dependent kinases (Cdks) are master regulators of the cell cycle that orchestrate the cell division and differentiation programs. Cdk1 is essential to drive cell division and is required for the first embryonic divisions, whereas Cdks 2, 4 and 6 are dispensable for organogenesis but vital for tissue-specific cell development. Here, we illustrate an important role for Cdk4 in regulating early pancreas development. Pancreatic development involves extensive morphogenesis, proliferation and differentiation of the epithelium to give rise to the distinct cell lineages of the adult pancreas. The cell cycle molecules that specify lineage commitment within the early pancreas are unknown. We show that Cdk4 and its downstream transcription factor E2f1 regulate mouse pancreas development prior to and during the secondary transition. Cdk4 deficiency reduces embryonic pancreas size owing to impaired mesenchyme development and fewer Pdx1$^+$ pancreatic progenitor cells. Expression of activated *Cdk4*^{R24C} kinase leads to increased Nkx2.2$^+$ and Nkx6.1$^+$ cells and a rise in the number and proliferation of Ngn3$^+$ endocrine precursors, resulting in expansion of the β cell lineage. We show that E2f1 binds and activates the *Ngn3* promoter to modulate *Ngn3* expression levels in the embryonic pancreas in a Cdk4-dependent manner. These results suggest that Cdk4 promotes β cell development by directing E2f1-mediated activation of *Ngn3* and increasing the pool of endocrine precursors, and identify Cdk4 as an important regulator of early pancreas development that modulates the proliferation potential of pancreatic progenitors and endocrine precursors.

KEY WORDS: Cdk4, E2f1, Ngn3 (Neurog3), Pancreas development, Pdx1, Mouse

INTRODUCTION

Pancreas development involves the intricate and complex morphogenesis of dorsal and ventral buds on the primitive gut tube to form a highly branched pancreas organ containing multiple specialized cell types (Jorgensen et al., 2007; Kim and MacDonald, 2002; Murtaugh, 2007; Puri and Hebrok, 2010). Lineage commitment, which is referred to as the primary transition, takes place by embryonic day (E) 8.5 in mouse. This is followed at ~E12.5 by a differentiation program referred to as the secondary transition. The extensive morphogenesis of the epithelium that results in the mature pancreas is regulated by diverse signaling networks and by the activation of pancreas-specific transcription factors that specify the timing and cell type of pancreas differentiation (Puri and Hebrok, 2010). The mesenchyme plays a crucial, albeit complex, role in the early steps of pancreas organogenesis by secreting diffusible factors to permit pancreas growth (Attali et al., 2007; Gittes et al., 1996; Miralles et al., 1998; Miralles et al., 2006). In particular, the Fgf10-Notch axis is considered to play a crucial role in the expansion of the pancreatic progenitor pool (Bhushan et al., 2001; Dhawan et al., 2007). In addition, the TGFβ, Wnt, Hedgehog and EGF signaling pathways play central and interdependent roles in pancreas development (Edlund, 2002; Kemp et al., 2003; Kim and Hebrok, 2001; Murtaugh, 2007; Murtaugh and Melton, 2003; Puri and Hebrok, 2010).

The homeodomain transcription factor Pdx1 (Ipf1) is an important regulator of early pancreatic development (McKinnon and Docherty, 2001) and Pdx1 deficiency leads to pancreatic agenesis (Jonsson et al., 1994; Offield et al., 1996; Schwitzgebel et al., 2003; Stoffers et al., 1997). After the activation of Pdx1 expression, cells expressing the transcription factors Sox9, Isl1, Pax6, Nkx2.2, Neurod1 (Beta2) and neurogenin 3 (Ngn3; also known as Neurog3) appear, which resembles the signature of an endocrine precursor cell type (Bernardo et al., 2008; Lioubinski et al., 2003; Sander et al., 2000; Wilson et al., 2003). Ngn3 is essential for the specification of a common precursor for the four pancreatic endocrine cell types, as mice with a targeted disruption of the *Ngn3* locus fail to develop endocrine cell lineages (Gradwohl et al., 2000). Moreover, cell lineage tracing revealed that Ngn3-expressing cells give rise to all types of islet endocrine cells and function as progenitors rather than self-renewing stem cells (Gu et al., 2002; Schonhoff et al., 2004). Further, Heimberg and colleagues recently showed that partial duct ligation can activate the differentiation of Ngn3-expressing progenitors into functional β cells in the adult pancreas, thereby illustrating a β cell-specific role for Ngn3 (Xu et al., 2008).

The cell cycle machinery receives growth factor signals and regulates the quiescence, proliferation, differentiation, senescence and apoptosis programs of all cells (Malumbres and Barbacid, 2005; Rane and Reddy, 2000; Satyanarayana and Kaldis, 2009). The retinoblastoma (Rb1) phospho-protein negatively regulates the cell cycle by sequestering E2F family transcription factors (van den Heuvel and Dyson, 2008). A

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family of cyclin-dependent kinases (Cdks) promotes S-phase progression and mitosis by sequentially phosphorylating Rb1 on several serine/threonine residues thereby rendering the protein inactive (Satyanarayana and Kaldis, 2009). Cdks are negatively regulated by the Ink4 and Cip/Kip families of cyclin-dependent kinase inhibitors (Ckis). Using mice with genetically modified *Cdk4* loci, we have previously shown that Cdk4 regulates β cell mass (Mettus and Rane, 2003; Rane et al., 2002; Rane et al., 1999; Rane and Reddy, 2000). *Cdk4*$^{-/-}$ mice exhibit β cell hypoplasia and develop diabetes, whereas *Cdk4*$^{R24C/R24C}$ mice (*Cdk4*$^{R/R}$ mice), which inherit the p16^{Ink4a} (Cdkn2a)-insensitive Cdk4^{R24C} kinase, exhibit β cell hyperplasia. We recently showed that Cdk4 enhances β cell replication within adult islets and activates progenitors within the adult pancreatic ductal epithelium in response to partial pancreatectomy (Lee et al., 2010). We found that Cdk4 promotes islet β cell replication, a mechanism that is presumed to play a primary role in regulating β cell mass. Further, we observed that Cdk4 catalyzes the recruitment of quiescent cells within the islets and the ductal epithelium to participate in the regenerative process. Interestingly, the regeneration response within the ductal epithelium displayed hallmarks of early pancreatic development, such as the rapid proliferation of ductal epithelial cells and a significant increase in cells that express Pdx1 (Lee et al., 2010). Moreover, we observed increased insulin-positive cells in the ductal epithelium and a significant enhancement in the number of duct-associated small islet-like clusters (ICCs) (Lee et al., 2010). In addition, several recent studies have underscored the important roles of other cell cycle regulators in β cell biology (Annicotte et al., 2009; Cozar-Castellano et al., 2006; Fajas et al., 2004; Fiaschi-Taesch et al., 2009; Georgia and Bhushan, 2004; Georgia and Bhushan, 2006; Georgia et al., 2010; Georgia et al., 2006; Krishnamurthy et al., 2006; Kushner et al., 2005; Rachdi et al., 2006; Uchida et al., 2005; Zhang et al., 2005).

Although the cell cycle regulators that regulate adult β cell proliferation are relatively well characterized (Butler et al., 2007; Cozar-Castellano et al., 2006; Heit et al., 2006; Kushner, 2006; Rane and Reddy, 2000), those that control embryonic pancreas development remain obscure (Dhawan et al., 2007). Here, we examined whether Cdk4 participates in embryonic pancreas development. We find that Cdk4 orchestrates endocrine pancreas development by influencing the proliferation of Pdx1^{+} pancreatic progenitors and by promoting the expansion of endocrine precursors during embryogenesis via E2f1-mediated regulation of *Ngn3*.

MATERIALS AND METHODS

Mice

Cdk4$^{+/+}$, *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} mice were maintained as described (Rane et al., 1999). Timed matings were carried out, with E0.5 as the day of discovery of a vaginal plug. For BrdU labeling, pregnant females were injected intraperitoneally with 50 μg BrdU/g body weight, and embryos were harvested 1 hour after injection. Animal protocols were approved by the NIH ACUC.

Wholemount immunofluorescence

The dissected tissue from *Cdk4* wild-type mice at E11.5 was fixed in 4% paraformaldehyde overnight at 4°C and then transferred to 70% ethanol overnight at 4°C. Tissues were immersed in 50% methanol in PBS for 1 hour at room temperature and then permeabilized in 1% Triton X-100 in PBS for 2 hours at room temperature, blocked with CAS-BLOCK (00-8020, Invitrogen) for 2 hours at room temperature and immunostained overnight at 4°C with anti-rabbit Cdk4 (sc-260, Santa Cruz) and anti-goat Pdx1 (ab47383, Abcam) antibodies in 0.5% Triton X-100 in CAS-BLOCK. Tissues were incubated with secondary antibodies and DAPI for 2 hours at room temperature and immersed in 1:2 benzyl alcohol:benzyl benzoate and mounted on a concave slide. Images were obtained using an LSM 510 confocal microscope (Zeiss).

Immunostaining assays

Mouse tissues were harvested, paraffin sectioned and immunostained overnight at 4°C with the following primary antibodies: goat anti-Pdx1 (ab47383, Abcam), rabbit anti-Pdx1 (AB3503, Millipore), goat anti-vimentin (AB1620, Millipore), mouse anti-Ngn3 (F25A1B3, DSHB at University of Iowa), mouse anti-Isl1 (40.2D6, DSHB), mouse anti-Nkx2.2 (74.5A5, DSHB), mouse anti-Nkx6.1 (F64A6B4, DSHB), mouse anti-E-cadherin (610181, BD Biosciences), rabbit anti-Cdk4 (sc-260, Santa Cruz), rabbit anti-E2f1 (ab94888, Abcam), guinea pig anti-insulin (A0564, Dako), rabbit anti-glucagon (A0565, Dako), rabbit anti-somatostatin (A0566, Dako), goat anti-pancreatic polypeptide (Ppy; EB06805, Everest Biotech), rabbit anti-Ki67 (NCL-Ki67p, Leica) and mouse anti-BrdU (M0744, Dako). Slides were imaged on a LSM 510 confocal microscope (Zeiss). For immunostaining of Ngn3 at P1, antigen retrieval was performed by heating tissue sections in 10 mM sodium citrate buffer (pH 6.0). Images were scanned by ScanScope (Aperio, CA, USA). To estimate the total β cell mass, the weight of the pancreas was determined. The relative β cell volume was obtained by point counting using immunohistochemistry for insulin. ScanScope software was used to measure β cell and total pancreas area. The absolute mass of the pancreatic β cells was estimated from the equation: β cell area:total area=β cell mass:total pancreas weight.

Cell culture and luciferase reporter assays

Embryonic pancreas was harvested at E14.5 and digested with 0.2 mg/ml Liberase RI (1815032, Roche) for 8 minutes at 37°C to generate fetal pancreatic (FP) cells. FP cells were grown in DMEM with 10% fetal bovine serum. For transient transfection, 0.02 μg of luciferase reporter plasmid DNA (*E2f1*-Luc or pFox*Ngn3*-Luc) was transfected together with pCMV Renilla-TK with or without 0.2 μg E2f1 expression plasmid. For transfection, we used Fugene6 (11814443001, Roche) and 1-2×10^{4} cells/well. These cells were usually derived from three to four embryonic pancreases for a four-well plate. Luciferase assays were performed 48 hours after transfection using the Dual-Luciferase Reporter System (E1910, Promega). Analyses were repeated three times in duplicate.

Real-time PCR

Total RNA was prepared from E12.5 pancreases and FP cells using the RNAqueous-Micro Kit (1931, Ambion). Reverse transcription PCR reagents were used to generate cDNAs from 1 μg of DNaseI-treated RNA. PCR reactions were performed and analyzed using the Applied Biosystems Fast 7500 real-time PCR system and SDS software. Relative changes were calculated by the comparative ΔΔCt method, in which 18S rRNA was used for normalization. Reactions were performed in triplicate and relative amounts of cDNA were normalized to 18S rRNA.

Chromatin immunoprecipitation (ChIP) assay

We used 10-15 embryonic pancreases at E15.5 per genotype. Embryonic pancreases were homogenized in lysis buffer [15 mM Hepes-Cl pH 7.6, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.34 M sucrose, 0.15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine and protease inhibitor (11836170001, Roche)] and cross-linked with 1% formaldehyde (Chaya and Zaret, 2004). The cross-linked chromatin was sheared using a Bioruptor 200 (Diagenode) for 45 minutes and the lysate was centrifuged at 10,000 ***g*** at 4°C for 12 minutes. A total of 150 μl of purified chromatin (1/20th of the total chromatin from one adult pancreas) in 118 μl of ChIP dilution buffer (101265, Active Motif) was incubated with 1-3 μg of negative control IgG (mouse IgG, sc-2025, Vector Labs) or polyclonal anti-E2f1 (sc-251, Santa Cruz) antibody overnight at 4°C and then incubated with protein G-agarose beads (Active Motif) for 2 hours at 4°C. Bound chromatin was eluted from the beads, formaldehyde cross-linking was reversed, and the immunoprecipitated DNA used for PCR analysis (for primers, see Table S3 in the supplementary material). Quantification of the binding to various promoter regions was performed by densitometry.

Statistical analysis

Standard error of the mean (s.e.m.) was used for determining statistical significance. The entire pancreas anlagen (minimum three animals of each genotype) were used to obtain a representative average of the number of endocrine or hormone-positive cells. Positive cells were counted every fourth section throughout the pancreas. The average cell number per section was determined for each individual pancreas (all sections containing pancreatic tissue were examined). The ratio (percentage) of single-positive cells to double-positive cells was measured by counting antigen-expressing cells. Mean differences were tested for statistical significance by Student's *t*-test.

RESULTS

Cdk4 is required for growth and morphogenesis of the developing pancreas

Wholemount immunofluorescence showed that Cdk4 is localized in the pancreatic epithelium and mesenchyme of the E11.5 wild-type mouse embryo (Fig. 1A,B and see Movie 1 in the supplementary material). To examine the role of Cdk4 in pancreas development, we examined embryos prior to and after the secondary transition: at E12.5 before endocrine differentiation occurs and further at E14.5 to evaluate the post-secondary transition phenotype when endocrine cells differentiate in exponentially increasing numbers, predominantly towards β cells (Murtaugh and Melton, 2003). Double immunofluorescence staining of the E12.5 pancreas using antibodies against Cdk4 and the epithelial cell marker E-cadherin (E-cad; cadherin 1 – Mouse Genome Informatics) revealed that Cdk4 is expressed in the pancreatic epithelium and mesenchyme. We detected robust Cdk4 staining in the *Cdk4* wild-type (*Cdk4*$^{+/+}$; Fig. 1C) and *Cdk4*$^{R24C/R24C}$ (*Cdk4*^{R24C}; Fig. 1E) pancreas, whereas, as expected, we did not detect Cdk4 expression in the *Cdk4*-null pancreas (*Cdk4*$^{-/-}$; Fig. 1D). Further, co-immunostaining for Pdx1 and Cdk4 at E12.5 and E14.5 in *Cdk4* wild-type pancreas revealed that Cdk4 is expressed in Pdx1^{+} progenitor cells (Fig. 1F-I and see Fig. S1 in the supplementary material). Morphometric analysis of the E-cad^{+} pancreatic epithelium area showed a significantly reduced sectional area in E12.5 *Cdk4*$^{-/-}$ as compared with *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} pancreas (Fig. 2A-D and see Table S1 in the supplementary material).

Proliferation and differentiation of Pdx1^{+} progenitor cells in the epithelium of the developing pancreas give rise to the cells that comprise the endocrine, exocrine and ductal pancreas (Gu et al., 2002; Jorgensen et al., 2007; Kim and MacDonald, 2002; Puri and Hebrok, 2010). We observed proliferating Cdk4^{+} Pdx1^{+} cells in E12.5 and E14.5 wild-type pancreas (see Fig. S2 in the supplementary material). To estimate the proliferation of Pdx1^{+} pancreatic progenitors, we conducted BrdU-incorporation assays at E12.5. The level of proliferation of Pdx1^{+} cells, as assessed by BrdU^{+} cells, was similar in *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} pancreas (Fig. 2E,G,H), suggesting that activation of Cdk4 is not essential for inducing proliferation of Pdx1^{+} pancreatic progenitors at the primary transition. However, compared with *Cdk4*$^{+/+}$ pancreas, we observed a significantly decreased proliferation of Pdx1^{+} progenitors in the *Cdk4*$^{-/-}$ pancreas (Fig. 2E,F,H), consistent with the reduction in pancreas size. At E12.5, the majority of the E-cad^{+} epithelial cells were also Pdx1^{+}. Immunofluorescence analysis using the proliferation marker Ki67 revealed reduced proliferation of E-cad^{+} epithelial cells in *Cdk4*$^{-/-}$ as compared with *Cdk4*$^{+/+}$ pancreas, whereas significantly increased proliferation was observed in the *Cdk4*^{R24C} pancreas (see Fig. S3 and Table S1 in the supplementary material). The total numbers of proliferating cells measured by Ki67, as compared with those measured by BrdU labeling, were higher in all the three genotypes (see Table S1 in the supplementary material). Ki67 antigen is a cell cycle-related nuclear protein that is expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M). By contrast, BrdU labels cells that are exclusively in S phase. Typically, Ki67^{+} cells are more abundant than BrdU^{+} cells. Further, these observations suggested that, whereas Cdk4 loss was detrimental, Cdk4^{R24C} kinase induced the proliferation of pancreatic epithelial cells. TUNEL staining of the E12.5 *Cdk4*$^{-/-}$ pancreas did not show elevated apoptosis, arguing against a role for apoptosis in the reduced area of the *Cdk4*$^{-/-}$ pancreas (data not shown).

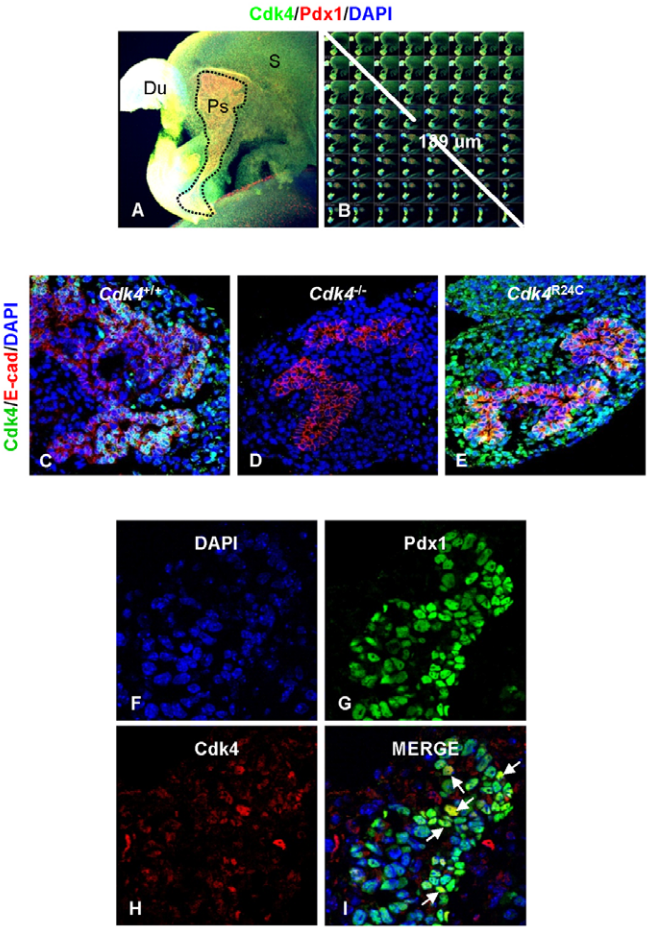

Fig. 1. Cdk4 expression in the developing embryonic pancreas. (**A**,**B**) Wholemount immunofluorescence analysis of Cdk4 expression in the E11.5 pancreas. The entire pancreas from an E11.5 wild-type mouse was subjected to immunofluorescence assay using antibodies against Cdk4 and Pdx1 and analyzed by confocal microscopy (A). The data were acquired as a movie (see Movie 1 in the supplementary material) and the 64 optical sections spanning 0-189 μm (B) were acquired on Zeiss software and reconstructed as a composite image (A). The image shows that Cdk4 is expressed in Pdx1^{+} and Pdx1^{-} cells in the pancreas (outlined). Ps, pancreas; S, stomach; Du, duodenum. (**C-E**) Embryonic pancreas from *Cdk4*$^{+/+}$ (C) and *Cdk4*^{R24C} (E) but not *Cdk4*$^{-/-}$ (D) mice express Cdk4 in the pancreatic epithelium and mesenchyme. The pancreases were co-immunostained with antibodies against Cdk4 and E-cad. (**F-I**) Cdk4 is expressed in Pdx1^{+} pancreatic progenitors. Arrows indicate Cdk4^{+} Pdx1^{+} cells in *Cdk4*$^{+/+}$ pancreas. In all sections, DAPI identifies nuclei.

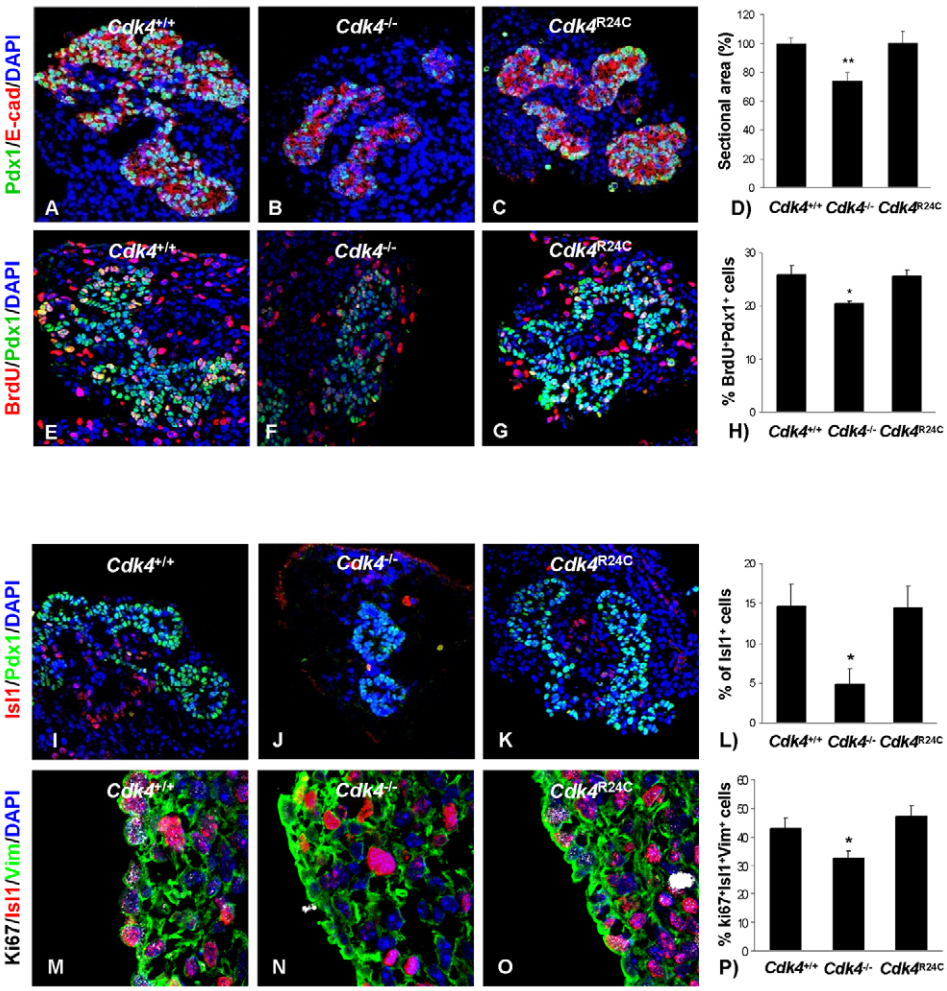

Fig. 2. Cdk4 is important for proliferation of Pdx1⁺ pancreatic progenitors and mesenchymal development in E12.5 embryos. (**A-D**) Absence of Cdk4 results in reduced pancreatic sectional area as confirmed by Pdx1 and E-cad immunostaining of *Cdk4*$^{+/+}$ (A), *Cdk4*$^{-/-}$ (B) and *Cdk4*^{R24C} (C) mouse pancreas, followed by morphometric analysis (D) to calculate the relative pancreatic epithelial E-cad⁺ sectional area (%). The results are normalized to wild type. (**E-H**) Compared with *Cdk4*$^{+/+}$ (E) and *Cdk4*^{R24C} (G) embryos, the *Cdk4*$^{-/-}$ embryos (F) exhibit decreased numbers of BrdU⁺ Pdx1⁺ pancreatic progenitor cells (H). (**I-P**) Cdk4 loss results in reduced Isl1⁺ numbers and reduced proliferation of Isl1⁺ Vim⁺ mesenchymal cells. (I-L) Absence of Cdk4 results in reduced numbers of Is1⁺ cells as confirmed by Pdx1 and Isl1 immunostaining of *Cdk4*$^{+/+}$ (I), *Cdk4*$^{-/-}$ (J) and *Cdk4*^{R24C} (K) pancreas, followed by morphometric analysis (L) to calculate the relative numbers of Isl1⁺ cells. (M-P) Compared with *Cdk4*$^{+/+}$ (M) and *Cdk4*^{R24C} (O) embryos, the *Cdk4*$^{-/-}$ embryos (N) exhibit decreased numbers of Ki67⁺ Isl1⁺ Vim⁺ cells (P). *, $P<0.05$; **, $P<0.001$; Student's *t*-test. Error bars indicate s.e.m.

The mesenchyme plays an important role in the early steps of pancreas organogenesis by secreting diffusible factors to permit pancreas growth (Attali et al., 2007; Gittes et al., 1996; Miralles et al., 1998; Miralles et al., 2006), and the Fgf10-Notch signaling pathway plays a crucial role in expansion of the pancreatic progenitor pool (Bhushan et al., 2001; Dhawan et al., 2007). We examined whether the depletion of the pancreatic progenitors and their proliferation deficits in the embryonic *Cdk4*$^{-/-}$ pancreas could be due to defects in the pancreatic mesenchyme. We observed that Isl1⁺ cells were significantly reduced in the *Cdk4*$^{-/-}$ pancreas, whereas the numbers in the *Cdk4*^{R24C} pancreas were similar to those seen in *Cdk4*$^{+/+}$ pancreas (Fig. 2I-L). Next, we examined the proliferation potential of Isl1⁺ cells that were positive for Ki67 and the mesenchymal marker vimentin (Vim). The numbers of Ki67⁺ Isl1⁺ Vim⁺ cells were significantly reduced in the *Cdk4*$^{-/-}$ pancreas, whereas they were unchanged in the *Cdk4*^{R24C} pancreas (Fig. 2M-P).

Finally, we assayed by real-time RT-PCR the transcript levels of *Fgf10*, *Hes1* and *p57* (*Cdkn1c* – Mouse Genome Informatics), which have been implicated in regulating mesenchymal cues with respect to the proliferation of pancreatic progenitors (Bhushan et al., 2001; Dhawan et al., 2007; Georgia et al., 2006). Whereas no changes in the transcript levels of *Fgf10* and *Hes1* were seen, we observed increased levels of *p57* transcripts in the E12.5 *Cdk4*$^{-/-}$ pancreas (see Fig. S4A in the supplementary material). By contrast, the levels of *Fgf10*, *Hes1* and *p57* were unchanged in the E14.5 *Cdk4*$^{-/-}$ pancreas, as compared with the *Cdk4*$^{+/+}$ pancreas (see Fig. S4B in the supplementary material). However, levels of *Fgf10* and *Hes1* transcripts were significantly increased, and that of *p57* significantly decreased, in the E14.5 *Cdk4*^{R24C} pancreas (see Fig. S4B in the supplementary material). Interestingly, we observed increased numbers of Pdx1⁺ BrdU⁺ cells in the E14.5 *Cdk4*^{R24C} (41.7±4.3%) as compared with the *Cdk4*$^{+/+}$ (29.3±0.8%) pancreas.

Taken together, these observations suggest that proper mesenchyme development is dependent on optimal Cdk4 expression. It is possible that the deficit in pancreatic progenitor proliferation and the reduced pancreatic size of the *Cdk4*$^{-/-}$ embryonic pancreas could be at least in part attributable to a defective pancreatic mesenchymal compartment.

Cdk4 promotes the proliferation of Ngn3⁺ endocrine precursors

The majority of insulin-producing β cells in the fetal pancreas arise during the secondary transition, a wave of morphogenesis and differentiation that starts at E13.5 (Jorgensen et al., 2007; Kim and MacDonald, 2002). To evaluate the contribution of Cdk4 to endocrine pancreas development towards the β cell lineage, we examined cells expressing Nkx2.2, Nkx6.1 and Ngn3. The NK-homeodomain proteins Nkx2.2 and Nkx6.1 are considered β cell competence factors. Nkx2.2 is initially expressed broadly in the pancreatic bud and becomes progressively restricted to islet cells during the secondary transition (reviewed by Jorgensen et al., 2007;

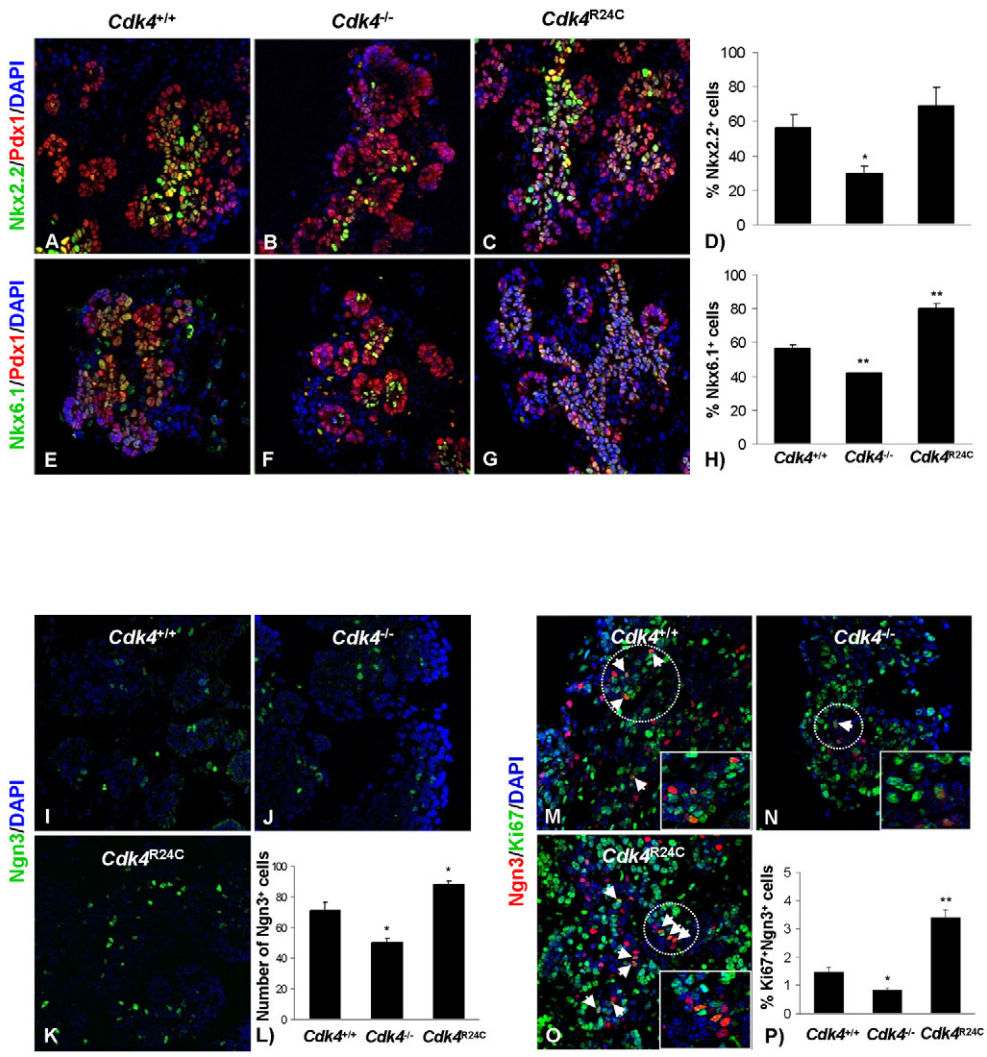

Fig. 3. Cdk4 promotes proliferation of Ngn3$^+$ endocrine precursors in E14.5 pancreas. (**A-H**) Nkx2.2-expressing (A-D) and Nkx6.1-expressing (E-H) cells within the Pdx1$^+$ cell population are reduced in *Cdk4*$^{-/-}$ mouse embryos (B,F), whereas they are increased in *Cdk4*^{R24C} embryos (C,G), as compared with *Cdk4*$^{+/+}$ embryos (A,E). The percentage of Pdx1$^+$ progenitors that are Nkx2.2$^+$ (D) and Nkx6.1$^+$ (H) is presented. (**I-L**) Compared with the *Cdk4*$^{+/+}$ pancreas (I,L), the *Cdk4*^{R24C} pancreas (K,L) shows increased numbers of Ngn3$^+$ endocrine precursors, whereas the absence of Cdk4 results in a reduced population of Ngn3$^+$ cells (J,L). (**M-P**) Numbers of Ki67$^+$ Ngn3$^+$ cells are reduced in *Cdk4*$^{-/-}$ (N,P) and increased in *Cdk4*^{R24C} (O,P) as compared with *Cdk4*$^{+/+}$ (M,P) pancreas. Arrows indicate Ki67$^+$ Ngn3$^+$ cells. Insets show the circled area at higher magnification. *, $P<0.05$; **, $P<0.001$; Student's *t*-test. Error bars indicate s.e.m.

Sussel et al., 1998; Sander et al., 2000). Disruption of *Nkx2.2* in mice leads to incomplete differentiation of β cells (Sussel et al., 1998), although Nkx2.2 also plays a role in α cell and PP cell development. The Nkx6.1 expression pattern initially resembles that of Nkx2.2, although its expression later becomes limited to β cells alone (Jensen et al., 1996). Disruption of *Nkx6.1* in mice leads to loss of β cell precursors and blocks β cell neogenesis during the secondary transition (Jensen et al., 1996; Rudnick et al., 1994). We examined the percentage of Nkx2.2$^+$ (Fig. 3A-D) and Nkx6.1$^+$ (Fig. 3E-H) cells relative to Pdx1$^+$ cells in the pancreas from *Cdk4*$^{+/+}$, *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} mice at E14.5. Compared with those observed in the *Cdk4*$^{+/+}$ pancreas, the percentage of Nkx2.2$^+$ and Nkx6.1$^+$ cells was significantly reduced in the *Cdk4*$^{-/-}$ pancreas. By contrast, the numbers of Nkx2.2$^+$ and Nkx6.1$^+$ cells were significantly increased in the *Cdk4*^{R24C} pancreas (Fig. 3D,H and see Table S2 in the supplementary material).

Immunofluorescence assays on E14.5 wild-type pancreas revealed rare Cdk4$^+$ Ngn3$^+$ cells (see Fig. S5A in the supplementary material). Interestingly, we observed increased numbers of Cdk4$^+$ Ngn3$^+$ cells in the E14.5 *Cdk4*^{R24C} as compared with the *Cdk4*$^{+/+}$ pancreas (see Fig. S5A,B in the supplementary material). *Cdk4*$^{-/-}$ pancreas displayed a significant reduction of the total number of Ngn3$^+$ cells, whereas a significant increase in total Ngn3$^+$ cells was observed in the *Cdk4*^{R24C} pancreas (Fig. 3I-L and see Table S2 in the supplementary material). To address whether Cdk4 controls the proliferation of Ngn3$^+$ endocrine precursors, double immunolabeling for Ngn3 and the proliferation marker Ki67 was performed. The proliferation of Ngn3$^+$ cells (Ngn3$^+$ Ki67$^+$) was suppressed in the *Cdk4*$^{-/-}$ pancreas and, by contrast, significantly elevated in the *Cdk4*^{R24C} pancreas (Fig. 3M-P and see Table S2 in the supplementary material). Together, these findings reveal a requirement for Cdk4 for the optimal expansion of the endocrine precursor pool during and after the secondary transition.

Cdk4 controls β cell differentiation and mass

We hypothesized that the paucity of Nkx2.2$^+$, Nkx6.1$^+$ and Ngn3$^+$ endocrine precursors in the *Cdk4*$^{-/-}$ embryonic pancreas might negatively affect β cell proliferation and differentiation during the period following the secondary transition. By implication, we postulated that increased numbers of Nkx2.2$^+$, Nkx6.1$^+$ and Ngn3$^+$ endocrine precursors and elevated proliferation of Ngn3$^+$ cells might promote β cell development in the *Cdk4*^{R24C} pancreas. At postnatal day (P) 1, compared with that seen in the *Cdk4*$^{+/+}$ pancreas (Fig. 4A,D), we observed an increase in proliferating β cells in the *Cdk4*^{R24C} pancreas (Fig. 4C,D). By contrast, BrdU$^+$ insulin$^+$ cells were not observed in the P1 *Cdk4*$^{-/-}$ pancreas (Fig. 4B,D). Consistent with this, at P1 β cell mass and numbers were

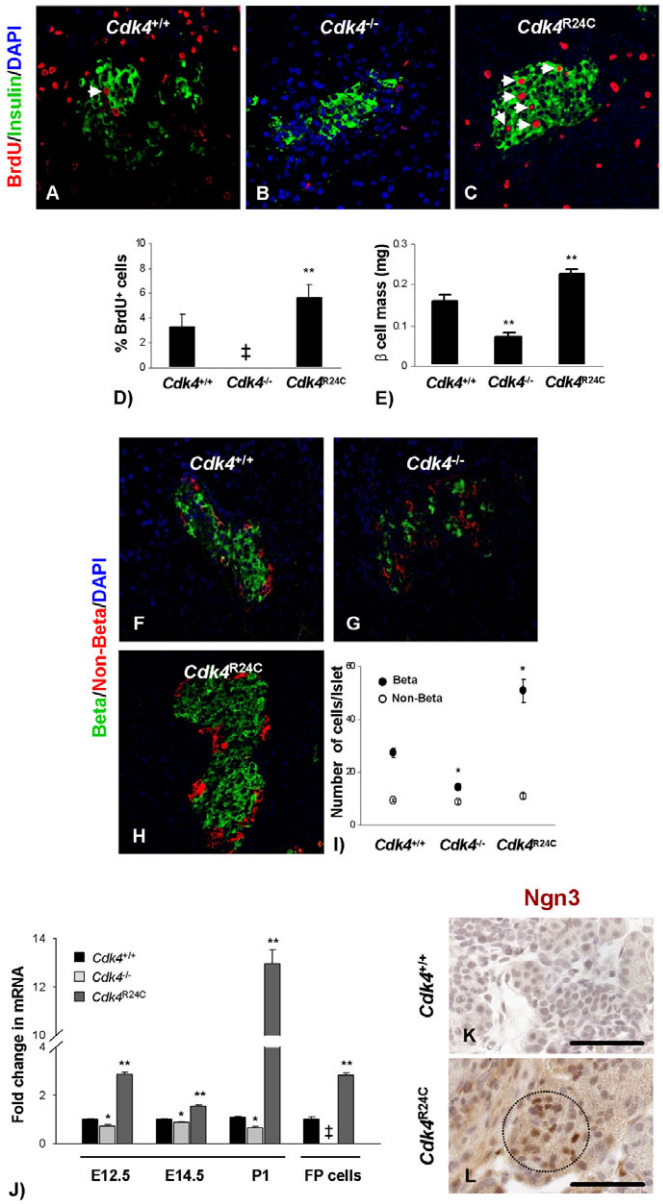

Fig. 4. Cdk4 controls β cell proliferation and mass during embryogenesis. (**A-D**) At P1, BrdU$^+$ Ins$^+$ cells are not observed (‡) in *Cdk4*$^{-/-}$ pancreas (B,D), whereas numbers are increased in *Cdk4*^{R24C} (C,D) as compared with *Cdk4*$^{+/+}$ (A,D) pancreas. Arrows indicate BrdU$^+$ Ins$^+$ cells. (D) Percentage of BrdU$^+$ Ins$^+$ cells in total Ins$^+$ cells. (**E**) Cdk4 deficiency results in a reduced β cell mass and the *Cdk4*^{R24C} pancreas shows a significant increase in β cell mass as compared with *Cdk4*$^{+/+}$ pancreas at P1. (**F-I**) Numbers of insulin-expressing β cells are reduced in *Cdk4*$^{-/-}$ (G,I) and increased in *Cdk4*^{R24C} (H,I) as compared with *Cdk4*$^{+/+}$ (F,I) pancreas at P1. The numbers of non-β cells expressing glucagon, somatostatin and pancreatic polypeptide (Ppy) are comparable in the islets of all genotypes. (I) The numbers of cells in islets from P1 mice of all three genotypes expressing insulin (closed circles) or non-β cell hormones (glucagon, somatostatin and Ppy, open circles) are shown. (**J**) Real-time PCR detection of *Ngn3* transcripts from embryonic (E12.5 and E14.5), postnatal (P1) pancreases and fetal pancreatic (FP) cells (after 3 days of culture) from *Cdk4*$^{+/+}$, *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} pancreas. *Ngn3* transcripts are undetectable (‡) in FP cells from *Cdk4*$^{-/-}$ mice. Fold change is relative to expression in *Cdk4*$^{+/+}$. (**K**,**L**) Detection of Ngn3$^+$ cells at P1 in *Cdk4*^{R24C} pancreas (L, circled) but not *Cdk4*$^{+/+}$ pancreas (K). *, $P<0.05$; **, $P<0.001$; Student's *t*-test. Error bars indicate s.e.m. Scale bars: 50 μm.

significantly reduced in the *Cdk4*$^{-/-}$ pancreas but significantly elevated in the *Cdk4*^{R24C} pancreas (Fig. 4E-I). The numbers of non-β cells within islets were similar in the P1 pancreas of all three genotypes, indicating that the *Cdk4* status does not affect the population of non-β cells. In agreement, compared with a β cell:non-β cell ratio of 2.95:1 in the P1 *Cdk4*$^{+/+}$ pancreas, this ratio increased to 4.64:1 in the *Cdk4*^{R24C} pancreas and decreased to 1.66:1 in the *Cdk4*$^{-/-}$ pancreas (Fig. 4I).

Cdk4 promotes β cell progenitor expansion via E2f1-mediated *Ngn3* promoter activation

Real-time RT-PCR assays revealed reduced expression of *Ngn3* transcripts in *Cdk4*$^{-/-}$ pancreas at E12.5, E14.5 and P1, compared with that observed in *Cdk4*$^{+/+}$ pancreas (Fig. 4J). By contrast, we observed a significant increase in *Ngn3* transcript levels in *Cdk4*^{R24C} pancreas (Fig. 4J). Consistent with these results, fetal pancreatic (FP) cells harvested from E14.5 embryos showed significantly elevated expression of *Ngn3* mRNA in *Cdk4*^{R24C} compared with *Cdk4*$^{+/+}$ FP cells, whereas *Ngn3* expression was undetectable in *Cdk4*$^{-/-}$ FP cells (Fig. 4J). These *Ngn3* mRNA expression levels are consistent with the reduced and increased numbers of Ngn3$^+$ cells in the *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} embryonic pancreas, respectively (Fig. 3I-L). The increase in *Ngn3* transcript levels in the P1 *Cdk4*^{R24C} pancreas was confirmed by immunohistochemistry for Ngn3$^+$ cells (Fig. 4K,L). By contrast, we failed to detect Ngn3$^+$ cells in the wild-type pancreas at P1. Taken together, these observations suggest that Cdk4 activation induces Ngn3 expression after birth, which in turn stimulates endocrine cell progression and, in particular, the proliferation of β cells.

Cdk4 phosphorylates its principal substrate Rb1, which results in the release of Rb1-associated E2F transcription factors that, in turn, transactivate or repress target gene promoters (Rane and Reddy, 2000; Satyanarayana and Kaldis, 2009). Our observations of a decrease and increase in *Ngn3* transcripts in *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} pancreas, respectively, led us to hypothesize that the effects of Cdk4 might be mediated via E2F-dependent regulation of the *Ngn3* promoter. Detection of rare E2f1$^+$ Ngn3$^+$ cells in E14.5 wild-type pancreas were further suggestive of a nexus between E2f1 and Ngn3 (see Fig. S5C in the supplementary material). Owing to technical limitations of the antibodies, we were unable to perform triple staining foe Cdk4, E2f1 and Ngn3. Interestingly, we observed an increased number of Cdk4$^+$ Ngn3$^+$ and E2f1$^+$ Ngn3$^+$ cells in the E14.5 *Cdk4*^{R24C} compared with the *Cdk4*$^{+/+}$ pancreas (see Fig. S5 in the supplementary material). In addition to double-positive cells, we observed Cdk4$^+$ cells adjacent to Ngn3$^+$ cells (see Fig. S6 in the supplementary material). Our repeated attempts to examine the proliferating Ngn3$^+$ cells that were positive for Cdk4 and E2f1 by triple staining (Cdk4$^+$ Ngn3$^+$ Ki67$^+$ and E2f1$^+$ Ngn3$^+$ Ki67$^+$) were unsuccessful owing to antibody limitations and we are unable to conclusively identify proliferating Ngn3$^+$ cells that express Cdk4 and/or E2f1.

We also developed a pancreatic rudiment culture system wherein FP cells from E14.5 *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} embryos were studied. Unfortunately, even after repeated attempts, we were unable to establish *Cdk4*$^{-/-}$ FP cells, primarily owing to their poor viability and inability to survive the culture conditions. *Cdk4*$^{+/+}$ FP cells were transfected with human *NGN3* promoter-driven luciferase reporter and plasmids expressing E2f1, E2f2 or E2f3. We observed that E2f1, but not E2f2 or E2f3, strongly activated the *NGN3*-luciferase reporter in *Cdk4*$^{+/+}$ FP cells (Fig. 5A). Moreover, compared with *Cdk4*$^{+/+}$ FP cells, *Cdk4*^{R24C} FP cells exhibited

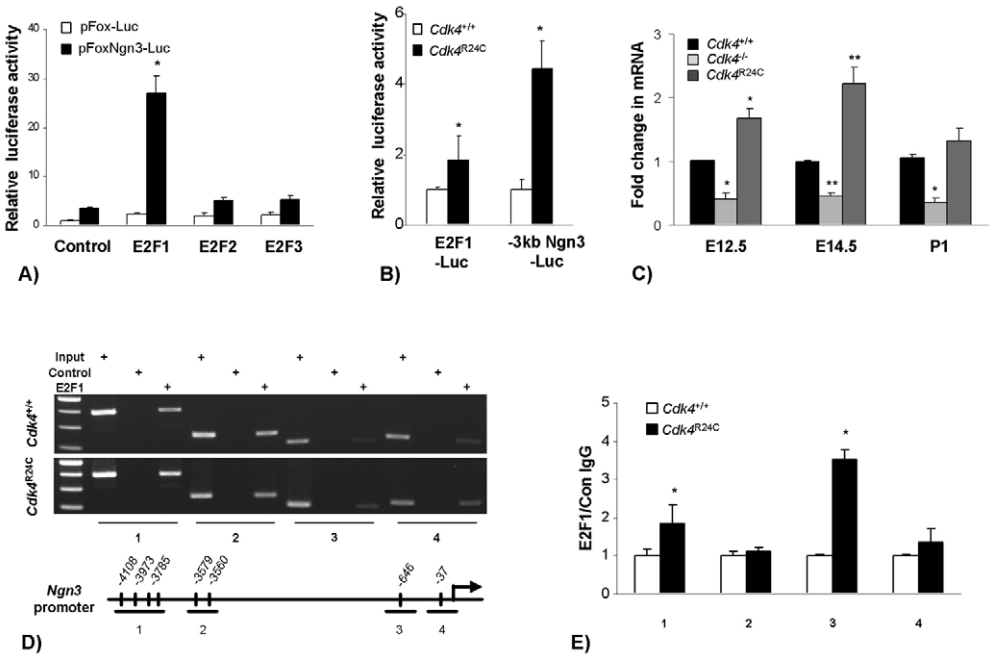

Fig. 5. Cdk4 promotes β cell progenitor expansion via E2f1-mediated *Ngn3* promoter activation. (**A**) The human *NGN3*-luciferase reporter (pFox*Ngn3*-Luc) was cotransfected into FP cells from *Cdk4*$^{+/+}$ mice together with plasmids expressing E2f1, E2f2 and E2f3 or the empty plasmid as control. Luciferase activities were determined 48 hours after transfection and are expressed relative to the activity in cells transfected with the promoterless reporter construct (pFox-Luc) and an empty expression plasmid. Results are expressed as mean ± s.e.m. of data from experiments performed in duplicate on at least three separate occasions. (**B**) The E2f1-luciferase reporter (*E2f1*-Luc) and pFox*Ngn3*-Luc (-3kb Ngn3-Luc) were transfected into FP cells derived from *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} mice. Luciferase activities were determined 48 hours after transfection and the activity in FP cells from *Cdk4*^{R24C} mice is expressed relative to that in *Cdk4*$^{+/+}$ FP cells. Results are expressed as the mean ± s.e.m. of duplicate experiments performed on three separate occasions. (**C**) Real-time PCR detection of *E2f1* transcripts from embryonic (E12.5 and E14.5) and postnatal (P1) pancreases from *Cdk4*$^{+/+}$, *Cdk4*$^{-/-}$ and *Cdk4*^{R24C} embryos. Fold change is relative to the expression in *Cdk4*$^{+/+}$. (**D**,**E**) ChIP assays were performed by immunoprecipitating cross-linked chromatin with anti-E2f1 or with control IgG from E15.5 pancreases from *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} mice. Four fragments of the mouse *Ngn3* promoter (1-4, underlined beneath the base number in D) were amplified by PCR from the precipitates or the input DNA. For quantification, relative occupancy was calculated as the value of the E2f1 immunoprecipitated sample relative to that of the negative IgG control for each of the four fragments (E). All data represent the mean ± s.e.m. of three independent experiments.*, $P<0.05$; **, $P<0.001$; Student's *t*-test.

increased E2f1 activity, as evidenced by enhanced *E2f1*-luciferase reporter activity (Fig. 5B). Consistent with this, real-time PCR analyses using RNA isolated from E12.5, E14.5 and P1 pancreas revealed elevated *E2f1* transcripts in E12.5 and E14.5 pancreas from *Cdk4*^{R24C} mice (Fig. 5C). By contrast, a strong suppression of *E2f1* expression was observed in *Cdk4*$^{-/-}$ pancreas (Fig. 5C). Importantly, we observed a significant 4- to 5-fold activation of the *NGN3*-luciferase reporter in *Cdk4*^{R24C} FP cells as compared with that seen in *Cdk4*$^{+/+}$ FP cells (Fig. 5B).

Sequence analyses revealed the presence of multiple E2F-binding elements in the full-length mouse *Ngn3* promoter (Fig. 5D). To examine whether E2f1 occupies the *Ngn3* promoter, we conducted chromatin immunoprecipitation (ChIP) assays using E15.5 pancreases from *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} mice and an anti-E2f1 antibody. Owing to limitations in the amount of tissue, we were unable to conduct similar experiments using embryonic pancreas tissue from *Cdk4*$^{-/-}$ mice. Four fragments of the *Ngn3* promoter were amplified from the E2f1-immunoprecipitated or the input DNA (Fig. 5D) and relative occupancy of E2f1 on the *Ngn3* promoter in *Cdk4*$^{+/+}$ and *Cdk4*^{R24C} embryonic pancreas was determined (Fig. 5E). The results showed a stronger binding of E2f1 to the *Ngn3* promoter in *Cdk4*^{R24C} embryonic pancreas, as compared with the *Cdk4*$^{+/+}$ embryonic pancreas (Fig. 5D,E). These results support the notion of E2f1-mediated activation of *Ngn3* expression.

Together, these results suggest that the Cdk4-E2f1 axis promotes *Ngn3* transcription and the subsequent proliferation of Ngn3^{+} cells. In addition, we observed that cyclin D2 (*Ccnd2*) levels are moderately increased in the *Cdk4*$^{-/-}$ pancreas at E12.5, whereas no changes in *Ccnd2* was seen at E14.5 (see Fig. S4 in the supplementary material). Further, the *p27* (*Cdkn1b* – Mouse Genome Informatics) Cki transcript levels were reduced at E12.5 in the *Cdk4*^{R24C} pancreas and tended to be lower (but not significantly so) at E14.5 in the *Cdk4*^{R24C} pancreas (see Fig. S4 in the supplementary material). It is possible that reduction of the Cdk inhibitors (*p57* and *p27*; see Fig. S4 in the supplementary material) might contribute to the elevated Cdk4 effect on the expansion of Ngn3^{+} cells.

DISCUSSION

Early pancreas development is an intricate cascade of cellular events that program the dorsal and ventral pancreatic buds into the highly organized pancreas organ (Jorgensen et al., 2007; Puri and Hebrok, 2010). The proliferation of pancreatic progenitors and their differentiation into diverse pancreatic cell types is suggestive of the involvement of cell cycle proteins during pancreatic development. Cdk4 is expressed broadly within the epithelium and mesenchyme of the embryonic pancreas and in the adult pancreas, whereas other Cdks are either rare or undetectable (Cdk6) or are expressed

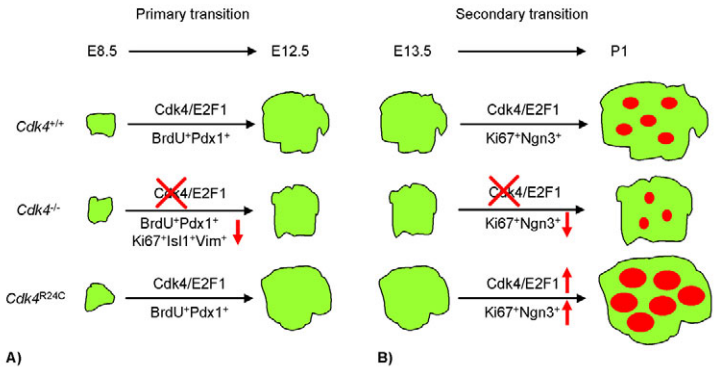

Fig. 6. Model of Cdk4-dependent regulation of embryonic pancreas development. Pancreatic buds originate by ~E8.5 in rodents and by E12.5 lineage commitment occurs, which is referred to as the 'primary transition' when the synergistic action of several transcription factors gives rise to committed precursors that differentiate into the exocrine, duct and endocrine lineage cells. The 'secondary transition' takes place after E13.5, when a program of Ngn3-regulated endocrine differentiation yields β cells. (**A**) Our results suggest that Cdk4 participates in determining the size of the embryonic pancreas (green) prior to the secondary transition by regulating the proliferation of Pdx1$^+$ progenitors and limiting the numbers and proliferation potential of Is1$^+$ Vim$^+$ mesenchymal cells. (**B**) During secondary transition, loss of Cdk4 represses the proliferation of Ngn3$^+$ endocrine precursor cells (red ovals). By contrast, activated Cdk4^{R24C} kinase promotes the proliferation of Ngn3$^+$ endocrine precursor cells via E2f1-mediated activation of the *Ngn3* promoter.

primarily in the adult pancreas (Cdk2). We, and others, previously described an important role for Cdk4 in the regulation of postnatal β cell mass, in which we observed that loss of Cdk4 results in a severely reduced β cell mass, whereas activation of the Cdk4 pathway resulted in β cell hyperplasia (Mettus and Rane, 2003; Rane et al., 2002; Rane et al., 1999; Rane and Reddy, 2000; Tsutsui et al., 1999). More recently, we have shown that Cdk4 enhances adult β cell regeneration in response to partial pancreatectomy by stimulating the replication of pre-existing β cells within islets and by activating progenitors within the pancreatic ductal epithelium (Lee et al., 2010). In the prior report, we stated that the morphology of the P1-P2 *Cdk4*$^{-/-}$ pancreas appeared to be similar to that of the *Cdk4*$^{+/+}$ pancreas (Rane et al., 1999). These results were based on examination of Hematoxylin and Eosin-stained pancreas and did not involve insulin immunohistochemistry to detect islet mass or quantify β cell numbers. Further, we observed similar blood glucose levels in P1-P2 *Cdk4*$^{-/-}$ and *Cdk4*$^{+/+}$ mice, which together with the pancreas morphology data led us to conclude that Cdk4 functions primarily as a regulator of postnatal β cells.

The studies reported in this manuscript provide new information on the role of Cdk4 in embryonic pancreas development (Fig. 6). Interestingly, *Cdk4*$^{-/-}$ pancreas at E12.5 has a reduced sectional area due to a limitation in the number of proliferating Pdx1$^+$ pancreatic progenitors. In addition, we find that Cdk4 plays an important role in optimal pancreatic mesenchymal development, wherein Cdk4 loss reduces the number and proliferation of mesenchymal cells. Thus, it is possible that the deficit in pancreatic progenitor proliferation and the reduced pancreatic size of the *Cdk4*$^{-/-}$ embryonic pancreas could be, at least in part, attributable to a defective pancreatic mesenchyme compartment. By contrast, the *Cdk4*^{R24C} mutation does not, per se, promote the proliferation of Pdx1$^+$ pancreatic progenitors at E12.5 and we do not observe an increased sectional area when compared with *Cdk4*$^{+/+}$ E12.5 pancreas. However, at E14.5 in the *Cdk4*^{R24C} pancreas, we observe increased transcript levels of *Fgf10* and *Hes1*, which are known to promote the proliferation of pancreatic progenitors (Bhushan et al., 2001; Georgia et al., 2006). The Notch signaling mediator Hes1 represses *p57* (Georgia et al., 2006) and *Ngn3* (Lee et al., 2001) transcription and promotes pancreatic progenitor proliferation while suppressing endocrine differentiation. Loss of p57 results in an increase in the proliferation of Pdx1$^+$ pancreatic progenitors, suggesting that p57 limits the division capacity of these cells (Georgia et al., 2006). We observe a reduced Pdx1$^+$ pancreatic progenitor pool in *Cdk4*-deficient mice, which suggests that Cdk4 is important for the proliferation of Pdx1$^+$ cells. Thus, it appears that p57 and Cdk4 serve as a 'brake' and 'accelerator', respectively, with regard to the proliferation competence of Pdx1$^+$ pancreatic progenitors.

There are several points that distinguish the study by Georgia et al. (Georgia et al., 2006) from our work as reported here. The expression of p57 appears to diminish at E12.5, when only a few pancreatic epithelial cells are detected, and p57 expression is absent during secondary transition (Georgia et al., 2006). Thus, p57 expression in pancreatic progenitors is limited to a short period of pancreas development and primarily serves to regulate the pancreatic progenitors. By contrast, Cdk4 expression appears strong prior to E12.5, and persists at E14.5, P1 and beyond, when endocrine differentiation occurs. In the majority of cell types Cdk4 does not serve as a substrate for p57 kinase inhibitory action, although we cannot exclude the possibility that p57 represses Cdk4 in pancreatic progenitors. It is possible that the p57 reduction at E12.5 might allow an increase in Cdk4 activity in the embryonic pancreas. In support of this, we observe that *p57* transcript levels are reduced in the E14.5 *Cdk4*^{R24C} pancreas. It is plausible that the *Cdk4*^{R24C} mutation supports the maintenance of Pdx1$^+$ pancreatic progenitors during later stages of pancreas development, and we indeed observe increased numbers of Pdx1$^+$ BrdU$^+$ cells in the E14.5 *Cdk4*^{R24C} pancreas. Further, we have shown that, in response to partial pancreatectomy, the adult *Cdk4*^{R24C} pancreas is able to activate presumptive Pdx1$^+$ pancreatic progenitors in the ductal epithelium as part of the regenerative response (Lee et al., 2010).

Cdk4 loss reduces, whereas *Cdk4*^{R24C} expression enhances, the numbers and proliferation of Ngn3$^+$ endocrine precursors. It is also likely that the limitation in the numbers and proliferation of Pdx1$^+$ pancreatic progenitors contributes to the deficit of the Ngn3$^+$ endocrine precursor pool in the *Cdk4*$^{-/-}$ pancreas. These results reveal a crucial role played by Cdk4 during the endocrine cell differentiation program by targeting *Ngn3*. The observation that

Cdk4 activation results in such a substantial increase in the proliferation of Ngn3$^+$ cells, which are generally considered to harbor a low mitotic potential, underscores the importance of Cdk4 in targeting endocrine precursors. *Ngn3* is required for the development of the four endocrine cell lineages of the pancreas, and mice with a disrupted *Ngn3* locus fail to develop endocrine cells (Gradwohl et al., 2000). Considering this notion, it is intriguing that, although the Cdk4-E2f1 axis targets *Ngn3*, the resultant effect is specifically restricted to β cells. It is plausible that Cdk4-E2f1-dependent activation of *Ngn3* induces the proliferation and expansion of endocrine precursors that ultimately become β cells. We observe an increased number of Cdk4$^+$ Ngn3$^+$ and E2f1$^+$ Ngn3$^+$ cells in the E14.5 *Cdk4*^{R24C} pancreas (see Fig. S5 in the supplementary material). Further, in addition to double-positive cells, we observe Cdk4$^+$ cells adjacent to Ngn3$^+$ cells (see Fig. S6 in the supplementary material), which supports the possibility that Cdk4 performs both cell-autonomous and non-cell-autonomous roles in endocrine differentiation. However, owing to technical limitations of the antibodies, we were unable to perform triple staining for Cdk4, E2f1 and Ngn3 and were unsuccessful in determining the numbers of proliferating Ngn3$^+$ cells that express Cdk4 and/or E2f1.

Interestingly, transgenic mice that overexpress *Ngn3* under the *Pdx1* promoter exhibit increased α cells at E12.5, although those cells do not express the proliferation markers Pcna and Ki67 (Schwitzgebel et al., 2000). We find Ngn3$^+$ Ki67$^+$ cells in our mouse models at E14.5, which further led us to speculate that proliferation-competent Ngn3$^+$ cells might be precursors of β cells, instead of other islet cell types. Thus, we suggest that all Ngn3$^+$ endocrine precursors might not be equivalent in terms of their final commitment and that differences in their proliferation potential might determine their eventual differentiation to specific endocrine cell types, such as α or β cells. Indeed, Schwitzgebel et al. have proposed that both α cells and β cells independently derive from Ngn3$^+$ endocrine precursors (Schwitzgebel et al., 2000). Further, Sander et al. demonstrated that glucagon-expressing α cells do not express Nkx6.1, which acts either downstream of, or in parallel to, Ngn3 during early pancreas development, suggesting that Nxk6.1$^+$ cells are already determined to a β cell fate (Sander et al., 2000). Our data show that the *Cdk4*^{R24C} embryonic pancreas has an increased number of Nkx6.1$^+$ cells, whereas these cells are decreased in the *Cdk4*$^{-/-}$ embryonic pancreas, which further agrees with the concept that Cdk4 may be dispensable for the expansion of non-β cells within the islets. Taken together, we suggest that a Cdk4-E2f1-dependent increase in the number and proliferation of Ngn3$^+$ endocrine precursors contributes, at least in part, to expansion of the β cell compartment. Like *Cdk4*$^{-/-}$ mice, mice deficient in Ccnd2 have defects in β cell proliferation (Georgia and Bhushan, 2004). However, Ccnd2 appears to be dispensable during embryonic development and its expression appears to be restricted to the epithelium and is not observed in the mesenchyme, nor in differentiated endocrine cells (Georgia and Bhushan, 2004). By contrast, Cdk4 is expressed in the pancreatic epithelium and mesenchyme in addition to colocalizing with differentiated endocrine cells. However, the loss of Ccnd2 results in deficits in the postnatal β cell compartment (Georgia and Bhushan, 2004), similar to that reported previously for Cdk4-deficient mice (Rane et al., 2002; Rane et al., 1999), suggesting that Ccnd2 and Cdk4 might collaborate in the adult β cell phenotype.

We suggest that Cdk4 utilizes its downstream signaling transcription factor E2f1 to promote the proliferation of Ngn3$^+$ endocrine precursors. In addition to pancreatic endocrine precursors, Ngn3 is expressed in enteroendocrine progenitor cells of the gut and in neuronal progenitor cells in the neural tube, although it is not known whether E2f1 regulates *Ngn3* transcription in these cells. We believe that understanding the regulators of the expansion of pancreatic progenitors or endocrine precursors, specifically those destined for β cell differentiation, is crucial. Further, the upstream regulators of *Ngn3* are relatively obscure. It is known that *Ngn3* transcription is activated by Sox9 and repressed by Hes1 transcription factors (Lee et al., 2001; Lynn et al., 2007). We show that E2f1 activates the *Ngn3* promoter, resulting in elevated Ngn3 expression and enhanced Ngn3$^+$ endocrine precursor cell numbers in *Cdk4*^{R24C} embryonic pancreas. The finding that *Ngn3* transcripts are under the control of the Cdk4-E2f1 pathway is consistent with in vivo findings of depleted β cell mass in mice that lack Cdk4 (Mettus and Rane, 2003; Rane et al., 1999; Rane and Reddy, 2000) and E2f1 (Fajas et al., 2004). *E2f1*-deleted mice have a primarily postnatal pancreatic growth phenotype (Fajas et al., 2004). E2f1 expression is observed in E16.5 and adult but not in E12.5 pancreas (Fajas et al., 2004), which is suggestive of a post-secondary transition role for E2f1. However, these studies employed in situ hybridization techniques and re-examination using E2f1 antibody-based assays along with colocalization with pancreatic markers is needed to unequivocally ascertain the importance of E2f1 during pancreatic organogenesis. It has been reported that E2f1 plays a role in β cell function by regulating the expression of Kir6.2 (Kcnj11 – Mouse Genome Informatics), a key component of the ATP-sensitive K$^+$ channel involved in the regulation of glucose-induced insulin secretion (Annicotte et al., 2009).

Together, these findings demonstrate that E2f1 promotes β cell mass expansion via activation of *Ngn3* expression and accentuates β cell function via activation of *Kir6.2*. In conclusion, our results provide a mechanistic explanation for the unique role played by Cdk4 in early pancreas development and, specifically, in the proliferation competence of pancreatic progenitors and endocrine precursors.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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