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# An RMCE-derived cyan fluorescent protein reporter allele for Pdx1

**Leah A. Potter**[a,b], **Eunyoung Choi**[a,b], **Susan B. Hipkens**[a], **Christopher V.E. Wright**[a,c], and **Mark A. Magnuson**[a,b,*]

[a]Vanderbilt Center for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

[b]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232

[c]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

## Summary

Fluorescent protein (FP) reporter alleles are useful both for identifying and purifying specific cell populations in the mouse. Here, we report the generation of mouse embryonic stem cells that contain a *Pdx1* loxed cassette acceptor ($Pdx1^{LCA}$) allele and the use of recombinase-mediated cassette exchange (RMCE) to derive mice that contain a $Pdx1^{CFP}$ (Cerulean) reporter allele. Mice with this allele exhibited cyan fluorescence within the previously well-characterized *Pdx1* expression domain in posterior foregut endoderm. Immunolabeling showed that endogenous Pdx1 was co-expressed with CFP at all time points examined. Furthermore, fluorescence-activated cell sorting (FACS) was used to isolate CFP-positive cells from E11.5 and E18.5 embryonic tissues using both 405 and 445 nm lasers, although the latter resulted in a nearly 50-fold increase in emission intensity. The $Pdx1^{CFP}$ allele will enable the isolation of specific foregut endoderm and pancreatic cell populations, both alone and in combination with other FP reporter alleles.

### Keywords for indexing

pancreas; posterior foregut endoderm

## Introduction

The pancreas consists of exocrine cells, which secrete digestive enzymes into the small intestine via pancreatic ducts, and Islets of Langerhans, which contain five different types of endocrine cells that regulate blood glucose homeostasis. In mice, the pancreas arises from both dorsal and ventral evaginations from the posterior foregut endoderm beginning around embryonic day (E) 9.0 (Guz *et al.*, 1995; Jonsson *et al.*, 1994; Offield *et al.*, 1996). As development proceeds, the pancreatic epithelial buds expand into the surrounding mesenchyme, partially fuse following rotation of the gut, and undergo complex expansion during which a plexus of cells is transformed into an organized series of ducts and both acinar and islet endocrine cells are formed.

*Corresponding author: 9465 MRB-IV, 2213 Garland Avenue, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, Tel: 615.322.7006, Fax: 615.332.6645, mark.magnuson@vanderbilt.edu.

Between E9.5 and approximately E12.5 the pancreatic epithelium consists of a heterogeneous population of multipotent progenitor cells (MPCs) that give rise to the endocrine, exocrine and ductal cell types found in the adult organ (Gu *et al.*, 2002; Kawaguchi *et al.*, 2002; Zhou *et al.*, 2007). The allocation of undifferentiated MPCs into specific lineages is a complex process orchestrated by numerous signaling pathways (Gittes, 2009; Jensen, 2004). *Pancreatic and duodenal homeobox 1* (*Pdx1*) plays a central role in this process and is essential for both the expansion and differentiation of pancreatic MPCs (Chiang and Melton, 2003; Jonsson *et al.*, 1994; Krapp *et al.*, 1998; Offield *et al.*, 1996). *Pdx1* expression begins around E8.5 in cells of the posterior foregut epithelium, some of which go on to form the pancreas. At later stages of development, *Pdx1* is highly expressed in β cells where it is essential for normal insulin secretion (Melloul *et al.*, 1993).

Numerous lines of mice expressing green fluorescent protein (GFP) (Chalfie *et al.*, 1994; Heim *et al.*, 1995; Shimomura *et al.*, 1962) under control of a variety of gene loci, including *Pdx1* (Micallef *et al.*, 2005), have been reported. However, the utility of some of these reporter alleles, including that of the $Pdx1^{GFP}$ allele, is limited by a single spectral profile which prevents their combinatorial use. Indeed, the isolation of many distinct cell populations by fluorescence-activated cell sorting (FACS) requires the simultaneous use of two or more FPs with spectrally distinct excitation and emission profiles (Heikal *et al.*, 2000; Nagai *et al.*, 2002; Rizzo *et al.*, 2004; Shaner *et al.*, 2004).

Recently, we described a strategy for generating loxed cassette acceptor (LCA) alleles (Chen *et al.*, 2011). Mouse embryonic stem (mES) cells containing an LCA allele allow for the use of recombinase-mediated cassette exchange (RMCE) to generate a wide variety of allelic variants. Here, we describe 1) the generation of a mES cell line containing a $Pdx1^{LCA}$ allele, and 2) the use of these cells to derive mice expressing Cerulean, an improved cyan fluorescent protein (CFP), under control of the *Pdx1* locus. As expected, the expression of $Pdx1^{CFP}$ mirrors that of *Pdx1* during both early and late embryogenesis. We anticipate that mES cells containing the $Pdx1^{LCA}$ allele will be useful for deriving additional mutant *Pdx1* alleles and that the $Pdx1^{CFP}$ mice will be useful alone or in combination with other FP-tagged alleles for isolating specific cell populations during pancreas development.

## Results and Discussion

### Generation of a $Pdx1^{LCA}$ allele

BAC recombineering and gene targeting were used to generate mES cells with a $Pdx1^{LCA}$ allele, as shown in Figure 1a. In this allele, an 8.6 kb region of the *Pdx1* locus, containing four previously characterized conserved regulatory regions termed Areas I – IV (Gittes, 2009; Pan and Wright, 2011), as well as exon 1, were replaced with a dual positive-negative selection cassette flanked by lox71 and lox2272 sites (Araki *et al.*, 2002; Chen *et al.*, 2011). Southern blot analysis using probes on both the 5′ and 3′ ends of the mutated region confirmed the desired homologous recombination events (Figure 1b).

### Derivation of a $Pdx1^{CFP}$ allele by RMCE

To validate the functionality of the $Pdx1^{LCA}$ allele, we next made an exchange vector that replaced coding sequences in exon one of the *Pdx1* gene with a nuclear-localized CFP (Cerulean) (Rizzo *et al.*, 2004), thereby generating a Pdx1-null allele. A portion of the rabbit *β-globin* gene, containing both intronic and polyadenylation sequences, was placed downstream of the CFP coding sequences (Chen *et al.*, 2011; Westwood *et al.*, 1993). In addition, the exchange vector contains a *Pgk-hygromycin resistance* ($Hygro^R$) cassette, flanked by tandem flippase recognition target (FRT) sites. RMCE into the $Pdx1^{LCA}$ was achieved through the use of a staggered positive-negative selection strategy (Long *et al.*,

2004) after co-electroporation of the exchange vector and a Cre-expression plasmid. Chimeric mice were generated by injection of an exchanged clone (Figure 1c) into E3.5 mouse blastocysts. After germline transmission, mice containing the *Pdx1*$^{CFP+HygroR}$ allele were bred with FLPe-expressing transgenic mice to remove the FRT-flanked *Hygro*$^{R}$ cassette, thereby generating the *Pdx1*$^{CFP}$ allele.

## Expression pattern of *Pdx1*$^{CFP}$ by whole mount fluorescence microscopy

To demonstrate the expression of CFP, we first utilized whole mount fluorescence microscopy (Figure 2). In *Pdx1*$^{CFP/+}$ embryos, CFP expression was easily observable beginning at E9.5 in both the dorsal and ventral endoderm, consistent with the pattern previously determined from a *Pdx1*$^{lacZ}$ insertion allele (Offield *et al.*, 1996). Between E10.5 to E11.5, CFP expression in both the dorsal and ventral pancreatic buds was brighter than that of the caudal stomach and duodenum, normal domains of Pdx1 expression. During this developmental stage, the expression domain of *Pdx1* contains a subpopulation of pancreatic MPCs (Gu *et al.*, 2002; Pan and Wright, 2011). As development proceeds, CFP expression persisted at a high level in the pancreatic epithelium and at a lower level in the caudal stomach and duodenum.

## Expression pattern of *Pdx1*$^{CFP}$ by immunohistochemical analysis

To determine whether CFP and Pdx1 were co-expressed, we performed immunohistochemical analysis using tissues from multiple developmental stages (Figure 3a and data not shown). Both Pdx1 and CFP were detected in the dorsal and ventral endodermal evaginations of the posterior foregut at E9.5. From E10.5 to E11.5, Pdx1 and CFP were detected throughout the pancreatic epithelium and expression was also observed in the stomach and duodenum. From E12.5 – E14.5, CFP immunofluorescence co-localized with Pdx1 throughout the branching epithelium, and by E15.5, high levels of Pdx1 and CFP were evident in periodic clusters throughout the epithelium with lower levels throughout the epithelium. At E18.5, high levels of Pdx1$^{CFP}$ expression were restricted primarily to the developing β cells as evident by the co-localization with insulin-expressing cells (Figure 3b). Additionally, lower levels of CFP expression were co-localized with amylase, indicative of Pdx1 expression in the acinar cells, again in accordance with previous reports (Figure 3b) (Guz *et al.*, 1995; Wu *et al.*, 1997). At each developmental stage, expression the *Pdx1*$^{CFP}$ allele paralleled that of endogenous Pdx1 expression, consistent with the *Pdx1*$^{CFP}$ allele faithfully recapitulating expression of *Pdx1* (Guz *et al.*, 1995; Leonard *et al.*, 1993; Miller *et al.*, 1994; Offield *et al.*, 1996; Ohlsson *et al.*, 1993). While perdurance of certain FP reporter alleles has been reported, (Burlison *et al.*, 2008; Viotti *et al.*, 2011) this was not observed at any of the time points examined in this study which may simply reflect the maintained expression of Pdx1 in numerous cell types of the pancreatic epithelium.

## FACS analysis of *Pdx1*$^{CFP}$ cells

Given that the optimal excitation wavelength for Cerulean is 433 nm (Rizzo *et al.*, 2004), we determined the effect of using either a 405 nm or 445 nm laser, both of which are commercially available, for sorting *Pdx1*$^{CFP}$-expressing cells. As shown in Figure 4, the 445 nm laser resulted in a broader range of emission intensity using embryonic tissues at E11.5 and E18.5. Cyan fluorescence observed using the 405 nm laser was minimally distinguishable above cellular autofluorescence in wild-type embryos. However, CFP-expressing cells excited by the 445 nm laser displayed approximately 50-fold higher fluorescence intensity. At E11.5, both high- and low-intensity CFP fluorescence was observed by FACS (Figure 4a), which is similar to the high- and low-expression patterns evident by immunolabeling in the pancreatic epithelium and the stomach/duodenal epithelium, respectively. Cells exhibiting higher levels of CFP fluorescence primarily

represented cells of the pancreatic epithelium as indicated by *Ptf1a* expression (Figure 4c). In addition, high- and low-intensity CFP fluorescence was observed at E18.5 (Figure 4b). An analysis of *Insulin* expression revealed that cells displaying high levels of CFP fluorescence at E18.5 were predominantly pancreatic β cells (Figure 4d).

### Conclusions

The ability to isolate *Pdx1*-expressing cells by using mice containing the $Pdx1^{CFP}$ reporter allele at different times during pancreas development will facilitate identification and functional characterization of both gene regulatory networks and cell signaling mechanisms critical for the differentiation, growth, and maturation of the pancreas. The $Pdx1^{CFP}$ allele can be used in combination with green and yellow fluorescent protein reporters, such as the previously reported $Ngn3^{GFP}$ and $Ptf1a^{YFP}$ alleles, although mice that express red FPs, such as mCherry or mApple, may be preferable due to better spectral separation of cyan and red fluorescence. Finally, mouse ES cells containing the $Pdx1^{CFP+HygroR}$ allele may also be useful for detecting *Pdx1* gene expression in cultured cells that have undergone directed differentiation.

## Materials and Methods

### Gene targeting vector

The gene-targeting vector used to generate the $Pdx1^{LCA}$ allele was made by gap repair in *E. coli* beginning with clone 228-B22 from the RP22 BAC library and contains long and short homology arms of 8.1 kb and 3.6 kb, respectively. The targeting replaced an 8.6 kb region of the Pdx1 gene, spanning from −6586 to +2024 bp, with a mouse *phosphoglycerol kinase* promoter (*Pgk*) driving expression of a *puromycin resistance-Δthymidine kinase* fusion gene (*puΔTK*) and a bacterial EM7 promoter driving expression of a *kanamycin resistance* gene (EM7-$Kan^R$). Both selectable markers were flanked with tandemly-oriented lox71 and lox2272 sites. An MC1-driven *diphtheria toxin A* gene (*DTA*) was placed outside the long homology arm to select against nonrecombinant clones.

### RMCE exchange vector

To facilitate assembly of the $Pdx1^{CFP+HygroR}$ exchange vector, a basal exchange vector, termed $Pdx1^{Ex1}$, containing the 8.6 kb of *Pdx1* gene sequence absent in the $Pdx1^{LCA}$ allele flanked by lox61 and lox2272 sites, was generated. Coding sequences for CFP (Cerulean) tagged with 3 copies of an SV40 nuclear localization sequence (NLS) and followed by rabbit *β-globin* intronic and 3′ UTR sequences were amplified by PCR then cloned into the basal exchange vector. A *Pgk*-driven *hygromycin resistance* ($Hygro^R$) sequence, flanked by tandem flippase recognition target (FRT) sites, was cloned into the 5′ end of the exchange vector for positive selection following RMCE.

### Gene targeting in mouse ES cells

Gene targeting was performed following standard protocols. In brief, 200 μg of the targeting vector was linearized with NotI and electroporated into $35 \times 10^6$ TL-1 mouse ES cells. Following puromycin selection, homologous recombination was verified by Southern blot analysis. Clones that had undergone the desired homologous recombination events were identified by a band of 9.0 kb after digestion with XmaI and hybridization with a 5′ probe and a band of 4.7 kb after digestion with SphI and hybridization with a 3′ probe.

### RMCE

RMCE was performed using a staggered, positive-negative selection strategy as previously described (Long *et al.*, 2004). In brief, mES cells (clone 2F11) containing the $Pdx1^{LCA}$, were

electroporated with equal amounts of $Pdx1^{CFP+HygroR}$ and pBS185, a Cre-expressing plasmid. Clones surviving selection with both hygromycin and gancyclovir were analyzed for cassette exchange by PCR using primers that spanned either the lox66/71 site on the 5′ end or the lox2272 site on the 3′ end. On the 5′ end, the combination of a 5′-TGAGATTGTATATTGCGGTGCA and 5′-ACGAGACTAGTGAGACGTGCTACT primer results in a band size of 660 bp after RMCE and on the 3′ end, use of 5′-TGAGCAATTCCAAGCAGCTGGA and 5′-ACCTTGCAGTCCTTCTGAAGT results in a 481 bp band for the exchanged allele and a 426 bp band in the wild type allele.

## Mouse strains

Germline transmission was achieved following the microinjection of clone 2F11:1B1 mES cells into E3.5 C57BL/6 blastocysts. The FRT-flanked $Hygro^{R}$ sequence was removed by breeding with Tg(ACTFLPe)9205Dym mice. The resulting $Pdx1^{CFP}$ allele was maintained on an outbred background. Embryos were isolated from wild-type CD-1 females crossed with $Pdx1^{CFP/+}$ mice where the presence of vaginal plug at noon was considered as E0.5. Experimental protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee.

## Immunolabeling and imaging

Immunolabeling was performed as previously reported (Burlison *et al.*, 2008). Primary antibodies were diluted in 1% BSA in PBS as follows: guinea pig anti-Pdx1 (C.V.E. Wright), 1:2500; chicken anti-GFP (Invitrogen), 1:1000; goat anti-amylase (Santa Cruz Biotechnology), 1:1000; guinea pig anti-insulin (Linco), 1:1000. Secondary antibodies were diluted in 1% BSA in PBS as follows: donkey anti-chicken DyLight 488 (Jackson ImmunoResearch), 1:500; donkey anti-guinea pig Cy3 (Jackson ImmunoResearch), 1:1000; donkey anti-goat Alexa Fluor 647 (Invitrogen), 1:1000. Sections were counterstained with DAPI and cover slips mounted using Aqua/Poly Mount (Polysciences, Inc.). Images were acquired using an Axioplan2 microscope (Zeiss) with a QImaging RETIGA EXi camera. Whole mount imaging of $Pdx1^{CFP}$ embryos was performed using a Leica MZ 16 FA stereoscope with a QImaging RETIGA 4000R camera.

## FACS analysis

$Pdx1^{CFP/+}$ embryos were identified by direct fluorescence and dissected tissues were dissociated using Accumax (Sigma). Following filtration through a 35 μm cell strainer (BD Biosciences) and centrifugation, cells were resuspended in FACS medium [L15 medium (Invitrogen) containing 1 mg/ml BSA, 10 mM HEPES pH 7.4, 1% penicillin/streptomycin, and 37.5 ng/ml DNase I] or FACS medium with 7-Amino-Actinomycin D (7-AAD; Invitrogen). Cells were analyzed and isolated using an Aria III (BD Biosciences). CFP was excited using either a 405 nm or 445 nm laser and emission detected with a 470 nm long pass and 510/80 bandpass filter.

## RNA Isolation and semi-quantitative RT-PCR

Total RNA was isolated using TRIzol LS (Invitrogen), DNase-treated (Ambion), and column-purified (Zymo Research). Total RNA was reverse transcribed (High Capacity cDNA Archive kit; ABI), and PCR was performed using 1 ng of cDNA template. *Ptf1a* was detected using the following primers: 5′ – CGAATTGCCACGGATCACT and 5′ – CCCGGAAGGACGAATGG. *Insulin* was detected using the following primers: 5′ – CCACCCAGGCTTTTGTCAAA and 5′ – CCCAGCTCCAGTTGTTCCAC.

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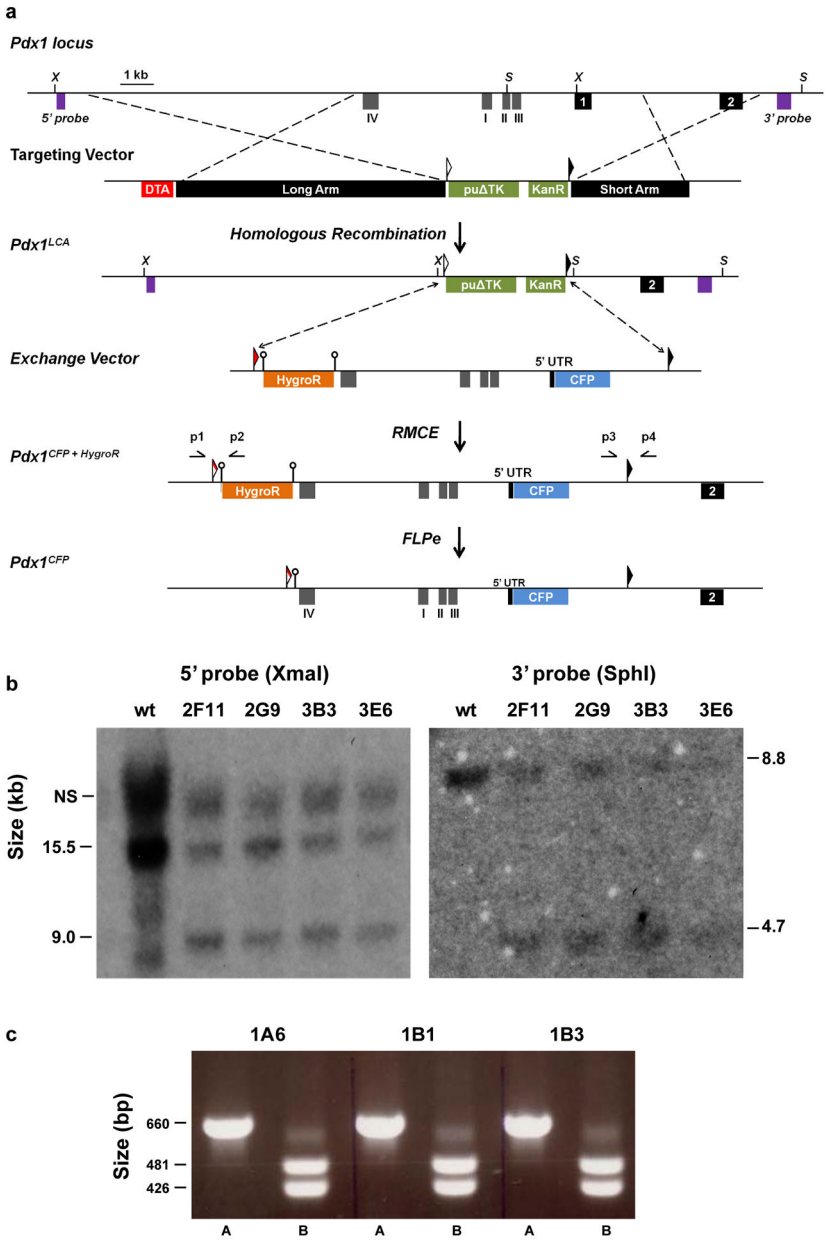


**Figure 1. Overall scheme for generating the Pdx1$^{CFP}$ allele**

(a) Diagram of the wild type *Pdx1* locus, gene targeting vector, *Pdx1*$^{LCA}$ allele, exchange vector, *Pdx1*$^{CFP+HygroR}$ allele, and *Pdx1*$^{CFP}$allele. Homologous recombination resulted in replacement of an 8.6 kb region of the *Pdx1* locus containing exon 1 and Areas I – IV with both a *Pgk*-driven *puromycin resistance-Δthymidine kinase* fusion gene (*puΔTK*) and an EM7-driven *kanamycin resistance (Kan*$^{R}$*)* gene flanked by lox71 (open triangle) and lox2272 (black triangle) sites. The exchange vector contained lox66 (red triangle) and lox2272 sites flanking a nuclear-localized CFP sequence followed by a rabbit *β-globin* polyadenylation sequence. A *Pgk*-driven *hygromycin resistance* (*Hygro*$^{R}$) cassette flanked by FRT sites (open circles) was used as a positive selectable marker during RMCE. Mice containing the *Pdx1*$^{CFP+HygroR}$ allele were bred with FLPe-expressing transgenic mice to remove the FRT-flanked *Hygro*$^{R}$ cassette. Restriction sites: XmaI (X) and SphI (S). Primer locations: p1, p2, p3 and p4. (b) Southern blot analysis of four puromycin resistant ES cells

using probes indicated in panel (a). The *Pdx1*$^{LCA}$ allele was detected by the presence of a 9.0 kb and 4.6 kb band on the 5′ and 3′ ends, respectively. NS: nonspecific band. (c) PCR analysis on both the 5′ (lane A, p1 and p2) and 3′ (lane B, p3 and p4) ends of three *Pdx1*$^{CFP+HygroR}$ exchanged clones. Properly exchanged clones were identified by 660 and 481 bp bands on the 5′ and 3′ ends, respectively.

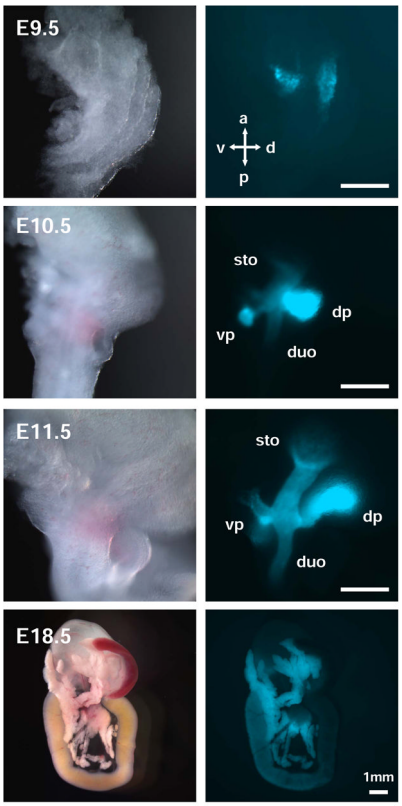


**Figure 2. Whole mount fluorescent microscopy of Pdx1$^{CFP/+}$ embryos**

CFP fluorescence was observed in dissected *Pdx1$^{CFP/+}$* embryos as early as E9.5 in the dorsal and ventral endoderm, consistent with previous reports detailing the expression pattern of Pdx1. From E10.5 to E11.5, CFP expression was detected in the dorsal and ventral pancreatic buds at a higher intensity as compared to the expression visualized in the caudal stomach and duodenum. Throughout development, CFP expression persisted at higher levels in the pancreatic epithelium than in the caudal stomach and duodenum. Scale bar = 250 μm unless otherwise noted. Dorsal pancreas (dp), ventral pancreas (vp), stomach (sto), duodenum (duo), anterior (a), posterior (p), dorsal (d), ventral (v).

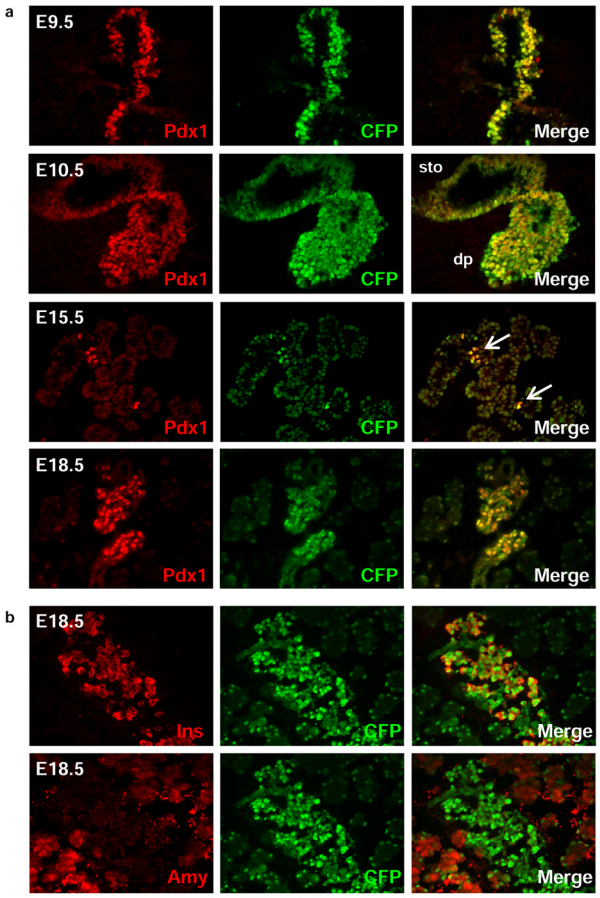


**Figure 3. Immunofluorescent analysis of Pdx1$^{CFP/+}$ embryos**

(a) Co-expression of Pdx1 and CFP at E9.5 throughout the dorsal and ventral endoderm. At E10.5, Pdx1 and CFP expression are co-localized in the pancreatic epithelium with expression also observed in other posterior foregut derivatives, such as the stomach. By E15.5, high levels of Pdx1 and CFP were evident in scattered clusters (arrows) throughout the epithelium with lower levels throughout the epithelium. At E18.5, Pdx1 and CFP are co-localized in cells displaying both high and low levels of expression. Stomach (sto), dorsal pancreas (dp). DAPI: nuclear counterstain. (b) At E18.5, Pdx1$^{CFP}$ expression is restricted primarily to developing β cells, as indicated by co-expression with insulin, with lower levels of CFP expression observed in the acinar cells, as indicated by co-expression with amylase.

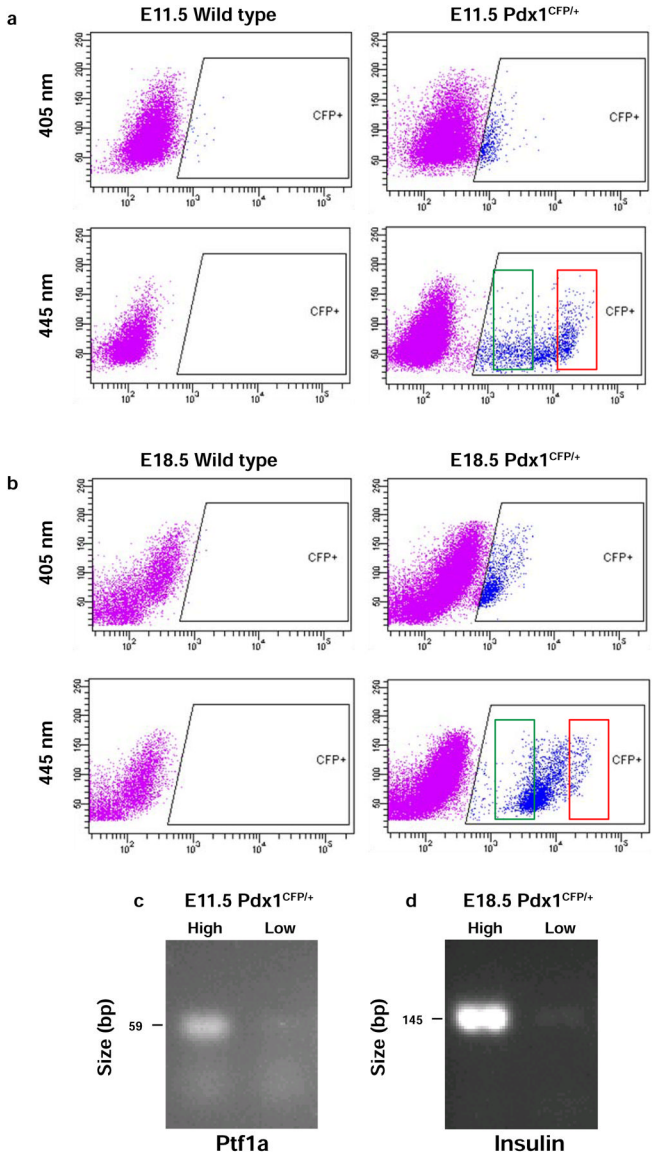


**Figure 4. Analysis of Pdx1$^{CFP/+}$ embryos by fluorescence-activated cell sorting (FACS)**

The emission intensity of CFP following excitation with either 405 nm or 445 nm was compared. E11.5 embryos (a) and E18.5 embryos (b) were used to isolate CFP-positive cells. The fluorescence intensity observed following excitation with 405 nm laser (top panels) is minimally distinguishable above cellular autofluorescence seen in the wild-type embryos (left panels); whereas a 445 nm laser (bottom panels) provided more optimal excitation of CFP. Both high- and low-expressing cells, indicated by red and green boxes, respectively, were isolated from both E11.5 embryos (a) and E18.5 embryos (b). (c) A higher level of *Ptf1a* gene expression was detected in cells showing high- versus low-intensity CFP fluorescence consistent with their origin in the pancreatic epithelium. (d) Similarly, a higher level of *Insulin* gene expression was observed in high- versus low-intensity CFP fluorescence cells at E18.5 consistent with them being pancreatic β cells.