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Generation of Mice with a Conditional Null Allele for Wntless

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Summary

The Wnt-signaling pathway is necessary in a variety of developmental processes and has been implicated in numerous pathologies. Wntless (Wls) binds to Wnt proteins and facilitates Wnt sorting and secretion. Conventional deletion of *Wls* results in early fetal lethality due to defects in body axis establishment. To gain insight into the function of *Wls* in later stages of development, we have generated a conditional null allele. Homozygous germline deletion of *Wls* confirmed prenatal lethality and failure of embryonic axis formation. Deletion of *Wls* using *Wnt1-cre* phenocopied Wnt1 null abnormalities in the midbrain and hindbrain. In addition, conditional deletion of *Wls* in pancreatic precursor cells resulted in pancreatic hypoplasia similar to that previously observed after conditional β-*catenin* deletion. This Wls conditional null allele will be valuable in detecting novel Wnt functions in development and disease.

Keywords

Wnt; Evi; Gpr177; Sprinter; Wnt transporter

INTRODUCTION

The vertebrate Wnt family consists of 19 secreted, cysteine-rich glycoproteins essential for embryogenesis and homeostasis. The ability of Wnts to reach their target cell is dependent on proteins responsible for processing and secretion of Wnt proteins. One of these *Wntless* (*Wls*) (also known as *Evenness Interrupted* or *Sprinter*) encodes a seven-pass transmembrane protein and was orginally described in *Drosophila* and *C. elegans. Wls* is evolutionarily conserved in vertebrates and invertebrates with a Blast search revealing only 1 homologue in *C. elegans*, mice, and humans (Banziger *et al.*, 2006; Bartscherer *et al.*, 2006; Goodman *et al.*, 2006). *Wls* mutant *Drosophila* larvae exhibit higher levels of Wg antigen than wild-type cells, suggesting that it is still expressed, but not secreted (Banziger *et al.*, 2006; Bartscherer *et al.*, 2006). Although it is possible that Wls might also be involved in lipid modifications, insights into Wls function have confirmed the role of Wls as a Wnt

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cargo receptor and thereby essential for Wnt secretion (Belenkaya *et al.*, 2008; Franch-Marro *et al.*, 2008; Pan *et al.*, 2008; Port *et al.*, 2008; Yang *et al.*, 2008).

Previous studies have often relied on β-*catenin* deletion to understand the impact of canonical Wnt signaling in different processes. However, due to the dual function of β-catenin in both canonical Wnt signaling and cell adhesion, it has sometimes been difficult to separate canonical pathway-dependent effects. Because Wls appears to control the release of all Wnts, the utilization of *Wls* conditional mutants would be a powerful tool allowing spatial and temporal control of Wnt secretion both during development and in disease models. Deletion of *Gpr177*, the mouse orthologue of *Wls*, results in embryonic lethality due to impairment of the developing body axis (Fu *et al.*, 2009). Therefore, we generated a *Gpr177*, or *Wls*, conditional null allele and affirmed its use as a means to study the loss of multiple Wnts by pheno-copying previously characterized Wnt-dependent development of the body axis, brain, and pancreas.

To test the role of the *Wls* gene in controlling Wnt signaling in mouse, we generated a conditional null allele using the Cre-LoxP and Flp-Frt systems. The targeting strategy is shown in Figure 1a. The *Frt* site-flanked neo cassette was placed in intron 1. One *LoxP* site was placed in front of the ATG start site in the 5′ untranslated region, and the other was placed after the *Frt* site-flanked neo cassette. Successful removal of the Neo cassette by crossing offspring that inherited the targeted allele with the flippase mouse was assessed by PCR using primers 2 and 4 (P2 and P4) (Fig. 1b).

The germline null allele was generated by crossing $Wls^{fl/fl}$ with the *EIIa-cre* transgenic line (Lakso *et al.*, 1996). Recombination of the *LoxP* sites using *EIIa-cre* results in the removal of exon1. PCR genotyping using primers 1 and 4 (P1 and P4) on E7.5 embryos generated from crosses of heterozygotes gave the expected amplification products (Fig. 1c). *Wls* floxed mice and *Wls* heterozygotes ($Wls^{+/fl}$; *EIIa-cre* and $Wls^{-/fl}$) were viable and had no obvious phenotype (data not shown). No *Wls* homozygous null pups or embryos were recovered after E8.5. Morphological analysis of E7.5 $Wls^{+/+}$ embryos confirmed that these embryos had undergone gastrulation (Fig. 2a). In addition, histological analysis demonstrated the presence of all three germ layers (Fig. 2c). At E8.5 $Wls^{+/+}$ embryos displayed well-developed headfolds (Fig. 2e). In contrast, *Wls* germline homozygotes appeared arrested at E6 as they did not progress past the egg cylinder stage and by morphological criteria lacked mesoderm (Fig. 2b,d). At E8.5, 9 of 10 $Wls^{-/-}$ embryos were in the process of being reabsorbed. $Wls^{-/-}$ embryos that did survive until this stage maintained the morphological appearance of an egg cylinder (Fig. 2f). Perhaps, the earliest described Wnt phenotype comes from a loss of Wnt3 (Liu *et al.*, 1999). Wnt3 null mice develop a normal egg cylinder, but fail to form a primitive streak or mesoderm. Our data confirms the recent finding of Fu *et al.* (2009) that germline *Wls* deletion results in the loss of primitive streak and mesoderm formation and is also very similar to Wnt3 deletion and β-catenin loss of function homozygotes (Fu *et al.*, 2009; Huelsken *et al.*, 2000; Liu *et al.*, 1999).

Mice deficient in Wnt1 display loss of a significant portion of the developing brain (Echelard *et al.*, 1994; McMahon and Bradley, 1990). A similar but more severe phenotype has been observed in mice that exhibit conditional deletion of β-catenin in neural crest cells (Brault *et al.*, 2001). To assess the consequences of Wls loss-of-function in the dorsal midline of the diencephalon, we conditionally deleted *Wls* using *Wnt1-cre*. Heterozygotes ($Wls^{+/fl}$; *Wnt1-cre* and $Wls^{-/fl}$) did not exhibit any obvious phenotype (data not shown). Mutant embryos ($Wls^{-/fl}$; *Wnt1-cre*) were recovered at Mendelian ratios, but mutant mice were not recovered postnatally. The mutant phenotype was obvious as early as E9.5 due to the presence of a shortened neural tube (Fig. 3j). Nonneuronal tissues appeared normal. At

E10.5 and E11.5, *Wls* wild-type littermates (*Wls*$^{+/fl}$) had a discernible forebrain (telencephalon and diencephalon), midbrain, and hind-brain (metencephalon and myelencephalon) (Fig. 3a,c). The isthmus was prominent. In contrast, mutant mice exhibited a shortened anteroposterior axis and no apparent isthmus, suggesting loss of midbrain and anterior hindbrain, that is similar to that observed in Wnt1 mutants (Fig. 3b). Additionally, a malformed forebrain was observed in whole-mount embryos as early as E11.5 (Fig. 3d). Histological sections at E10.5 verified deletion of much of the midbrain and of regions of metencephalon (Fig. 3f). Furthermore, E12.5 sections exhibited the obvious loss of midbrain and hindbrain structures including the cerebellum and choroid plexus and a severely truncated forebrain (Fig. 3h). These results parallel those observed in mice with *Wnt1-cre* mediated deletion of β-catenin (Brault *et al.*, 2001). In addition, although no fore-brain phenotypes have been described in either Wnt1 or Wnt3a mutants, combined deletion has been shown to result in a loss of forebrain structures (Ikeya *et al.*, 1997). These findings thus validate the use of the *Wls* conditional allele in studying Wnt-signaling pathways and argue that it is a means to overcome ligand redundancy.

To determine whether canonical Wnt function was in fact impaired in *Wls* mutant mice, we took advantage of the *TOPGAL* reporter line that expresses lacZ in response to activation of the canonical Wnt pathway (DasGupta and Fuchs, 1999). At E9.5, intense X-gal staining was present in regions of the midbrain and hindbrain (Fig. 3i). In mutant embryos, X-gal staining was absent in the head region with the exception of the otic vesicle (Fig. 3j). At E10.5, X-gal staining was more prominent in the dorsal forebrain (Fig. 3k), a region that has not been shown to express Wnt1, but does express other Wnt ligands (Parr *et al.* 1993). E10.5 mutants did not exhibit any central nervous system (CNS) X-gal staining (Fig. 3l). This suggests that the loss of Wls in the dorsal midline of the diencephalon, where *Wnt1-cre* is expressed, may have a secondary effect on Wnt ligand expression in the telencephalon.

A number of Wnts are expressed during pancreas development including Wnt2b, Wnt4, Wnt5a, and Wnt7b (Heller *et al.*, 2002). With the exception of the Wnt5a mutant, which exhibits defects in islet formation, none of the aforementioned Wnt gene mutants display a pancreas phenotype (Kim *et al.*, 2005). Although there is some debate on the effects of conditional *Pdx1-cre*-mediated deletion of β-*catenin* in pancretic precursors due to the *Pdx1-cre* line used, all studies showed degrees of reduction in pancreas size (Dessimoz *et al.*, 2005; Murtaugh *et al.*, 2005; Wells *et al.*, 2007). To determine whether Wls might be required for pancreas development, we conditionally deleted *Wls* in pancreatic precursor cells using a *Pdx1-cre* previously described (Wells *et al.*, 2007). Early *Pdx1-cre* lineage tracing using *Rosa26-Lacz* reporter mice showed robust expression throughout the developing pancreas at E10.5 (Spence *et al.* 2009). Heterozygotes (*Wls*$^{+/fl}$; *Pdx1-cre* and *Wls*$^{-/fl}$) did not display any obvious pancreas abnormalities (data not shown). However, mice with conditional deletion of *Wls* were recovered postnatally, but the mutant pancreas was extremely hypoplastic (data not shown). At E15.5, the dorsal and ventral pancreatic lobes had formed in the mutant, although, overall, the pancreas was severely hypoplastic relative to wild-type control littermates (Fig. 4a). Histological analysis of E15.5 embryos revealed developing acinar, endocrine, and ductlike tissue in wild-type and mutant embryos (Fig. 4b,c). All three tissues appeared to be reduced in the mutant pancreas (Fig. 4e). To investigate the impact of deletion of *Wls* on endocrine pancreas development, we stained the E15.5 pancreas for glucagon and insulin. Similar to the phenotype observed with the *Pdx1-cre* β-*catenin* deletion, endocrine cell specification appeared unaffected by loss of *Wls* (Fig. 4f,g) (Wells *et al.*, 2007). These findings further validate the use of the *Wls* allele to study the effects of Wnt-signaling pathways.

Taken together, these data demonstrate that the *Wls* allele is a functional conditional null. We anticipate it to be a valuable tool for use where precise spatial and temporal control of

Wnt activity loss-of-function is advantageous. In addition, it will be useful to uncover Wnt ligand redundancy.

MATERIALS AND METHODS

Animal Maintenance and Use

Animals were housed in a pathogen-free vivarium in accordance with institutional policies. Gestational age was determined through detection of a vaginal plug. At specific gestational ages, dams were anesthetized with isoflurane and embryos removed by hysterectomy.

Generation of *Wls*^{flox} Allele

The *Wls*^{fl} allele was generated using conventional gene targeting methods. The *Frt* site-flanked neo gene of the targeting vector was excised in vivo using a flippase expressing mouse line (Rodriguez *et al.*, 2000). Primers for genotyping of the *Wls*^{fl} allele were as follows: P1, CTTCCCTGCTTCTTTAAGCGTC; P2, AGGCTTCGAACG TAACTGACC; P4, CTCAGAACTCCCTTCTTGAAGC. The PCR protocol used was 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s and a final extension of 72°C for 7 min. The use of primers P2 and P4 produce bands of 411 bp for the wild-type allele and 556 bp for the *Wls*^{fl} Neo deleted allele. The use of primers P1 and P4 produce bands of 1,625 bp for the wild-type allele and 410 bp for the *Wls*$^{-}$ allele. The stock number for this mouse that will soon be available at Jackson Laboratory is 012888.

Mouse Lines

The following transgenic mice used in this study were generated previously: *EIIa-cre* (Lakso *et al.*, 1996), *Wnt1-cre* (Danielian *et al.*, 1998), *TOPGAL* (DasGupta and Fuchs, 1999), and *Pdx1-cre* (Wells *et al.*, 2007). The *Wls* floxed mice were crossed to the *EIIa-cre*, *Wnt1-cre*, and *Pdx1-cre* mice to generate conditional mice where *Wls* would be deleted in the germline, neural crest cells, or pancreatic progenitor cells, respectively. All mouse lines used in this study were genotyped by PCR using primers and protocols described previously. Yolk sacs from staged embryos or tail tips were digested overnight at 55°C in lysis buffer and genomic DNA extracted using a Kingfisher 96 Magnetic Particle Processor.

Immunofluorescence Staining

Pancreata were dissected in PBS and fixed in formalin. Pancreata were then processed in paraffin wax and sectioned at 4 μm. Primary antibody incubation was performed overnight at 4° followed by secondary antibody incubation for 1.5 h at room temperature. Antibody identities and dilutions are as follows: chicken anti-insulin (Abcam), guinea pig anti-glucagon (ABR) both used at 1:1,000, goat anti-chicken 488 (Jackson ImmunoResearch), and donkey anti-guinea-pig Cy5 (Jackson ImmunoResearch). Figures in this work were prepared digitally using Canvas 8.0 and Creative Suite 3.0 software.

X-gal Staining

Staged embryos expressing lacZ reporter genes were fixed for 30 min using X-gal fixative (1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl2, 5 mM EGTA, and 0.01% NP-40) and washed twice with 1× PBS/0.02% NP-40 for 15 min. Embryos were then stained with X-gal solution (5 mM $K3Fe(CN)_6$, 5 mM $K4Fe(CN)_6$, 1 M $MgCl_2$, 0.01% NP-40, 1 mg/ml X-gal) overnight, postfixed with 4% paraformaldehyde for 1 h, cryoprotected, and 10 μM cryosections prepared.

Hematoxylin and Eosin Staining

For CNS staining, whole fetal samples were fixed in 4% paraformaldehyde overnight, cryoprotected in 15% then 30% sucrose before embedding and freezing in OCT. E7.5 and E8.5 embryos and pancreata were dissected in PBS and fixed in formalin and then processed in paraffin wax. Eight micromolar sections were cut and stained with hematoxylin and eosin.

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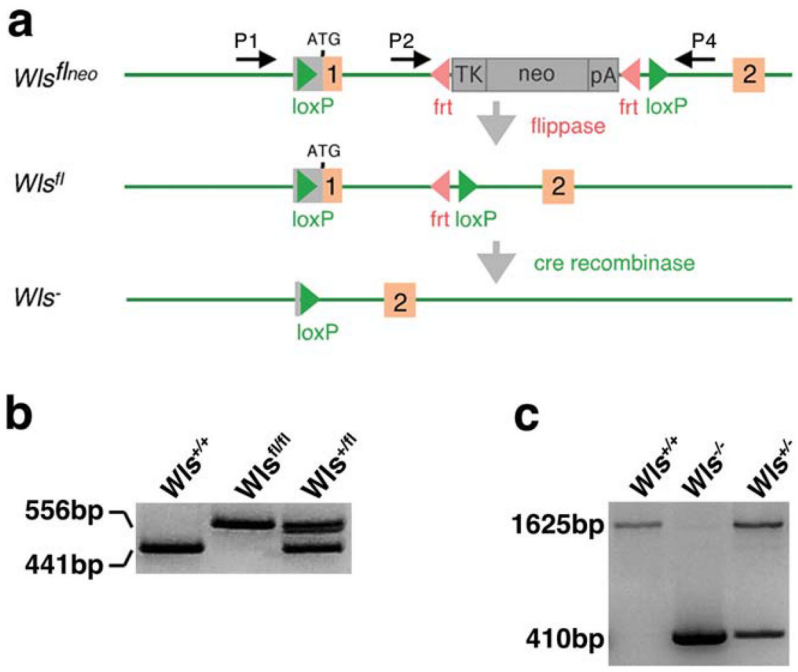

FIG. 1.

Generation of the *Wls* conditional allele. (**a**) Orange boxes correspond to exons. Red arrowheads are *Frt* sites. Green arrowheads denote *LoxP* sites. Recombination of the *Frt* sites flanking the Neo results in the floxed *Wls* allele. Recombination of the *LoxP* sites removes the ATG start site in exon 1 resulting in the *Wls* null (*Wls*$^{-}$) allele. P1, P2, and P4 represent the location of the primers used for *Wls* genotyping. (**b**) Successful Flp recombination was confirmed using PCR analysis to detect a 441-bp WT band and a 556 bp *Wls*^{fl} Neo deleted band (using primers P2 and P4). (**c**) Cre recombination was validated using PCR analysis to detect a 1,625-bp WT band and a 410-bp null band (using primers P1 and P4).

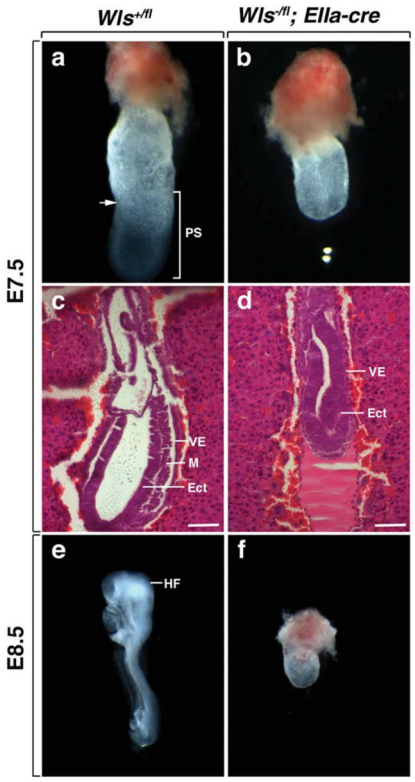

FIG. 2.

Deletion of germline *Wls* inhibits mouse embryogenesis. (**a, b**) Morphological analysis of embryos dissected at E7.5. The wild-type embryo has the characteristics of an early neural plate stage embryo with evidence of primitive streak formation. $Wls^{-/-}$ mutants resemble egg cylinders. The arrow denotes the embryonic-extra-embryonic junction at the anterior side. (**c, d**) Histological analysis of embryos dissected at E7.5. Wild-type embryos exhibit endoderm, mesoderm, and ectoderm. $Wls^{-/-}$ mutants display endoderm and ectoderm, but no mesoderm. Scale bars represent 100 μm. (**e**) Control embryo at E8.5 with a normal embryonic axis. (**f**) At E8.5 $Wls^{-/-}$ mutants still resemble egg cylinders. VE, visceral endoderm; M, mesoderm; Ect, ectoderm, PS, primitive streak; HF, head-fold.

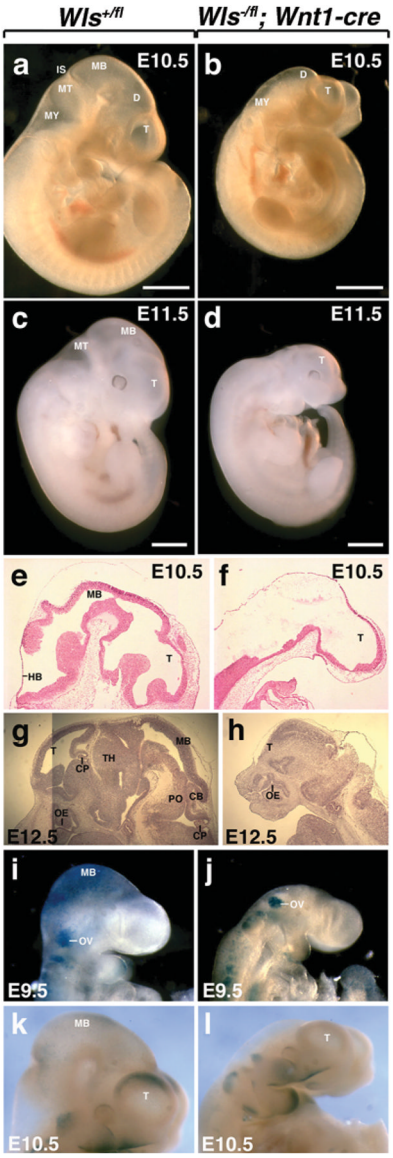

FIG. 3.

Deletion of *Wls* in the dorsal midline of the diencephalon using *Wnt1-cre*. (**a, b**) Morphological analysis of embryos dissected at E10.5. $Wls^{+/fl}$ embryos exhibit a forebrain, midbrain and hindbrain. $Wls^{-/fl}$; *Wnt1-cre* embryos fail to develop a midbrain. (**c**) $Wls^{+/fl}$ embryos dissected at E11.5. (**d**) $Wls^{-/fl}$; *Wnt1-cre* embryos at E11.5 show defective development of midbrain, hindbrain and forebrain. Histologic comparison of WT (**e, g**) and mutant embryos (**f, h**). Mutant embryos display loss of midbrain and hindbrain formation at E10.5 (f). At E11.5, mutant embryos additionally lack a forebrain choroid plexus and display a modified forebrain (h). β-catenin/Wnt activity in the brain of $Wls^{+/fl}$ (**i** and **k**) and $Wls^{-/fl}$; *Wnt1-cre* (**j** and **l**) embryos stained for X-gal. Scale bars represent 1 mm. T, telencephalon; D, diencephalon; MB, midbrain; MT, metencephalon; MY, myencephalon; IS, isthmus; HB, hindbrain; CP, choroid plexus; OE, olfactory epithelium; TH, thalamus; PO, pons; CB, cerebellum; OV, otic vessicle.

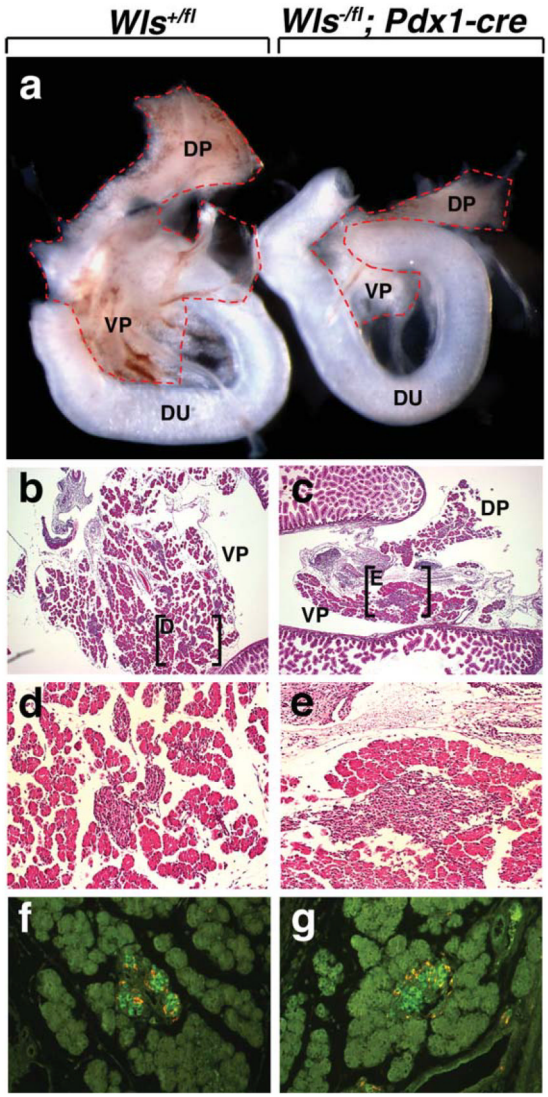

FIG. 4.

Deletion of *Wls* in pancreatic precursor cells using *Pdx1-cre*. (**a, b**) Morphological analysis of pancreata dissected at E15.5. Both $Wls^{+/fl}$ and $Wls^{-/fl}$; *Pdx1-cre* pancreata exhibit a dorsal and ventral pancreas. $Wls^{-/fl}$; Pdx1 pancreata are severely hypoplastic (**b**). Histologic comparison of WT (b, **d, f**) and mutant pancreata (**c, e, g**). Low-power magnification of both $Wls^{+/fl}$ and $Wls^{-/fl}$; *Pdx1-cre* pancreata reveal the presence of acinar cells and Islets of Langerhans (b and c). Higher power magnification of the boxed regions in b and c (d and e, respectively). (f, g) E15.5 islets immunostained for insulin (green) and glucagon (orange). DP, dorsal pancreas; VP, ventral pancreas; Du, duodenum.