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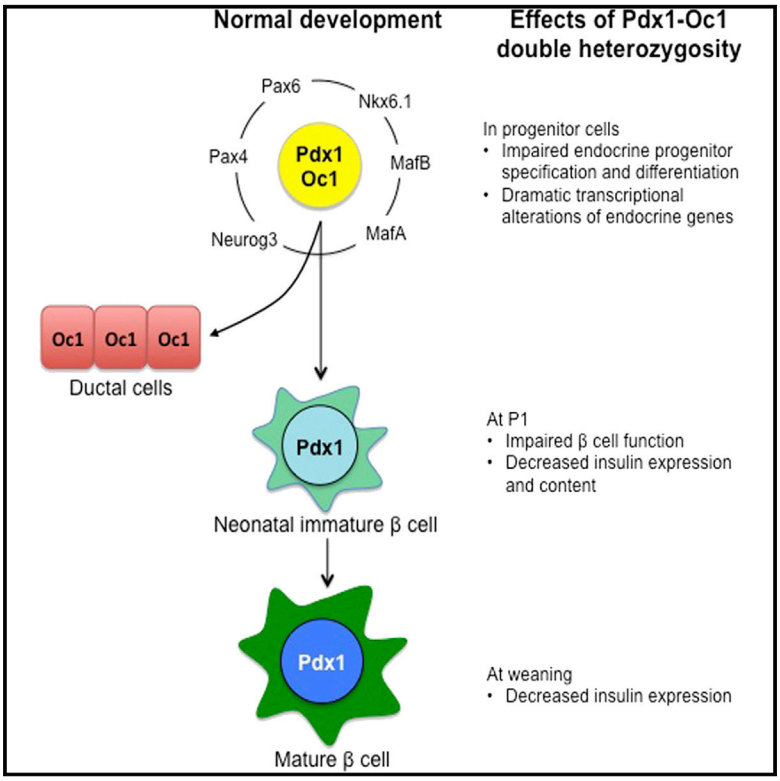
Cell Reports

Threshold-Dependent Cooperativity of Pdx1 and Oc1 in Pancreatic Progenitors Establishes Competency for Endocrine Differentiation and β-Cell Function

Graphical Abstract



Highlights

- Gene expression changes in Pdx1-Oc1 double heterozygotes are distinct from single hets
- Double hets have decreased endocrine differentiation at the secondary transition
- Compensatory mechanisms occur at late gestation to restore endocrine mass
- Double heterozygosity results in persistent impairment in β-cell function

Authors

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In Brief

Developmental defects, even subtle ones, can predispose individuals to adult disease. Henley et al. demonstrate that combined reduction in levels of the pancreatic transcription factors Pdx1 and Oc1 during development impair differentiation of insulin-producing β cells, linking modest embryonic changes to potential susceptibility to diabetes later in life.

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Threshold-Dependent Cooperativity of Pdx1 and Oc1 in Pancreatic Progenitors Establishes Competency for Endocrine Differentiation and β-Cell Function

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SUMMARY

Pdx1 **and** ***Oc1*** **are co-expressed in multipotent pancreatic progenitors and regulate the pro-endocrine gene** ***Neurog3*****. Their expression diverges in later organogenesis, with Oc1 absent from hormone+ cells and Pdx1 maintained in mature β cells. In a classical genetic test for cooperative functional interactions, we derived mice with combined** ***Pdx1*** **and** ***Oc1*** **heterozygosity. Endocrine development in double-heterozygous pancreata was normal at embryonic day (E)13.5, but defects in specification and differentiation were apparent at E15.5, the height of the second wave of differentiation. Pancreata from double heterozygotes showed alterations in the expression of genes crucial for β-cell development and function, decreased numbers and altered allocation of Neurog3-expressing endocrine progenitors, and defective endocrine differentiation. Defects in islet gene expression and β-cell function persisted in double heterozygous neonates. These results suggest that** ***Oc1*** **and** ***Pdx1*** **cooperate prior to their divergence, in pancreatic progenitors, to allow for proper differentiation and functional maturation of β cells.**

INTRODUCTION

Knowledge gained from developmental biology has been instrumental in deriving glucose-responsive, insulin-secreting pancreatic β cells from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) to generate a cell-based therapy for the treatment of diabetes (Bruin et al., 2015; Pagliuca et al., 2014; Russ et al., 2015). Of particular interest are signaling molecules and transcriptional regulators that direct the β-cell fate or generate fully functional β cells. Many elegant single gene inactivation studies have revealed critical roles for specific transcription factors in different stages of pancreas development and endocrine differentiation. However, few studies have analyzed the functional consequences of combinatorial genetic manipulations of structurally unrelated pancreas transcription factors during development (Burlison et al., 2008; Courtney et al., 2013; Shih et al., 2015), Here, we report on the genetic and functional cooperativity of the Pdx1 and Oc1 transcription factors and the requirement for a combined threshold of activity in setting up a genetic program for endocrine differentiation and β-cell function.

Pancreatic and duodenal homeobox 1 (Pdx1) is required for pancreas development, endocrine differentiation, and mature β-cell function in mouse and human (Gao et al., 2014; Jonsson et al., 1994; Lammert et al., 2001; Offield et al., 1996; Stoffers et al., 1997b, 1997c, 1998). *Pdx1* is initially expressed in the mouse posterior foregut endoderm at embryonic day (E)8.5, expanding into the antral stomach, rostral duodenum, and common bile duct by E11.5 and maintained at high levels in mature β cells (Guz et al., 1995; Jonsson et al., 1994; Offield et al., 1996; Wu et al., 1997). In addition, the burst of β-cell proliferation that occurs just prior to birth requires Pdx1 (Gannon et al., 2008). Beginning at late gestation and continuing into the early postnatal period, β cells undergo gene expression changes associated with functional maturation, including the acquisition of tightly controlled glucose-stimulated insulin secretion (Artner et al., 2010; Nishimura et al., 2006; Stolovich-Rain et al., 2015). In adult mice, Pdx1 regulates β-cell function and survival (Brissova et al., 2002; Dutta et al., 1998; Gauthier et al., 2009; Kulkarni et al., 2004; Sachdeva et al., 2009; Waeber et al., 1996). The crucial role for Pdx1 in endocrine-lineage development and postnatal β-cell function is underscored by the identification of

diabetes-causing *PDX1* mutations in humans (Hani et al., 1999; Macfarlane et al., 2000; Stoffers et al., 1997a, 1998).

Onecut 1 (*Oc1;* also known as hepatic nuclear factor 6; *Hnf6*) is expressed more broadly in the developing endoderm and plays roles in the developing liver and pancreas (Jacquemin et al., 2000, 2003; Samadani et al., 1996; Zhang et al., 2009). Expression of the endocrine-progenitor transcription factor, *neurogenin 3* (*Neurog3*), is nearly undetectable in $Oc1^{-/-}$ embryos, which are diabetic at birth, with a near complete loss of all pancreatic endocrine cell lineages (Jacquemin et al., 2000). Oc1 binds to an upstream enhancer from the *Neurog3* gene (Jacquemin et al., 2000), suggesting that *Neurog3* is a direct transcriptional target of Oc1. Unlike *Pdx1*, *Oc1* is not expressed in differentiated, hormone-positive endocrine cells, but its expression persists in ducts and acinar cells into adulthood (Pekala et al., 2014; Prévot et al., 2012; Rausa et al., 1997; Zhang et al., 2009). Overexpression of *Oc1* in the developing pancreas results in an increase in Neurog3-positive cells (Wilding Crawford et al., 2008). However, its downregulation in the endocrine lineage is essential: maintained *Oc1* expression prevents β-cell maturation, most likely by directly inhibiting expression of the β-cell transcription factor, *MafA* (Yamamoto et al., 2013), and results in diabetes (Gannon et al., 2000; Tweedie et al., 2006).

Pdx1 and *Oc1* are coexpressed in multipotent pancreatic progenitors (MPCs) in the early pancreatic bud and later in the undifferentiated, bipotential duct/endocrine cell pool located within the "trunk" domain of the pancreatic epithelium. Pdx1 and Oc1 each activate *Neurog3* expression and our in vitro evidence suggests that a physical interaction between these two factors involving the Pdx1 C terminus promotes endocrine specification. Pdx1 occupies an evolutionarily conserved *Neurog3* enhancer at E13.5 and, in reporter assays, Pdx1 transactivation via this enhancer was significantly enhanced by Oc1. Mice homozygous for a *Pdx1* allele with a premature C-terminal truncation ($Pdx1^{\Delta C/\Delta C}$) display a global reduction in endocrine lineages and decreased numbers of $Neurog3^{+}$ progenitors at E13.5 (Oliver-Krasinski et al., 2009).

We hypothesized that the Pdx1-Oc1 interaction is critical at multi- or bipotent stages to promote the specification of pancreatic endocrine progenitors by regulating *Neurog3* and other developmentally important genes. To assess the significance of the Pdx1-Oc1 interaction in vivo, we generated animals globally heterozygous for either or both genes. To date, no developmental phenotype for either single heterozygous animal has been reported. At E13.5, double heterozygotes showed normal numbers of glucagon+ and Neurog3+ cells, suggesting that the first wave of endocrine differentiation is unaffected. By E15.5 at the height of the secondary wave of differentiation, the numbers of Neurog3+ endocrine progenitors and insulin+ and glucagon+ cells were reduced. Whole transcriptome analysis at E15.5 revealed a dramatic and unique impact of Pdx1-Oc1 heterozygosity on the endocrine compartment. Later stages of endocrine differentiation and function, well after the normal downregulation of Oc1 in the endocrine lineage, were also defective in double heterozygotes. Thus, Pdx1 and Oc1 cooperate to promote endocrine specification and subsequent functional maturation of β-cells, most likely by establishing a state of competency in progenitors earlier in development that allows for later steps in endocrine differentiation to be realized.

RESULTS

Combined *Pdx1* and *Oc1* Heterozygosity Has a Broad Effect on the Transcriptional Network Regulating Endocrine Development

To determine the effect of combined global heterozygosity for *Pdx1* and *Oc1* on pancreas development, we analyzed the transcriptome of pancreata from control (WT), $Pdx1^{lacZ/+}$ (which carry one null allele of *Pdx1* containing a lacZ cassette; Offield et al., 1996), $Oc1^{+/-}$, and $Pdx1^{lacZ/+}$;$Oc1^{+/-}$ (hereafter: double heterozygous, DH) animals using RNA-sequencing (RNA-seq; see Supplemental Information). We performed our analysis at E15.5 since all pancreatic lineages are present at this stage and the greatest number of Neurog3+ cells can be detected (Gradwohl et al., 2000). A total of 2,331 genes were differentially expressed in at least one of the three experimental genotypes (Figure 1A; Table S1). Expression of 102 genes was altered in $Pdx1^{lacZ/+}$ pancreata (Figure 1B). $Oc1^{+/-}$ pancreata showed the greatest number of gene expression changes, with more than 2,000 genes affected. In contrast to *Pdx1* single-heterozygotes (SH), β-cell genes such as *Ins1, Ins2,* and *Iapp* were increased in $Oc1^{+/-}$ SH, consistent with a role for Oc1 in suppressing β-cell differentiation (Tweedie et al., 2006).

The transcriptome of *Pdx1-Oc1* double heterozygotes showed a pattern of gene dysregulation distinct from either SH transcriptome. Of the 257 genes affected in DH, 153 genes were specifically altered in DH compared to WT (Figure 1B), including key transcriptional regulators *MafA*, *MafB*, *NeuroD1*, and *Nkx6.1*, providing strong support for functional cooperation between these two transcription factors to regulate a distinctive genetic program. Furthermore, the shift of gene expression patterns of $Oc1^{+/-}$ SH versus DH or $Pdx1^{lacZ/+}$ SH versus DH (Figure 1A) suggests the Pdx1-Oc1 interaction acts cooperatively or antagonistically at the level of broad categories of genes.

To assess systematic changes in expression of genes involved in canonical signaling pathways, disease and biological function categories, and molecular networks of genes altered in the three experimental genotypes, we performed comparison-enrichment analysis using Ingenuity Pathway Analysis (IPA). The top gene ontology categories ascribed to genes altered in $Oc1^{+/-}$ or in $Pdx1^{lacZ/+}$ SH pancreata were "cancer", "embryonic development" and "cellular development", or "gene expression", respectively (Table S2). The genes altered in DH pancreata clustered primarily in the "endocrine system development and function", "carbohydrate metabolism", and "endocrine system disorders" categories (Figure 1C; Table S2). The "endocrine system development and function" category was enriched for genes associated with "quantity of endocrine cells" and "quantity of beta and alpha islet cells" (Table 1), indicating a possible impact on endocrine progenitors and endocrine differentiation. Genes in these categories clustered less strongly in $Pdx1^{lacZ/+}$ or $Oc1^{+/-}$ SH because the vast majority of genes were specifically altered in the DH data set and not in SH pancreata (Figures 1C and 1D; Table 1).

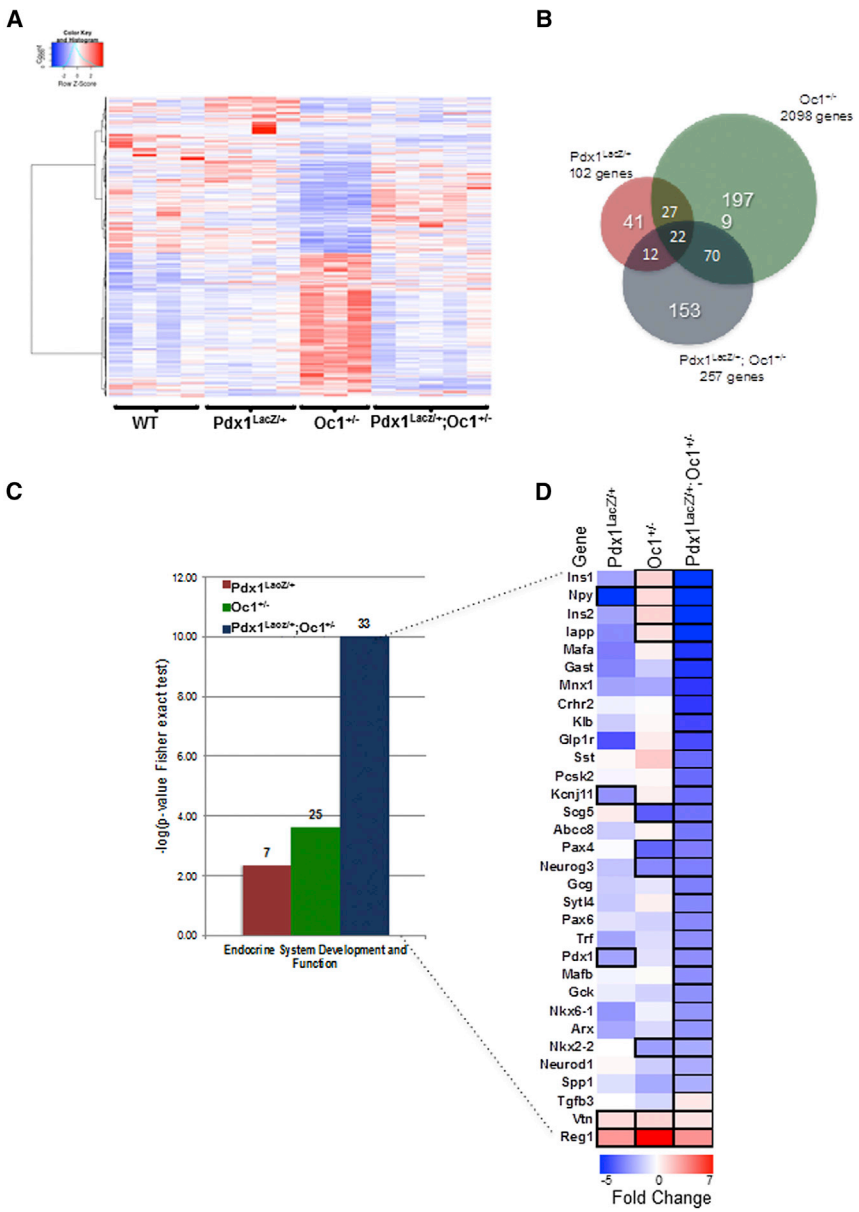

Figure 1. Combined Heterozygous Reduction in *Pdx1* and *Oc1* Gene Dosage Has a Broad Impact on the Transcriptional Network of Endocrine Pancreas Progenitors

(A–D) Hierarchical clustering of 2,331 differentially expressed genes in individual pancreata at E15.5 from $Pdx1^{LacZ/+}$ (n = 4), $Oc1^{+/-}$ (n = 3), and $Pdx1^{LacZ/+}$; $OC1^{+/-}$ mice (n = 4) (A); Venn diagram depicting the number of altered genes in the $Pdx1^{LacZ/+}$, $Oc1^{+/-}$, and $Pdx1^{LacZ/+}$; $Oc1^{+/-}$ (B); and endocrine system development and function gene ontology category in each genotype, according to negative log of p value from Fisher exact test. The numbers above each column represent the number of genes enriched in each category (C) and the heatmap of endocrine development and function genes (D). The up- or downregulated genes with false discovery rate less than 0.1 and fold change higher than 0.5 versus WT are highlighted with bold black borders. See also Tables S1 and S2.

at the level of activation of *Neurog3.* Expression of *Neurog3* and *Pax4* was decreased in *Oc1* SH and DH, while *neuropeptide Y* (*NPY*) was decreased similarly in the *Pdx1* SH and DH compared with WT, indicating that some genes show sensitivity to reductions in either *Pdx1* or *Oc1* gene dosage alone.

In DH pancreata, we also observed large decreases in expression of genes encoding proteins involved in multiple aspects of glucose-stimulated insulin secretion, glucose metabolism (*Gck* and *G6pc2*), K_{ATP} channel components (*Abcc8* and *Kcnj11*), vesicle trafficking (*Scg5* and *Sytl4*), G protein coupled receptors (*Glp1r* and *Ffar1*), and transmembrane proteins (*Klb*) (Figure 1D). These findings are likely related to the decreased number of differentiated endocrine cells in DH at this developmental age (see below). Although the majority of changes were associated with downregulation of gene expression, some genes were upregulated in all three mutant genotypes, including vitronectin (*Vtn*), which is increased in delaminating endocrine-committed cells (Cirulli et al., 2000) and inhibits insulin production and secretion (Kaido et al., 2006), and regenerating islet-derived 1 (*Reg1*), which is associated with islet regeneration (Kobayashi et al., 2000; Terazono et al., 1988).

The largest effects of DH on transcription were observed in pancreatic endocrine-hormone expression. Insulin was most affected in the DH pancreata, being significantly reduced compared to both WT and SH animals (Figure 1D). mRNA levels of other islet hormones (*Gcg*, *Iapp*, and *Sst*) were also decreased in the DH animals by 30%–50% compared to WT or SH pancreata, suggesting an overall synergistic action of Pdx1 and Oc1 on the entire endocrine compartment. Within the gene set significantly altered in DH pancreata, we also observed an impressive array of transcription factors crucial for development of the endocrine pancreas. The decreased expression of *MafA*, *MafB*, *Pax4*, *Pax6*, *Mnx1*, *Nkx2.2*, and *Nkx6.1* suggests that simultaneous reduction in expression of both *Pdx1* and *Oc1* acts at multiple levels of endocrine differentiation and not just

Embryonic Endocrine Progenitor Specification and Endocrine Cell Maturation during the Secondary Transition Are Impaired by Double *Pdx1-Oc1* Heterozygosity

The E15.5 RNA-seq data suggested that endocrine lineage commitment and differentiation were impaired in DH pancreata. Specifically, expression of *Neurog3,* the critical endocrine

Table 1. Clustering of Genes in Each Endocrine System Development and Function Gene Ontology Category in Each Experimental Genotype

Endocrine System Development and Function Annotation[a]	p Value	Molecules
$Pdx1^{LacZ/+}$		
Migration of beta islet cells	4.38×10^{-3}	Vtn
Transmembrane potential of beta islet cells	4.38×10^{-3}	Kcnj11
Development of enteroendocrine cells	8.74×10^{-3}	Atoh1
Replication of beta islet cells	1.31×10^{-2}	Reg1a
Formation of parathyroid gland	1.74×10^{-2}	Aldh1a7
Glucuronidation of beta-estradiol	1.74×10^{-2}	Nr1i2
Differentiation of beta islet cells	2.17×10^{-2}	Slc2a2
$Oc1^{+/-}$		
Glucose tolerance	2.34×10^{-4}	Abcb4, Atf4, C19orf10, Cbl, cckbr, Cyp2J2, Ffar3, Fxyd2, Grb10, Hnf4a, Htr2c, htr3a, Hyou1, Iapp, ins, Irs2, Klf15, Mir-802, Nrob2, Nr1h4, Pcbd1, Rbp1, Serp1, Srebf1, and Wnt10b
$Pdx1^{LacZ/+}$; $Oc1^{+/-}$		
Quantity of endocrine cells	9.28×10^{-11}	Arx, Gast, Ins, Ins1, Kcnj11, Mafb, Mnx1, Neurog3, Nkx6-1, Pax4, Pax6, and Pcsk2
Quantity of beta islet cells	2.40×10^{-10}	Arx, Ins, Ins1, Kcnj11, Mnx1, Nkx6-1, Pax4, and Pcsk2
Quantity of alpha islet cells	2.79×10^{-6}	Arx, Ins, Ins1, and Pcsk2
Entry into cell-cycle progression of endocrine cell lines	2.53×10^{-5}	Ins1, Sst, and Tf
Concentration of corticosterone	2.77×10^{-5}	Crhr2, Gck, Glp1r, Ins, Klb, Npy, and Scg5
Glucose tolerance	1.43×10^{-4}	Abcc8, Glp1r, Iapp, Ins, Mafa, Pdx1, Sytl4, and Tgfb3
Replication of beta islet cells	3.55×10^{-4}	Gast and Reg1a
Differentiation of endocrine cells	1.10×10^{-3}	Nkx6-1, Pax4, and Pdx1
Area of islets of Langerhans	1.17×10^{-3}	Ins and Ins1
Differentiation of beta islet cells	1.17×10^{-3}	Nkx6-1 and Pdx1
Regeneration of islet cells	1.17×10^{-3}	Nkx6-1 and Pcsk2
Formation of islet cells	2.42×10^{-3}	Pax6 and Pdx1
Quantity of delta islet cells	2.42×10^{-3}	Arx and Pcsk2
Synthesis of hormone	2.88×10^{-3}	Abcc4, Crhr2, Cyp11a1, Ins, Pax6, and Vip
Proliferation of beta islet cells	3.98×10^{-3}	Iapp, Ins, Nkx6-1, and Pax4
Quantity of enteroendocrine cells	6.15×10^{-3}	Arx and Pax4
Steroidogenesis of hormone	7.54×10^{-3}	Abcc4, Crhr2, Cyp11a1, Ins, and Vip
Size of beta islet cells	8.60×10^{-3}	Pcsk2 and Pdx1
Synthesis of corticosterone	8.60×10^{-3}	Apoa1 and Cyp11a1
Activation of parathyroid gland	1.09×10^{-2}	Casr
Arrest in organogenesis of pancreas	1.09×10^{-2}	Pdx1
Binding of hypothalamus	1.09×10^{-2}	Pyy

[a]See also Figure 1 and Tables S1 and S2.

specification transcription factor, and *Pax6,* a pan-endocrine-lineage transcription factor downstream of Neurog3, were down-regulated. Pancreas development is asynchronous and multiple developmental stages can be observed at a single time point (Guney and Gannon, 2009). To obtain cellular resolution of gene expression changes, we quantified the number of cells expressing either Neurog3 or Pax6 at E15.5. DH pancreata had approximately 50% fewer $Neurog3^{+}$ endocrine progenitors than WT (Figures 2A and 2C). These data support our hypothesis that combined Pdx1 and Oc1 deficiency leads to reduced endocrine lineage specification. Despite a reduction in *Neurog3* mRNA expression in *Oc1* SH (Figure 1D), the number of Neurog3+ cells in *Oc1* SH was not statistically significantly affected compared to WT (Figure 2C), suggesting a decrease in *Neurog3* expression per cell in *Oc1* SH. Although the reduction in Pax6+ endocrine precursor cells did not achieve statistical significance (Figures 2B and 2D), the ratio of Neurog3+ cells to Pax6+ cells at E15.5 was similar among genotypes (Figure S1), supporting an overall decrease in the number of specified and committed endocrine cells. In contrast, at E13.5, Neurog3+ progenitor numbers were not affected in SH or DH pancreata (Figure S2A). Pax6+ and glucagon+ cells were also normal at this stage

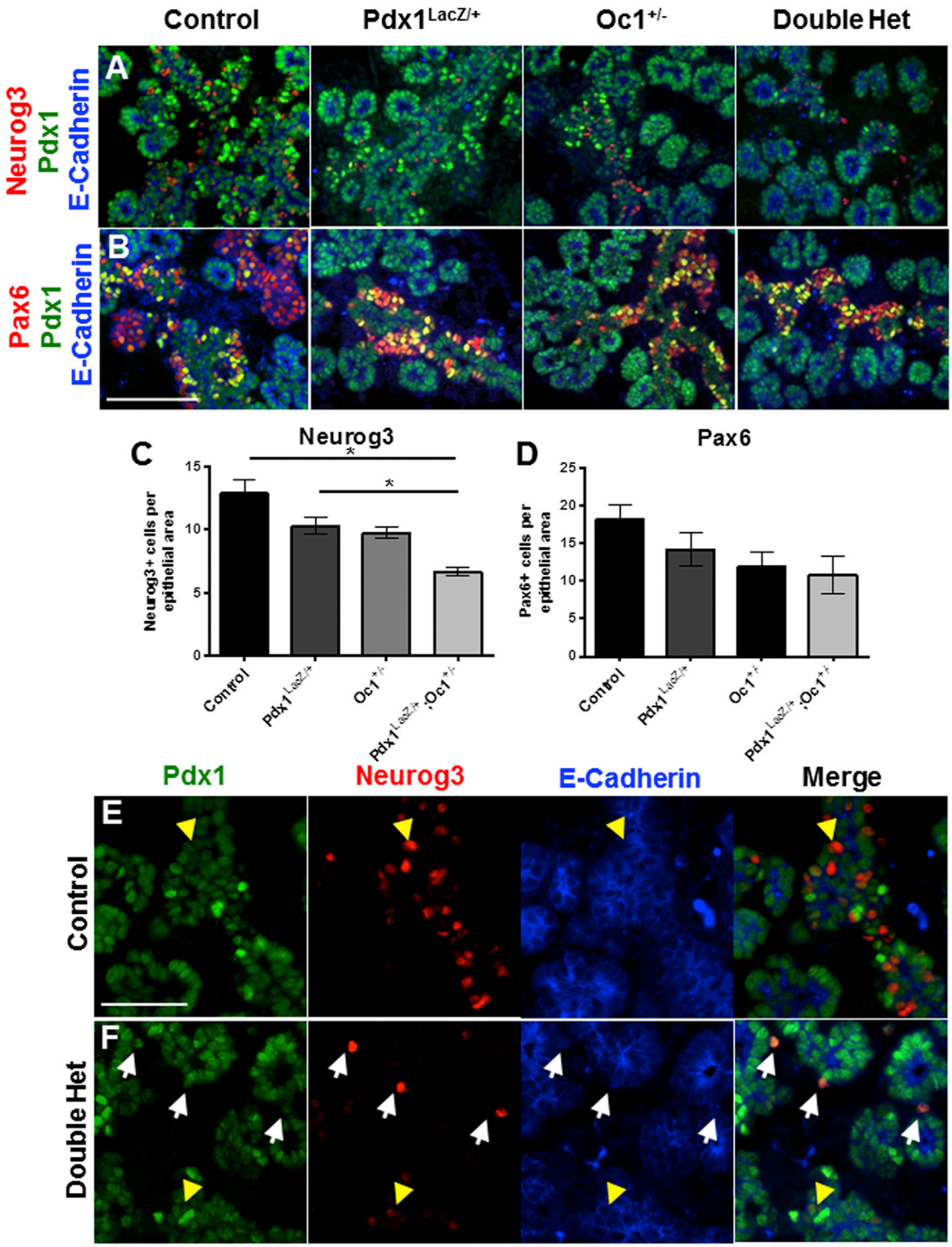

Figure 2. Reduced Number and Altered Location of *Neurog3*-Expressing Endocrine Progenitors in DH Mice at E15.5

(A, B, E, and F) WT, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$, and DH pancreata were immunolabeled for Neurog3 (A, E, and F) or Pax6 (in red) (B), Pdx1 (green), and E-cadherin (blue). (C and D) Quantification for Neurog3 (C) and Pax6 (D). The white arrows: delaminated Neurog3^{+} progenitors and yellow arrowheads: Neurog3^{+} progenitors within developing trunk.

(A and B) 20× magnification. The scale bar represents 100 μm. (E and F) 40× magnification. The scale bar represents 100 μm. p value for all marked comparisons was < 0.05 by one-way ANOVA with Tukey correction (*p = 0.0019). See also Figure S1.

(Figures S2B and S2C; too few insulin+ cells were detected to quantify). Thus, the impact of decreased Pdx1-Oc1 dosage appears to be restricted to the second wave of endocrine differentiation that gives rise to cells within the mature islets of Langerhans (Guney and Gannon, 2009).

During normal pancreas development, *Neurog3* expression initiates within a subset of bipotential trunk epithelial cells (Beucher et al., 2012) and becomes elevated in cells destined to undergo commitment to the endocrine lineage. It is thought that the Neurog3^{hi} cells give rise to hormone-expressing cells after delaminating from the ductal epithelium (Villasenor et al., 2008). Closer examination of DH pancreata at E15.5 revealed that, in contrast to WT pancreata, fewer Neurog3+ cells could be found within the pancreatic trunk epithelium. A greater proportion of the Neurog3+ cells present were instead located adjacent to the epithelium in DH compared with the other three genotypes; these extra-truncal Neurog3+ cells seemed to express high levels of Neurog3 (Figures 2E and 2F, white arrows compared to yellow arrows).

To characterize the impact of Pdx1-Oc1 cooperativity on the endocrine compartment during the definitive second wave of endocrine differentiation, we examined markers of differentiated endocrine lineages at E15.5 including the hormones insulin and glucagon. DH pancreata showed significant decreases in the numbers of both insulin+ and glucagon+ cells (Figures 3A and 3B). Only DH pancreata showed a reduction in glucagon+ cell number (Figure 3B), whereas similar decreases in the number of insulin+ cells were observed in *Pdx1* SH and DH (Figure 3A). While previous studies have shown decreased β-cell function and impaired glucose homeostasis in *Pdx1* SH postweaning, here, we show that defects in the β-cell lineage exist in *Pdx1* SH during embryonic development. Defective β-cell development could contribute to the susceptibility to mature onset diabetes of the young (MODY) and adult-onset diabetes observed in mice and humans with heterozygous *Pdx1* mutations (Dutta et al., 1998; Hani et al., 1999; Macfarlane et al., 2000; Sachdeva et al., 2009; Stoffers et al., 1997c, 1998; Weng et al., 2001). Taken together, these data reveal a reduction in the numbers of emerging hormone-expressing cells at E15.5 in mice with combined reduction in *Pdx1* and *Oc1* gene dosage.

In light of the transcriptional impact of Pdx1 and Oc1 reduction on markers of α and β cell maturation (Figure 1), we assessed the number of cells expressing the "large Maf" transcription factors, MafA and MafB. *MafB* is activated soon after endocrine progenitor delamination in both glucagon+ and insulin+ cells and is downregulated postnatally in insulin+ cells in mice. MafA expression initiates later, specifically in insulin+ cells, and is maintained in these cells. Despite measurable decreases in MafA and MafB transcripts (Figure 1D), we observed no differences in the total number of MafA+ or MafB+ cells at E15.5 (Figures 3C and 3D). Normal cell numbers in spite of reduced transcripts suggests that MafA and MafB expression per cell is decreased. When we compared the numbers of MafA/B+ cells to the number of insulin/glucagon-expressing cells, we found a significant increase in the number of Maf+/hormone cells in DH pancreata (Figure S3). These Maf+ cells may derive from Neurog3+ cells generated prior to E13.5 that fail to gain mature hormone expression in a timely fashion. Taken together, these data further establish that the simultaneous decrease in *Pdx1-Oc1* dosage preferentially affects the endocrine progenitor program during the second wave of endocrine differentiation.

Impaired Terminal Differentiation and Function of Hormone+ Cells with Double *Pdx1-Oc1* Heterozygosity

To determine whether the developmental defects in DH persist after birth, we examined early postnatal stages physiologically and morphologically. Immediately at birth, prior to feeding, there were no significant differences in body weight or blood glucose levels (Figure S2). However, with the start of feeding at postnatal day 1 (P1), DH animals failed to increase in body weight compared to the other genotypes (Figure 4A). At P1, DH pups exhibited elevated *ad lib* blood glucose compared to WT and *Oc1*$^{+/-}$ animals (Figure 4B). We therefore analyzed whether DH neonates had reduced α- or β-cell mass, consistent with our observations of decreased insulin+ and glucagon+ cells at E15.5. There was a strong trend toward reduced α- and β-cell mass at P1 in *Pdx1* SH and DH, but this was not statistically significant (Figures 4G and 4H). Islet morphology (Figures 4C–4F) and α:β cell ratio were unchanged at P1 (Figure 4I). However, there was a dramatic and significant decrease in total pancreatic insulin protein content in DH when compared with WT or either SH (Figure 4J). These data suggest reduced insulin production per β cell and indicate a functional β-cell defect in DH mice that persists after birth in a cell population in which Pdx1 and Oc1 no longer colocalize.

A postnatal functional defect in DH islets is also supported by islet gene expression data at P1 (Figure 5). Taqman low density array (TLDA) analysis revealed that P1 DH islets had undetectable levels of insulin and glucagon mRNA; expression of these hormones was unaffected in islets from SH (Figure 5A). qRT-PCR using primers spanning the insulin and glucagon transcripts confirmed the decrease in insulin and glucagon in DH islets at P1 (Figure S5; Table S4). *Sst* mRNA expression was not changed in any genotype at P1. Expression of several key islet transcription factor genes that regulate either insulin or glucagon expression was substantially increased specifically in DH, suggesting attempts at compensation. However, expression of *Pax4,* a critical β-cell differentiation factor (Sosa-Pineda et al., 1997), was undetectable (Figure 5B), consistent with the reduction in insulin+ cells and the decrease in *Pax4* expression in DH at E15.5 detected by RNA-seq. Similarly, expression of genes involved in glucose sensing and hormone-granule exocytosis was also increased (Figure 5C). The bone morphogenetic protein (BMP) inhibitor *Sostdc1* was increased in DH P1 islets (Figure 5C), possibly contributing to the impaired islet function (Henley et al., 2012), as autocrine BMP activity was shown to be important for glucose-stimulated insulin secretion (Goulley et al., 2007).

We also observed increased expression of *Ctgf*, a β-cell-derived growth factor that is critical for embryonic β-cell proliferation and capable of inducing proliferation of embryonic α- and β-cells (Crawford et al., 2009; Guney et al., 2011; Riley et al., 2015) (Figure 5C). The dramatically increased *Ctgf* expression in DH suggested increased α- and/or β-cell proliferation as a mechanism for restoring α- and β-cell mass by birth. Indeed,

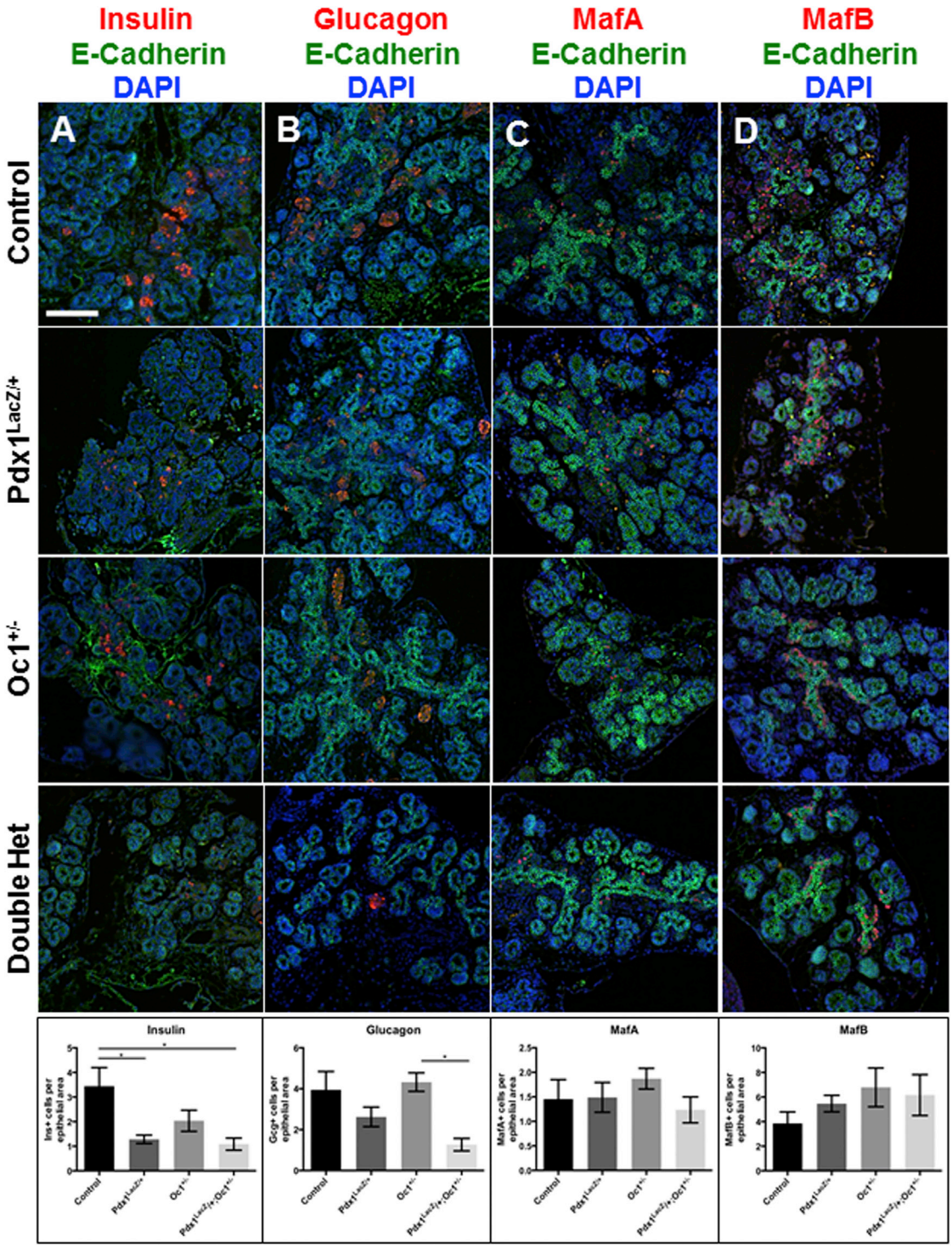

Figure 3. Defective Differentiation of DH Hormone-Expressing Cells at E15.5

(A–D) WT, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$, and DH pancreata were immunolabeled for insulin (A), glucagon (B), MafA (C), or MafB (D) in red, E-Cadherin (green), and DAPI (blue). The images are at 20× magnification. The scale bar represents 100 μm. p value for all marked comparisons was < 0.05 by one-way ANOVA with Tukey correction (*p < 0.05). See also Figure S3.

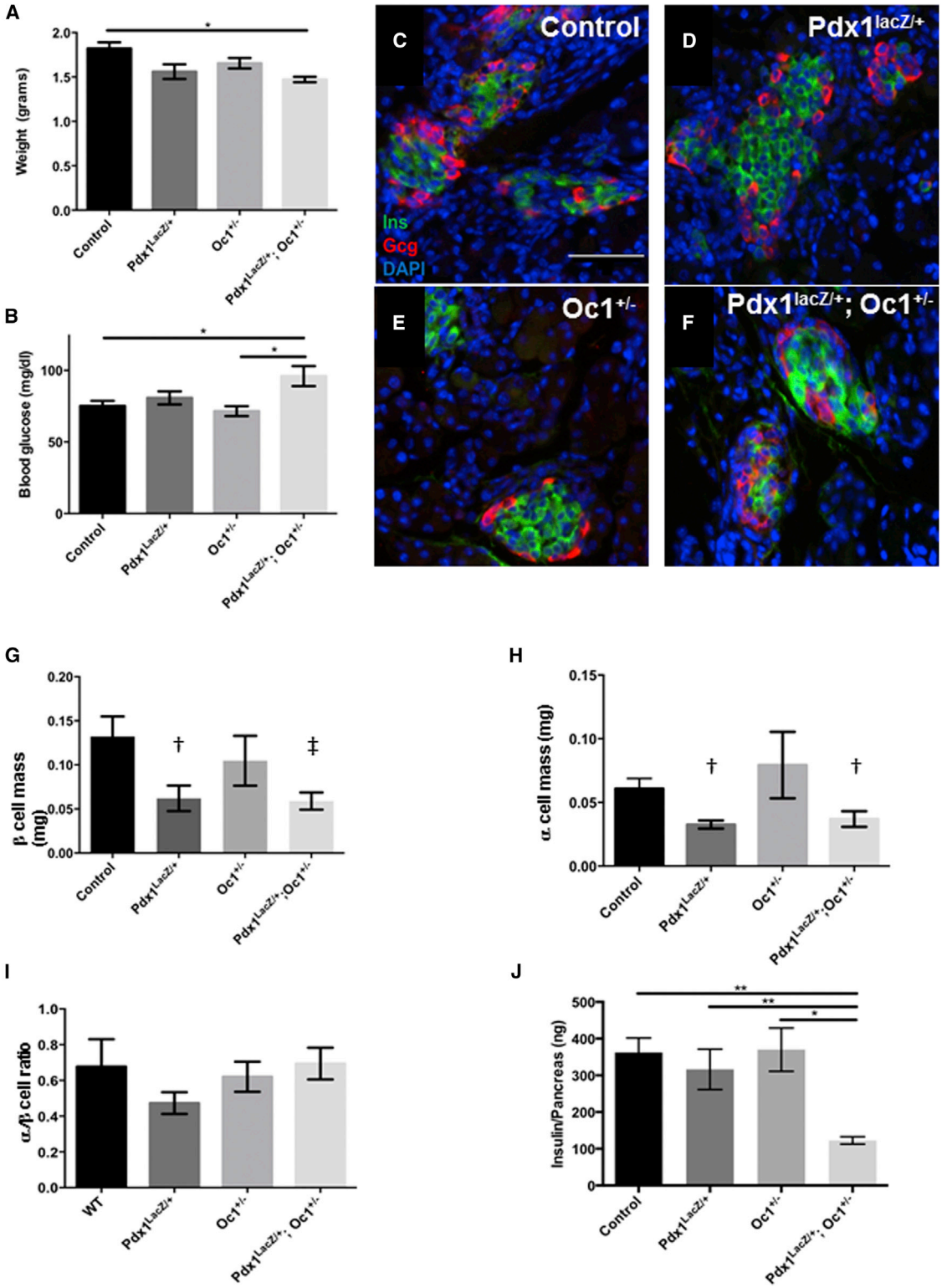

Figure 4. Defects in Glucose Homeostasis and Islet Gene Expression in DH Mice at P1

(A and B) Body weight (A) and *ad lib* feeding blood glucose measurements (B).

(C–F) Insulin (green) and glucagon (red) immunolabeling of pancreatic sections from WT (C), SH (D and E), and double heterozygotes (F).

(G–I) β cell mass (G), α cell mass (H), and α/β cell ratio (I).

(J) Total pancreatic insulin content (†$p > 0.10$, ‡$p = 0.069$, *$p < 0.05$, and **$p < 0.01$ by one-way ANOVA with Tukey correction).

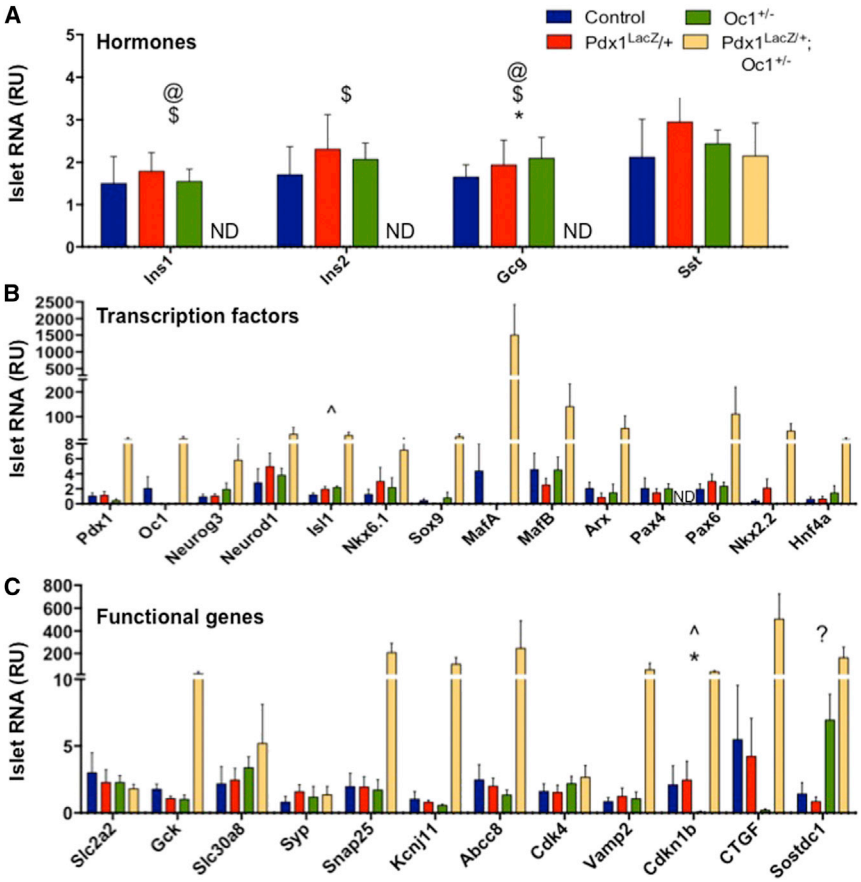

Figure 5. Combined Pdx1 and Oc1 Heterozygosity Leads to Dramatic Alterations in Neonatal Islet Gene Expression

(A–C) WT, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$, and *Pdx1*$^{LacZ/+}$; *Oc1*$^{+/-}$ islets were analyzed for gene expression of hormones (A), endocrine-associated transcription factors (B), and secretory functional genes (C). p value for marked comparisons was < 0.05 by Kruskal-Wallace Test followed by two-tailed Student's t test (*, WT versus *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; #, WT versus *Pdx1*$^{LacZ/+}$; ^, WT versus *Oc1*$^{+/-}$; @, *Pdx1*$^{LacZ/+}$ versus *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; $, *Oc1*$^{+/-}$ versus *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; and ?, *Pdx1*$^{LacZ/+}$ versus *Oc1*$^{+/-}$) (not detected = ND). See also Figure S5.

we detected a significant increase in α- and β-cell proliferation specifically in DH pancreata at E18.5 (Figure 6).

Restoration of Normal Glucose Homeostasis, but Persistent Gene Expression Defects, in DH Animals at Weaning

We next examined the DH phenotype at weaning when β cells become functionally fully mature (Nishimura et al., 2006; Stolovich-Rain et al., 2015). At this time point, DH animals had normal body weight (data not shown) and normal fasting blood glucose (Figure S6A). *Ad lib* blood glucose levels were elevated in *Pdx1* SH (Figure S6B), consistent with the adult phenotype in the literature (Ahlgren et al., 1998; Brissova et al., 2002; Dutta et al., 1998; Johnson et al., 2003). DH animals showed no statistically significant difference in *ad lib* feeding blood glucose levels (Figure S6B). When challenged with glucose during an intraperitoneal glucose tolerance test at 3 weeks of age, DH animals were not glucose intolerant (Figure S6C).

Since the physiological phenotype of DH animals appeared to resolve by weaning, we examined islet gene expression at 4 weeks of age. Although expression of insulin could be detected in DH islets at this age, it was still significantly reduced compared with all other genotypes (Figure 7A). At this time point, expression of *Sst* was also reduced in DH islets. Expression levels of islet transcription factors that were elevated in DH at P1 were normalized at 4 weeks of age, with the exception of *MafA*, which remained significantly decreased (Figure 7B). Expression of genes involved in glucose sensing and insulin secretion was also restored to normal levels in DH islets at 4 weeks (Figure 7C). *Ctgf* expression remained slightly elevated, while *Sostdc1* levels were normal (Figure 7C). Expression of the remaining genes that were abnormal at P1 was normalized by weaning.

Oc1 is also expressed in liver and previous studies show that glycogenolysis and gluconeogenesis are impaired in the absence of Oc1 in the liver (Jacquemin et al., 1999, 2000). To determine whether a liver phenotype of Oc1 SH could be contributing to the normalization of glucose tolerance in DH mice by weaning, we examined glycogen deposition. Indeed, *Oc1* SH mice have increased glycogen deposition in the liver postweaning compared to WT. *Pdx1* SH have reduced hepatic glycogen compared with WT, likely due to decreased insulin output associated with the known impairment in β-cell function in these animals. DH mice have glycogen deposits that more closely resemble WT animals (Figure S7). Thus, the effects of the two genotypes on the liver appear to counteract one another, and it is possible that the "normalization" of DH glucose tolerance at weaning reflects compensation by the postnatal liver *Oc1* heterozygous phenotype rather than resolution of the endocrine pancreas developmental defect. The persistence of hormone gene expression defects in 4-week-old DH islets supports this concept.

DISCUSSION

Pdx1 and *Oc1* are co-expressed in MPCs very early in pancreas development and our previous work suggested that these two factors cooperate to regulate transcription of the endocrine progenitor transcription factor *Neurog3*. We hypothesized that a threshold of cooperative *Pdx1* and *Oc1* activity is required for realization of the endocrine program from MPCs. Transcriptome analyses of DH pancreas at E15.5 supported our hypothesis, revealing a highly compromised islet differentiation program with reduced expression of several key endocrine lineage

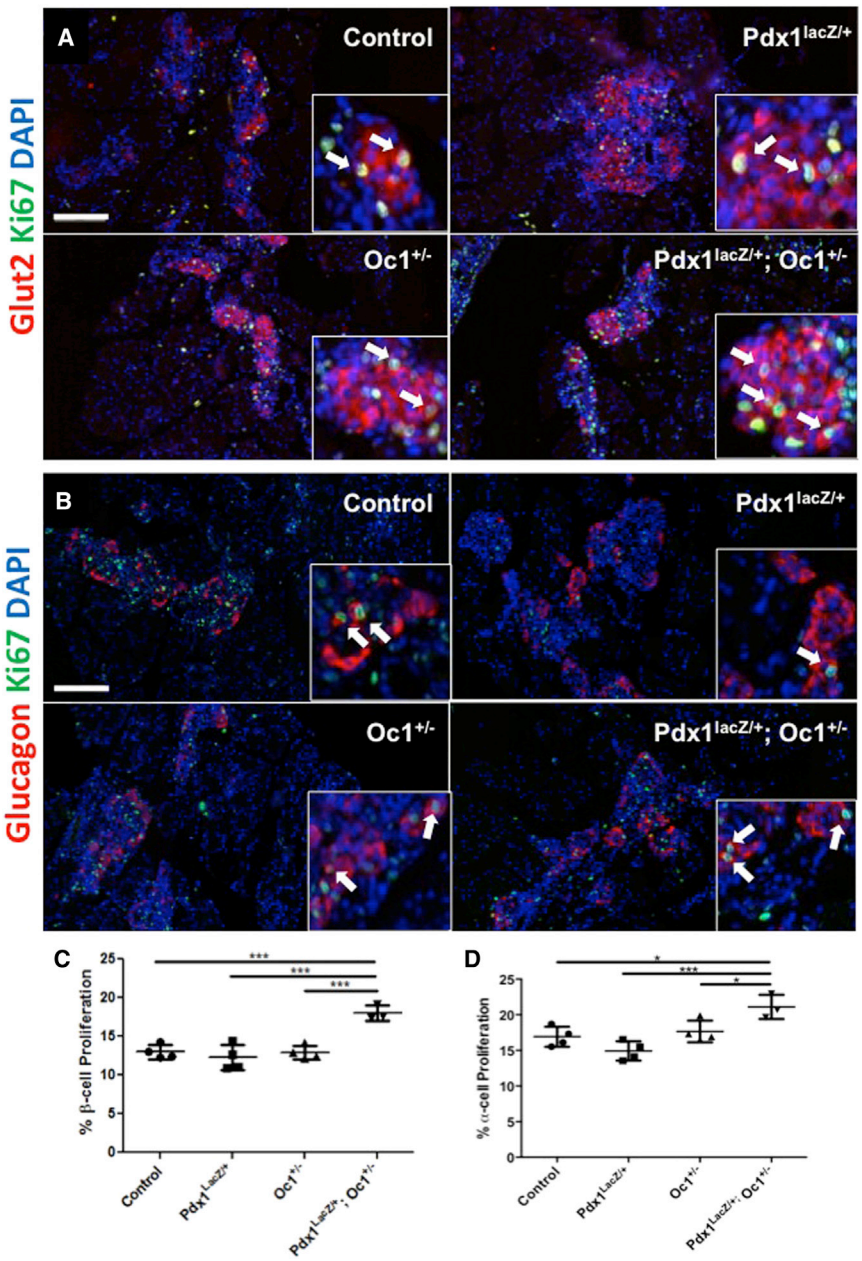

Figure 6. Increased α- and β-Cell Proliferation at E18.5 in DH Pancreata

(A and B) Representative images of proliferating β cells (A; red: Glut2 and green: Ki67) and α cells (B; red: glucagon and green: Ki67) at E18.5. α-cell proliferation at E18.5. The arrows point to proliferating hormone+ cells.

(C and D) Quantification of β-cell proliferation (C), and quantification of α-cell proliferation (D). The scale bar represents 100 μm (*p < 0.05; ***p < 0.001).

transcription factors, including *Neurog3* and *Pax6*, as well as multiple genes involved in mature β-cell function. Complementary morphological and physiological studies point to distinct effects of combined Pdx1 Oc1 reduction in endocrine progenitor specification and maturation with long-term effects on gene expression and function.

Our findings highlight a specific role for combined Pdx1 and Oc1 activity in establishing the endocrine progenitor program during the second wave of endocrine differentiation. Decreased dosage of *Pdx1-Oc1* impacted Neurog3+ cell numbers and subsequent endocrine differentiation at E15.5, but not at E13.5. Since the duration of the Neurog3+ state is short (estimated to be ~12 hr) (Bankaitis et al., 2015), the reduced numbers of Neurog3+ cells at E15.5 suggest that endocrine progenitors are more sensitive to *Pdx1-Oc1* dosage after E13.5. The normal number of glucagon+ and Pax6+ cells at E13.5, which derive from even earlier Neurog3+ progenitors (Johansson et al., 2007), supports this timeline of events. The relative increase in the proportion of delaminated cells expressing high Neurog3 levels in DH pancreata suggests that Pdx1 and Oc1 synergize to regulate the timing of transition from a Neurog3^{lo} to Neurog3^{hi} cell. The significance of duration of the Neurog3^{lo} state is currently unclear, but may affect subsequent steps in endocrine maturation.

The morphologic and transcriptomic analyses at E15.5 further suggest that Pdx1-Oc1 reduction leads to defective maturation of α- and β-cell lineages. This could occur either directly by decreased dosage of *Pdx1 and Oc1* and/or indirectly due to decreased expression of maturational and other endocrine cell markers per cell. The persistence of gene expression defects and elevated blood glucose at P1 indicate that the endocrine maturation program is not being completed successfully by birth in DH embryos.

Our results suggest that the combined activity of two structurally unrelated transcription factors within a progenitor-cell population affects subsequent differentiated cell populations (α and β cells) in which the two factors are not coexpressed. Expression of *Pdx1* and *Oc1* initially overlaps in MPCs and in bipotential duct/endocrine progenitors in the pancreatic epithelial trunk, but they diverge with Oc1 silenced and Pdx1 maintained almost exclusively in the pro-β cell lineage, as endocrine cells become specified. It is possible that the cooperative activity of Pdx1 and Oc1 in progenitors primes the cells for subsequent steps of the differentiation program. This notion that transcription factors can have temporally separated effects on cell behavior is not novel, as deletion of *Hnf4α* in the embryonic liver affects gene expression in differentiated hepatocytes long after its expression is downregulated (Kyrmizi et al., 2006). Similarly,

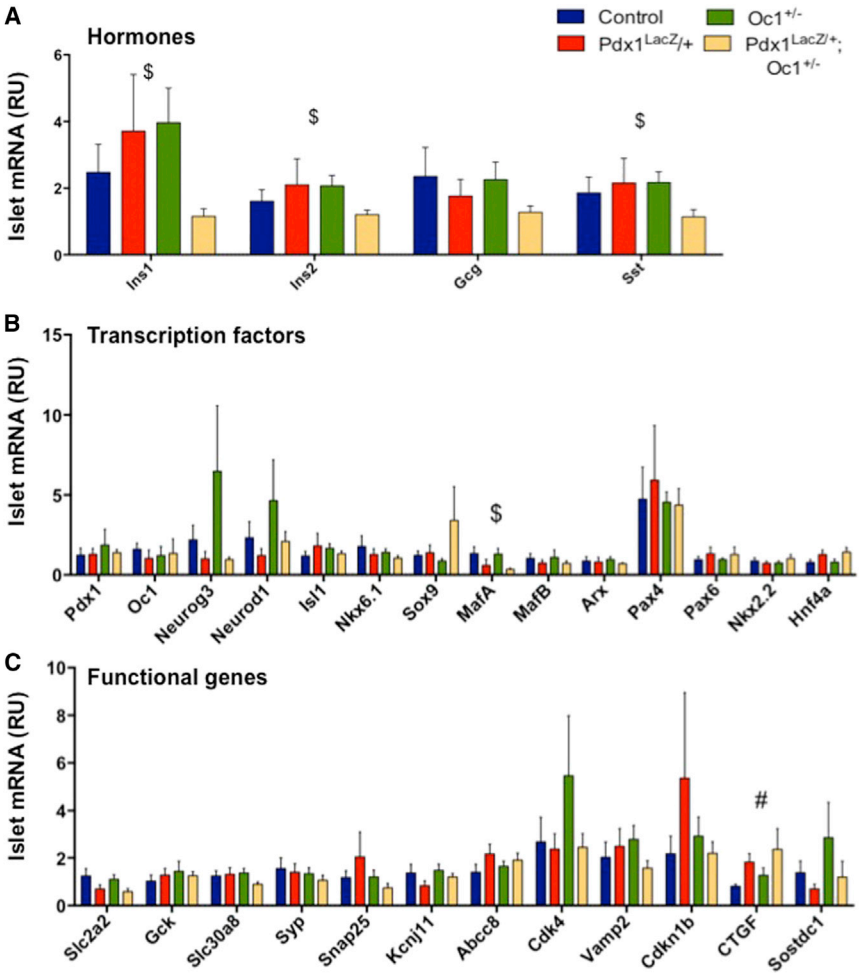

Figure 7. Early Reductions in Pdx1 and Oc1 Lead to Persistent Alterations in Islet Gene Expression at Weaning

(A–C) WT, Pdx1$^{LacZ/+}$, Oc1$^{+/-}$, and DH islets were analyzed for gene expression of hormones (A), endocrine-associated transcription factors (B), and secretory functional genes (C) at P28. p value for marked comparisons were < 0.05 by Kruskal-Wallace Test followed by two-tailed Student's t test (#, WT versus Pdx1$^{LacZ/+}$; $, Oc1$^{+/-}$ versus Pdx1$^{LacZ/+}$;Oc1$^{+/-}$). See also Figure S6.

the FoxD3 transcription factor, which is expressed in adult, quiescent β cells is required for β-cell proliferation during pregnancy despite being absent from maternal β cells during pregnancy (Plank et al., 2011). Our preferred model is that Pdx1 and Oc1 cooperate in multi- or bipotent pancreatic progenitors to establish a competency state that is realized in later stages of differentiation. Consistent with the Pdx1-Oc1 interaction occurring during a critical time sensitive period, inactivation of one *Oc1* allele in *Pdx1* heterozygotes later in development, using a later-acting pancreas-specific Cre driver line, did not impact endocrine function (unpublished data).

The transcriptome of DH islets at P1 suggests potential mechanisms for the partial recovery of insulin-expressing cells by birth, but also for the persistent defect in β-cell function. The reduced hormone expression could result in a feedback loop that upregulates expression of regulatory transcription factors to promote further endocrine differentiation. Additionally, the Oc1 target *Ctgf* is essential for embryonic β-cell proliferation; increased *Ctgf* expression in embryonic β cells induces both α- and β-cell proliferation, resulting in increased α- and β-cell mass at birth (Guney et al., 2011). The dramatic increase in *Ctgf* expression in DH islets likely increased α- and β-cell proliferation, thereby increasing α- and β-cell mass by P1. It is also possible that decreased insulin expression itself stimulates increased β-cell proliferation during late gestation. Mice with targeted disruption of both insulin genes in early development exhibit increased β-cell proliferation at E18.5, similar to the DH animals (Duvillié et al., 2002). Reduction in insulin and glucagon expression persists at P1, which likely contributes to impaired glucose homeostasis. In addition, the increase in *Sostdc1* could impair β-cell function. Sostdc1 is a BMP inhibitor and autocrine BMP signaling was suggested to enhance insulin secretion and glucose homeostasis (Goulley et al., 2007). Indeed, inactivation of *Sostdc1* enhances glucose-stimulated insulin secretion and glucose homeostasis (Henley et al., 2012). We were surprised to observe that glycemic control is restored in the majority of DH animals by weaning. *Sostdc1* expression was no longer elevated and most islet transcription factors, including *Pax4*, had returned to normal expression levels. However, decreases in insulin and glucagon expression persisted, now along with reduced *MafA* expression. Loss of MafA is associated with impaired β-cell function in adult mice (Artner et al., 2010; Zhang et al., 2005). Thus, it is likely that islets from DH animals still have reduced functionality.

Taken together, our results suggest that Pdx1 and Oc1 cooperate within pancreatic MPCs or bipotential trunk cells to promote endocrine specification and to establish a permissive state that allows for later steps of endocrine differentiation and function. Together, these two transcription factors initiate a network of gene expression beyond simple activation of the endocrine progenitor determinant, *Neurog3*. The concerted action of Oc1 and Pdx1 is critical for the timely functional maturation of endocrine cells. Their cooperative role in ESC or iPSC differentiation toward functional β cells has not been explored and should be considered.

EXPERIMENTAL PROCEDURES

Mutant and Transgenic Mice

Pdx1^{XSLacZ} (*Pdx1*^{LacZ}) animals are described in Offield et al. (1996). *Oc1* floxed mice are described in Zhang et al. (2009). The *Pdx1-Cre* and *Protamine-Cre*

(Prm-Cre) transgenes are described in Hingorani et al. (2003) and O'Gorman et al. (1997). The Rosa26-EYFP allele is described in Srinivas et al. (2001). Mice were on a mixed genetic background, were maintained on a 12 hr light/dark cycle, and provided food and water *ad libitum* (except where indicated). All mouse experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. Genotyping was performed using tail or ear punch DNA and the primer sets listed in Table S3.

Tissue Dissection, Preparation, and Histology

The morning of the vaginal plug was defined as E0.5. Digestive organs were fixed for 1–4 hr in 4% paraformaldehyde (PFA) at 4°C, dehydrated, cleared in Citrisolv (Fisher) or xylenes, and embedded in paraffin. Livers were fixed 24 hr in 4% PFA at 4°C, dehydrated, cleared in xylenes, and embedded in paraffin. For frozen embedding, tissue was fixed as above and placed in 30% sucrose overnight. Following 30 min in 50/50 FSC22 (frozen section compound, Leica) and 30% sucrose, frozen tissues were embedded in 100% FSC22 and frozen on dry ice. Paraffin embedded and FSC22 embedded tissues were cut at 5 μm and 7 μm, respectively. Paraffin-embedded tissues were deparaffinzed in Citrisolv or xylenes and rehydrated; frozen tissues were allowed to thaw for 30 min and permeabilized in 0.1% Triton 2 × 15 min in 1× PBS. Detection of MafA, MafB, glucagon, Pax6, and synaptophysin required antigen retrieval. Neurog3 immunolabeling required amplification with PerkinElmer tyramide tissue amplification. Antibody information is found in Supplemental Information. X-gal staining was performed as previously described (Wu et al., 1997). Periodic acid Schiff staining was performed following manufacturer's protocol (Sigma-Aldrich). Fluorescent and bright field images were captured using an Olympus BX41 microscope, the Aperio ScanScope microscope and slide scanner, or Nikon 600. Digital images were captured and quantified using MagnaFire software (Optronics Engineering), ImageScope software of the Aperio software suite for the insulin, glucagon, MafA, and MafB, or MetaMorph software for the Neurog3 and Pax6.

β Cell Mass, α/β Cell Area, and α:β Ratio

β cell mass was analyzed as in Riley et al. (2015). For α- or β-cell area, whole pancreata were serially sectioned at 5 μm and sections every 250 μm immunolabeled for insulin and glucagon. At least 1%–2% of the entire pancreas was imaged. Slides were imaged using MetaMorph or a macro built in Genie (Aperio System). Proportional insulin/glucagon-positive area was calculated by adding the insulin-positive and glucagon-positive area of each section and dividing it by the total pancreas area of each section. α:β cell ratio was calculated by dividing the number of glucagon-positive cells by the number of insulin-positive cells. Total endocrine area was calculated by measuring total area staining positive for synaptophysin as in Zhang et al. (2009).

Quantitative Real-Time PCR and TaqMan Low Density Array

Islet isolation required collagenase digestion of whole pancreas at 37°C and hand-picking of islets from exocrine tissue. Islets were placed immediately in 500 μl TRIzol reagent or RNAlater (Ambion), lysed by vortexing or homogenized using a Tissuemiser (Fisher Scientific), and RNA was isolated using the RNAqueous (Ambion) or RNeasy Micro/Mini Kits (QIAGEN). RNA concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent) at Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core. cDNA generated from neonatal islets required amplification with the SMARTer Pico PCR cDNA Synthesis Kit (Clontech). cDNA was prepared from 50–350 ng islet or pancreas RNA using the Superscript III First-Strand Synthesis System (Invitrogen). Real-time reactions were carried out in technical duplicates with iQ SYBR Green Supermix (Bio-Rad) on a CFX Real-Time PCR Detection System (Bio-Rad) in the Vanderbilt Molecular and Cellular Biology Resource Core. Primers used for hormone gene expression are listed in Table S3. TLDA required 150–300 ng cDNA. Genes were analyzed using TaqMan Universal PCR Mastermix (with UNG, Applied Biosystems) on custom-designed TLDA cards using a 7900HT Fast Real-Time PCR System. Data were analyzed using SDS RQ Study software (Applied Biosystems, Life Technologies). All samples were run in triplicate.

Pancreatic Insulin Content and Glucose Homeostasis

Pancreatic insulin content was measured as described in Zhang et al. (2009). Intraperitoneal glucose tolerance tests (IPGTT) were performed as in Henley et al. (2012).

Statistics

Results are expressed as mean + SEM. Statistical significance was calculated by Student's t test, one-way ANOVA with Tukey correction, or two-way ANOVA where applicable. $p < 0.05$ was considered significant.

ACCESSION NUMBER

The accession number for the RNA-seq data reported in this paper is GEO: GSE77896.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.040.

AUTHOR CONTRIBUTIONS

K.D.H. and P.A.K. planned and conducted experiments, analyzed the results, and contributed to manuscript writing under the supervision of M.G. D.E.S. planned and conducted experiments, analyzed the results, and contributed to manuscript writing under the supervision of D.A.S. K.-J.W. performed the bioinformatic analysis of the RNA-seq data set and supported the manuscript preparation. C.V.E.W. provided conceptual input and data interpretation and helped prepare the manuscript. D.A.S. and M.G. guided the project and wrote the manuscript.

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