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Preferential reduction of β cells derived from Pax6- MafB- pathway in *MafB* deficient mice

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Abstract

During pancreatic development insulin$^+$ cells co-express the transcription factors MafB and Pax6, and transition from a MafA$^-$ to MafA$^+$ state. To examine the role of *Pax6* and *MafB* in the development of β cells, we analyzed embryonic pancreata from *Pax6*- and *MafB*- deficient mice. *Pax6* deficiency, as manifest in the *Pax6*$^{Sey\text{-}Neu}$ allele, reduced not only the number of cells expressing insulin or glucagon, but also the number of MafB, PDX-1 and MafA expressing cells. We show that *MafB* can directly activate expression of insulin and glucagon, and a MafB protein engineered to contain N248S mutation in the *MafB* (*kr*^{ENU}) results in significantly reduced activation. Furthermore, pancreata from *MafB* deficient (*kr*^{ENU}/*kr*^{ENU}) mice exhibited reduced number of cells expressing insulin, glucagon, PDX-1 and MafA, with only a minor reduction in MafB expressing cells. *MafB* deficiency does not affect endocrine specification but does affect the lineage commitment of the endocrine cells and their maturation. Similar to *Pax6* deficient mice, *MafB* deficient mice showed reductions both in insulin and glucagon expressing cells and in the ability of MafB and PDX-1 expressing cells to activate expression of these hormones. However, *MafB* deficient mice exhibited no effect on Pax6 expression. These results suggest that MafB may function as a downstream mediator of Pax6 in regulating the specification of insulin and glucagon expressing cells. Interestingly, the remaining insulin$^+$ cells in these knockouts preferentially express Hb9, suggesting the existence of an alternate pathway for the generation of insulin expressing cells, even in the absence of *Pax6* and *MafB* function. Thus, *Pax6* acts upstream of *MafB*, which in turn may trigger the expression of insulin and regulate the *PDX-1* and *MafA* expression required for β-cell maturation.

Keywords

MafB; *MafA*; *Pax6*; *Sey*; *Kreisler*; insulin gene transcription factor; pancreatic development; endocrine differentiation; pancreatic islets

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Introduction

To develop reliable sources of glucose-responsive β-cells for the treatment of diabetes, it is essential to understand how β-cells form during development. The pancreas develops as a dorsal and ventral evagination from the developing foregut that expresses the homeodomain transcription factor PDX-1 (Edlund, 2002; Leonard et al., 1993; Miller et al., 1994; Ohlsson et al., 1993). PDX-1 is essential for the normal proliferation and differentiation of embryonic pancreatic precursors (Edlund, 2002; Jonsson et al., 1994; Offield et al., 1996). Another transcription factor, Ngn3, regulates the differentiation of endocrine cells (Gradwohl et al., 2000). In addition, several other transcription factors play critical roles in both pancreatic development and endocrine differentiation (Collombat et al., 2006; Grapin-Botton and Melton, 2000; Jensen, 2004; Kim and MacDonald, 2002; Murtaugh, 2007). For example, transcription factors Arx and Pax4 play critical roles in regulating specification of endocrine progenitors towards α- or β- and δ-cell fates (Collombat et al., 2005; Collombat et al., 2006; Collombat et al., 2007). Pax6 is required for maintaining these hormone-expressing cells and for regulating specification of ghrelin-expressing ε-cells (Ashery-Padan et al., 2004; Heller et al., 2005; Wang et al., 2004). In addition, members of the Nkx and Maf families regulate the differentiation of hormone positive cells.

The identification of MafA as a β-cell specific basic leucine zipper (bZIP) transcription factor has initiated the systematic characterization of Maf factors in pancreatic development and in β–cell function (Olbrot et al., 2002; Kataoka et al., 2002; Matsuoka et al., 2003). MafA (formerly known as RIPE3b1) is a glucose-responsive insulin gene transcription factor that binds to the insulin Maf responsive element (MARE) and activates insulin gene expression (Sharma and Stein, 1994; Olbrot et al., 2002; Kataoka et al., 2002; Matsuoka et al., 2003). Another Maf factor, MafB [also known as Kreisler (Krml1/MafB)], is expressed in pancreatic endocrine cells and regulates differentiation of several cell types (Eichmann et al., 1997; Manzanares et al., 1997; Moriguchi et al., 2006; Sieweke et al., 1996). The gene was first identified as the locus mutated in the Kreisler (kr) mouse, and a second ethylnitrosourea (ENU) induced mutation [kr^{ENU} (also known as $MafB^{ENU}$)] in this gene has been described (Cordes and Barsh, 1994). MafB, expressed only in α-cells in adults, is expressed in the insulin positive cells during pancreatic development (Artner et al., 2006; Nishimura et al., 2006). We proposed that endocrine progenitors after committing to the insulin positive lineage continue a further differentiation/maturation process. Early insulin$^+$ cells express MafB, and following the induction of PDX-1, mature into insulin$^+$ and MafA$^+$ cells (Nishimura et al., 2006). *MafA* deficient Mice have normal pancreatic islets at birth, but the ratio of β to α cells gradually reduce after birth, resulting in glucose intolerance by 8–12 weeks (Zhang et al., 2005). This phenotype suggests a critical role of *MafA* in the maturation step required for the function and survival of β cells. The importance of this maturation step is highlighted by a recent publication on the differentiation of human ES cells into insulin$^+$ cells (D'Amour et al., 2006). Although these insulin$^+$ cells expressed MafB, they did not maintain the expression of PDX-1 and did not secrete insulin in response to glucose (D'Amour et al., 2006). We hypothesize that these cells may not have switched from MafB$^+$MafA$^-$ to MafB$^-$MafA$^+$ state. It is likely that the lack of MafA expression prevents early insulin$^+$ cells from expressing the downstream targets of *MafA* that are required for the insulin synthesis and secretion (Kato et al., 2006; Wang et al., 2007). Thus, elucidation of the mechanisms that regulate the conversion of insulin$^+$ cells into mature functional β-cells should facilitate our ability to generate glucose-responsive, insulin-producing cells for cell-based diabetes therapy.

In the present study, we characterized the roles of *Pax6* and *MafB* in the differentiation of insulin expressing cells. Loss of either *Pax6* or *MafB* function results in reduced numbers of cells that express insulin, glucagon, PDX-1 or MafA, and decreases the ability of MafB$^+$ and

PDX-1^{+} cells to express insulin. Our results suggest that *MafB* may function as a downstream mediator of *Pax6* in regulating the formation of insulin and glucagon positive cells. We present data supporting the initiation of insulin expression in the endocrine progenitors by at least two pathways: the formation of insulin-expressing cells from one pathway requires the function of *Pax6* and *MafB*, while specification of the remaining insulin-expressing cells may occurs via a *Pax4-Hb9* dependent pathway. These results suggest that *MafB* can regulate the initiation of insulin expression in some, but not all, endocrine progenitors destined to express insulin.

Material and methods

Construction of expression vectors

Various expression vectors were constructed by conventional molecular biology techniques. Insulin promoter luciferase reporter constructs −238 WT LUC and its derivative mutant 121–22m LUC have been described previously (Harrington and Sharma, 2001). The glucagon promoter luciferase reporter GLU LUC was kindly provided by Dr. Dan Drucker (Toronto, Canada); the PDX-1 promoter luciferase reporter (Eto et al., 2007), by Dr. Melissa Thomas (Massachusetts General Hospital, Boston, MA). The expression vectors pcDNA MafA, pCMVSport6 MafB and pcDNA cMaf have been described previously (Nishimura et al., 2006). pCMVSport6 kr^{ENU} expression vector was generated by PCR amplification of homozygous kr^{ENU} mouse genomic DNA with oligonucleotide primers (5′-GACCCGCCAGGACTCACAGAAA-3′and 5′-CCGCGCAACAGCTACCCACTA-3′) for 35 cycles of 30 sec at 94.0C, 40 sec at 62.3C and 1 min at 72C. PCR product was digested with AflII and PvuII and was used to replace the corresponding fragment from pCMVSport6 MafB to give full-length kr^{ENU} cDNA containing a point mutation at base pair 743 in the DNA binding region of *MafB*. Constructs were confirmed by sequencing.

Luciferase Assays

HeLa cells were transfected with 1μg of reporter constructs of −238 WT LUC, 121–22m LUC, GLU LUC or PDX-1 promoter, 1 μg of pSVβ-gal plasmid (Promega, Madison, WI) as an internal control and 1 μg of indicated expression vector. Whole cell extracts were prepared and luciferase activity was measured as previously described (Nishimura et al., 2005).

Animals

Mice with mutation in *kreisler/MafB* generated by ethylnitrosourea (ENU) induced chemical mutagenesis have been reported earlier (Cordes and Barsh, 1994). Mutagenesis results in a point mutation in the DNA-binding region of MafB that changes amino acid 248 from asparagine to serine (*kr^{ENU}* or *MafB^{ENU}*) leading to compromised DNA binding affinity of kr^{ENU} to Maf Response Element (MARE)(Sadl et al., 2002). Heterozygous mice with the ENU-induced *kr^{ENU}* allele were received from Dr. Greg Barsh (Stanford, CA), were maintained on C57Bl/6J background for more than 10 generation and used in this study. Small eye mutant (*Pax6$^{Sey\text{-}Neu}$*) mice (Glaser et al., 1994; Hill et al., 1991; Sander et al., 1997), also generated by chemical mutagenesis, have a point mutation in a splice donor site of 3′ end of the homeobox that results in incorrect splicing and truncated Pax6 protein lacking the carboxy-terminal 115 amino acids corresponding to the transcriptional activation domain. The day of vaginal-plug discovery was designated as embryonic day E 0.5.

Immunohistochemistry and quantification

Immunohistochemical and immunofluorescent analyses were performed as described previously (Nishimura et al., 2006). The primary antibodies used were: guinea pig anti-insulin (Linco, Billerica, MA); guinea pig anti-glucagon (Linco); rabbit anti-somatostatin (Santa Cruz Biotechnology, CA); rabbit anti-pancreatic polypeptide (Linco); rabbit anti-Ghrelin (Phoenix

Pharmaceuticals, Belmont, CA); rabbit Hb9 antibody (provided by Dr. S. Pfaff); rabbit anti-MafB (Bethyl Laboratories, Montgomery, TX); mouse Nkx2.2 (Developmental Studies Hybridoma Bank, Iowa City, IA); rabbit anti-Nkx6.1 (provided by Dr. P. Serup); mouse Neurogenin3 (provided by Drs. Serup and Madsen, Beta Cell Biology Consortium); goat anti-PDX-1 (provided by Dr. C. Wright); rabbit anti-Pax6 (Covance, Princeton, NJ). Rabbit anti-MafA antibody was described previously (Nishimura et al., 2006). For amplification, biotinylated anti-rabbit, anti-mouse or anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) were used at a 1:400 dilution followed by streptavidin-conjugated Texas red (1:400) or streptavidin-conjugated Alexa fluor 488 (1:400) (Molecular Probes, Eugene, OR). The secondary antibodies were: FITC- or Texas red-conjugated anti-rabbit, anti-mouse or anti-guinea pig (Jackson ImmunoResearch). Nuclear counterstaining was performed by DAPI mounting medium (Vector, Burlingame, CA). For the double staining using rabbit anti-MafB and rabbit anti-Ghrelin antibodies, pancreatic sections were first stained with rabbit anti-MafB antibody using secondary biotinylated anti-rabbit antibody followed by streptavidin-conjugated Alexa flour 488, and images of areas expressing MafB were taken. The same imaged sections were next stained with rabbit anti-Ghrelin antibody and Texas red conjugated anti-rabbit secondary antibody and images of the same areas were taken again to examine co-expression of MafB and Ghrelin. The results of immunohistochemistry and quantification were derived from several replicates of at least three embryonic pancreases for each genotype. Every 10^{th} section of embryonic pancreas was analyzed for immunofluorescent hormone-positive areas as a proportion of epithelial area. For total number of transcription factor$^+$ cells (for Figure 2A), every 10^{th} section was stained with DAB and counted. For number of transcription factor$^+$ insulin$^+$ cells per total number of indicated transcription factor$^+$ cells or total number of insulin$^+$ cells, cells were counted from at least three different pancreatic blocks. Immunofluorescence data were collected as confocal images on Zeiss LSM410 (Zeiss, Thornwood, NY); all DAB-stained images were analyzed on an Olympus BH2 bright field microscope. Images were quantitated using NIH Image J software.

Western blot analysis

Cell lysates used in luciferase assays were subjected to 10% SDS-PAGE and gels were transferred to polyvinyldenedifluoride membrane filters (BioRad Laboratories, Hercules, CA). Western bolt analyses were performed using MafB and β-actin antibody (Santa Cruz Biotechnology, CA) and detected with horseradish peroxidase using ECL reagents (Amersham).

Results

Pax6 deficiency results in reduced numbers of MafA, MafB and PDX-1 expressing cells

Previously, we showed that Pax6$^+$ insulin$^+$ cells transition from a MafB$^+$ MafA$^-$ stage to a mature MafA$^+$ MafB$^-$ phenotype (Nishimura et al., 2006). An association between Pax6 and Maf factors has been reported in different systems (Reza et al., 2002; Reza and Yasuda, 2004). Pax6 deficiency results in the inability of endocrine cells to express markers of terminal differentiation and maturation (Ashery-Padan et al., 2004). Hence, we examined the role of *Pax6* in regulating the expression and function of Maf factors during pancreatic development. E15.5 *Pax6$^{Sey\text{-}Neu/Sey\text{-}Neu}$* (subsequently referred as *Sey^{Neu}/Sey^{Neu}*) pancreata completely lack Pax6 expressing cells (antibody recognizes C-terminal domain of Pax6), while wild type littermates have significant numbers of Pax6$^+$ cells, some of which co-expressed insulin (Suppl. Figure 1). As reported earlier (Sander et al., 1997; Wang et al., 2004), loss of *Pax6* results in a significant reduction in insulin and glucagon-expressing cells but does not affect the pancreatic expression of Nkx transcription factors at E15.5. Nkx6.1 expression in the pancreas marks the area of endocrine differentiation and, as shown in Suppl. Figure 1, was unaffected by loss of *Pax6* function. Similarly, expression of the pro-endocrine gene Ngn3 was not

affected in *Pax6* deficient mice (Suppl. Figure 1), consistent with the earlier observation (Ashery-Padan et al., 2004; Wang et al., 2004) that *Pax6* is not required for the initiation of endocrine differentiation.

We next examined whether the loss of Pax6 affected the expression of Maf factors. The number of cells in *Pax6*-deficient (Sey^{Neu}/Sey^{Neu}) E15.5 pancreas expressing MafB, PDX-1 (induced PDX-1 expression during secondary transition or PDX-1^{high}) and MafA (Figures 1B–D, F–H) were reduced. Some cells still expressed these transcription factors demonstrating the presence of a *Pax6* independent mechanism for their expression. The number of insulin$^+$ cells expressing these three transcription factors was significantly reduced in Sey^{Neu}/Sey^{Neu} mice (Figures 1B–D, F–H). Thus, the loss of *Pax6* function not only reduces the expression of terminal hormone markers but also reduces the expression of the transcription factors implicated in the terminal differentiation process.

We next examined whether expression of MafB, PDX-1^{high} and MafA were maintained in insulin$^+$ cells in the absence of *Pax6* function (Figures 1J–L, N–P). As reported previously (Sander et al., 1997), at E17.5 the number of insulin$^+$ cells remained low in the Sey^{Neu}/Sey^{Neu} embryos. Additionally, MafA and PDX-1 expression was restricted to insulin$^+$ cells, and the number of cells expressing these transcription factors was reduced (Figures 1K, L, O, P); most Nkx6.1$^+$ cells expressed insulin, with only occasional Nkx6.1$^+$ insulin$^-$ cells (Figures 1I, M). We also observed a greater reduction in insulin$^+$ and MafB$^+$ cells in the Sey^{Neu}/Sey^{Neu} embryos at E17.5 (Figure 1N) than at E15.5 (Figure 1F).

Pax6-deficiency reduces the proportion of MafB$^+$ and PDX-1 ^{high} cells expressing insulin

Our observation that MafB$^+$ pancreatic cells are present in the Sey^{Neu}/Sey^{Neu} embryos (Figures 1F, N) is consistent with the conclusion of Artner and colleagues (Artner et al., 2006) that *Pax6* and *Pax4* are not essential for MafB expression. To determine whether *Pax6* deficiency reduces only the number of cells expressing *MafB*, *PDX-1* and *MafA* or has additional effects on their function, we quantified stained E15.5 pancreatic sections (Figure 2). The number of MafB$^+$ cells was significantly reduced in Sey^{Neu}/Sey^{Neu} pancreas (Figures 1F, 2A). While the number of Nkx6.1$^+$ cells did not change, those of insulin$^+$, MafB$^+$ and MafA$^+$ cells were reduced by about 75% (Figures 2A), clearly demonstrating that loss of Pax6 function significantly affects MafB$^+$ and MafA$^+$ cells.

Since *Pax6* is not required for the specification of endocrine cells but it regulates their ability to acquire final differentiation markers (Suppl. Figure 1, Ashery-Padan et al., 2004; Heller et al., 2005; Wang et al., 2004), we analyzed in E15.5 pancreas whether the loss of *Pax6* expression affected 1) the ability of MafB$^+$, PDX-1^{high} and MafA$^+$ cells to express insulin and 2) the ability of remaining insulin$^+$ cells to express normal levels of these factors. MafA was expressed only in insulin$^+$ cells, while several MafB$^+$ and PDX-1^{high} cells did not express insulin. Similarly, several insulin$^+$ cells did not express these transcription factors (Figures 1). Quantification was performed to determine the proportion of transcription factor$^+$ cells expressing insulin (Figure 2B) and the proportion of insulin$^+$ cells expressing different transcription factors (Figure 2C), as depicted in the Venn diagrams. Nearly 65–70% of MafB$^+$ cells (390 MafB$^+$, 273 Ins$^+$ and 259 MafB$^+$Ins$^+$ cells; 259 out of 390), 75–80% of PDX-1^{high} cells (346 PDX-1^{high}, 324 Ins$^+$ and 276 PDX1^{high}Ins$^+$ cells) and all MafA$^+$ cells (179 MafA$^+$, 268 Ins$^+$ and 179 MafA$^+$Ins$^+$ cells) expressed insulin in wild type pancreas whereas loss of *Pax6* function significantly reduced the ability of MafB$^+$ (~35%)(125 MafB$^+$, 58 Ins$^+$ and 49 MafB$^+$Ins$^+$ cells; 49 out of 125) and PDX-1^{high} (~45%)(82 PDX-1^{high}, 58 Ins$^+$ and 49 PDX1^{high}Ins$^+$ cells) cells to co-express insulin. Importantly, while the number of insulin$^+$ cells was reduced in Sey^{Neu}/Sey^{Neu} pancreas, there was no change in the proportion of MafA$^+$ cells expressing insulin (100%)(50 MafA$^+$, 81 Ins$^+$ and 50 MafA$^+$Ins$^+$ cells), which suggests that MafA is expressed only after insulin$^+$ cells have reached

a latter maturation stage. Next, we quantified the proportion of insulin$^+$ cells expressing these transcription factors (Figure 2C). Unlike the reduction in the proportion of MafB$^+$ and PDX-1^{high} cells expressing insulin in the absence of *Pax6*, the proportion of insulin$^+$ cells expressing these different transcription factors in wild type and *Sey^{Neu}/Sey^{Neu}* mice remained unchanged. Nearly 95%, 75% and 65% of insulin$^+$ cells were MafB$^+$, PDX-1^{high} and MafA$^+$, respectively, in both wild type and *Pax6* deficient mice (Figure 2C). This observation suggests that the remaining insulin$^+$ cells derived from a *Pax6* independent pathway appear to undergo normal maturation.

Pax6 deficiency reduces the proportion of MafB$^+$ cells expressing glucagon

Since *Pax6* deficiency reduced the number of MafB$^+$ cells that co-express insulin, we also used *Sey^{Neu}/Sey^{Neu}* mice to examine whether the MafB$^+$ cells that did not express insulin instead expressed glucagon. At E12.5, Nkx6.1 and PDX-1, two transcription factors that mark the developing pancreatic epithelium at this stage, are not expressed in the majority of glucagon$^+$ cells in either wild type or *Sey^{Neu}/Sey^{Neu}* pancreas (Figures 3A, B, E, F). It is important to note that at E12.5, PDX-1 expression was not affected by *Pax6* loss of function. Thus, the reduced PDX-1 in older *Sey^{Neu}/Sey^{Neu}* embryos (Figures 1, 2) may reflect the ability of *Pax6* to selectively regulate the induction of PDX-1 expression (PDX-1^{high}) in insulin$^+$ cells during secondary transition.

The quantification of glucagon$^+$ and MafB$^+$ cells at E12.5 and 15.5 provides important insights into the roles of *Pax6* and *MafB* in the induction of hormone expression and in the differentiation of early endocrine cells. At E12.5 in wild type pancreas, the majority of MafB$^+$ cells co-express glucagon, and conversely the majority of glucagon$^+$ cells are MafB$^+$ (260 MafB$^+$, 251 Glu$^+$ and 239 MafB$^+$Glu$^+$ cells). The number of MafB$^+$ cells at E12.5 in *Sey^{Neu}/Sey^{Neu}* pancreata was extremely low, but all MafB$^+$ cells co-expressed glucagon (7 MafB$^+$, 37 Glu$^+$ and 7 MafB$^+$Glu$^+$ cells), and a number of glucagon$^+$ cells did not express MafB (Figure 3G). At E15.5, with the induction of insulin expression, nearly 40% MafB$^+$ cells expressed glucagon in wild type (500 MafB$^+$, 204 Glu$^+$ and 198 MafB$^+$Glu$^+$ cells), and this number decreased to less than 10% in *Sey^{Neu}/Sey^{Neu}* pancreata (216 MafB$^+$, 48 Glu$^+$ and 15 MafB$^+$Glu$^+$ cells) (Figure 3I). Nearly 65% of MafB$^+$ cells at E15.5 express insulin in wild type pancreas (Figure 2B), suggesting that most of the MafB$^+$ cells at E15.5 in wild type either express insulin or glucagon. Together, these results (Figures 2, 3) suggest increased numbers of MafB$^+$ cells that do not express either insulin or glucagon in *Sey^{Neu}/Sey^{Neu}* pancreas at E15.5. Deficiency of Pax6 (Heller et al 2005) and *Nkx2.2* (possibly via reducing *Pax6* expression) (Prado et al 2004) changes cell-fate of endocrine precursors to ghrelin expressing ε-cells at the expense of the other endocrine cell types. We observed a similar increase in ghrelin only expressing ε-cells in Pax6 deficient mice, but the ghrelin-expressing ε-cells did not express MafB (Suppl. Figure 2).

MafB can activate insulin and glucagon gene expression

To determine whether MafB itself plays an important role in the differentiation of endocrine cells, we first examined the ability of ENU induced N248S mutation in the MafB coding region (kr^{ENU}) to activate the insulin and glucagon promoters. Two plasmid constructs were made from wild-type MafB plasmid (pCMV Sport MafB), one carrying the ENU induced N248S mutation (krENU) and another lacking the C-terminal DNA binding and bZIP domain (ΔCMafB) (Figure 4A). All three plasmids transfected into HeLa cells expressed a protein recognized by anti- MafB antibody in Western blots (Figure 4B). Quantification with normalization to β-actin (Figure 4C) indicated that kr^{ENU} is expressed at a level slightly higher than the wild-type MafB protein. Co-transfection of these plasmids with insulin (Figure 4D) or glucagon (Figure 4E) promoter luciferase reporter constructs demonstrated that wild type MafB activated both insulin and glucagon promoters, as previously reported (Nishimura et al.,

2006; Zhao et al., 2005). ΔCMafB was ineffective in inducing expression of these genes, and kr^{ENU} showed significant impairment in its ability to activate insulin and glucagon gene expression (Figures 4D, E). Co-transfecting these expression plasmids with the insulin reporter construct -122.121m Luc (Harrington and Sharma, 2001) containing a mutated insulin MARE significantly reduced the MafB and kr^{ENU} mediated activation of luciferase, suggesting that kr^{ENU} retains some residual activity to regulate insulin expression via its cognate binding site. Thus, in principal, kr^{ENU} mice should represent a model of *MafB* deficiency, but not absence, of *MafB* function.

MafB deficiency results in reduction of insulin$^+$ and glucagon$^+$ cells

In adult mouse pancreas MafB expression is restricted to α-cells, but during embryonic development MafB expression is also seen in insulin$^+$ cells (Nishimura et al., 2006). Since our results indicate a correlation between reduced MafB expression and the number of insulin$^+$ and glucagon$^+$ cells in Sey^{Neu}/Sey^{Neu} mice (Figures 1–3), we hypothesized that MafB might regulate the differentiation of both α- and β-cells. To test this hypothesis we examined the effect of *MafB* deficiency on endocrine differentiation using kr^{ENU}/kr^{ENU} mice. At E15.5, *MafB* deficiency did not affect pancreatic appearance but insulin$^+$ and glucagon$^+$ cells were drastically reduced (Figure 5A, B). As a percentage of total epithelial area, insulin$^+$ and glucagon$^+$ area were reduced to nearly 55% and 60%, respectively (Figures 5C, D). Thus, as in Sey^{Neu}/Sey^{Neu} mice, *MafB* deficiency results in a reduction, but not complete loss, of insulin$^+$ and glucagon$^+$ cells.

MafB deficiency does not affect the initiation of endocrine differentiation

We next examined the mechanism underlying the reduced number of insulin$^+$ and glucagon$^+$ cells in kr^{ENU}/kr^{ENU} mice. During pancreatic development, the transcription factor Ngn3 marks the progenitors that give rise to endocrine cells, while Pax6 marks cells further along the endocrine differentiation pathway. Nkx2.2 is expressed in early epithelial progenitors and subsequently becomes restricted to endocrine cells. Pancreatic sections from E15.5 wild type and kr^{ENU}/kr^{ENU} mice were immunostained for Ngn3, Nkx2.2 and Pax6 (Figures 6). Although kr^{ENU}/kr^{ENU} mice have reduced insulin$^+$ cells, the number of Ngn3$^+$ cells was unaffected (Figures 6A, D), suggesting that *MafB* deficiency does not affect the specification of endocrine differentiation. Similarly, *MafB* deficiency did not affect Nkx2.2$^+$ or Pax6$^+$ cell number (Figures 6B, C, E, F). These results show that the kr^{ENU} mutation does not affect pancreatic precursors or formation of endocrine cells and that the reduction in the number of insulin$^+$ and glucagon$^+$ cells (Figures 5 and 6) occurs in the absence of any change in the levels of Pax6$^+$ cells (Figures 6C, F).

Effect of MafB deficiency on MafB$^+$, PDX-1^{high} and MafA$^+$ cells

Whether the homozygous kr^{ENU} mutation affected the maturation of insulin$^+$ cells was addressed next. Since the kr^{ENU} allele expresses a mutant protein recognized by the MafB antibody (Figure 4B), we examined the effect of *MafB* deficiency on MafB expression. In the kr^{ENU}/kr^{ENU} pancreas at E15.5 Nkx6.1$^+$ cells were not affected by *MafB* deficiency (Figures 7B, F), insulin$^+$ MafB$^+$, insulin$^-$ MafB$^+$ cells and occasional insulin$^+$ MafB$^-$ cells were seen, and the total number of MafB$^+$ cells showed a modest increase (~20%) compared to the wild type (Figure 7A, E). Unlike the effect of *MafB* deficiency on its own expression, the number of PDX-1^{high} and MafA$^+$ cells were reduced (Figure 7C, D, G, H), suggesting that deficient *MafB* function was sufficient to reduce, but not eliminate, cells expressing PDX-1^{high} and MafA. At E15.5, nearly 60% of MafB$^+$ cells (175 MafB$^+$, 110 Ins$^+$ and 105 MafB$^+$Ins$^+$ cells) expressed insulin in wild type, but this number dropped to ~25% in kr^{ENU}/kr^{ENU} (238 MafB$^+$, 68 Ins$^+$ and 65 MafB$^+$Ins$^+$ cells) (Figure 7I). This observation suggests that a functionally compromised MafB isoform (kr^{ENU}) prevents a significant proportion of

differentiating endocrine cells from expressing insulin. The deficiency of *MafB* function also resulted in a reduced proportion of PDX-1^{high} cells co-expressing insulin (128 PDX-1^{high}, 122 Ins^{+} and 92 PDX1^{high}Ins^{+} cells in wild type vs. 107 PDX-1^{high}, 90 Ins^{+} and 65 PDX1^{high}Ins^{+} cells in *kr*^{ENU}/*kr*^{ENU} pancreas). This reduced ability of MafB^{+} and PDX-1^{high} cells to co-express insulin in *MafB* deficient mice was similar to that observed in *Sey*^{Neu}/*Sey*^{Neu} mice (Figure 2). In *kr*^{ENU}/*kr*^{ENU} pancreas all MafA^{+} cells were insulin^{+}, but not all insulin^{+} cells expressed MafA (75 MafA^{+}, 111 Ins^{+} and 75 MafA^{+}Ins^{+} cells in wild type vs. 60 MafA^{+}, 92 Ins^{+} and 60 MafA^{+}Ins^{+} cells *kr*^{ENU}/*kr*^{ENU} pancreas). Interestingly, the proportion of insulin^{+} cells co-expressing the different transcription factors was unaltered in wild type and *kr*^{ENU}/*kr*^{ENU} mice (Figure 7J): nearly 95%, 75% and 65% of insulin^{+} cells co-expressed MafB, PDX-1^{high} and MafA, respectively.

The ability of MafB to directly activate PDX-1 expression was examined by co-transfecting −4.5kb PDX-1:luciferase plasmid (Eto et al., 2007) and either MafA, MafB or cMaf plasmids in HeLa cells (Figure 7K). MafA, MafB and cMaf were all capable of activating PDX-1 expression, consistent with the reported ability of MafA to induce PDX-1 expression (Samaras et al., 2003).

Unlike Pax6, MafB deficiency does not affect the cell-fate decision of endocrine cells

As stated above, *Sey*^{Neu}/*Sey*^{Neu} and *kr*^{ENU}/*kr*^{ENU} mice show very similar pattern of reductions of insulin^{+} and glucagon^{+} cells. Loss of *Pax6* function results in endocrine cells that do not express insulin or glucagon and that acquire an alternate cell-fate with ghrelin expression (Prado et al 2004; Heller et al., 2005). However, in E15.5 *kr*^{ENU}/*kr*^{ENU} the reduction in insulin^{+} and glucagon^{+} cells was not accompanied by an increase in somatostatin^{+} or ghrelin^{+} cells (Figure 8 and Artner et. al., 2007). This finding is consistent with our observation in *Sey*^{Neu}/*Sey*^{Neu} mice that the ghrelin^{+} ε cells do not express MafB (Suppl. Figure 2). Thus, *MafB* deficiency, unlike *Pax6* deficiency, does not trigger endocrine cells to acquire alternate cell-fates.

An increased proportion of the remaining insulin^{+} cells in kr^{ENU}/kr^{ENU} pancreata express Hb9

The presence of insulin^{+} and glucagon^{+} cells in *Sey*^{Neu}/*Sey*^{Neu} and *kr*^{ENU}/*kr*^{ENU} mice suggests the induction of these hormones can occur via a pathway independent of *Pax6* or *MafB* function. Two parallel pathways have been proposed to trigger the formation of insulin^{+} cells: one involving *Nkx2.2* and *Pax4* in regulating the Hb9 and PDX-1 dependent expression of insulin, and a second involving Nkx2.2, Pax6 and PDX-1 (Wang et al., 2004). Wang and colleagues reported that at E14.5, Pax6 deficiency had no obvious effect on Hb9 expression; we also observed cells expressing Hb9 in our Pax6 deficient mice at E15.5 (Suppl. Figure 3). Since our data suggest that *MafB* functions downstream of *Pax6*, we examined whether *MafB* deficiency differentially affected the formation of insulin^{+} cells from either the *Nkx2.2-Pax6* or *Pax4-Hb9* pathways by quantifying immunostained E15.5 wild type and *kr*^{ENU}/*kr*^{ENU} pancreata. Here, *MafB* deficiency increased the proportion of insulin^{+} cells expressing Hb9 but had no effect on the ability of Hb9^{+} cells to co-express insulin (220 Hb9^{+}, 227 Ins^{+} and 164 Hb9^{+}Ins^{+} cells in wild type vs. 139 Hb9^{+}, 111 Ins^{+} and 104 Hb9^{+}Ins^{+} cells *kr*^{ENU}/*kr*^{ENU} pancreas) (Figures 9). The increased proportion of insulin^{+} cells expressing Hb9 in *kr*^{ENU}/*kr*^{ENU} mice suggests that these remaining insulin^{+} cells may be specified via the *Pax4-Hb9* pathway.

Discussion

Previously we proposed that the maturation of insulin^{+} cells during embryonic development requires a switch from an insulin^{+} MafB^{+} state to an insulin^{+} MafA^{+} state via an intermediate

PDX-1^{high} stage. Here we use *Pax6* and *MafB* deficient mice to demonstrate that the deficiency of either *Pax6* or *MafB* function results in reduced number of cells expressing insulin, glucagon, PDX-1 and MafA (Figures 1, 3, 5, 7). Comparable proportions of Ngn3 and MafB expressing cells in *kr*^{ENU}/*kr*^{ENU} and wild type pancreata (Figures 6, 7) suggest that similar to *Pax6* deficiency (Ashery-Padan et al., 2004), MafB deficiency does not affect the initiation of endocrine differentiation, but affects expression of markers of terminal differentiation. The reduced function of either *Pax6* or *MafB* results in an increased proportion of MafB^{+} insulin^{-} and PDX-1^{high} insulin^{-} cells, while the expression of MafA, although reduced, is always restricted to the cells that express insulin (Figures 2, 7), suggesting that *PDX-1* and *MafA* function downstream of *Pax6* and *MafB*. The loss of *Pax6* function reduces the numbers of MafB expressing cells (Figures 1, 2), while the deficiency of *MafB* activity has no effect on Pax6 expression (Figure 6). Taken together, these results suggest that *MafB* can be a downstream mediator of *Pax6* function or that *Pax6* requires *MafB* for inducing the expression of insulin and glucagon. However, the conversion of endocrine precursors into ghrelin^{+} cells does not occur in *MafB* deficient mice (Figure 8, and Artner et al., 2007) as observed in the absence of *Pax6* function (Heller et al., 2005; Prado et al., 2004, and Suppl. Figure 2). Thus, the suppression of ghrelin^{+} ε-cells occurs at the level of *Pax6* expression, and *MafB* does not appear to have a direct role in regulating the formation of ε^{-}cells.

Analysis of *Nkx2.2*, *Pax6* and *Pax4* knockout mice suggested that specification of insulin^{+} cells involves two distinct pathways; one requiring *Nkx2.2*, *Pax4*, *Hb9* and *PDX-1*, and a second involving *Nkx2.2*, *Pax6* and *PDX-1* (Wang et al., 2004). Deficiency of MafB function in *kr*^{ENU}/*kr*^{ENU} mice led to reduction of insulin^{+} and glucagon^{+} cells (Figure 5) as in *Pax6* deficient mice (Sander et al., 1997; Wang et al., 2004; Ashery-Padan et al., 2004; Figures 1,3). These observations suggest that insulin and glucagon expression in some endocrine precursors is independent of *Pax6* and *MafB* function. A similar reduction in the proportion of hormone^{+} cells found in *MafB* knockout mice (Artner et al., 2007) suggests that the residual activity of the *kr*^{ENU} allele does not regulate the formation of remaining hormone^{+} cells. We also observed in *MafB* deficient mice an increased proportion of remaining insulin^{+} cells expressing Hb9 (Figure 9). This increase could be due to the selective lack of insulin^{+} cells derived from the *Pax6-MafB* pathway in these *kr*^{ENU}/*kr*^{ENU} mice. Thus, our results provide strong support to the two parallel pathways model proposed by Sosa-Pineda and colleagues for the formation of insulin^{+} cells (Wang et al., 2004).

The presence of increased numbers of MafB^{+} insulin^{-} cells in *Sey*^{Neu}/*Sey*^{Neu} mice (Figure 2) suggests that either *Pax6* function is essential for MafB to activate insulin expression or *Pax6* initiates insulin expression with MafB required for further maturation of insulin^{+} cells. However, in *kr*^{ENU}/*kr*^{ENU} mice the number of Pax6^{+} cells was unchanged while that of insulin^{+} cells was reduced (Figure 6). Thus, it is most likely that in the presence of *Pax6* (or following its action), MafB initiates the expression of insulin in some hormone^{-} endocrine precursors. Although *Pax6* and *MafB* are required for the formation of only some insulin^{+} cells, they are expressed in most, if not all, insulin^{+} cells, suggesting a role for these factors even in insulin^{+} cells derived from a *Pax6-MafB* independent pathway. Since maturation of insulin^{+} cells accompanies their transition from MafB to MafA expression (Nishimura 2006), MafB may have two distinct roles during endocrine differentiation: one essential for regulating the differentiation of insulin^{+} cells via the *Pax6-MafB* pathway, and the other in the maturation of *Pax4-Hb9* derived insulin^{+} cells. Additional studies are needed to confirm the consequence of a loss of *MafB* or *Pax6* function on the maturation of these latter insulin^{+} cells.

The increased proportion of insulin^{+} Hb9^{+} cells in *kr*^{ENU}/*kr*^{ENU} mice would suggest that the induction of insulin expression in the *Pax4-Hb9* pathway does not depend on MafB expression. Interestingly, a recent *MafB* knockout study (Artner et al., 2007) suggested that MafA was required to specify the remaining insulin^{+} cells. However, since MafA is expressed only in

insulin$^+$ cells in *kr^{ENU}/kr^{ENU}* mice and many insulin$^+$ cells are MafA$^-$ (Figure 7), it is unlikely that MafA induces insulin expression in *MafB* knockout mice. MafA, at the most, might be responsible for a subpopulation of insulin$^+$ cells (MafA$^+$ cells), while another factor is required to trigger insulin expression in MafA$^-$ cells. It is important to note that MafA expression remained restricted to insulin$^+$ cells in both *Sey^{Neu}/Sey^{Neu}* and *kr^{ENU}/kr^{ENU}* mice, and *MafA* knockout mice (Zhang et al., 2005) show no reduction in insulin$^+$ cells at birth. It seems most likely that MafA expression is initiated only after the induction of insulin expression in endocrine precursors, which suggests that in *kr^{ENU}/kr^{ENU}* mice a factor other than MafA or MafB induces insulin expression in cells differentiating via the *Pax4-Hb9* pathway. While our analysis of *MafB* deficient mice generally agrees with a recent characterization of *MafB* knockout mice (Artner et al., 2007), our data do not support a role for PDX-1^{high} upstream of *MafB* function nor a role for *MafA* in inducing insulin expression. Additionally, by analyzing both *MafB* and *Pax6* deficient mice, the current study extends our understanding of the role of *MafB* in pancreatic development. Our results strongly suggest that *MafB* functions downstream of *Pax6* and is most likely responsible for the *Pax6* dependent loss of insulin$^+$ and glucagon$^+$ cells.

Analysis of *Sey^{Neu}/Sey^{Neu}* mice demonstrates a role of *Pax6* and *MafB* in the differentiation of glucagon$^+$ cells. The majority of glucagon positive cells present before the secondary transition express MafB (Figure 3), consistent with the expression of Pax6 in the early glucagon$^+$ cells reported earlier (Heller et al., 2004;Sander et al., 1997). It is likely that the MafB$^+$ glucagon$^-$ cells present at E12.5 (Figure 3) represent the insulin$^+$ MafB$^+$ cells seen at this stage (Nishimura et al., 2006). At E15.5, due to the increase in insulin$^+$ MafB$^+$ cells, the proportion of MafB$^+$ cells expressing glucagon decreased. Yet, the proportion of glucagon$^+$ cells expressing MafB remained unchanged at 95% in wild type mice (Figures 2, 3), suggesting that either the induction of glucagon expression in a small proportion of endocrine precursors in wild type mice does not require MafB function or that *MafB* is turned on rapidly after the induction of glucagon.

Loss of *Pax6* function significantly reduced the number of MafB$^+$ cells, resulting in a significant proportion of glucagon$^+$ cells not expressing MafB at both E12.5 and E15.5 (Figure 3). This finding suggests either the existence of a *Pax6-MafB* independent pathway for the induction of glucagon or a lack of sustainability of MafB in these glucagon$^+$ cells in the absence of Pax6 function. The reduction in the proportion of glucagon$^+$ cells expressing MafB in *Sey^{Neu}/Sey^{Neu}* mice was opposite of no effect of *Pax6* deficiency on the ability of insulin$^+$ cells to express MafB (Figures 2, 3). This observation suggests differences in the specification and maturation of glucagon$^+$ cells and insulin$^+$ cells. Since the specification and maturation process of glucagon$^+$ cells does not require the function of *PDX-1* and *MafA*, it is likely that *MafB* does not regulate the maturation process and only regulates initiation of glucagon expression. A recent study showing different actions of *Nkx2.2* in rescuing glucagon and insulin-expressing cells in *Nkx2.2* knockout mice (Doyle et al., 2007), supports the suggestion that the transcription factors that regulate differentiation of both insulin and glucagon expression, use distinct mechanisms in each endocrine cell type. Similarly, *MafB* may only be involved in specification of glucagon$^+$ cells whereas it may have a dual role in the specification and maturation of insulin$^+$ cells.

Inhibiting the proper maturation of insulin$^+$ cells has functional consequences. It has been shown that *MafA* knockout mice develop diabetes due to postnatal reduction in β-cell number (Zhang et al., 2005). Inhibition of *MafA* function in insulin-producing cells via gene knock-down also resulted in reduced expression of genes involved in insulin synthesis and secretion (Wang et al., 2007). MafA regulates the expression of granuphilin, critical for docking the insulin vesicle to the plasma membrane (Kato et al., 2006). Furthermore, insulin$^+$ cells derived from human embryonic stem cells expressed MafB but not PDX-1 and probably not MafA

(D'Amour et al., 2006); this lack could contribute to their inability to secrete insulin in response to glucose and further highlights the importance of the maturation process.

Previously we proposed that during maturation, insulin$^+$ cells undergo a switch from an insulin$^+$ MafB$^+$ state to an insulin$^+$ MafA$^+$ state via an intermediate PDX-1^{high} stage. In this study, we observed that nearly 95%, 75% and 65% of insulin$^+$ cells in wild type, *Sey^{Neu}/Sey^{Neu}*, and *kr^{ENU}/kr^{ENU}* mice expressed MafB, PDX-1 and MafA, respectively (Figures 2, 7). The observed gradation in expression of these three transcription factors in insulin$^+$ cells as well as the ability of MafB to activate PDX-1 expression further supports our hypothesis that maturation of insulin$^+$ cells proceeds from a MafB$^+$ to MafA$^+$ state after the induction of PDX-1^{high}. Results from *Pax6* and *MafB* deficient mice further support such sequential requirement for *MafB*, *PDX-1* and *MafA* functions in the maturation process. An analysis of conditional *PDX-1* knockout in developing endocrine cells will be necessary to confirm whether the *MafB* is upstream of PDX-1^{high} function and *MafA* is downstream of this gene. Knowledge of the precise function of these transcription factors during the maturation of insulin$^+$ cells will play a crucial role in generating glucose responsive insulin producing cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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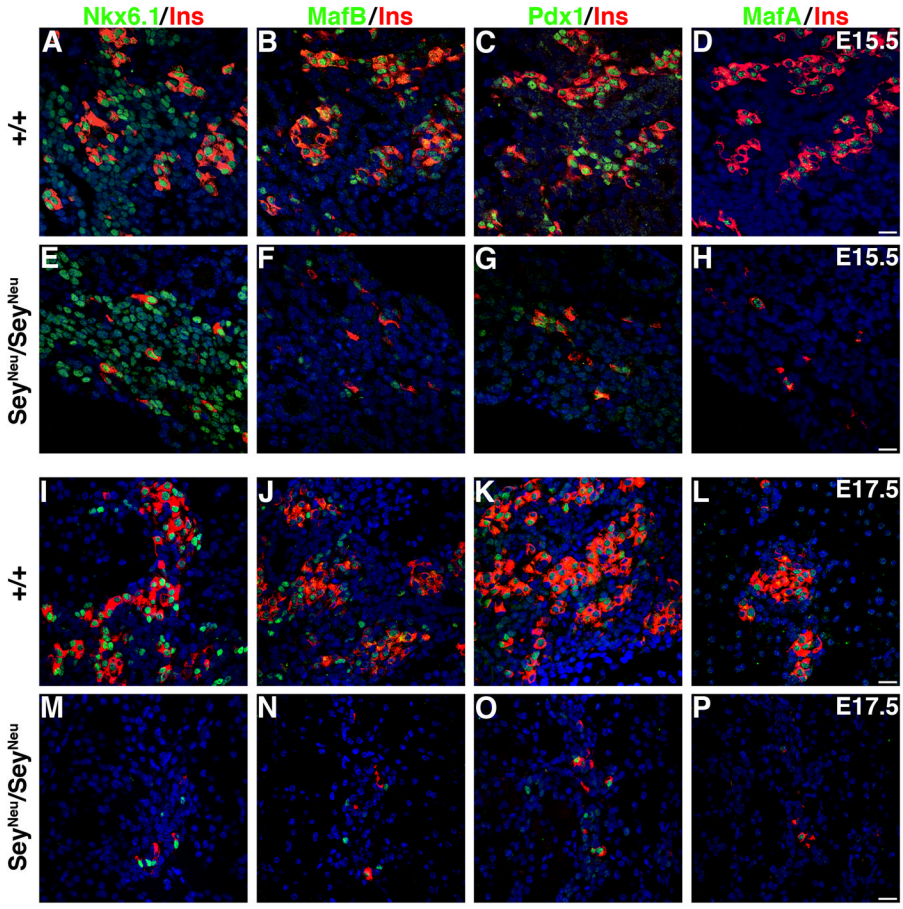

Figure 1. Sey^{Neu}/Sey^{Neu} embryos have reduced expression MafB, PDX-1 and MafA

Adjacent sections from E15.5 wild type (**A–D**) and Sey^{Neu}/Sey^{Neu} (**E–H**) embryonic pancreata (n = 6 each) were stained to detect the expression of Nkx6.1, MafB, PDX-1 and MafA in green and insulin in red. In wild type pancreas adjacent sections show cells expressing Nkx6.1, MafB, PDX-1 and MafA, but in Sey^{Neu}/Sey^{Neu} embryos MafB, PDX-1 and MafA expression was significantly reduced. E17.5 pancreata from wild type (**I–L**) and Sey^{Neu}/Sey^{Neu} (**M–P**) embryos (n = 3 each) were stained for Nkx6.1, MafB, PDX-1 and MafA in green and insulin in red. At this stage loss of *Pax6* function results in significant reduction in the number of $insulin^+$ cells, and the majority of $Nkx6.1^+$, $MafB^+$, $PDX\text{-}1^{high}$ and $MafA^+$ cells are restricted to these $insulin^+$ cells. Bars: 20μm.

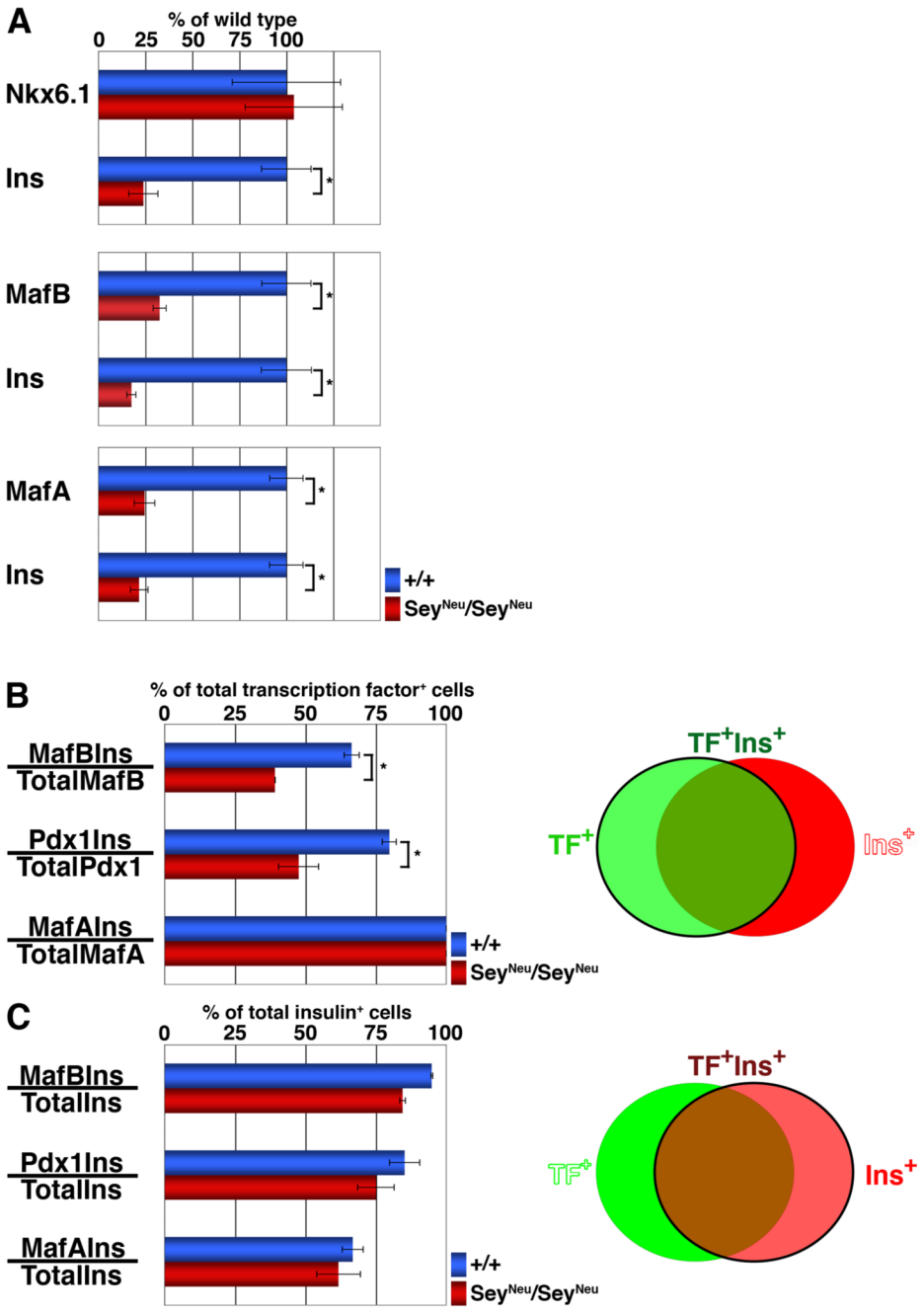

Figure 2. Pax6-deficiency reduces the proportion of MafB$^+$ and PDX-1$^+$ cells that express insulin

Quantification of immunostained E15.5 embryonic pancreatic sections from wild type and *Sey^{Neu}/Sey^{Neu}* embryos (n = 6 each). (**A**) Total number of Nkx6.1$^+$, MafB$^+$ and MafA$^+$ cells in wild type and *Sey^{Neu}/Sey^{Neu}* embryonic pancreas were determined along with the number of insulin$^+$ cells in the same sections. In *Sey^{Neu}/Sey^{Neu}* embryonic pancreas, the numbers of MafB$^+$, MafA$^+$ and insulin$^+$ cells were significantly reduced (p = 0.03, 0.01 and 0.01 respectively), while the number of Nkx6.1$^+$ cells remained unchanged (p = 0.46). (**B**) The ability of transcription factor expressing cells to express insulin was determined by quantifying the proportion of transcription factor$^+$ insulin$^+$ cells per total number of transcription factor$^+$ cells, as represented by cells in green circle in adjacent Venn diagram. Proportions of

MafB^{+}Ins^{+} cells / total MafB^{+} cells and PDX-1^{+}Ins^{+} cells / total PDX-1^{+} cells in *Sey^{Neu}/Sey^{Neu}* pancreas were significantly less than the wild-type ($p = 0.005$ and 0.01 respectively), while all of the MafA^{+} cells express insulin. (**C**) To determine the ability of insulin^{+} cells to undergo further maturation in the absence of *Pax6* function, the proportion of insulin^{+} cells expressing these transcription factors was quantified, as represented by cells in red circle in adjacent Venn diagram. The proportion of MafB^{+} Ins^{+}, PDX-1^{+}Ins^{+} and MafA^{+}Ins^{+} cells to the total Ins^{+} cells was not significantly different between wild type and *Sey^{Neu}/Sey^{Neu}* mice.

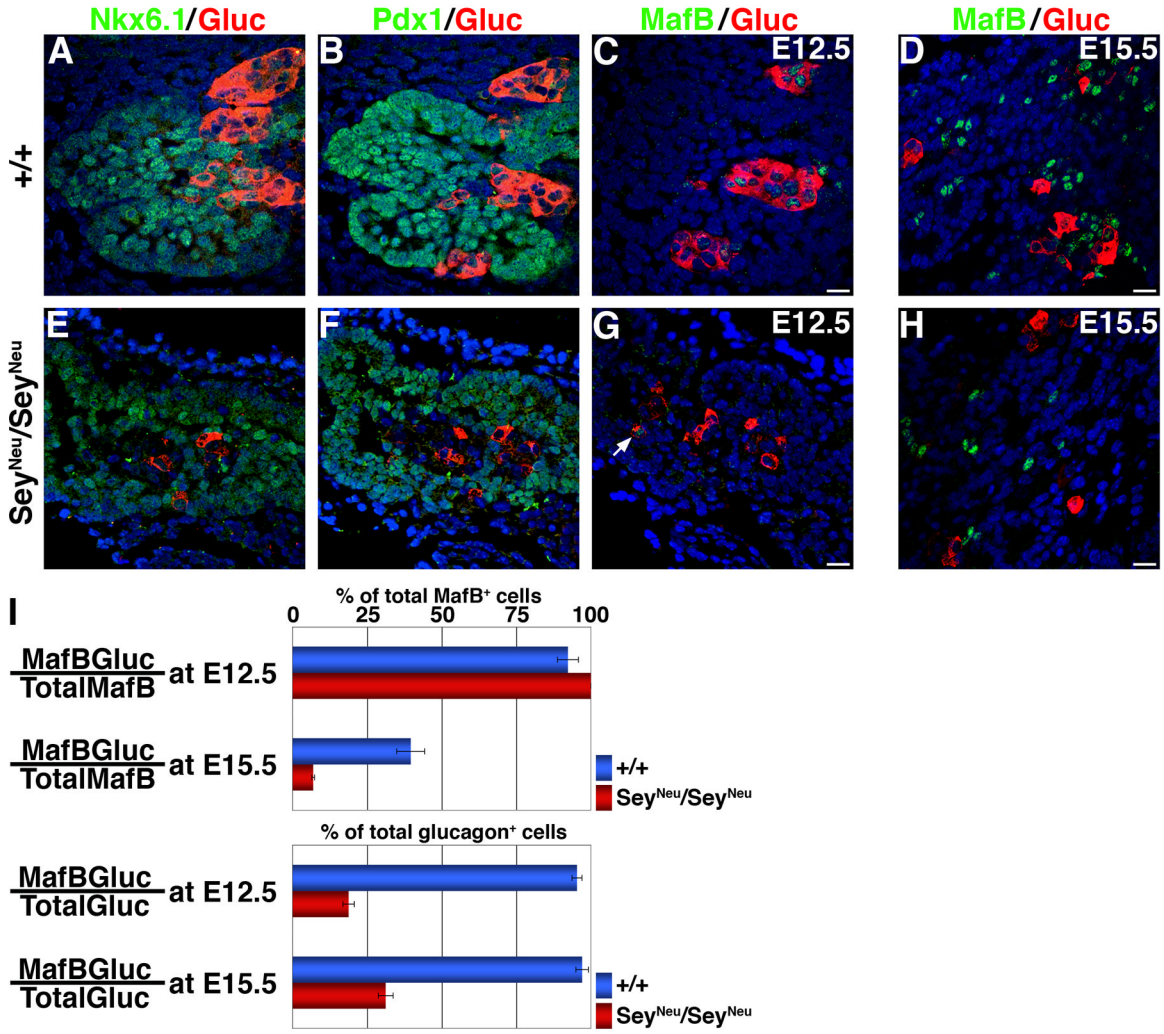

Figure 3. Pax6-deficiency reduces the proportion of MafB$^+$ cells expressing glucagon

Adjacent sections from E12.5 wild type (**A–C**) and *Sey^{Neu}/Sey^{Neu}* (**E–G**) (n = 5 wild type, 4 mutant) pancreata were stained to detect Nkx6.1, PDX-1 and MafB expression in green and glucagon in red. In addition, sections from E15.5 embryonic pancreata (**D, H**) were stained for MafB in green and glucagon in red. In E12.5 *Sey^{Neu}/Sey^{Neu}* pancreata Nkx6.1 and PDX-1 expression was normal, while the expression of MafB and glucagon expression was reduced at both E12.5 and E15.5. Arrow denotes MafB$^+$glucagon$^+$ cells in *Sey^{Neu}/Sey^{Neu}* pancreas at E12.5. Bars: 20μm. (**I**) Proportion of MafB$^+$glucagon$^+$ cells to total number of MafB$^+$ or glucagon$^+$ cells were quantified from wild type and *Sey^{Neu}/Sey^{Neu}* pancreatic sections from E12.5 and E15.5. Absence of *Pax6* function reduces the proportion of glucagon$^+$ cells that express MafB at both E12.5 and E15.5, while the proportion of MafB$^+$ cells expressing glucagon is reduced only in E15.5 pancreata.

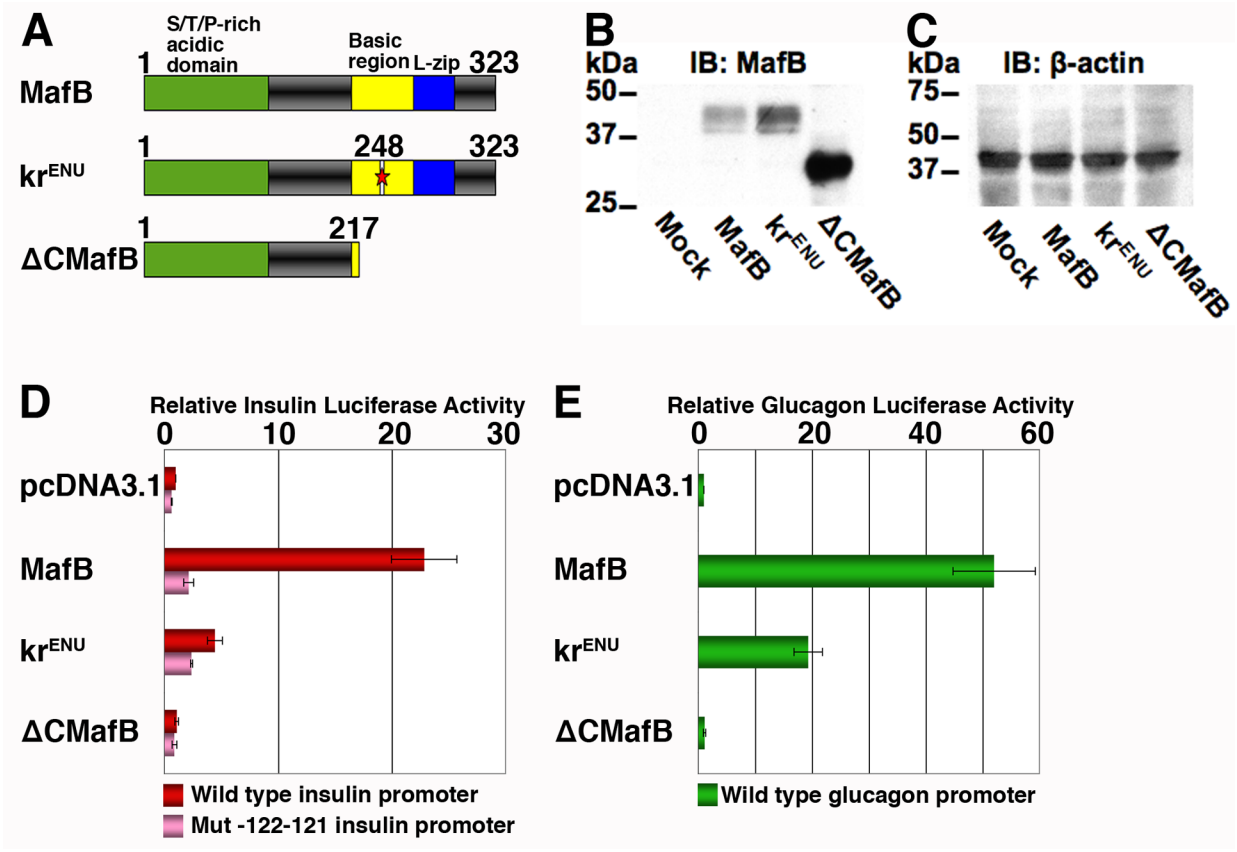

Figure 4. The kr^{ENU} allele encodes a reduced ability to activate insulin and glucagon gene expression

(**A**) Schematics of wild type MafB and its kr^{ENU} mutant and deletion derivatives showing activation and DNA binding domains. (**B, C**) Western blot analyses to detect MafB protein expression from various MafB constructs. Lysates used in transient transfection assays in (**D**) and (**E**) were subjected to Western blot to detect expression of MafB (**B**) and β-actin (**C**). (**D**) Insulin promoter:Luciferase reporter constructs, wild type (−238 WT LUC, *red*) and insulin promoter with mutation in −121−122bp (−121−122m LUC, *pink*), and (**E**) glucagon promoter:Luciferase reporter constructs (GLU LUC, *green*) were transfected into HeLa cells with the indicated expression plasmids and pSVβ-gal as an internal control. Luciferase and β-gal activities were determined. Results are presented relative to activity of wild-type luciferase construct ± S.E. (n = 4)

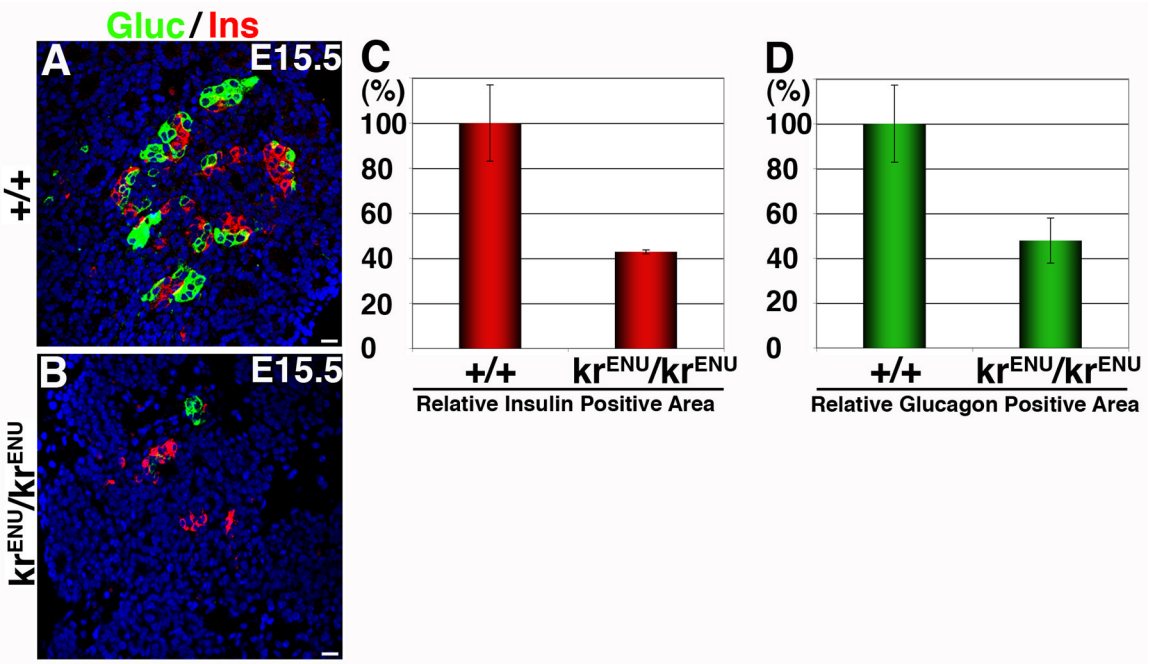

Figure 5. MafB deficiency reduces insulin$^+$ and glucagon$^+$ cells

(**A, B**) Pancreas from E15.5 wild type (+/+) and *MafB* mutant (kr^{ENU}/kr^{ENU}) (n = 4 wild type, 4 mutant) embryos were stained for glucagon in green and insulin in red. Immunostained sections were used to quantify the proportion of insulin (**C**) and glucagon (**D**) positive areas per pancreatic epithelial area as described in Materials and Method. Bars: 20μm.

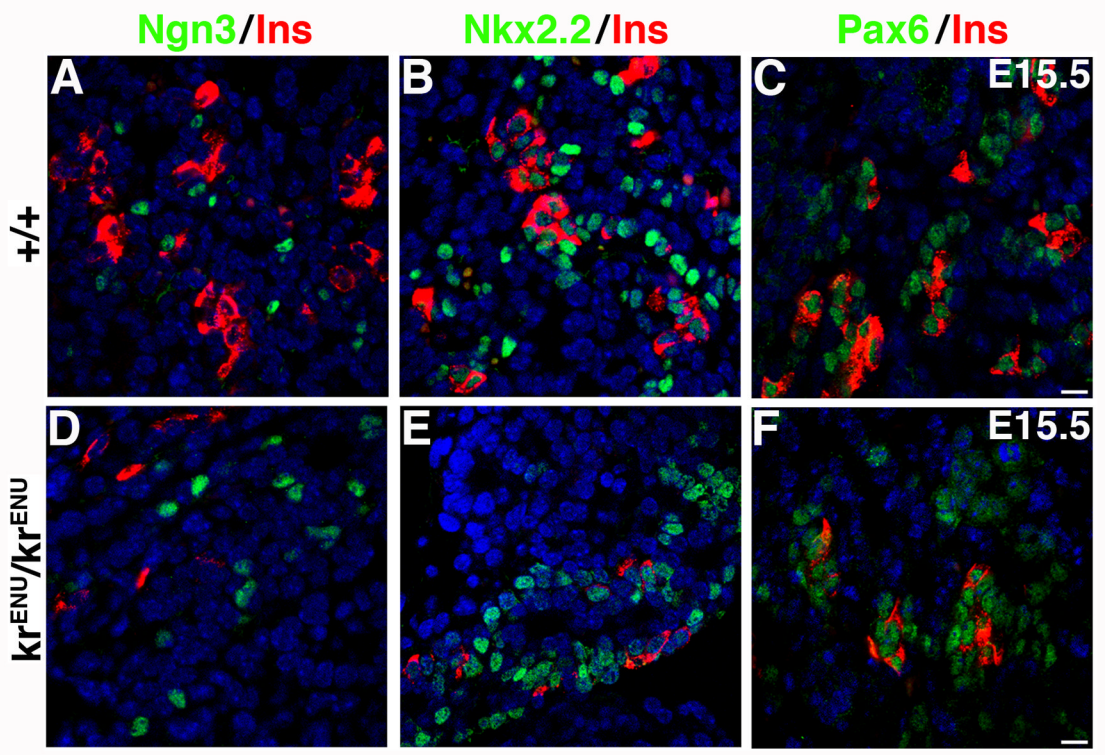

Figure 6. MafB deficiency does not inhibit the initiation of endocrine differentiation

Sections from E15.5 pancreata from wild type and kr^{ENU}/kr^{ENU} embryos were stained for Ngn3, Nkx2.2 and Pax6 in green and insulin in red. Expression of insulin was reduced in kr^{ENU}/kr^{ENU} pancreata, but Ngn3, Nkx2.2 and Pax6 expression was similar to that in the wild type embryos. Bars: 20μm.

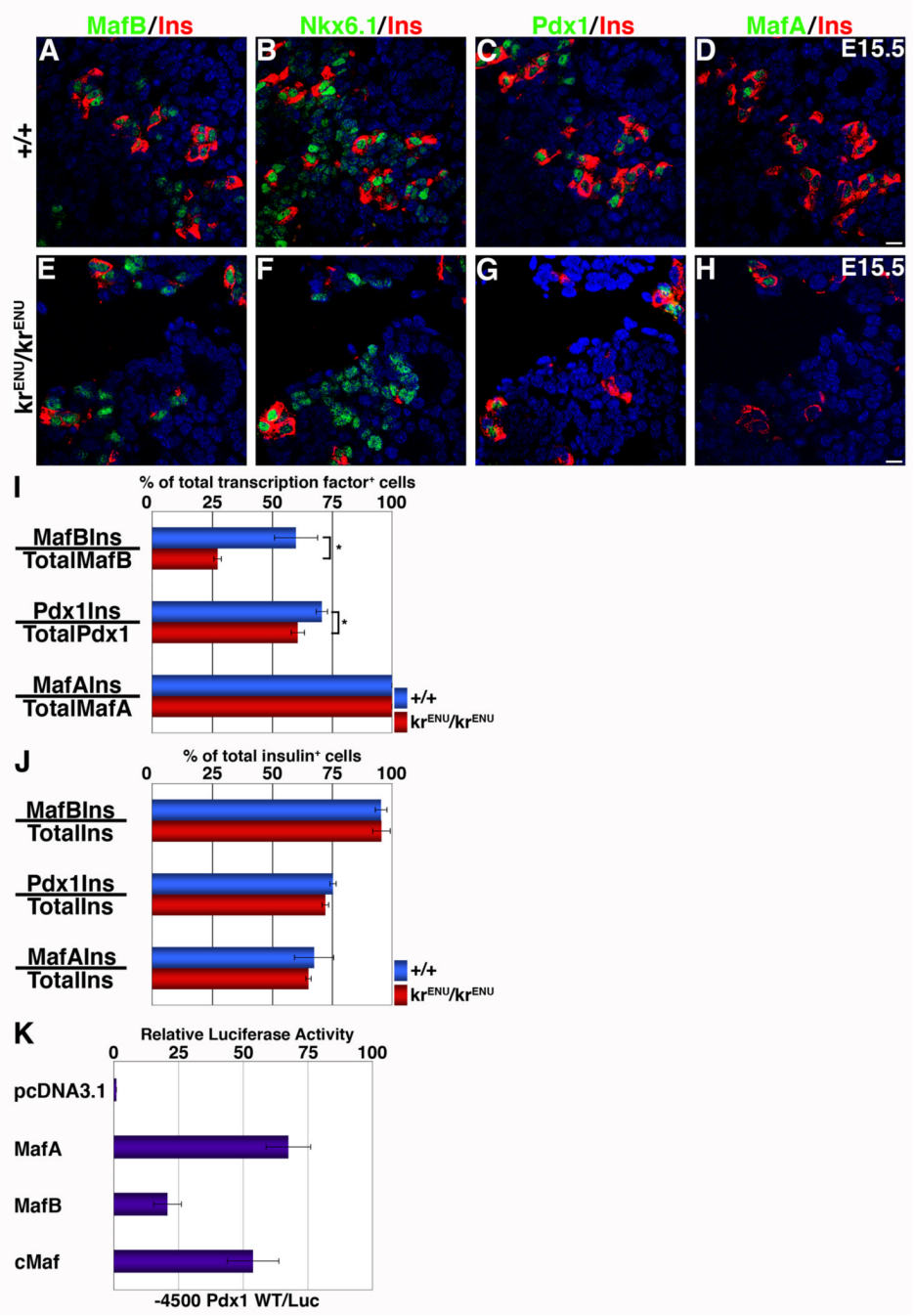

Figure 7. MafB deficiency reduces PDX-1, MafA and insulin expression and impairs the ability of MafB$^+$ and PDX-1$^+$ cells to express insulin

(**A–H**) Adjacent sections from E15.5 wild type and *kr^{ENU}/kr^{ENU}* (n = 4 wild type, 3 mutant) pancreata were stained for MafB, Nkx6.1, PDX-1 and MafA in green and insulin in red. In *kr^{ENU}/kr^{ENU}* sections the number of cells expressing PDX-1, MafA and insulin were reduced compared to wild type pancreata. Bars: 20μm. (

I, J) Stained E15.5 pancreatic sections from wild type and kr^{ENU}/kr^{ENU} embryos were quantified to determine the proportion of transcription factor$^+$ cells expressing insulin (**I**), and the proportion of insulin$^+$ cells expressing different transcription factors (**J**). (**I**) Proportions of MafB$^+$Ins$^+$ cells / total MafB$^+$ cells and PDX-1$^+$Ins$^+$ cells / total PDX-1$^+$ cells in *kr^{ENU}/*

kr^{ENU} were significantly reduced, while all MafA^{+} cells expressed insulin. (**J**) The proportion of insulin^{+} cells expressing MafB, PDX-1 or MafA was not significantly different between wild type and kr^{ENU}/kr^{ENU}. (**a**) MafB and other large Maf factors can activate PDX-1 expression. A −4.5kb PDX-1 promoter:luciferase reporter construct was transfected into HeLa cells with the indicated expression plasmids and pSVβ-gal was used as an internal control. Results are presented relative to the activity of a wild-type luciferase construct transfected with the pcDNA3.1 ± S.E. (n = 3).

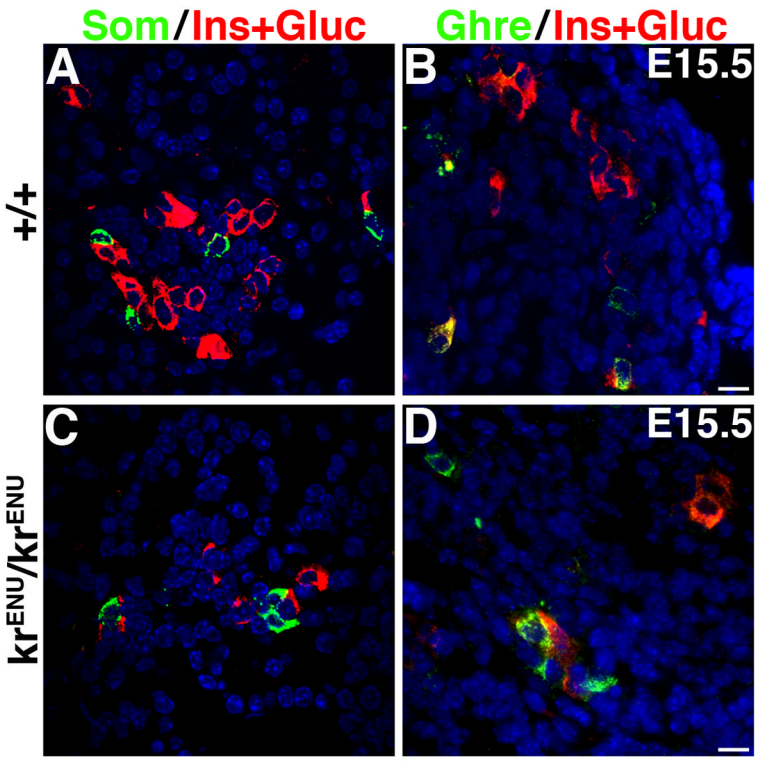

Figure 8. MafB deficiency does not induce the formation of ε-cells

Sections from E15.5 wild type (+/+) and kr^{ENU}/kr^{ENU} pancreata were stained to detect somatostatin (Som) or ghrelin (Ghre) in green and a mixture of antibodies that recognize insulin and glucagon in red. In spite of reduction in the number of insulin and glucagon expressing cells, the number of somatostatin^{+} and ghrelin^{+} cells is unchanged in kr^{ENU}/kr^{ENU} embryos. Also, ghrelin^{+} glucagon^{-} ε-cell numbers were similar in pancreata from wild type and kr^{ENU}/kr^{ENU} embryos.

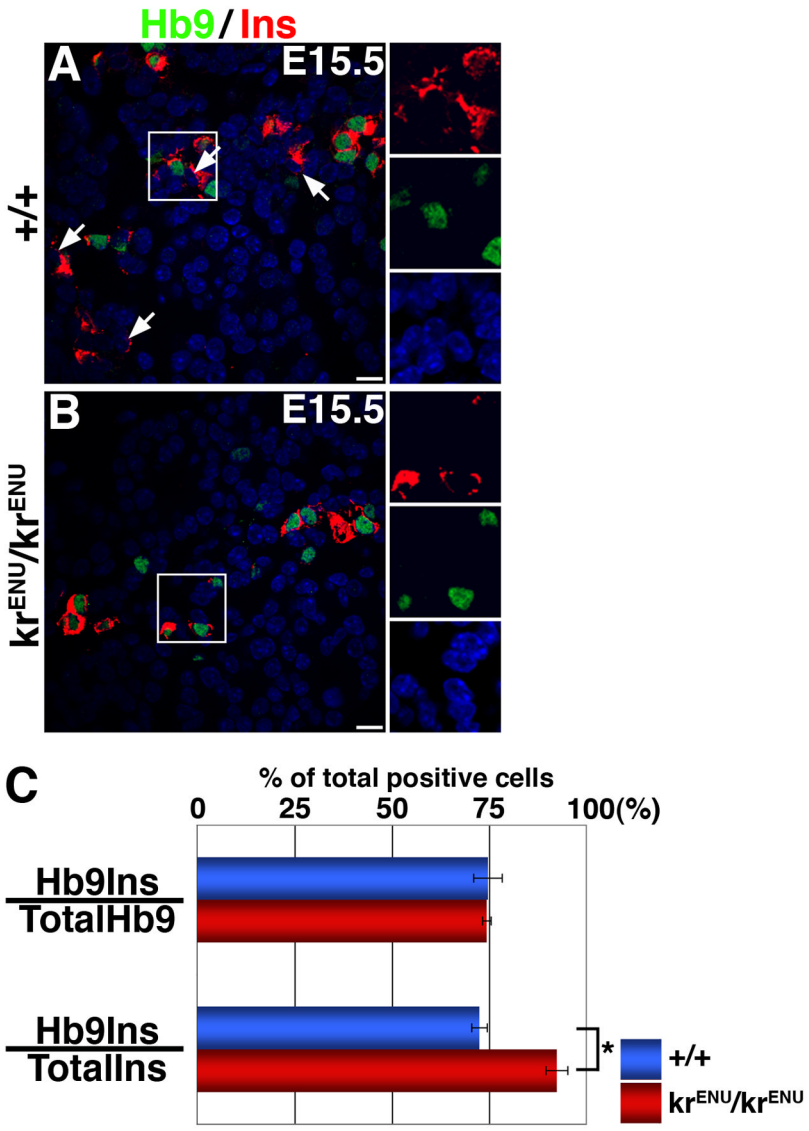

Figure 9. An increased proportion of insulin$^+$ cells in the MafB deficient pancreata co-express Hb9

(**A, B**) E15.5 sections from wild type and kr^{ENU}/kr^{ENU} pancreata were stained for Hb9 in green and insulin in red. Hb9$^-$ insulin$^+$ cells and Hb9$^+$ insulin$^+$ cells co-exist in the wild type pancreas while the number of Hb9$^-$ insulin$^+$ cells is reduced in kr^{ENU}/kr^{ENU} pancreas. Bars: 20μm. (**C**) Quantification of Immunohistochemical data. The proportion of Hb9$^+$ cells expressing insulin did not change between the kr^{ENU}/kr^{ENU} and wild type mice. However, the proportion of insulin$^+$ cells that co-express Hb9 in these mice is significantly increased ($p = 0.02$).