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GATA6 Plays an Important Role in the Induction of Human Definitive Endoderm, Development of the Pancreas, and Functionality of Pancreatic β Cells

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SUMMARY

Induced pluripotent stem cells were created from a pancreas agenesis patient with a mutation in *GATA6*. Using genome-editing technology, additional stem cell lines with mutations in both *GATA6* alleles were generated and demonstrated a severe block in definitive endoderm induction, which could be rescued by re-expression of several different GATA family members. Using the endodermal progenitor stem cell culture system to bypass the developmental block at the endoderm stage, cell lines with mutations in one or both *GATA6* alleles could be differentiated into β-like cells but with reduced efficiency. Use of suboptimal doses of retinoic acid during pancreas specification revealed a more severe phenotype, more closely mimicking the patient's disease. *GATA6* mutant β-like cells fail to secrete insulin upon glucose stimulation and demonstrate defective insulin processing. These data show that GATA6 plays a critical role in endoderm and pancreas specification and β-like cell functionality in humans.

INTRODUCTION

Pancreatic agenesis is a rare congenital disease caused by a mutation in *PDX1* (Stoffers et al., 1997), *GATA4* (Shaw-Smith et al., 2014), or most commonly *GATA6* (Chao et al., 2015; De Franco et al., 2013; Lango Allen et al., 2012; Stanescu et al., 2014). The majority of *GATA6* mutations leading to pancreatic agenesis are de novo heterozygous mutations. Some *GATA6* mutations have incomplete penetrance as determined by patients having identical mutations to pancreatic agenesis patients, but displaying either adult-onset diabetes or an absence of pancreatic abnormalities (Bonnefond et al., 2012; De Franco et al., 2013). The majority of pancreatic agenesis patients also display a combination of other defects including congenital heart defects, gut abnormalities, and intrauterine growth retardation (Chao et al., 2015).

GATA6 belongs to a six-member family of transcription factors that bind to the consensus sequence (A/T)GATA(A/G). GATA1, GATA2, and GATA3 are mainly expressed in hematopoietic cell lineages, while GATA4, GATA5, and GATA6 are predominantly expressed in the heart, gonads, and endodermal-derived tissues (Viger et al., 2008). GATA6 is known to regulate endodermal gene expression and development of endoderm-derived organs (Molkentin, 2000). In mice, GATA6 is expressed in the primitive streak, heart, lung, intestine, gonads, adrenal, and pancreatic tissues (Koutsourakis et al., 1999; Liu et al., 2002). Within the adult pancreatic tissue, GATA6 is expressed in both the exocrine tissue and the islets of Langerhans (Sartori et al., 2014).

In contrast to the severe disease phenotype found in humans with *GATA6* heterozygous mutations, *GATA6* heterozygous mice are fertile and phenotypically normal. Homozygous GATA6 null mice are embryonic lethal (Morrisey et al., 1998). Using tetraploid complementation, GATA6 has been shown to be essential for extra-embryonic endoderm development explaining the embryonic lethality (Koutsourakis et al., 1999; Zhao et al., 2005); however, GATA6 null cells can contribute to the definitive endoderm. Analysis of a loss of GATA6 in pancreas progenitors or adult β cells has demonstrated minimal impact on endocrine function, with normal numbers of β cells and no overt signs of diabetes despite a mild impact on endoplasmic reticulum stress (Carrasco et al., 2012; Martinelli et al., 2013; Sartori et al., 2014; Xuan et al., 2012).

Due to the major differences in phenotype between human and murine GATA6 disease models, human pluripotent

stem cells (PSCs) offer an alternative system for the in vitro study of GATA6. With recent developments in the genome-editing field, the use of clustered regularly interspaced short palindromic repeats (CRIPSR)/CAS9 technology (Ran et al., 2013) has enabled PSCs to become an even more powerful model system as mutant and control isogenic lines can be made to avoid confounding results due to differing genetic backgrounds.

Here, we study *GATA6* mutant human PSCs. Induced pluripotent stem (IPS) cells were generated from a previously described pancreatic agenesis patient having a heterozygous *GATA6* mutation (Stanescu et al., 2014). Using genome editing, PSC lines with mutations in both alleles of *GATA6* were generated and failed to differentiate into definitive endoderm due to a block at the primitive streak stage of development. Re-expression of GATA6 or other GATA family members restored this defect. Using endodermal progenitor (EP) cells as a tool to bypass the endoderm defect, pancreatic β cell differentiation was examined. We found that all mutant lines maintained the ability to differentiate into pancreatic β-like cells but that these cells were functionally defective in glucose responsiveness. Finally, we show that limiting retinoic acid (RA) signaling during pancreas induction in the *GATA6* mutant lines led to a dramatic decrease in pancreas specification and β cell generation. These data suggest that human GATA6 plays a critical role in endoderm development and functionality of pancreatic β-like cells.

RESULTS

Establishment of GATA6 PSC Lines

To study the role of GATA6 in human development, mutant and control PSC lines were generated by standard reprogramming and CRISPR/Cas genome editing. An iPS cell line was generated from cells of a previously described patient expressing a heterozygous *GATA6* mutation (Stanescu et al., 2014). The 4 base pair (bp) duplication in the second exon of *GATA6* causes a frameshift mutation resulting in a truncated protein (Figures 1A and 1B). This patient-derived iPS cell line, is designated $IPS^{+/indel}$ (Table S1). To generate cell lines expressing mutations in both alleles of *GATA6*, CRISPR/Cas9 genome editing was performed using the $IPS^{+/indel}$ cell line and the Mel1-INS-GFP (Micallef et al., 2012) embryonic stem (ES) cell line (Figure 1C). The $IPS^{+/indel}$ cell line was used to maintain genetic background identity to the patient's cells for experimental comparison. The Mel1-INS-GFP ES cell line (designated $ES^{+/+}$, Table S1) was used for two reasons in addition to confirming phenotypes in a second genetic background. First, the Mel1-INS-GFP line allows easy purification of β-like cells as it contains a GFP reporter in the insulin locus. Second, to assist with inducible gene expression studies, we generated a Mel1-INS-GFP sub-line that constitutively expressed the reverse tet transactivator (rtTA) targeted to the *AAVS1* safe harbor locus (Figures S1A and S1B) using a previously described methodology (Hockemeyer et al., 2009; Tiyaboonchai et al., 2014). For CRISPR/Cas genome editing, the guide RNA (gRNA) was designed to target *GATA6* near the patient mutation site (Figure 1B) creating frameshift insertion and/or deletion (INDEL) mutations in both alleles of *GATA6* (Table S1 and Figure S1C). The genome-edited patient iPS cell line is designated $IPS^{indel/indel}$ and the genome-edited Mel-INS-GFP line is designated $ES^{indel/indel}$ (Table S1). To generate an isogenic control for the $IPS^{+/indel}$ line, genome editing was used to correct the mutation, and this line is designated $IPS^{+/+}$ (Table S1). The $IPS^{+/indel}$ line was confirmed for pluripotency (Figure S2) and all genome-edited PSC lines were confirmed to have a normal karyotype (Figure S1D).

As GATA6 is expressed in the primitive streak and not in PSCs, protein and transcript levels were examined in control and mutant cells utilizing a protocol that induces definitive endoderm (Cheng et al., 2012; D'Amour et al., 2005). Using western blot analysis, full-length GATA6 protein was detected in the control lines, $ES^{+/+}$, and $IPS^{+/+}$, and in the patient line, $IPS^{+/indel}$, that expressed one normal allele of *GATA6* (Figure 1C). While the *GATA6* transcript contains two alternative start sites (Brewer et al., 1999), we predominantly observe the smaller isoform. The patient line, $IPS^{+/indel}$, also expressed one mutant allele of *GATA6* that generated a truncated GATA6 protein of ~35 kDa, the only form of GATA6 detected in the compound heterozygous mutant lines, $IPS^{indel/indel}$ and $ES^{indel/indel}$ (Figure 1C). Using flow cytometry, GATA6 protein was also quantified by mean fluorescence intensity (MFI). We found that the truncated GATA6 protein in the $IPS^{indel/indel}$ line was expressed at significantly lower levels (0.27 ± 0.06) relative to the $IPS^{+/indel}$ (0.97 ± 0.13) and $IPS^{+/+}$ (normalized to 1) lines (Figure 1D). The same results were observed in the $ES^{indel/indel}$ line, with significantly decreased levels of GATA6 (0.30 ± 0.01-fold) compared with the $ES^{+/+}$ line (Figure S3D).

In addition to GATA6, GATA4 is another member of the GATA family that is upregulated during primitive streak and definitive endoderm induction (Arceci et al., 1993; Czysz et al., 2015). Both *GATA4* and *GATA6* transcript levels were measured by qPCR in definitive endoderm cells. *GATA6* levels were significantly decreased in the $IPS^{indel/indel}$ cell line compared with the $IPS^{+/+}$ cell line (Figure 1E), suggesting that GATA6 may be part of a self-regulatory feedback loop. *GATA4* was decreased in a dose-dependent manner in the $IPS^{+/indel}$ and $IPS^{indel/indel}$ lines (Figure 1F). A time-course analysis of both transcripts during endoderm differentiation in $IPS^{+/+}$ cells showed that

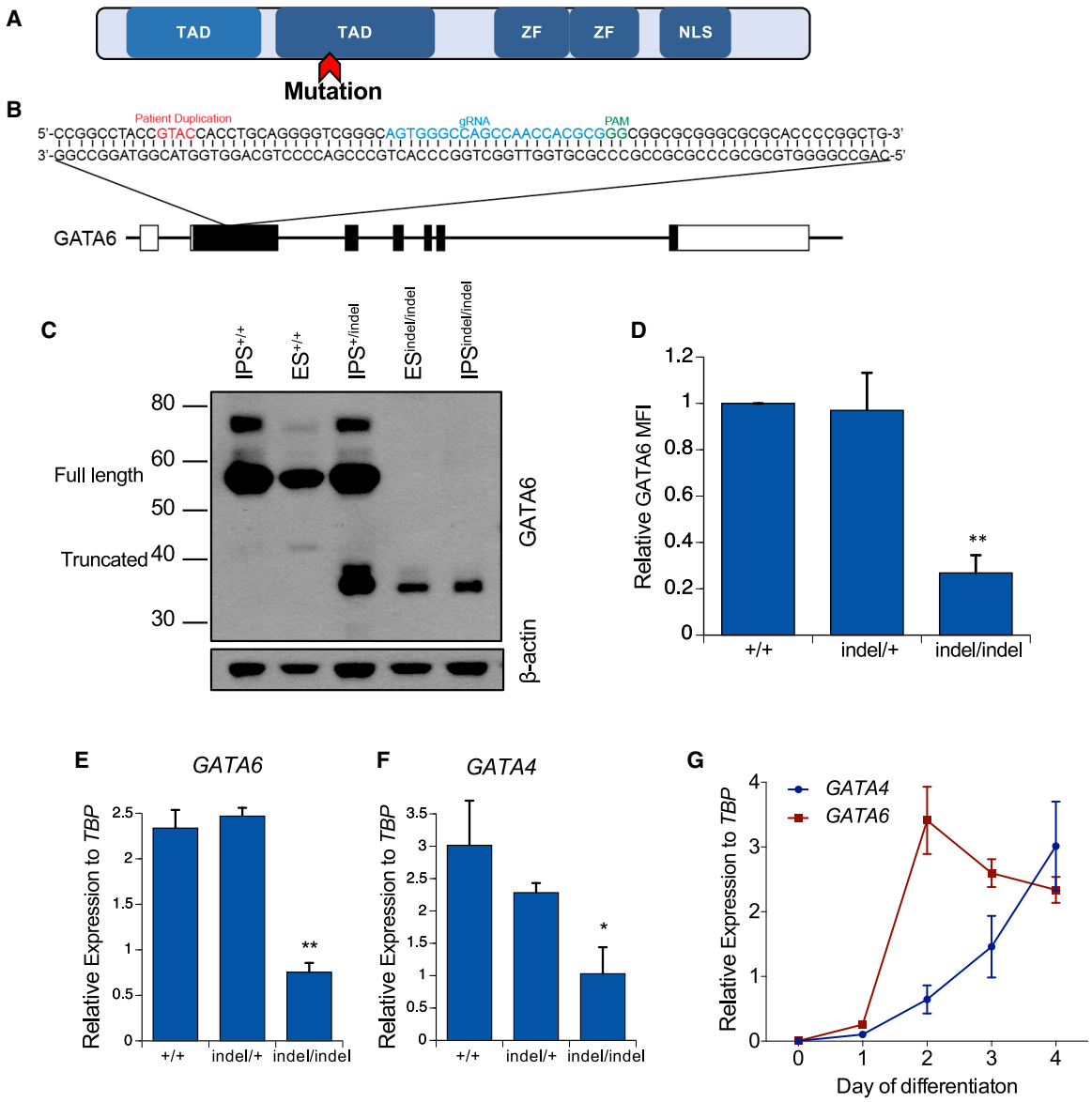


Figure 1. Generation of PSC Lines with GATA6 Mutations

(A) Schematic of GATA6 protein with site of patient mutation (red arrow).

(B) Schematic of *GATA6* gene with location of patient mutation (red) and site of gRNA (blue) with the PAM sequence (green) indicated.

(C) Western blot of GATA6 protein from PSC lines differentiated toward definitive endoderm for 4 days.

(D) Mean fluorescence intensity (MFI) of samples examined in (C) by intracellular flow cytometry (n = 5 per cell line).

(E) *GATA6* expression of samples examined in (C) by qRT-PCR analysis (n = 3 per cell line).

(F) *GATA4* expression of samples examined in (C) by qRT-PCR analysis (n = 3 per cell line).

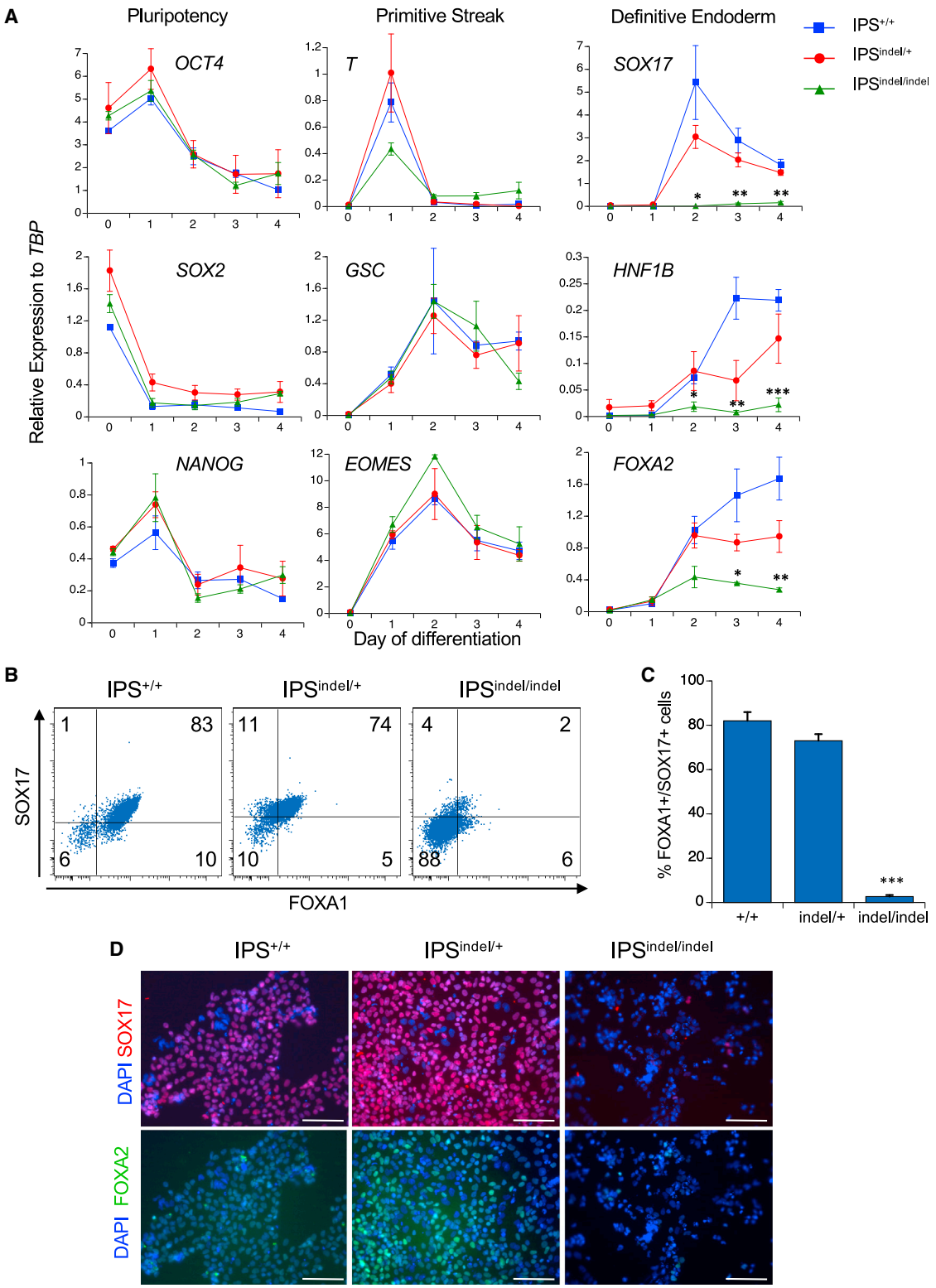
(G) Time-course analysis of *GATA4* and *GATA6* expression during definitive endoderm differentiation of control cells by qRT-PCR (n = 3).

For all statistical analysis, *p < 0.05, **p < 0.01.

GATA6 is expressed at higher levels and more rapidly than *GATA4* (Figure 1G). Together, these data suggest cross-talk between GATA6 and GATA4, and GATA6 may be maintaining expression of both itself and GATA4 during endoderm induction.

Definitive Endoderm Differentiation

To analyze the impact of GATA6 on differentiation to definitive endoderm, a time-course analysis was performed using a modification of established protocols (D'Amour et al., 2005; Kubo et al., 2004). The expression



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of developmentally regulated markers at different stages of differentiation was analyzed using the $IPS^{+/+}$, $IPS^{+/indel}$, and $IPS^{inde/indel}$ lines (Figure 2A). These experiments were also repeated in the $ES^{+/+}$ and $ES^{indel/indel}$ lines (Figure S3). In all lines, the pluripotency markers *NANOG*, *SOX2*, and *OCT4* were downregulated by day 2 of differentiation, and the primitive streak markers brachyury (*T*), goosecoid (*GSC*), and eomesodermin (*EOMES*) were expressed at the appropriate times. The definitive endoderm markers *SOX17*, *FOXA2*, and *HNF1B* failed to upregulate in the $IPS^{indel/indel}$ and $ES^{indel/indel}$ cell lines compared with the $IPS^{+/indel}$, $IPS^{+/+}$, and $ES^{+/+}$ lines (Figures 2A and S3A). By using intracellular flow cytometry to examine the co-expression of SOX17 and FOXA1, these data were confirmed and quantitated (Figures 2B, 2C, S3B, and S3C). Robust co-expression of SOX17 and FOXA1 was observed in $IPS^{+/+}$ (82% ± 4%), $ES^{+/+}$ (82% ± 7%) and $IPS^{+/indel}$ (75% ± 3%) lines. Compared with their respective control cells, there was a ~27-fold decrease in the $IPS^{indel/indel}$ (2.7% ± 0.7%) and a ~6-fold decrease in the $ES^{indel/indel}$ (14% ± 3%) lines. These data were confirmed in all cell lines by immunofluorescence staining for SOX17 and FOXA2 (Figures 2D and S3E). These data show that GATA6 is a critical transcription factor required during definitive endoderm specification in human cells.

To determine if GATA6 affected differentiation to the other two germ layers, established protocols were used to monitor differentiation of $ES^{+/+}$, $IPS^{+/indel}$, $ES^{indel/indel}$, and $IPS^{indel/indel}$ lines. All cell lines displayed similar differentiation efficiency to the mesoderm and ectoderm germ layers suggesting that GATA6 is not required (Figures S3H and S3I).

Rescue of Definitive Endoderm by GATA6

To confirm that GATA6 was responsible for the decrease in endoderm induction from the $ES^{indel/indel}$ line, GATA6 rescue experiments were performed. Because the $ES^{indel/indel}$ line constitutively expresses rtTA, a lentiviral vector containing the tet response element was used to express GATA6/RFP in a doxycycline (Dox)-inducible manner (Figure 3A). Adding Dox on day 1 of differentiation, robust co-expression of SOX17 and FOXA1 was observed in the $ES^{indel/indel}$ line only after rescue with full-length and not truncated GATA6 (Figures 3B and 3C). Compared with the empty vector which only expresses RFP, overexpression of the truncated GATA6 protein resulted in a decreased $ES^{indel/indel}$ cell differentiation to definitive endoderm, suggesting a possible dominant-negative activity of the truncated protein (Figures 3B and 3C). Gene expression analysis of other endodermal markers was also performed in cells expressing the *GATA6* and truncated *GATA6* transgenes by sorting the RFP-positive cells at day 5 of differentiation. The expression levels of *SOX17*, *FOXA2*, and *HNF1B* in GATA6-rescued $ES^{indel/indel}$ cells were comparable with normal levels in $ES^{+/+}$ cells differentiated to definitive endoderm (Figure 3E). Expression levels of these markers in $ES^{indel/indel}$ cells expressing truncated GATA6 were not statistically different to cells expressing the empty vector.

To address a temporal requirement of GATA6 during definitive endoderm specification, Dox was added at either the primitive streak (days 0–1) or the endoderm specification (days 2–3) stage of development (Figure 2A). RFP^+ cells were analyzed for the co-expression of SOX17 and FOXA1 at day 5 of differentiation (Figure 3D). The addition of Dox at days 0 and 1 resulted in 77% ± 4.5% and 82% ± 4.2% of $SOX17^+$ and $FOXA1^+$ cells while the addition of Dox at days 2 and 3 resulted in 50% ± 5.9% and 30% ± 4.0% in $SOX17^+$ and $FOXA1^+$ cells. These data show that robust endoderm specification requires GATA6 expression at the primitive streak stage.

Rescue of Definitive Endoderm by Other GATA Family Members

All members of the GATA transcription factor family share conserved DNA activation and zinc finger DNA-binding domains (Molkentin, 2000; Viger et al., 2008). To determine if other GATA family members can compensate for loss of GATA6, the same rescue experiments described above were performed using lentiviral vectors expressing GATA1, 3, and 4. GATA4 was chosen because it is functionally redundant with GATA6 in animal models (Holtzinger and Evans, 2005; Xuan et al., 2012). GATA1 and GATA3 are not typically expressed in definitive endoderm and were initially chosen as controls. Rescue with GATA1, GATA3, and GATA4 resulted in cells co-expressing 53% ± 5.4%, 70% ± 3.6%, and 76% ± 3.3% SOX17 and FOXA1, respectively. The rescue of definitive endoderm differentiation with GATA4 was expected, but the ability of GATA1

Figure 2. GATA6 Is Required for Definitive Endoderm Differentiation of PSC Lines

Pluripotent stem cell lines described in Figure 1B were differentiated into definitive endoderm.

(A) Time-course analysis of pluripotency (*OCT4*, *SOX2*, and *NANOG*), primitive streak (*T*, *GSC*, *EOMES*) and definitive endoderm (*SOX17*, *HNF1B*, and *FOXA2*) markers by qRT-PCR (n = 3 per time point per cell line).

(B) Analysis of the definitive endoderm markers FOXA1 versus SOX17 at day 4 of differentiation by intracellular flow cytometry.

(C) Quantitation of the percentage of $FOXA1^+SOX17^+$ cells in (B) (n = 5 per cell line).

(D) Immunofluorescence analysis of SOX17 and FOXA2 at day 4 of differentiation. Scale bar represents 100 μm.

For all statistical analysis, *p < 0.05, **p < 0.01, ***p < 0.001.

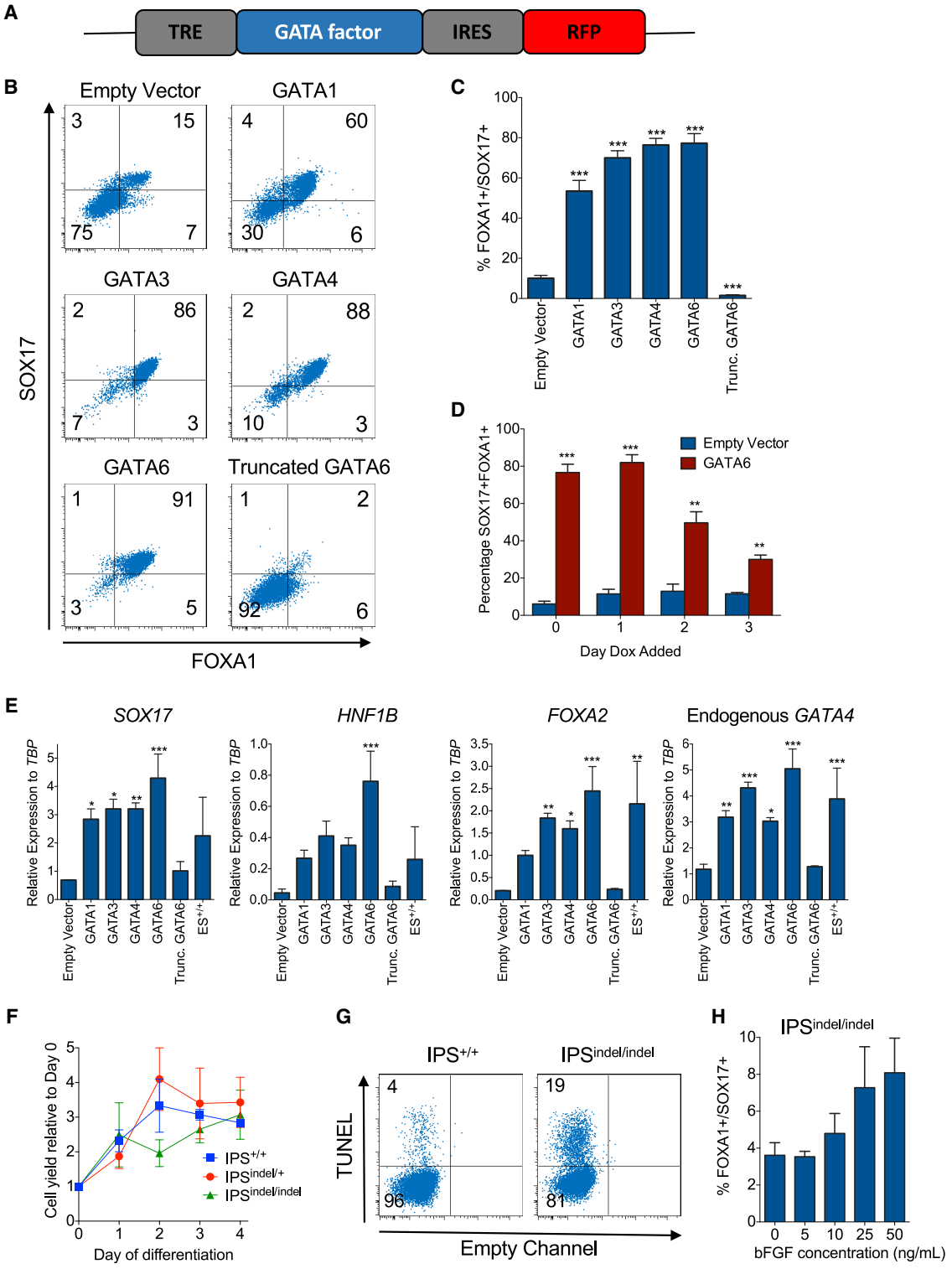

Figure 3. Rescue of Definitive Endoderm Differentiation

(A) A schematic of the doxycycline-inducible lentivirus that was used to infect ES$^{indel/indel}$ cells.

(B) ES$^{indel/indel}$ cells were transduced with lentivirus harboring *GATA1*, *GATA3*, *GATA4*, *GATA6*, truncated-*GATA6* transgenes, or an empty vector and differentiated into definitive endoderm. Doxycycline was added on day 1 of the differentiation to induce gene expression.

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and GATA3 to rescue definitive endoderm was not and suggests that any GATA family member, even those not typically expressed in endoderm, may compensate for GATA6. These data were confirmed by analysis of *SOX17*, *FOXA2*, and *HNF1B* on RFP$^+$ sorted cells (Figure 3E). As mutant ES$^{indel/indel}$ cells have significantly lower *GATA4* expression in definitive endoderm compared with control ES$^{+/+}$ cells (Figure 1F), we questioned whether other GATA family members were rescuing the differentiation by inducing *GATA4*. We show that any of the GATA factors can rescue expression of endogenous *GATA4* (Figure 3E), although further experimentation would be needed to formally demonstrate that the rescue was simply due to *GATA4* induction.

GATA6 Mutants Undergo Increased Apoptosis during Endoderm Induction that Can Be Rescued by Growth Factor Supplementation

During endoderm induction, a decrease in cell number at day 2 of differentiation was observed with the iPS$^{indel/indel}$ and ES$^{indel/indel}$ cell lines compared with the iPS$^{+/+}$ and ES$^{+/+}$ cell lines (Figures 3F and S4A). This time point directly follows primitive streak induction and is when GATA6 would begin to be expressed (Figure 1G). We hypothesized that in GATA6 mutants, cells attempting to commit to endoderm are lost. To determine whether apoptosis caused the decrease in cell number, TUNEL staining was performed on day 2 of differentiation. We found a 2.7- to 4.7-fold increase in the percentage of TUNEL-positive cells in the iPS$^{indel/indel}$ and ES$^{indel/indel}$ cell lines compared with the iPS$^{+/+}$ and ES$^{+/+}$ cell lines (Figures 3G and S4B) suggesting that cell death may be a factor contributing to the decreased cell numbers during definitive endoderm specification.

To determine whether a rescue of apoptosis would also rescue the defect in definitive endoderm differentiation of iPS$^{indel/indel}$ and ES$^{indel/indel}$ cells, increasing concentrations of the pro-survival growth factor basic fibroblast growth factor (bFGF) were used to treat the cells for 24 hr at day 1 of differentiation, prior to the observed decrease in cell number. Co-expression of FOXA1 and SOX17 was examined at the end of the definitive endoderm differentiation. While the efficiency of differentiation in the IPS$^{+/+}$ and ES$^{+/+}$ cell lines was unaffected by higher bFGF concentrations (Figures S4D and S4E), there was a partial rescue of the definitive endoderm differentiation in both the ES$^{indel/indel}$ and iPS$^{indel/indel}$ cell lines (Figures 3H and S4C), suggesting a cell survival defect in the ES$^{indel/indel}$ and iPS$^{indel/indel}$ cells.

β-like Cell Differentiation of GATA6 Mutants from Endodermal Progenitor Cells

The use of established differentiation protocols to study the role of GATA6 in pancreas development (Pagliuca et al., 2014; Rezania et al., 2014, 2012; Russ et al., 2015) was difficult due to the inability of the ES$^{indel/indel}$ and IPS$^{indel/indel}$ cell lines to efficiently form definitive endoderm. To overcome this developmental block, we established EP cells from the IPS cell allelic series as well as the ES$^{+/+}$ and ES$^{indel/indel}$ cell lines (Cheng et al., 2012). EP cells express *GATA6* at lower levels but *GATA4* and *GATA3* at higher levels compared with definitive endoderm, making the establishment of these lines feasible (Cheng et al., 2012). Cells were differentiated to definitive endoderm and CXCR$^+$ CKIT$^+$ cells were sorted for the generation of EP cell lines. All lines were characterized as EP cells displaying self-renewal capacity to greater than 20 passages (data not shown) and expression of appropriate markers including SOX17, FOXA1, FOXA2, EOMES, *TBX3, MSX2, MEIS2*, and *ID2* (Figure S5).

To study pancreatic cell fate, the EP cell lines were differentiated into β-like cells following our published protocol (Cheng et al., 2012). All EP cell lines generated β-like cells co-expressing C-peptide and PDX1 (Figure 4A). At the end stage of the adherent differentiation, cells were aggregated into suspension culture by dispase treatment to enrich for C-peptide$^+$ cells. Following the aggregation, the percentage of C-peptide$^+$ cells was comparable between

Representative intracellular flow cytometric analysis of FOXA1 versus SOX17 expression at day 5 of differentiation. Data shown are gated on cells expressing RFP.

(C) Quantitation of the percentage of FOXA1$^+$SOX17$^+$ cells in (B). (n = 4 per lentivirus).

(D) Quantitation of the percentage of FOXA1$^+$SOX17$^+$ cells in cells transduced with GATA6 or an empty vector control lentivirus with doxycycline addition beginning at various days during the differentiation. Data shown are gated on cells expressing RFP at day 5 of differentiation (n = 3 per time point per cell line).

(E) Cells treated and differentiated as described in (B) were cell-sorted for RFP expression at day 5 of differentiation and subjected to qRT-PCR analysis for *SOX17*, *FOXA2*, *HNF1B*, and endogenous *GATA4* expression levels (n = 3 per lentivirus).

(F) A time course of cell yield during definitive endoderm differentiation in the IPS cell allelic series (n = 3 per time point per cell line).

(G) Analysis of TUNEL staining by intracellular flow cytometry at day 2 of definitive endoderm differentiation.

(H) Quantitation of the percentage of FOXA1$^+$SOX17$^+$ cells for IPS$^{indel/indel}$ cells treated with different concentrations of bFGF from day 1 to 2 of definitive endoderm differentiation (n = 4 per condition).

For all statistical analysis, *p < 0.05, **p < 0.01, ***p < 0.001.

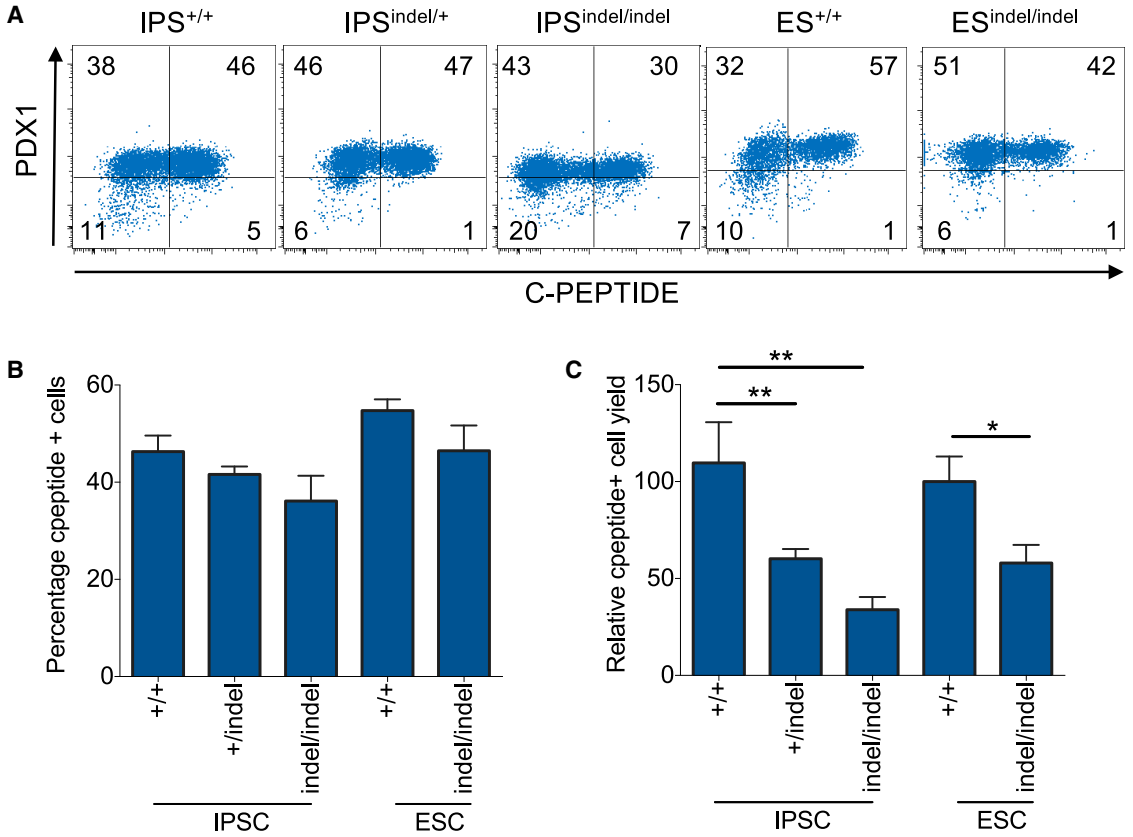

Figure 4. GATA6 Is Dispensable for Differentiation of Pancreatic β-like Cells from PSCs

EP cell lines were generated from two control, one heterozygous, and two compound heterozygous PSC lines and differentiated into β-like cells.

(A) Representative intracellular flow cytometric analysis of C-peptide versus PDX1 at day 15 of differentiation.

(B) Quantification of the percentage of C-peptide$^+$ cells from (A). Results are averaged from at least eight independent differentiations per cell line.

(C) Absolute yield of C-peptide$^+$ cells from (A). Results are an average from at least eight independent differentiations per cell line.

IPSC, induced pluripotent stem cells; ESC, embryonic stem cells. For all statistical analysis, *$p < 0.05$, **$p < 0.01$.

all lines in both genetic backgrounds (Figure 4B). The efficiency of differentiation, as calculated by absolute yield of C-peptide$^+$ cells per cell seeded relative to the respective controls of each genetic background, was found to be significantly lower in IPS$^{+/indel}$, IPS$^{indel/indel}$, and ES$^{indel/indel}$ cells (Figure 4C). Thus, while mutant *GATA6* EP cells can be differentiated into β-like cells, the efficiency of differentiation is lower compared with wild-type cells of the same genetic background.

Retinoic Acid as a Modulator of the Phenotype of Mutant GATA6 Cells

The patient from whom the IPS$^{+/indel}$ cells were derived was born with pancreas agenesis. The ability to generate β-like cells from the *GATA6* mutant lines in vitro was unexpected, and we hypothesized that our in vitro differentiation system may act to bypass the developmental defect. The differentiation protocol supplies various extrinsic signals necessary to drive pancreas specification and maturation into endocrine cells. Therefore, if GATA6 and/or GATA4 act in vivo to modulate, or are the target of one of these signals, it could explain the differences in phenotype between in vitro and in vivo development. Furthermore, if our hypothesis is correct, we predict that using limiting doses of these inductive signals might reveal a more severe phenotype in the *GATA6* mutant cell lines. We decided to focus our further studies on the pancreas induction stage from foregut endoderm, as defects here would be predicted to lead to pancreas agenesis. Virtually all differentiation protocols from either ES or EP cells utilize similar inductive signals at this stage including FGF, inhibition of sonic hedgehog, and RA signaling (Cheng et al., 2012; Kroon et al., 2008; Nostro et al., 2011; Rezania et al., 2013).

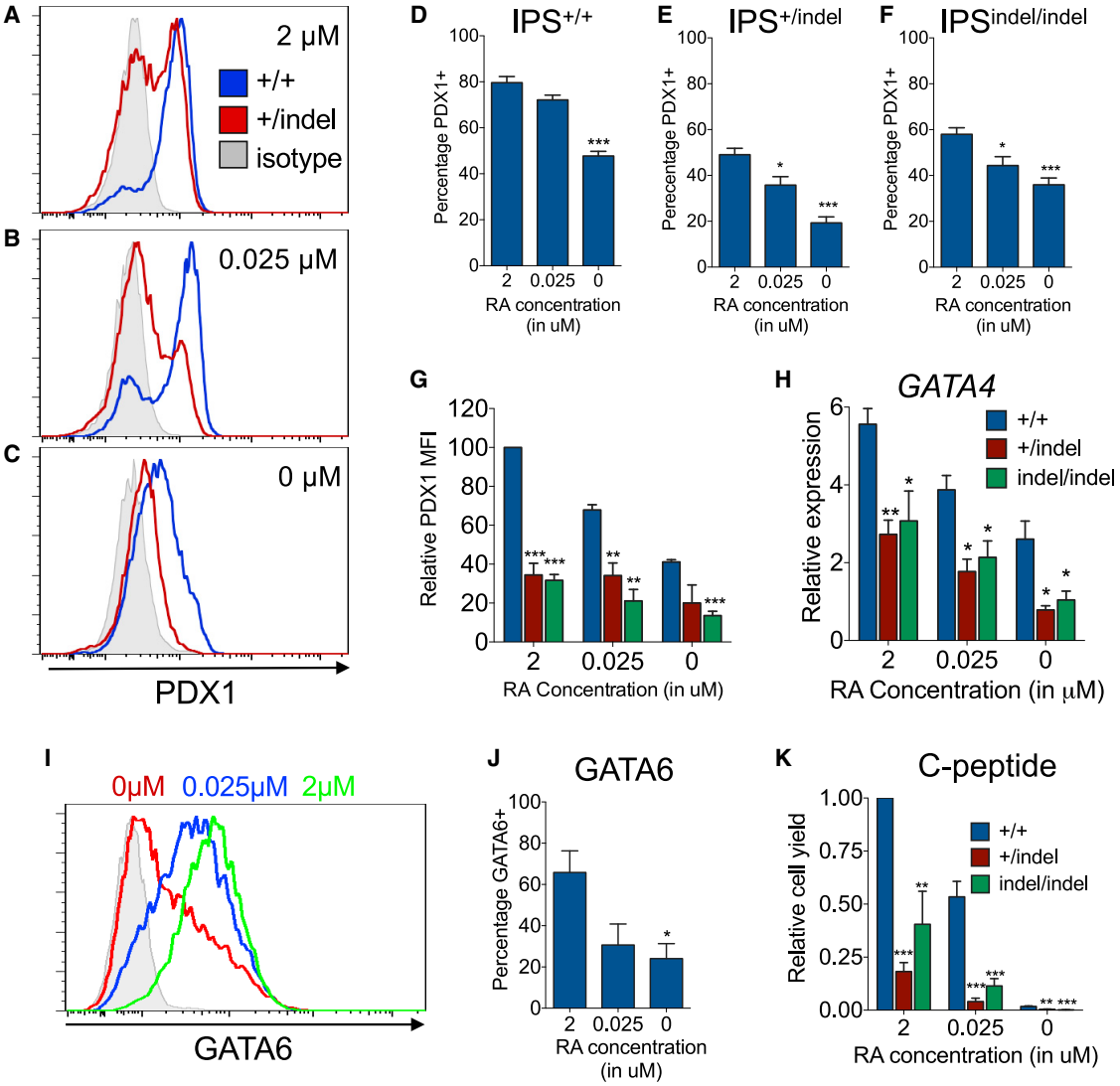

Figure 5. Retinoic Acid Acts as a Modulator of the GATA6 Phenotype

(A–E) Representative flow cytometry analysis of PDX1 on day 8 in IPS$^{+/+}$ and IPS$^{+/indel}$ pancreatic progenitors differentiated in (A) 2 μM, (B) 0.025 μM, or (C) 0 μM retinoic acid. Quantification of the percentage of PDX1^{+} cells on day 8 differentiated in 2, 0.025, or 0 μM retinoic acid for (D) IPS$^{+/+}$, (E) IPS$^{+/indel}$, and (F) IPS$^{indel/indel}$ cells (n = 5 for each cell line).

(G) Quantification of the mean florescence intensity (MFI) of PDX1 relative to the isotype control of each cell line with 2, 0.025, or 0 μM retinoic acid.

(H) *GATA4* qRT-PCR of samples from (D, E, and F) (n = 3 per line per condition).

(I) Representative flow cytometry analysis of GATA6 in IPS$^{+/+}$ pancreatic progenitors.

(J) Quantitation of percentage GATA6^{+} cells from (I) (n = 3 per condition).

(K) Quantification of the percentage of C-peptide^{+} cells on day 14 of differentiation in 2, 0.025, or 0 μM RA for IPS$^{+/+}$, IPS$^{+/indel}$ and IPS$^{indel/indel}$ cells. Cell yields are normalized to IPS$^{+/+}$ at 2 μM retinoic acid (n = 4 per cell line per condition).

For all statistical analysis, *p < 0.05, **p < 0.01, ***p < 0.001.

To determine whether loss of GATA6 affects pancreatic specification, induction of PDX1, a master regulator of pancreas development, was examined (Pan and Wright, 2011). Under standard differentiation conditions, IPS$^{+/+}$ EP cells more efficiently generate PDX1^{+} pancreatic progenitor cells (Figures 5A and 5D) compared with IPS$^{+/indel}$ (Figures 5A and 5E) and IPS$^{indel/indel}$ EP cells (Figure 5F). The mutant cells still generate a significant proportion of PDX1^{+} cells. To address whether high levels of exogenous signals provided in vitro allow *GATA6* mutant cells to

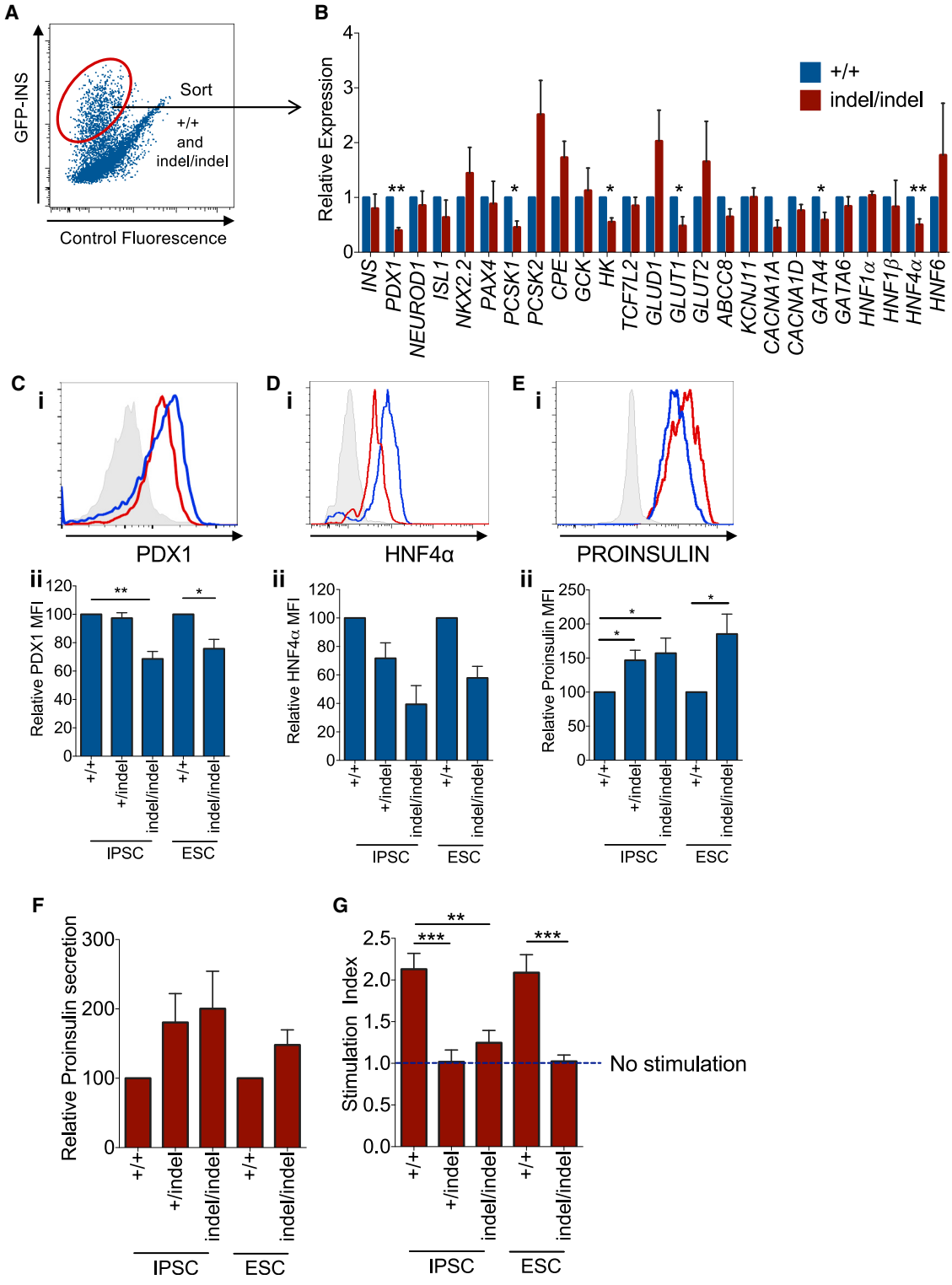

Figure 6. Gene Expression and Functional Analysis of C-Peptide$^+$ β-Like Cells

(A) The Mel1-INS-GFP cells contain a GFP reporter in the insulin locus. β-like cells from ES$^{+/+}$ and ES$^{indel/indel}$ cells were purified by cell sorting for GFP$^+$ cells and analyzed for gene expression (n = 4).

(B) qRT-PCR analysis for expression of genes important in β cell development, identity, and processing. Expression levels are normalized to ES$^{+/+}$ cells.

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more efficiently induce PDX1, limiting concentrations of FGF10, cyclopamine, and RA were tested during the differentiation in pilot studies. While lowering the concentrations of FGF10 or cyclopamine did not have a major impact on PDX1 induction (data not shown), lowering RA concentrations by 80-fold to 0.025 μM resulted in a statistically significant decrease in the percentage of $PDX1^+$ cells generated in the $IPS^{+/indel}$ and $IPS^{indel/indel}$ cell lines (Figures 5E and 5F). In contrast, the percentage of PDX1+ cells induced in the $IPS^{+/+}$ cell line was not significantly affected by the lower dose of RA, while the MFI of PDX1 was somewhat decreased (Figures 5B, 5D, and 5G). Adding no RA resulted in an even further decrease in PDX1 in the $iPS^{+/indel}$ and $iPS^{indel/indel}$ cells though $IPS^{+/+}$ cells were also affected (Figures 5A–5G). These experiments were repeated in $ES^{+/+}$ and $ES^{indel/indel}$ cells with similar findings (Figures S6A–S6D). Together, these data suggest that the addition of excess RA during in vitro differentiation may allow *GATA6* mutant cells to form pancreatic progenitors and subsequently β-like cells.

Previous findings have shown that GATA4 and GATA6 expression can be induced by RA (Arceci et al., 1993; Mauney et al., 2010). *GATA4* RNA and GATA6 protein levels were examined during the differentiation of $IPS^{+/+}$ cells with decreasing amounts of RA. A dose-dependent decrease in both *GATA4* and GATA6 was observed with decreased RA concentrations (Figures 5H–5J). Analysis of *GATA4* in GATA6 mutant lines also demonstrated a decrease in expression that was exacerbated with lower RA. These results were confirmed in the ES cell background (Figures S6D–S6F). These observations suggest that GATA4 is unable to compensate for loss of GATA6. Instead, *GATA4* is further downregulated by the loss of GATA6 and low-dose RA signaling. These findings offer a possible explanation for why *GATA6* mutant mouse models do not mimic the pancreas agenesis phenotype in humans as multiple organs examined in GATA6 null mice did not display decreases in *GATA4* (Walker et al., 2014; Zhao et al., 2005).

To determine the effects of RA dosage on β cell development, the EP cell lines were differentiated to β-like cells under different doses of RA during pancreatic induction. We examined the yield of C-peptide$^+$ β-like cells from the various mutant and wild-type IPS-derived EP lines. Confirming results in Figure 4C, under standard differentiation conditions, the $IPS^{+/indel}$ and $IPS^{indel/indel}$ cell lines display a reduced yield of C-peptide$^+$ cells (~60%–80% decreased), but in low RA conditions a more severe decrease in C-peptide$^+$ cells is seen in both mutant lines (~80%–95% decreased) (Figure 5K). Similar results were seen in the $ES^{+/+}$ and $ES^{indel/indel}$ cell lines (Figure S6H). These data confirm that differentiation of *GATA6* mutant stem cells in the context of a low dose of RA can more closely mimic the patient phenotype with a severe loss of pancreatic β-like cells.

Gene Expression Analysis of GATA6 Mutant β-like Cells

To analyze gene expression differences due to loss of GATA6, INS-GFP$^+$ β-like cells were sorted from the $ES^{+/+}$ and $ES^{indel/indel}$ cell lines and a panel of genes important for β cell function and development were assayed (Figure 6A). Significant differences were found in *PDX1*, *PCSK1*, *HK*, *GLUT1*, *GATA4*, and *HNF4α* (Figure 6B).

PDX1 was confirmed by flow cytometry to be significantly lower in $iPS^{indel/indel}$ and $ES^{indel/indel}$ cells compared with $IPS^{+/+}$ and $ES^{+/+}$ β-like cells. However, there was no difference in PDX1 in $IPS^{+/indel}$ cells compared with $IPS^{+/+}$ β-like cells (Figures 6Ci, 6Cii, and S7A). By flow cytometry, HNF4α trended toward a dose-dependent decrease within both the IPS and ES cell allelic series (Figures 6Di, 6Dii, and S7B). *PCSK1*, a gene involved in proinsulin to insulin processing (Steiner, 2004) was significantly lower in the $ES^{indel/indel}$ cells compared with the $ES^{+/+}$ cells, thus we

(C) (i) Representative intracellular flow cytometric analysis of PDX1 in $ES^{+/+}$ and $ES^{indel/indel}$ cells. (ii) Quantification of the mean fluorescence intensity of PDX1 standardized to the respective control of each genetic background. Experiments were repeated at least three times per cell line.

(D) (i) Representative intracellular flow cytometric analysis of HNF4α in $ES^{+/+}$ and $ES^{indel/indel}$ cells. (ii) Quantification of the mean fluorescence intensity of HNF4α relative to the respective isotype control of each genetic background. Experiments were repeated at least three times per cell line.

(E) (i) Representative intracellular flow cytometric analysis of proinsulin in $ES^{+/+}$ and $ES^{indel/indel}$ cells. (ii) Quantification of the mean fluorescence intensity of proinsulin in C-peptide-positive cells relative to C-peptide-negative cells. Experiments were repeated at least five times per cell line.

(F) Quantification of the ratio of proinsulin to insulin secreted per C-peptide positive cell. Secretion is normalized to the respective control ($^+/^+$) of each genetic background. Experiments were repeated at least five times per cell line.

(G) The stimulation index of C-peptide secretion on day 14 of differentiation in basal glucose (1 mM) and glucose (20 mM) conditions. The stimulation index is the ratio of C-peptide secreted during glucose stimulation to C-peptide secreted under basal glucose conditions. Stimulation index was calculated from a minimum of six differentiations (and at least ten replicates in total) for each cell line.

IPSC, induced pluripotent stem cells; ESC, embryonic stem cell. For all statistical analysis, *p < 0.05, **p < 0.01, ***p < 0.001.

hypothesize that there should be defective proinsulin processing and accumulation of proinsulin. Flow cytometry demonstrated a statistically significant, dose-dependent increase in proinsulin in the IPS and ES cell *GATA6* allelic series (Figures 6Ei, 6Eii, and S7C). In both the IPS and ES cell backgrounds, there was a trend toward increased proinsulin compared with insulin secretion in the mutant compared with the wild-type β-like cells (Figure 6F). Thus, GATA6 may be involved in insulin processing in EP-derived β-like cells and may regulate other key genes involved in β cell function such as *HNF4A* and *PDX1*.

Functional Analysis of GATA6 Mutant β-like Cells

To analyze the functionality of EP differentiated β-like cells, their responsiveness to glucose stimulation was determined. Upon glucose stimulation, the increase in C-peptide secretion of the $IPS^{+/+}$ EP-derived β-like cells was 2.1 ± 0.18-fold over basal levels (Figure 6G). The $IPS^{+/indel}$ and $IPS^{indel/indel}$ EP-derived β-like cells were unresponsive to glucose stimulation with stimulation indexes of 1.0 ± 0.14 and 1.2 ± 0.15, respectively. These results were corroborated in the ES cell background. Control $ES^{+/+}$ EP-derived β-like cells had a stimulation index of 2.1 ± 0.22-fold, while mutant $ES^{indel/indel}$ β-like cells were unresponsive with a stimulation index of 1.0 ± 0.08-fold (Figure 6G). In unstimulated conditions, all of the β-like cell lines within both allelic series, secreted similar amounts of basal C-peptide when normalized for the absolute number of C-peptide$^+$ cells per culture (Figure S7E). These data demonstrate that the levels of GATA6 are critical in determining the functional responsiveness of pancreatic β-like cells to glucose stimulation in an in vitro setting.

DISCUSSION

This study established a human in vitro PSC model system to study the role of GATA6 in the development of the pancreas and the functionality of β-like cells. Considering that GATA6 null mice are embryonic lethal due to a requirement for extra-embryonic endoderm (Morrisey et al., 1998), PSCs are advantageous because the extra-embryonic endoderm is unnecessary for their maintenance and differentiation. We have shown that while $IPS^{+/indel}$ cells with one normal allele have no defects in definitive endoderm specification, $ES^{indel/indel}$ and $IPS^{indel/indel}$ cells with two defective alleles failed to efficiently differentiate into definitive endoderm. The downregulation of pluripotency markers and the induction of primitive streak markers remained normal, suggesting a defect in the transition of cells from the primitive streak stage of development to definitive endoderm. Transgene expression of *GATA6* in the $ES^{indel/indel}$ cells rescued endoderm differentiation demonstrating the importance of GATA6 in definitive endoderm specification and that off-target effects of genome editing were not responsible for the observed phenotype.

In chimeric mouse models, GATA6 null cells can contribute to the formation of the primitive gut tube (Koutsourakis et al., 1999). The inability of human $ES^{indel/indel}$ and $IPS^{indel/indel}$ cells to generate definitive endoderm (Figures 2 and S3) suggest species-specific differences in the role of GATA6 during endoderm development. One explanation for this difference could be the timing of GATA6 induction in the human system. We observed that the expression of *GATA6* slightly preceded *GATA4* expression during endoderm induction from PSCs (Figure 1G). No other GATA factors were expressed at this time (data not shown). In mouse models, *GATA4* and *GATA6* are co-expressed in the primitive streak (Morrisey et al., 1997), therefore it is possible that in the murine system, GATA4 is compensating for GATA6, leading to the differences in phenotype.

In the mutant $ES^{indel/indel}$ and $IPS^{indel/indel}$ cell lines, there was decreased expression of both *GATA6* (Figures 1E and S3F) and *GATA4* (Figures 1F and S3G) during definitive endoderm differentiation, suggesting that GATA6 may regulate its own expression in addition to GATA4. A similar observation has been made in murine visceral and primitive endoderm of GATA6 knockout embryos (Morrisey et al., 1998). However, loss of GATA6 in the heart (Zhao et al., 2005) or the jejunum of embryos (Walker et al., 2014) did not lead to a decrease in GATA4 levels. These data support the possibility that GATA4 and GATA6 are differentially regulated in definitive endoderm and pancreatic lineages in human and mouse leading to the observed differences.

The defective definitive endoderm differentiation in both $ES^{indel/indel}$ and $IPS^{indel/indel}$ cells could be partially rescued by increased bFGF signaling (Figures 3H and S4C). During definitive endoderm differentiation, the decrease in cell number is transient, and it is possible that there may be cell proliferation of non-endodermal cell types that can fill the void left by loss of endodermal cells. Furthermore, there is a more severe deficiency in definitive endoderm differentiation of $IPS^{indel/indel}$ cells (Figure 2) than in $ES^{indel/indel}$ cells (Figure S3), suggesting genetic background influences the phenotype. In both genetic backgrounds, there is a consistent 2- to 3-fold increase of $SOX17^+/FOXA1^+$ endoderm with increasing amounts of bFGF, potentiating a partial rescue of the definitive endoderm phenotype and suggesting that apoptosis may only be a partial cause of the defect in endoderm specification with other mechanisms also playing a role.

Our data demonstrating that *GATA6* mutant stem cell lines could generate both pancreatic progenitors and β-like cells (Figures 4 and 5), even with decreased efficiency, was somewhat surprising as patients with *GATA6* mutations often have pancreas agenesis (Stanescu et al., 2014).

We identified RA signaling as an exogenous signal that may overcome the developmental block of GATA6 loss in the in vitro differentiation system. Consistent with previous reports, a decrease in the expression of both *GATA4* (Figures 5H and S6E) and GATA6 (Figures 5I and S6F) was observed when limiting amounts of RA were used during the differentiation of wild-type cells to pancreatic progenitors (Arceci et al., 1993; Mauney et al., 2010). Only when low doses of RA were used during the differentiation of indel/+ or indel/indel *GATA6* mutant cell lines was a more significant loss of both pancreatic progenitors and β-like cells observed (up to 95% loss; Figures 5K and S6H).

Heterozygous *GATA6* mutations have incomplete penetrance as displayed by different phenotypes in family members having identical mutations (Bonnefond et al., 2012). These differences in the agenesis phenotype could be partially explained by variability in the levels of endogenous RA signaling suggesting nutritional supplementation as a possible means to prevent pancreas agenesis by boosting RA signaling and regulating expression of GATA family members. We propose a model (Figure 7) to both explain our results as well as provide insights into the role that GATA6 may play during pancreas development in vivo. In wild-type cells, RA may induce the expression of GATA4 and GATA6, allowing levels to increase above the threshold required for the formation of pancreatic progenitors (Figure 7A). A decrease in endogenous RA signaling could decrease the expression of GATA4 and GATA6, but in the context of two normal *GATA6* alleles, sufficient levels of both factors are produced, resulting in the formation of pancreatic progenitors (Figure 7B). Similarly, the loss of one allele of *GATA6* in the context of high RA signaling leads to decreased GATA factor expression, but again GATA levels remain above a critical threshold for pancreatic progenitor formation (Figure 7C). Conversely, a combined loss of one allele of *GATA6* with limiting RA signaling could potentiate lower levels of GATA4, resulting in overall GATA factors falling below a critical level that may cause pancreas agenesis (Figure 7D).

The model systems described here are also important for studying the subset of diabetic patients without agenesis who have *GATA6* mutations. These systems provide a means to dissect the underlying disease mechanism during development and to study the role of GATA6 in endoderm and pancreatic development. The utilization of an intermediate progenitor population was essential for defining the role of GATA6 in pancreatic β-cell functionality, however, with the caveat that this may affect the phenotypes that could be observed. A future interest will be examination of pancreatic β cell function in cells expressing heterozygous *GATA6* mutations from patients who do not have pancreatic agenesis but present with adult-onset diabetes.

A complementary study confirms our findings that GATA6 is necessary for endoderm specification as well as the decreased efficiency of β-like cell generation from the *GATA6*$^{indel/+}$ PSC line (Shi et al., 2017). This other report did have one major discrepancy with our results; they did not see a defect in glucose-stimulated insulin secretion in *GATA6*$^{indel/+}$ β-like cells. This could be due to genetic background as they examined gene-edited ES cells, not pancreas agenesis patient derived IPSCs, or due to differences in the differentiation protocols. Future studies will be needed to further address the role of GATA6 in β cell function in the PSC system.

The use of PSCs has provided a powerful human-based system to study GATA6 mutations. Important insights have been gained into the role of GATA6 in early human definitive endoderm development, pancreatic progenitor specification, and pancreatic β cell functionality.

EXPERIMENTAL PROCEDURES

PSC Lines

The Mel1-INS-GFP ES cells were obtained from Ed Stanley and Andrew Elafanty at the Murdoch Children's Research Institute (Micallef et al., 2012). The CHOP.Panagenesis1 (patient IPS$^{+/indel}$) IPS cells were generated from a lymphoblastoid cell line (Stanescu et al., 2014) by reprogramming using episomal vectors (Okita et al., 2011) by the Stem Cell core at the Children's Hospital of Philadelphia. PSC maintenance was performed as described previously (Paluru et al., 2013).

Genome Editing Using CRISPR/Cas

To generate *GATA6* mutations in both alleles and correct the patient IPS$^{+/indel}$ cells, a gRNA was generated with the sequence 5′-AGT GGG CCA GCC AAC CAC GCG GG-3′ targeting the second exon of GATA6. For gene correction, a 200 bp oligonucleotide containing silent mutations in the gRNA sequence and a PstI restriction site in close proximity were transfected along with CAS9-GFP and gRNA. GFP^{+} cells were sorted and plated at clonal density. For INDEL mutations, single colonies were screened by PCR. For gene correction, single colonies were screened by PCR followed by a restriction digest with PstI and sequenced to confirm the correction. A more detailed protocol can be found in Supplemental Experimental Procedures.

Targeting the AAVS1 Locus

The *AAVS1* loci of Mel1-INS-GFP ES cells were targeted with a vector containing a chicken actin promoter driving the reverse rtTA using a published protocol (Tiyaboonchai et al., 2014).

Pancreatic β-like Cell Differentiation and Reaggregation

Differentiation was started 5 days after EP cells were split. Differentiation to pancreatic β-like cells was performed as previously described (Cheng et al., 2012; D'Amour et al., 2006) with modifications at the end stage of the protocol (details can be found in the

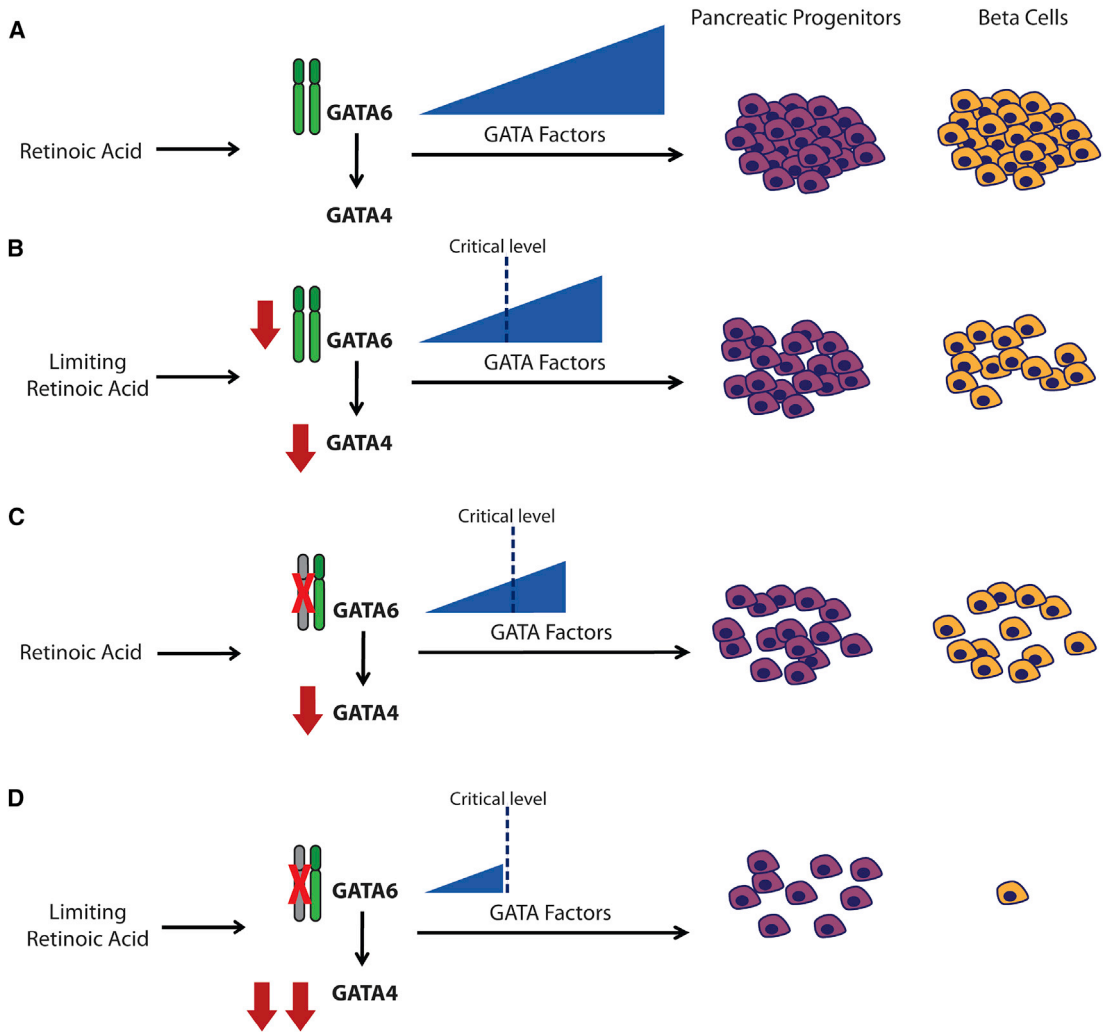

Figure 7. Model for the Incomplete Penetrance of GATA6 Heterozygous Mutations

(A) Under normal conditions, endogenous retinoic acid can activate the expression of both GATA6 and GATA4 leading to the development of pancreatic progenitors and β cells.

(B) Decreased endogenous retinoic acid signaling may lead to decreased GATA4 and GATA6 expression. Despite this decrease, the combined level of GATA4 and GATA6 remains above a critical threshold and is sufficient for pancreatic progenitor specification and β cell development.

(C) The loss of one allele of GATA6 in individuals with higher endogenous retinoic acid signaling. A loss of one allele of GATA6 may cause some decrease in the expression level of GATA4 but the level of combined GATA factors still remain above the critical level for pancreatic progenitor and β cell formation.

(D) A combination of lower endogenous retinoic acid signaling and a loss of one allele of GATA6 will lead to even lower levels of GATA4 expression. The levels of combined GATA factors may be insufficient to meet the minimum critical level necessary for the formation of pancreatic progenitors leading to pancreatic agenesis.

Supplemental Experimental Procedures). At day 13, cells were reaggregated following treatment with 1 mg/mL dispase for 15 min and replated into a low-adherent plate (Corning). The medium was changed every other day.

Glucose-Stimulated Insulin Secretion Assay

β-like cell aggregates were washed twice with Krebs-Ringer bicarbonate HEPES (KRBH) (details in Supplemental Experimental Procedures) and incubated at 37°C in 1 mL of KRBH for 1 hr. Cells were incubated in 500 μL of 1 mM glucose in KRBH for 20 min and stimulated in 500 μL of 20 mM glucose in KRBH for 20 min. Following each incubation, cells were centrifuged at 150 × *g* for 90 s. Supernatant was collected and stored at −20°C. C-peptide ELISA was performed using the ultra-sensitive C-peptide kit following the manufacturer's instructions (Mercordia). For the ELISA, supernatant was diluted 8- to 20-fold.

Statistical Analysis

Results from multiple experiments are expressed as the mean ± SEM. An unpaired two-tailed Student's t test for groups with equal variance was performed to determine p values. For experiments that tested different conditions on the same cell lines, a one-way ANOVA followed by Dunnett's multiple comparisons test was performed. All statistical analysis was performed on Prism version 6.0e for Mac (GraphPad Software). In the figures, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, and n denotes individual experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.026.

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