David E. Metzger,[1] Chengyang Liu,[2] Amin Sam Ziaie,[2] Ali Naji,[2] and Kenneth S. Zaret[1]

Grg3/TLE3 and Grg1/TLE1 Induce Monohormonal Pancreatic β-Cells While Repressing α-Cell Functions

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In the pancreas, α- and β-cells possess a degree of plasticity. In vitro differentiation of pluripotent cells yields mostly α- and polyhormonal β-like cells, indicating a gap in understanding of how functional monohormonal β-cells are formed and of the endogenous repressive mechanisms used to maintain β-cell identity. We show that the corepressor Grg3 is expressed in almost all β-cells throughout embryogenesis to adulthood. However, Grg3 is expressed in fewer nascent α-cells and is progressively lost from α-cells as endocrine cells mature into adulthood. We show that mouse *Grg3*$^{+/-}$ β-cells have increased α-specific gene expression, and *Grg3*$^{+/-}$ pancreata have more α-cells and more polyhormonal cells, indicating that Grg3 is required for the physiologic maintenance of monohormonal β-cell identity. Ectopic expression of Grg3 in α-cells represses *glucagon* and *Arx,* and the addition of Pdx1 induces *Glut2* expression and glucose-responsive insulin secretion. Furthermore, we found that Grg1 is the predominant Groucho expressed in human β-cells but acts functionally similarly to Grg3. Overall, we find that Grg3 and Grg1 establish a monohormonal β-cell identity, and Groucho family members may be useful tools or markers for making functional β-cells.

Much attention has been directed to generating functional pancreatic β-cells from other sources, such as embryonic stem cells, induced pluripotent stem cells, and the conversion of non-β-cell types. Developmental biology experiments have outlined the multistep differentiation process toward a functional β-cell (1,2). However, monohormonal, glucose-responsive β-cells are not readily produced in culture (3,4); thus, more focus is needed on how the pancreas develops monohormonal β-cells.

Repressive mechanisms often are used to prevent cells from attaining alternative fates and to maintain a cell's differentiated identity. The Groucho corepressor proteins (Gro/Grg/TLE) interact with many transcription factors, converting them to repressors. Although broadly expressed, Grouchos play many specific roles during invertebrate and vertebrate development (5–7).

Of the Groucho family members expressed in mouse pancreas, *Grg3* is the most abundant (8–10). *Grg3* is induced by *Ngn3* in nascent endocrine cells and is required for the delamination of endocrine progenitors from the pancreatic epithelium by repressing *E-cadherin* (8). Grg3 also interacts with Nkx2.2 in β-cells where it helps to specify the correct number of β-cells and maintains β-cell identity by recruiting HDAC1 and Dnmt3a to the *Arx* gene (11,12). Because the misexpression of *Arx* converts β-cells to α-cells (13), the Grg3-containing repressive complex that normally represses *Arx* expression in β-cells may help to prevent β-cell-to-α-cell conversion. However, whether Grg3 is the essential Groucho protein acting during β-cell induction and maturation is not known. Furthermore, Grg3 may interact with other transcription factors that repress the α-cell fate. For example, Groucho proteins have been shown to bind Nkx6.1 in the context of neural tube development (14), and Nkx6.1 can repress the α-cell fate (15).

[1]Institute for Regenerative Medicine, Institute for Diabetes Obesity and Metabolism, Department of Cell and Developmental Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA
[2]Department of Surgery, University of Pennsylvania Perelman School of Medicine, Smilow Center for Translational Research, Philadelphia, PA
Corresponding author: Kenneth S. Zaret, zaret@upenn.edu.

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Under near-total β-cell ablation, α-cells can convert to β-cells (16). Forced expression of the β-cell–specific transcription factor Pdx1 directs endocrine progenitors to the β-cell fate. However, ectopic Pdx1 expression in glucagon-positive α-cells fails to completely convert α-cells to β-cells (17), suggesting that additional transcriptional repression is required to complete the conversion phenotype.

We now find that *Grg3* is expressed higher and more frequently in β-cells throughout development than in α-cells and helps β-cells to become monohormonal. It does this in part by being recruited by Nkx6.1 to the *glucagon* promoter to repress expression in β-cells. We also found that Grg3 can act in synergy with Pdx1 to convert α-cells in vitro to a cell that secretes insulin upon glucose stimulation, a feature that ectopic Pdx1 was not able to perform alone. Groucho repression through Grg1/TLE1 also occurs in human β-cells. We show that Groucho/TLE corepressors may be useful sentinels of monohormonal β-cell formation as well as a useful tool along with other β-cell transcription factors to efficiently convert α-cells to functional β-cells.

RESEARCH DESIGN AND METHODS

Immunofluorescence

Immunofluorescence on OCT frozen sections was performed as previously described (8) with guinea pig-α-insulin (Abcam), mouse-α-glucagon (Beta Cell Biology Consortium [BCBC]), rabbit-α-Grg3 (18), rabbit-α-Grg1 (18), and mouse-α-Nkx6.1 (BCBC) antibodies. To assess the specificity of α-Grg3 and α-Grg1 on human islet sections, antibodies were incubated with immunizing peptide (18) for 1 h before application on sections. Cultured cells were fixed with 4% paraformaldehyde, permeabilized with 2% Triton X, blocked with 3% BSA, and probed with rabbit-α-Grg3, mouse-α-Nkx6.1, goat-α-FoxA2 (Santa Cruz Biotechnology), mouse-α-Flag (Sigma-Aldrich), guinea pig-α-insulin, mouse-α-Pdx1 (BCBC), C-peptide (Cell Signaling Technology), and Alexa Fluor conjugated secondary antibodies (Invitrogen). Staining intensity of Grg1 on human islet sections was determined by analyzing random images of 15 α-cells and 15 β-cells with ImageJ software. Images were taken at the same exposure, and the same threshold was set for each on ImageJ. Pixel area was then counted by ImageJ, and data are represented as an average of all images.

Endocrine Cell RNA Isolation

To isolate RNA from embryonic *Grg3*$^{+/+}$*;Ngn3-eGFP* and *Grg3*$^{+/-}$*;Ngn3-eGFP* (8,19,20) endocrine cells, we dissociated E17.5 pancreata with 0.05% trypsin/EDTA (Gibco) and fluorescence-activated cell–sorted green fluorescent protein (GFP)–positive cells directly into RLT buffer and isolated RNA with an RNeasy Mini Kit (Qiagen). To obtain RNA from neonatal α- and β-cells, yellow fluorescent protein (YFP)/GFP+ cells from *glucagon-Cre;R26R-YFP* (21) and *Pdx1-GFP* (22) pancreata, respectively, were similarly sorted. All sortings were performed by the University of Pennsylvania FACS Facility.

Mouse Genotyping

All animal studies were performed with Institutional Animal Care and Use Committee approval. Mice were genotyped by the following primer sets: *Grg3*$^{+/-}$*(LacZ)* 5′-TACTGTCGTCGTCCCCTCAAA-3′, 5′-ACTCCAACGCAG CACCATCAC-3′; *Ngn3-eGFP* 5′-ATACTCTGGTCCCCGTG-3′, 5′-TGTTTGCTGAGTGCCAACTC-3′, 5′-GAACTTGTGGCCGT TTACGT-3′; and *glucagon-Cre* 5′-TTGAAAATGCTTCTGTCC GTTTGC-3′, 5′-AACGAACCTGGTCGAAATCAGTG-3′.

Quantitative PCR for Gene Expression

RT-quantitative PCR (qPCR) was performed with the AgPath-ID One-Step RT-PCR Kit (Ambion) using the following TaqMan Gene Expression Assays (Applied Biosystems): *Gapdh* (Mm99999915_g1), *Tle3/Grg3* (Mm00437097_m1), *Tle1/Grg1* (Mm00495643_m1), *Gcg* (Mm00801712_m1), *Arx* (Mm00545903_m1), *Ins1* (Mm01259683_g1), *Ins2* (Mm00731595_gH), *Slc2a2/Glut2* (Mm00446229_m1), *Chga* (Mm00514341_m1), *Nkx6.1* (Mm00454962_m1), *hPdx1* (Hs00236830_m1), *hGapdh* (Hs02758991_g1), and *hTle3/Grg3* (Hs00183222_m1). Reactions were run on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and quantitated by RQ Manager software (Applied Biosystems) using the ΔΔCT method. Expression was normalized to *Gapdh*, except for adult pancreas, which was normalized with the panendocrine marker *Chga*.

Single-Cell Sort and Fluidigm PCR

Single GFP+ pancreatic endocrine cells from E17.5 Ngn3-eGFP embryos were sorted directly into a single well in a 96-well PCR plate. cDNA synthesis and amplification was performed using the CellsDirect One-Step qRT-PCR Kit (Invitrogen) along with TaqMan probes specific to *Gapdh*, *Grg3*, *Gcg*, *Arx*, *Ins1*, *Ins2*, *Chga*, *Sst* (Mm00436671_m1), *Ghrl* (Mm00445450_m1), and *Ppy* (Mm00435889_m1). In brief, the cells were sorted directly into CellsDirect 2X Reaction Mix. Subsequently, TaqMan probes and SuperScript III RT/Platinum Taq Mix were added to the wells, and the PCR plate was placed in a thermocycler to generate and amplify probe-specific cDNAs. Fluidigm PCR was performed by the University of Pennsylvania Molecular Profiling Facility by using these TaqMan probes on the BioMark HD System (Fluidigm). The PCR results were evaluated for whether gene expression was present in each cell.

Lentiviral Production, Cell Culture, Knockdown, and Ectopic Expression

Grg3-specific (V2LMM_242222 and V2LHS_18355) and nonsilencing short hairpin RNA (shRNA) (pGIPZ; OpenBiosystems), GFP-Grg3, GFP, Flag-Grg3, human-Pdx1 (23), and Nkx6.1 lentiviruses were produced as previously described (8). To generate the Nkx6.1 lentiviral construct, the Nkx6.1 cDNA-containing plasmid (MGC clone 8861038;

OpenBiosystems) was used as a PCR template, and the PCR product was digested and ligated into the PWPT vector (24). Lentivirus was produced in 293T-cells, and media were passed through a 0.45 μmol/L filter directly added to αTC1-6 and βTC6 cells. αTC1-6 and βTC6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose) with 10% FBS and penicillin-streptomycin. For *Grg3* knockdown in βTC6 cells, pGIPZ lentiviral media were directly added to cells. After 3 days, the media were replaced with fresh media. After 2 more days of culture, the cells were dissociated, and high-expressing GFP cells were sorted into RLT buffer to isolate RNA. For ectopic GFP-Grg3 expression in αTC1-6 cells, cells were cultured for 3 weeks and sorted by negative-, low-, and high-GFP–expressing cells into RLT buffer. For ectopic GFP-Grg1 expression in αTC1-6 cells, cells were transfected with pAcGFP1-Grg1 (18) using FuGENE 6 (Promega), cultured for 3 days, and sorted for negative-, low-, and high-GFP–expressing cells into RLT buffer to isolate RNA.

Chromatin Immunoprecipitation–qPCR

Chromatin immunoprecipitation (ChIP) was performed with the ChIP-IT Express Kit (Active Motif) using 10^7 cells per ChIP. For the Flag ChIP, αTC1-6 cells and βTC6 cells were infected with Flag-Grg3 lentivirus. ChIP was performed with mouse-α-Flag (Sigma-Aldrich) antibody on Flag-Grg3–infected and GFP-infected cells. For the Nkx6.1 ChIP, Nkx6.1 antibody (BCBC) was used in both αTC1-6 and βTC6 cells. ChIP-qPCR product was quantitated using $2^{(CTinput - CTChIP)}$. Results are shown as ChIP/input in relative units. PCR was performed on ChIP products for the *glucagon* promoter (5′-AAGCAGATGAGCAAAGTGAGTG-3′ and 5′-AGGCTGTTTAGCCTTGCAGATA-3′), *Hnf1β* promoter (5′-CTCTGGCAAGTCCCA ATCCC-3′ and 5′-CCAT GATCTCCACCATTAGGC-3′), and *Hnf6* promoter (5′-TT TGGGCCATGACATAGTTTC-3′ and 5′-CTTGCTACCTCCT GGTCTTCC-3′) using Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems).

Glucose Tolerance Tests

$Grg3^{+/+}$ and $Grg3^{+/-}$ mice were fasted overnight and then administered 2 mg/g glucose i.p. Blood glucose levels were measured with a Contour blood glucose meter (Bayer) after fasting, after injections, and at time points after injection.

Proximity Ligation Assays

Proximity ligation assay (PLA) was performed on cultured αTC1-6 and βTC6 cells using the Duolink kit (Olink Bioscience). Antibodies from various species were used to probe for potential interacting proteins. Plus and Minus secondary antibodies detecting various primary antibodies from various species were then used. Plus and Minus antibodies in proximity to each other interact to make a ligation and amplification reaction that can be visualized as red fluorescence foci. Rabbit-α-Grg3/mouse-α-Nkx6.1, rabbit-α-Grg3/mouse-IgG, rabbit-α-Grg3/goat-α-FoxA2, and rabbit-α-Grg3/goat-IgG antibody combinations were tested.

Insulin Secretion Tests

Equal numbers of αTC1-6 and βTC6 cells were cultured in 24-well plates in DMEM (1 g/L glucose) (Gibco) with 10% FBS. To test insulin secretion into the media, cells were incubated with 250 μL low-glucose (1 g/L) and high-glucose (4.5 g/L) serum-free DMEM for 1 h at 37°C. The media were then collected, and insulin content was assessed with an Ultrasensitive Mouse Insulin ELISA kit (Mercodia).

RESULTS

Biallelic Expression of *Grg3* Is Necessary for Establishing the Proper Ratio of β- and α-Cells

We previously quantified Grg3 expression in pancreatic endocrine cells in E15.5 and neonatal mice (8). We have now also analyzed α- and β-cells for Grg3 at E17.5 and in adult mice. Immunofluorescence of the E17.5 pancreas shows that the majority of insulin-positive β-cells express Grg3 (Fig. 1*A*, red arrows, inset magnification in *right panel*), whereas glucagon-positive α-cells either lacked Grg3 or had low levels of nuclear Grg3 (Fig. 1*A*, gray arrow). The specificity of the Grg3 antibody was validated by its lack of nuclear staining in $Grg3^{-/-}$ embryos and in the presence of peptide immunogen on wild-type embryos (8,18). In adult islets, we found that Grg3 was expressed

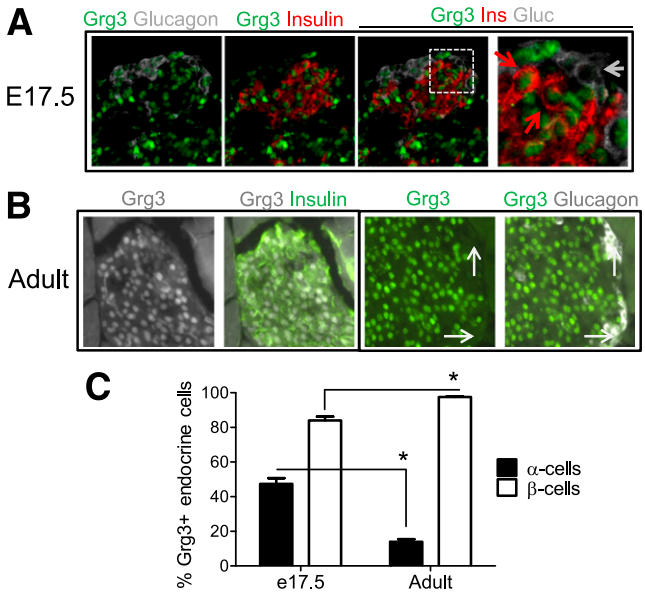

Figure 1—Grg3 is expressed in most β-cells but less frequently in α-cells. *A*: Triple immunofluorescence of E17.5 pancreas with Grg3 (green), insulin (red), and glucagon (gray) on the same section, showing that most insulin-positive cells are Grg3+ (red arrows), whereas many glucagon-positive cells are Grg3– or have low-level Grg3 expression (gray arrow). *B*: Immunofluorescence for Grg3, insulin, and glucagon in adult islets, showing that virtually all insulin-positive cells are Grg3+, whereas glucagon-positive cells are Grg3– (white arrows). *C*: The percentage of Grg3+ β-cells and α-cells was determined at E17.5 (4 animals; 588 α-cells and 932 β-cells analyzed) and adulthood (14 islets from 3 animals; 547 α-cells and 1,479 β-cells analyzed). *$P < 0.0001$.

in virtually every insulin-positive β-cell but was absent from the majority of glucagon-positive α-cells (Fig. 1*B*, white arrows). As endocrine maturation proceeds, the percentage of Grg3+ α-cells decreases from E17.5 (47%) to the adult (14%), whereas the percentage of Grg3+ β-cells slightly increases from E17.5 (84%) to the adult (98%) (Fig. 1*C*). Thus, Grg3 expression is dynamic during endocrine maturation, marking a large portion of α- and β-cells early in endocrine development but being restricted to the β-cell lineage and diminished in α-cells as they mature. In addition, we found that the Grg1 protein becomes strongly expressed in adult mouse β-cells but not in the embryonic or neonatal endocrine cells (Supplementary Fig. 1), suggesting that Grg3 plays a nonredundant role during early endocrine development, but Grg1 may then act redundantly with Grg3 in the adult mouse islet.

We sought to determine whether wild-type levels of Grg3 in β-cells are required for β-cell identity and maintenance. The majority of *Grg3*$^{-/-}$ embryos die by E14.5, and mutant embryos that survive have very few endocrine cells (8). However, analysis of rare Grg3$^{-/-}$ endocrine cells indicated that these cells are mainly bihormonal compared with monohormonal endocrine cells of wild-type littermates (Supplementary Fig. 2). To analyze later stages of endocrine development and maturation, we used *Grg3*$^{+/-}$ mice (8). We found that both E17.5 and 6-week-old *Grg3*$^{+/-}$ pancreata had a decrease in the percentage of insulin-positive β-cells compared with wild-type and an increase in glucagon-positive α-cells and bihormonal insulin-positive/glucagon-positive cells (Fig. 2*A–D*), with variability from islet to islet (Fig. 2*E–G*). Although we were unable to readily detect changes in *insulin 1/2* and *glucagon* gene expression in whole *Grg3*$^{+/-}$ pancreata, β-cell–specific *Glut2* was significantly reduced in *Grg3*$^{+/-}$ adult pancreata, consistent with a reduction of β-cells (Fig. 2*H*). We also found a significant induction of *somatostatin* gene expression in *Grg3*$^{+/-}$ pancreata (Fig. 2*I*). The decrease in β-cells, nonetheless, was not enough to cause overt hyperglycemia after a glucose challenge in 6-week-old and 12-month-old mice (Supplementary Fig. 3). In summary, robust levels of *Grg3* are needed to repress α-cell identity to maintain the correct ratio of β-cells to α-cells throughout development to adulthood.

We next assessed whether Grg3 represses α-cell genes at the transcriptional level. To enrich for the endocrine cell population, we sorted bulk GFP+ endocrine cells from *Ngn3-eGFP;Grg3*$^{+/-}$ and *Ngn3-eGFP;Grg3*$^{+/+}$ pancreata at E17.5 (Fig. 3*A*). GFP+ cells in the *Ngn3-eGFP* E17.5 pancreas represent descendants of Ngn3+ progenitors that recently expressed and then shut off *Ngn3* expression and now express one or another hormone gene; at E17.5, they mostly initiate *insulin* expression (20). We found that *Grg3*$^{+/-}$ GFP+ cells express significantly higher levels of the α-cell–specific transcription factor *Arx* (25) and *glucagon* (Fig. 3*B*). However, at E17.5, *insulin 1* and *insulin 2* were not affected (Fig. 3*B*). *Insulin 1/2* were not affected because more β-cells than α-cells are born at this time point (26), so a slight decrease in β-cell gene expression may not be evident or heterozygosity for *Grg3* is insufficient to decrement *insulin* mRNA steady-state levels at that time.

We also performed RT-qPCR on single endocrine cells by sorting E17.5 *Ngn3-eGFP;Grg3*$^{+/+}$ or *Ngn3-eGFP;Grg3*$^{+/-}$ endocrine cells and analyzing gene expression with Fluidigm PCR technology (Fig. 3*C*), probing for the expression of multiple endocrine genes in each cell. In this assay, we scored the presence of gene expression by determining whether a Ct value was achieved. We found that at E17.5, ~92% of *Grg3*$^{+/+}$ and 90% of *Grg3*$^{+/-}$ GFP+ cells were positive for *Chga*, a marker for the endocrine lineage (27). Sixty-four percent of *Grg3*$^{+/+}$ and 68% of *Grg3*$^{+/-}$ of the *Chga*+ endocrine cells expressed *insulin 1* and/or *insulin 2,* indicating that the majority of the endocrine cells sorted from the E17.5 developmental time point are β-cells (Fig. 3*D*). A previous study showed that single *insulin*-positive β-cells can coexpress non-β-cell hormone genes (28). We analyzed the *insulin*-positive cells for either monohormonal (*insulin* positive only) or polyhormonal (*insulin* positive plus other hormones expressed) status. We found that of the 14 *insulin*-positive *Grg3*$^{+/+}$ endocrine cells, only 1 (7%) coexpressed *glucagon* and the α-cell transcription factor *Arx* (Fig. 3*E*). However, in 13 *insulin*-positive *Grg3*$^{+/-}$ endocrine cells, 5 (38%) coexpressed *glucagon*, and 3 of those expressed *Arx* (Fig. 3*E*). This result recapitulates the change in gene expression of *glucagon* and *Arx* seen in bulk *Grg3*$^{+/+}$ versus *Grg3*$^{+/-}$ endocrine cells (Fig. 3*B*) and the increase in the bihormonal insulin-positive/glucagon-positive cells in *Grg3*$^{+/-}$ pancreata by immunofluorescence (Fig. 2). Of note, the expression of other non-β-endocrine cell hormones (*PP*, *SS*, and *ghrelin)* occurs more frequently in *insulin*-positive *Grg3*$^{+/-}$ cells than in *Grg3*$^{+/+}$ cells (Fig. 3*E*). Overall, the wild-type pancreas had more monohormonal *insulin*-positive cells (9 of 14 [64%]) than the *Grg3*$^{+/-}$ pancreas (3 of 13 [23%]), and the *Grg3*$^{+/-}$ pancreas had more polyhormonal *insulin*-positive cells (10 of 13 [77%]) than the *Grg3*$^{+/+}$ pancreas (5 of 14 [36%], $P = 0.037$) (Fig. 3*F*). The difference in extent of polyhormonal cells between the Fluidigm data here and the immunofluorescence data in Fig. 2 could reflect differences between mRNA and protein stability, indicating that wild-type levels of *Grg3* are needed physiologically to specify a monohormonal β-cell identity.

Grg3 Threshold Is Required for α-Cell Gene Repression In Vitro

We further sought to determine whether Grg3 helps to maintain the endocrine cell fate in culture. We used the mouse cell lines βTC6 (29) and αTC1-6 (30), which express robust levels of *insulin 1/2* and *glucagon*, respectively (Fig. 4*A*). We found that *Grg3* is expressed higher in βTC6 cells than in αTC1-6 cells (Fig. 4*A*). This expression pattern closely resembles that seen in neonates in GFP+ sorted *Pdx1-GFP* β-cells and YFP+ sorted *glucagon-Cre;*

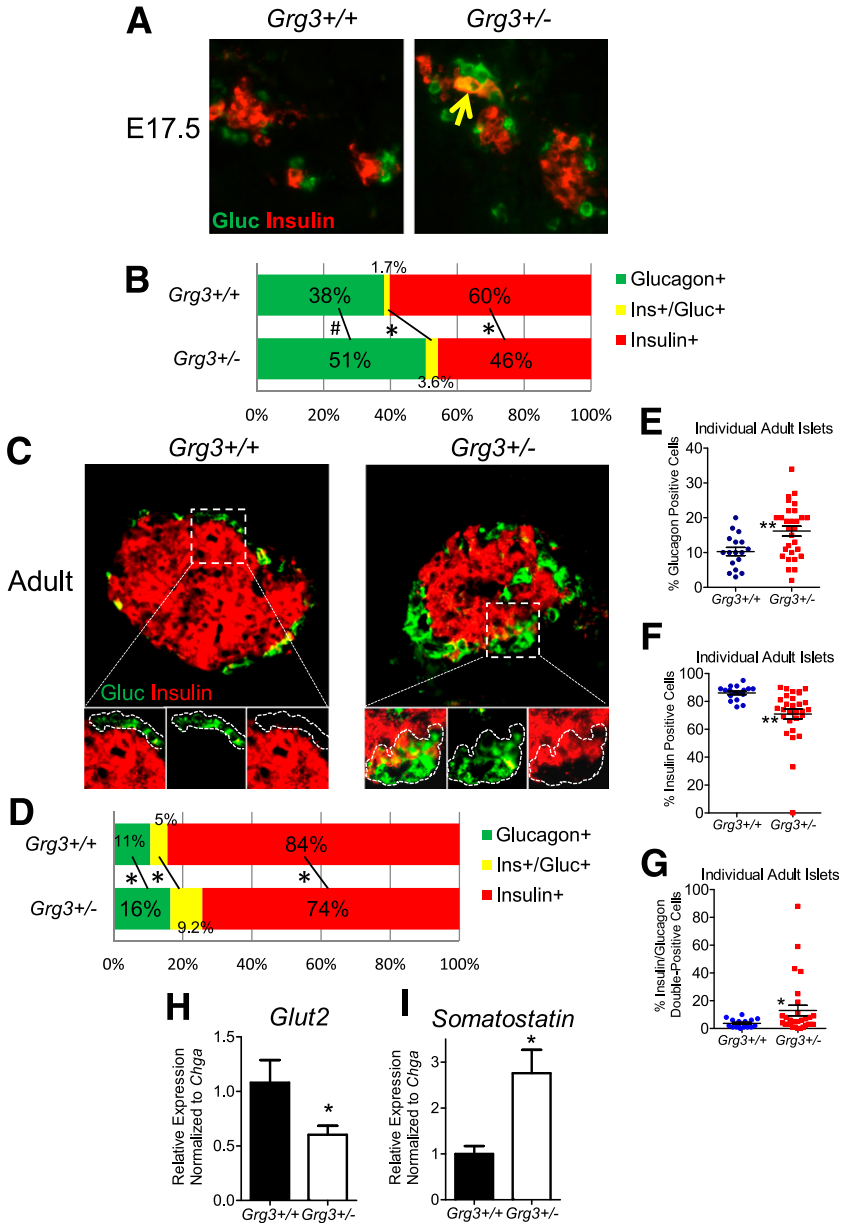

Figure 2—*Grg3*$^{+/-}$ pancreata have fewer β-cells and more α-cells than *Grg3*$^{+/+}$. *A*: E17.5 *Grg3*$^{+/+}$ and *Grg3*$^{+/-}$ pancreata stained for insulin and glucagon. The yellow arrow points out a double-positive (insulin-positive/glucagon-positive) cell in the *Grg3*$^{+/-}$ pancreas. *B*: Percentage of insulin-positive β-cells, glucagon-positive α-cells, and double-positive cells in *Grg3*$^{+/+}$ (4 animals, 1,402 cells analyzed) and *Grg3*$^{+/-}$ (4 animals, 2,000 cells analyzed) E17.5 pancreata. *C*: Adult *Grg3*$^{+/+}$ and *Grg3*$^{+/-}$ islets stained for insulin and glucagon. Boxed areas are enlarged to show single insulin or glucagon channel. *D*: Percentage of insulin-positive β-cells, glucagon-positive α-cells, and double-positive cells in *Grg3*$^{+/+}$ (3 animals, 2,551 cells analyzed) and *Grg3*$^{+/-}$ (3 animals, 3,264 cells analyzed) adult islets. The percentage of glucagon-positive (*E*), insulin-positive (*F*), and glucagon/insulin-positive (*G*) cells in individual islets. *Glut2* expression is significantly reduced in adult *Grg3*$^{+/-}$ pancreata (*H*), whereas *somatostatin* expression is increased (*I*). #$P = 0.053$, *$P < 0.05$, **$P < 0.01$.

R26R-YFP α-cells (Fig. 4*B*). To determine whether robust levels of *Grg3* are required for *glucagon* and *Arx* repression in βTC6 cells, we knocked down *Grg3* in βTC6 cells with two independent shRNAs introduced by lentivirus (Fig. 4*C*). Compared with a control shRNA, the *Grg3* shRNAs caused *glucagon* expression to increase and *Arx* expression to be induced (Fig. 4*C*). These results are similar to the in vivo data with *Grg3*$^{+/-}$ endocrine cells (Figs. 2 and 3).

To determine whether ectopic Grg3 in α-cells can repress *glucagon* and *Arx*, we overexpressed GFP and GFP-Grg3 in αTC1-6 cells by lentiviral infection. We found that short-term ectopic Grg3 greatly repressed both *glucagon* and *Arx* (Fig. 4*D*). Because in vivo results suggested that a certain threshold of Grg3 is needed for *glucagon* and *Arx* repression, we investigated whether this threshold is also necessary for repression in α-cells in vitro. After 3 weeks of culturing GFP-Grg3–infected αTC1-6 cells and sorting for negative

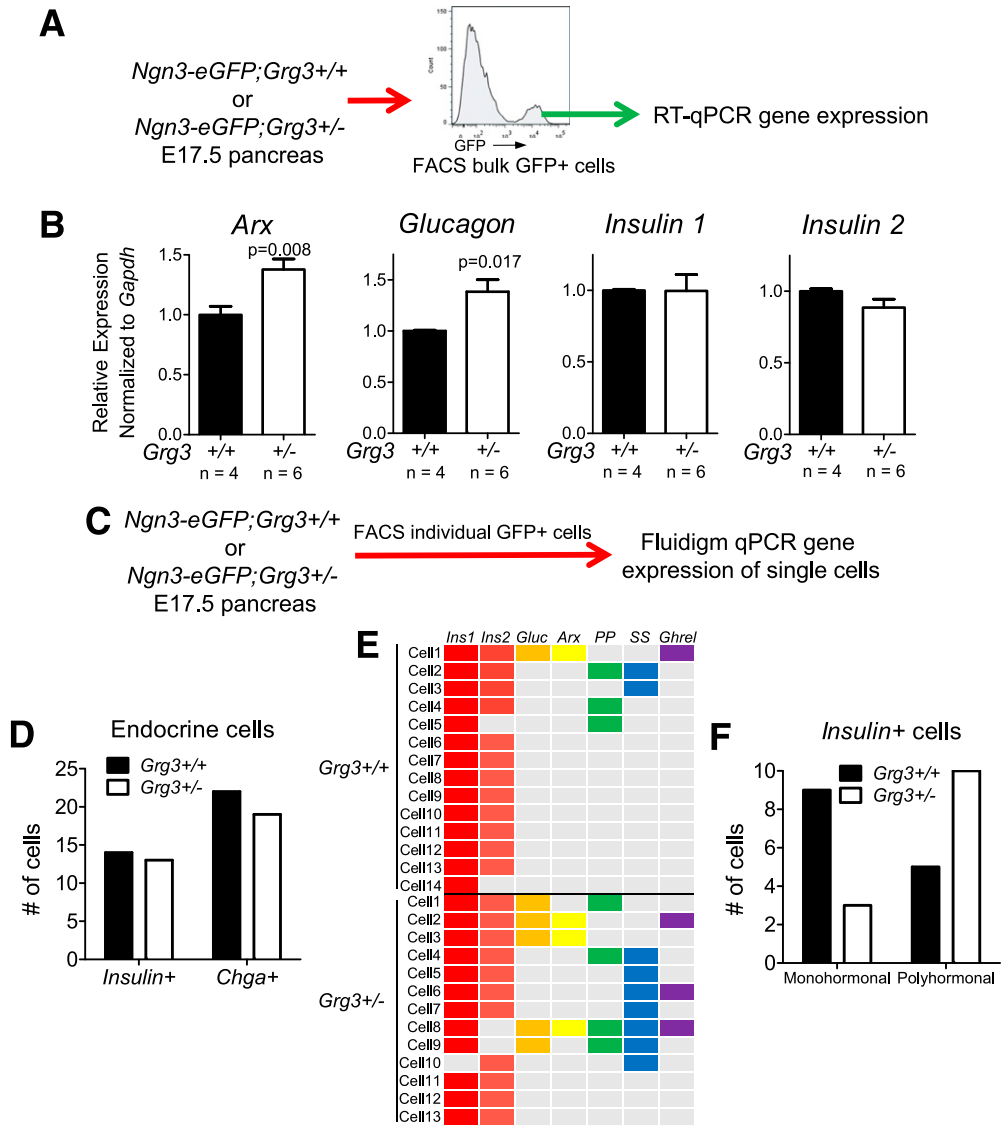

Figure 3—Robust *Grg3* expression helps to maintain a monohormonal β-cell identity. *A*: Strategy to isolate bulk endocrine cells from *Ngn3-eGFP;Grg3*$^{+/+}$ and *Ngn3-eGFP;Grg3*$^{+/-}$ E17.5 embryos for gene expression analysis. *B*: *Arx* and *glucagon* become derepressed in *Grg3*$^{+/-}$ versus *Grg3*$^{+/+}$ endocrine cells, but *insulin 1* and *insulin 2* remain unchanged. *C*: Strategy to isolate individual endocrine cells from *Ngn3-eGFP;Grg3*$^{+/+}$ and *Ngn3-eGFP;Grg*$^{+/-}$ E17.5 embryos for single-cell Fluidigm gene expression analysis. *D*: Of the *Chga*+ endocrine cells, 14 of 22 (64%) and 13 of 19 (68%) expressed *insulin* in *Grg3*$^{+/+}$ and *Grg*$^{+/-}$ pancreata, respectively. *E*: *Grg3*$^{+/+}$ and *Grg3*$^{+/-}$ *insulin 1*-positive and/or *insulin 2*-positive cells were analyzed for *glucagon* (*Gluc*), *Arx*, *pancreatic peptide* (*PP*), *somatostatin* (*SS*), and *ghrelin* (*Ghrl*) positivity. Filled-in boxes represent a positive call for the indicated gene in the indicated cell. *F*: *Grg3*$^{+/+}$ has more monohormonal *insulin*-positive cells (9 of 14 [64%]) than the *Grg3*$^{+/-}$ (3 of 13 [23%]) and has fewer polyhormonal *insulin*-positive (5 of 14 [36%]) cells than *Grg3*$^{+/-}$ (10 of 13 [77%]). One-tailed Fisher exact test $P = 0.037$. FACS, fluorescence-activated cell sorter.

or low-GFP-Grg3– and high-GFP-Grg3–expressing cells, we found that Grg3 repressed *glucagon* in a dose-dependent manner, but only a high level of Grg3 repressed *Arx* (Fig. 4*E*). Of note, in long-term cultures, high-GFP-Grg3–expressing cells significantly induced *insulin 2* expression but not *insulin 1* expression (data not shown), whereas both low- and high-GFP-Grg3–expressing cells significantly induced the β-cell–specific gene *Glut2* (Fig. 4*E*), suggesting that robust expression of Grg3 represses the α-cell program and, over time, allows for the induction of β-cell–specific *insulin 2* and *Glut2* gene expression.

Grg3 Directly Represses *Glucagon*

It has previously been reported that Grg3 is recruited to the *Arx* gene and required for its repression in β-cells (11). The dose-responsive repression of Grg3 on *glucagon* in αTC1-6 cells (Fig. 4*E*) and derepression of *glucagon* with reduced *Grg3* levels in β-cells (Figs. 3*B* and *E* and 4*C*) suggest that Grg3 also directly represses the *glucagon* gene. To test this, we performed ChIP on βTC6 and αTC1-6 cells that were infected with viruses encoding GFP and Grg3 fused to a Flag tag (Flag-Grg3) (Supplementary Fig. 4), as performed previously in liver cells

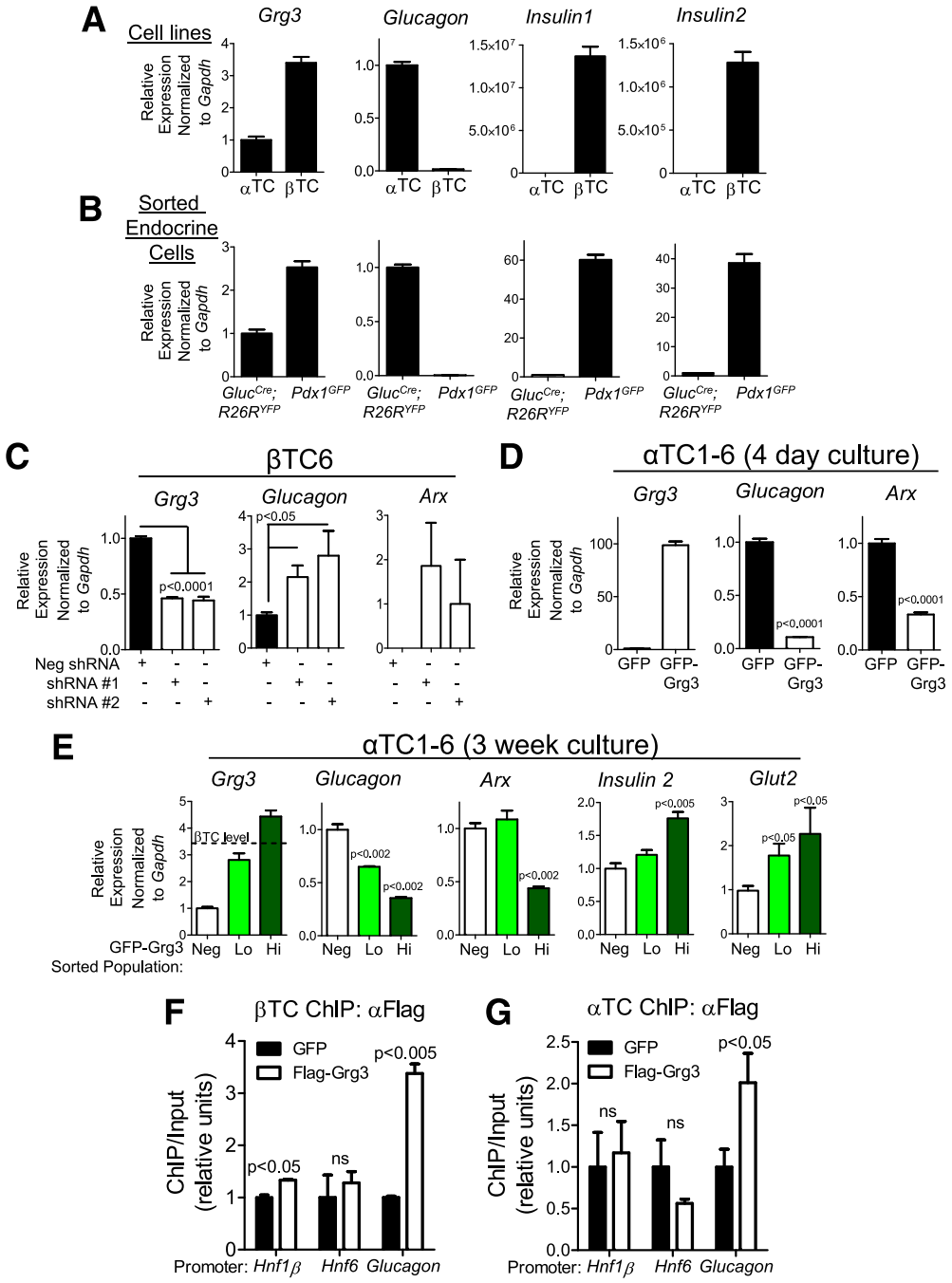

Figure 4—Grg3 directly represses *glucagon* and *Arx*. *A*: αTC1-6 and βTC6 cells were analyzed for *Grg3*, *glucagon*, *insulin 1*, and *insulin 2*. *B*: YFP+ α-cells sorted from *glucagon-Cre*;*R26R-YFP* neonates and GFP+ β-cells sorted from *Pdx1-GFP* neonates were analyzed for *Grg3*, *glucagon*, *insulin 1*, and *insulin 2*. *C*: *Grg3* was knocked down by two independent shRNAs in βTC6 cells, causing a derepression of *glucagon* and *Arx*. *D*: Ectopic expression of GFP-fused Grg3 (GFP-Grg3) through lentiviral infection for 4 days in αTC1-6 cells repressed *glucagon* and *Arx* compared with GFP-infected cells. *E*: GFP-Grg3 was stably expressed for 3 weeks in αTC1-6 cells and sorted for negative, low GFP, and high GFP expression, and *Grg3* expression increased with GFP intensity. *Glucagon* was repressed in a dose-dependent manner, whereas only the highest *Grg3* dose repressed *Arx* and induced *insulin 2*. *F*: βTC6 cells were infected with GFP or Flag-Grg3, and ChIP was performed using an α-Flag antibody. qPCR of the *glucagon*, *Hnf1β*, and *Hnf6* promoters indicates that Flag-Grg3 occupies the *glucagon* promoter, whereas the *Hnf1β* and *Hnf6* promoters are not bound. *G*: αTC1-6 cells were infected with GFP or Flag-Grg3, and ChIP was performed using an α-Flag antibody. Similarly, qPCR of the *glucagon* promoter shows that it is occupied in Flag-Grg3 cells. Each data point was repeated two to four times. ns, not significant.

(31). Using a Flag antibody on chromatin from GFP cells (serving as a negative genetic control) and Flag-Grg3 cells, we found Flag-Grg3 bound to the *glucagon* promoter in βTC6 cells (Fig. 4*F*). The fragment used for PCR analysis extends from 33 bp upstream of the transcription start and spans an Nkx6.1 binding site. Flag-Grg3 did

not bind to the same extent as *Hnf1β* and *Hnf6* promoters, genes whose expression was not seen to be affected by ectopic Grg3 (8). Similarly, ChIP for Flag in Flag-Grg3–infected αTC1-6 cells showed enrichment at the *glucagon* promoter over control-infected cells (Fig. 4*G*). These results coupled with the functional studies (Fig. 4*C–E*) indicate that Grg3 directly represses the *glucagon* gene.

Because Grg3 does not directly bind DNA, we sought to determine what recruits Grg3 to the *glucagon* promoter. Nkx6.1 is known to be a potent repressor of *glucagon* (32,33) and was found to bind Groucho corepressors in the context of neural tube development (14). We found that at E15.5, at E17.5, and in neonates, Grg3 was coexpressed with Nkx6.1 in the pancreas (Fig. 5*A–C*). By performing a PLA in βTC6 cells to probe for endogenous protein interactions in situ (34), we confirmed that Nkx6.1 and Grg3 interact (Fig. 5*D* and *E*). Using *Nkx6.1*+ βTC6 cells and *Nkx6.1*− αTC1-6 cells as a negative genetic control (Fig. 5*F*), we performed ChIP with an Nkx6.1 antibody. Comparing αTC1-6 cells to βTC6 cells, we found an enrichment of Nkx6.1 at the *glucagon* promoter but not at the *Hnf6* promoter in βTC6 cells (Fig. 5*G*), confirming previously published results (15,32,33).

Because Nkx6.1 is not present in α-cells to recruit Grg3 to the *glucagon* promoter, we hypothesized that other transcription factors known to be bound to the *glucagon* promoter in α-cells may recruit Grg3. FoxA factors have

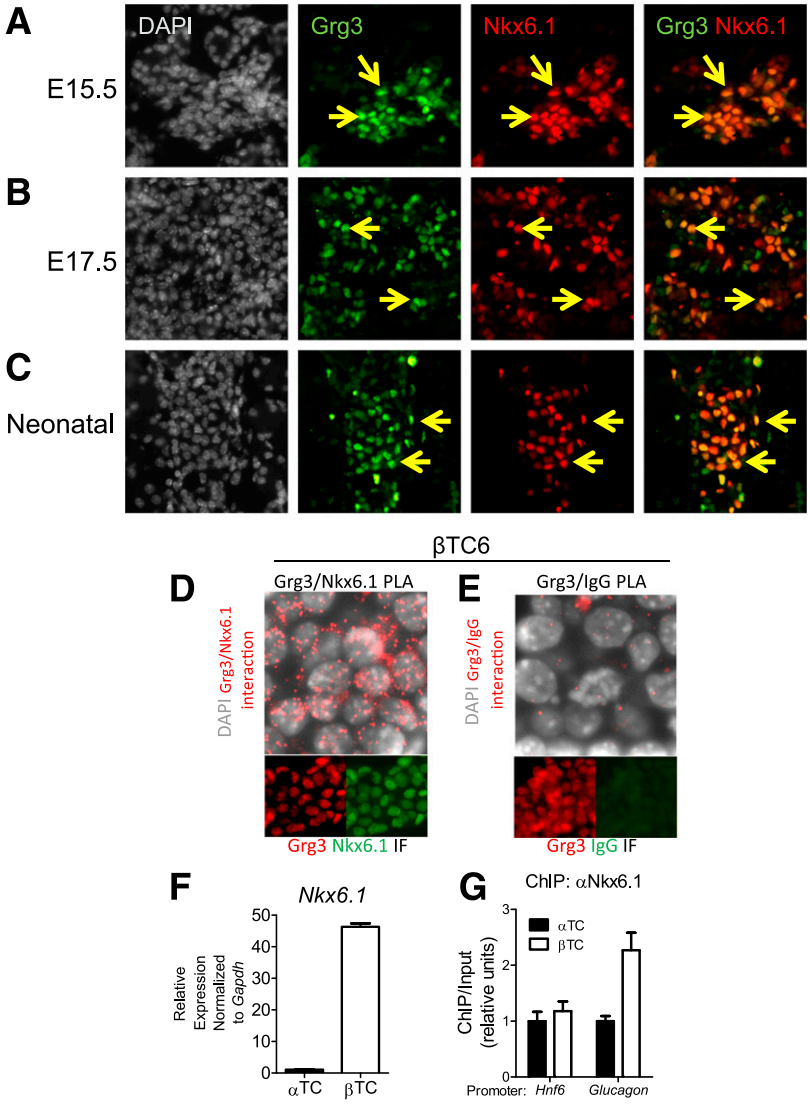

Figure 5—Grg3 is recruited to the *glucagon* promoter through an Nkx6.1 interaction. *A–C*: Immunofluorescence of Nkx6.1 and Grg3 on E15.5, E17.5, and neonatal pancreata. The yellow arrows indicate coexpressing cells. *D*: PLA demonstrates that endogenous Grg3 and Nkx6.1 interact in βTC6 cells; immunofluorescence indicates the specificity of the Grg3 and Nkx6.1 antibodies. *E*: Controls using Grg3 and IgG show a low, nonspecific signal; immunofluorescence indicates the specificity of the Grg3 and IgG antibodies. *F*: *Grg3* is expressed much more abundantly in βTC6 cells vs. αTC1-6 cells. *G*: ChIP was performed on αTC1-6 and βTC6 cells with an αNkx6.1 antibody, and qPCR was performed on *glucagon* and *Hnf6* promoters. Nkx6.1 occupies the *glucagon* promoter in βTC6 cells. IF, immunofluorescence.

been shown to directly activate the *glucagon* gene (35,36), and we have found previously that Grg3 interacts with FoxA factors in liver cells to repress the *albumin* gene (31). Similarly, we found that ectopic Grg3 in αTC1-6 cells (Supplementary Fig 5*A–F*) and endogenous Grg3 in βTC6 cells (Supplementary Fig. 5*G* and *H*) interact with FoxA2. Nkx2.2-Grg3 PLA was performed in βTC6 cells as a positive control (Supplementary Fig. 5*I* and *J*) because it has been previously shown that Nkx2.2 interacts with Grg3 in β-cells (10,11).

We also analyzed human islets for other members of the Groucho family (Grg1, Grg2, Grg3, Aes, and Grg6). Although we detected *Grg3/TLE3* expression by RT-qPCR, we were unable to detect nuclear human Grg3/TLE3 (97% homology to mouse) in human islets by immunofluorescence (Fig. 6*A–C*). However, of all the family members tested by immunofluorescence, only Grg1/TLE1 showed specific strong nuclear staining (Fig. 6*D–F*). Furthermore, we measured the intensity of Grg1/TLE1 staining in human α- and β-cells and found that β-cells expressed significantly higher amounts of Grg1/TLE1 than did α-cells (Fig. 6*E* and *G*). Additionally, RNA sequencing data of isolated human α- and β-cells (37) show that *Grg1/TLE1* is enriched 3.35-fold in β-cells versus α-cells (www.betacell.org). These findings suggest that human Grg1/TLE1 plays a similar role as mouse Grg3 in repressing α-cell–specific genes. To test whether Grg1 can function like Grg3, we ectopically expressed mouse Grg1 (98% homology to human) in αTC1-6 cells and found that Grg1 repressed *glucagon* and *Arx* expression in a dose-dependent manner (Fig. 6*H*). Overall, this finding suggests that Grg1/TLE1 is the predominant Groucho expressed in the human islet, not

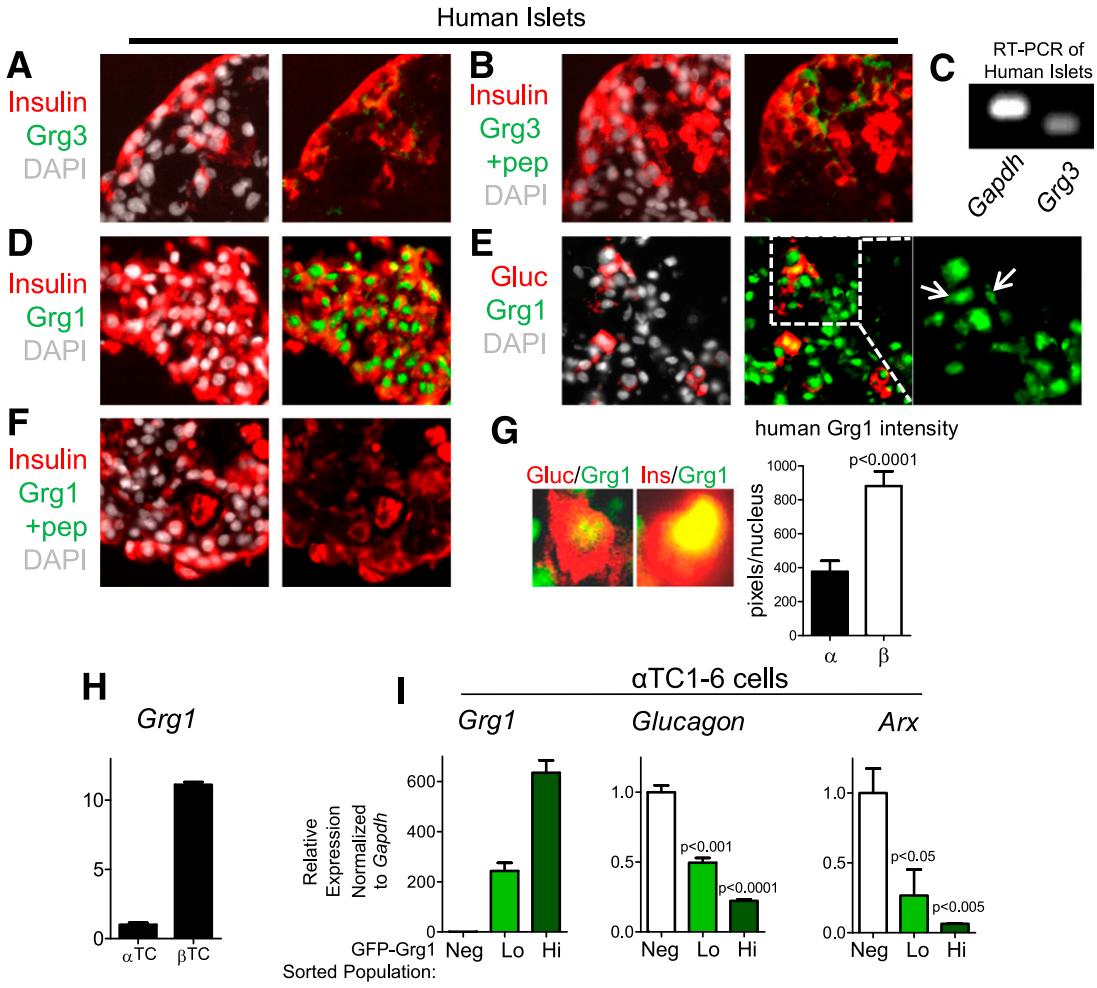

Figure 6—Grg1 is the predominant Groucho family member expressed in human islets. *A* and *B*: Nuclear Grg3 is not detectable in human islets (*A*), and nonspecific staining persists in the peptide-block control (*B*). However, *Grg3* mRNA is detected by RT-PCR (*C*). *D–F*: Nuclear Grg1 protein is expressed in both human β- (*D*) and α-cells, but note the weaker Grg1 staining of α-cells (white arrows) compared with other surrounding cells in the magnified view (*E*). Peptide-block controls indicate that the signal is specific (*F*). Quantitation of pixels staining the nucleus of single α-cells and β-cells (representative cells shown) indicate that Grg1 staining in β-cells is significantly more intense than in α-cells (*G*). *mGrg1* is more abundant in βTC6 cells than in αTC1-6 cells (*H*). *I*: αTC1-6 cells were transfected with GFP fused to Grg1 (GFP-Grg1) for 3 days, and cells were sorted based on GFP intensity. Ectopic GFP-Grg1 repressed *glucagon* and *Arx* in a dose-dependent manner in αTC1-6 cells.

Grg3/TLE3, and that Grg1 most likely plays a similar role as Grg3 in β-cells.

Groucho Repression Induces Glucose-Stimulated Insulin Secretion in α-Cells

The previously mentioned studies indicate that Grg3 ultimately helps to promote *insulin* expression while repressing α-cell–specific genes (Fig. 4*E*). We determined whether ectopic Nkx6.1 and Grg3 coexpression in α-cells can help to promote robust *insulin* expression and help to promote α-cell-to-β-cell conversion. Indeed, ectopic Grg3 and Nkx6.1 (Supplementary Fig. 6*A* and *B*) could each repress *glucagon* and *Arx,* although more markedly when they are coexpressed (Supplementary Fig. 6*C* and *D*). However, only ectopic Nkx6.1 could modestly induce *insulin 2* (Supplementary Fig. 6*E*), whereas *insulin 1* was not detectable in any of the samples (data not shown). This result was not surprising because Nkx6.1 has been shown to bind the *insulin* promoter (32). Of note, *insulin 2* was also reduced by ectopic Grg3 in these short-term cultures (Supplementary Fig. 6*E*); however, this is most likely indirect because Flag-Grg3 did not bind the *insulin 2* promoter (data not shown), and long-term GFP-Grg3 cultures induced *insulin 2* (Fig. 4*E*). We next hypothesized that Grg3 could help to promote α-cell-to-β-cell conversion in the presence of Pdx1, a known *insulin* gene inducer (38). Pdx1 has been used in several different contexts to promote β-cell conversion (17,39–41). However, ectopic Pdx1 introduced in α-cells during in vivo endocrine cell maturation failed to completely convert cells into β-cells because *Arx* was not silenced (17). Furthermore, previous studies found that Pdx1 expressed into α-cells in culture-induced *insulin* expression, but it was not clear whether insulin was secreted (23,42).

Ectopic Pdx1 introduced by lentivirus for 4 days (Fig. 7*A* and *B*) modestly repressed *glucagon* and failed to repress *Arx* but robustly induced *insulin 1* and *insulin 2* at the mRNA level (Fig. 7*C–F*). Pdx1 similarly expressed in α-cells with long-term high levels of GFP-Grg3 more efficiently repressed *glucagon* and *Arx* while also inducing both *insulin 1* and *insulin 2* mRNAs (Fig. 7*C–F*). Of note, Pdx1 expressed in α-cells only allowed for *insulin 1* and *insulin 2* mRNA expression but not insulin secretion into the media. By contrast, Grg3 ectopically expressed along with Pdx1 exhibited both *insulin 1* and *insulin 2* mRNA induction as well as glucose-sensitive insulin secretion (Fig. 7*G*). Immunofluorescence of αTC1-6 cells expressing GFP, GFP-Grg3, GFP/Pdx1, GFP-Grg3/Pdx1, and βTC6 confirmed Pdx1 protein expression and insulin production (Fig. 7*H*). However, only GFP-Grg3/Pdx1 cells and βTC6-positive control cells were positive for insulin C-peptide, suggesting that Grg3 promotes the production of mature insulin protein (Fig. 7*H*). Also important was that ectopic Grg3 repressed glucagon protein in both *GFP-Grg3* and *GFP-Grg3/Pdx1* αTC1-6 cells (Supplementary Fig. 7*A*) while inducing *Glut2* (Supplementary Fig. 7*B*). Note that SS and PP proteins were not accessed because their mRNA expression was already very low (data not shown). These data suggest that Grg3, along with other β-cell–specific transcriptional activators, would be useful to help to convert α-cells into functional monohormonal β-cells.

DISCUSSION

Our previous study showed that *Grg3* plays a pivotal role in the delamination of secondary transition endocrine progenitors from the pancreatic trunk epithelium (8), a step that has largely been overlooked in in vitro differentiation cultures. We now demonstrate with haploinsufficiency and knockdown studies that *Grg3* plays an additional role after delamination in establishing monohormonal β-cell identity, a characteristic of functional β-cells. The data also indicate that this function is performed by Grg1 in the human islet, not Grg3, suggesting that Grg3 for mouse and Grg1 for human can be used as sentinels of proper β-cell monohormonal differentiation for in vitro cultures.

A strategy for β-cell replacement is the in vivo conversion of pancreatic cells into functional β-cells (16,17,39,43), suggesting a pathway to regenerate β-cells in patients with diabetes who have reduced levels of β-cells. However, the results of these studies are still complex. Ectopic Pdx1 induced in endocrine progenitors converted α-cells into β-cells; however, Pdx1 induced in glucagon-positive α-cells failed to shut off the α-cell–specific transcription factor *Arx*, resulting in the incomplete conversion of α-cells into insulin-positive β-cells (17). Similarly, we found that ectopic Pdx1 expression in αTC1-6 cells induced *insulin 1/2* expression but failed to turn off *Arx* expression. Although we found that coexpression of both Pdx1 and Grg3 induced *insulin 1/2* less than ectopic Pdx1 alone, together, Pdx1 and Grg3, but not Pdx1 alone, repressed *Arx*, ultimately inducing glucose-sensitive insulin secretion as well as C-peptide positivity.

Of note, we found that most newly born endocrine cells at E15.5 express Grg3, but Grg3 expression gradually resolves to mainly β-cells as development proceeds (8). We propose that in the Yang et al. (17) study, the early ectopic Pdx1 expression in newly born Grg3+ endocrine cells allowed for a more efficient conversion toward a β-cell identity, whereas a later induction of Pdx1 in α-cells that lack or have low levels of Grg3 may not efficiently convert. The present data suggest that robust *Grg3* expression is needed for *Arx* repression, and only after long-term high *Grg3* expression in α-cells, along with *Arx* repression, does *insulin* become induced. Several studies have indicated an inverse correlation between *Arx* and *insulin* expression in vivo, suggesting that *Arx* repression is required for β-cell maintenance (12,13,25,44) and that Grg3 along with other β-cell–specific transcription factors may be important for the complete conversion to a functional β-cell. The data indicating that Grg1 is functionally redundant to Grg3 in repressing *glucagon* and *Arx* suggest a similar role for Grg1/TLE1 in human β-cells. Therefore,

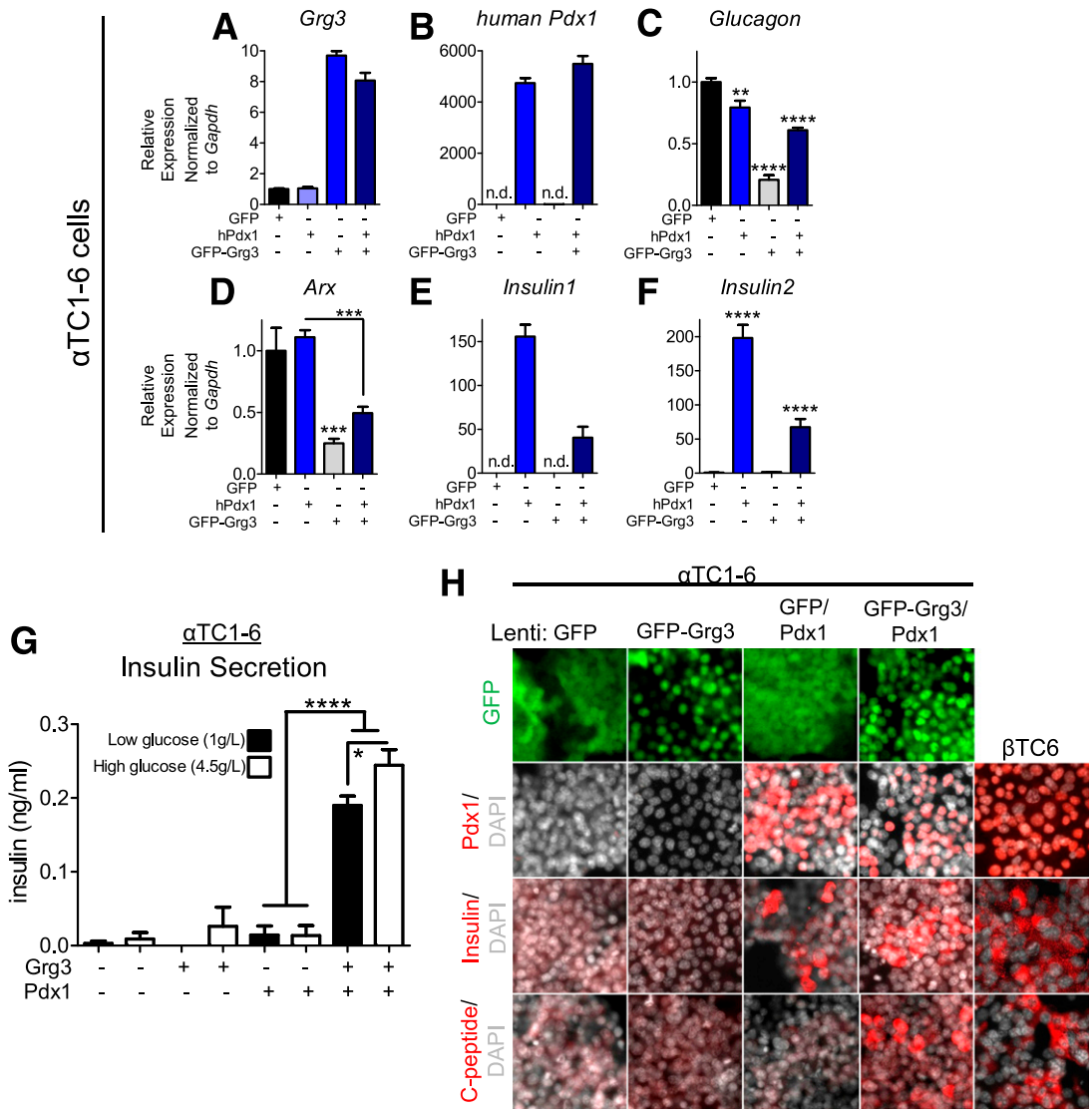

Figure 7—Grg3 works with Pdx1 to repress α-cell genes and induce glucose-stimulated insulin secretion. *A* and *B*: GFP-Grg3 is coexpressed with Pdx1 in αTC1-6 cells for 1 week. *C–F*: Pdx1 modestly represses *glucagon* and fails to repress *Arx* but induces abundant levels of *insulin 1* and *insulin 2*. GFP-Grg3 coexpressed with Pdx1 represses both *glucagon* and *Arx* while inducing *insulin 1* and *insulin 2*. *G*: αTC1-6 cells ectopically expressing GFP, GFP-Grg3, or Pdx1 fail to secrete insulin, but GFP-Grg3 coexpressed with Pdx1 induces glucose-stimulated insulin secretion. Note that the sensitivity of the secretion assay is 0.025 ng/mL. *H*: GFP- and GFP-Grg3–infected αTC1-6 cells are cotransduced with Pdx1 lentivirus. Immunofluorescence for Pdx1 confirms ectopic expression in αTC1-6 cells and positive control βTC6 cells. βTC6, GFP/Pdx1, and GFP-Grg3/Pdx1 αTC1-6 cells express insulin protein, whereas GFP and GFP-Grg3 cells do not. GFP-Grg3/Pdx1 αTC1-6 and βTC6 cells are positive for C-peptide, whereas GFP/Pdx1, GFP, and GFP-Grg3 αTC1-6 cells are not. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. n.d., not detectable.

Grg/TLE proteins may be necessary to complete the β-cell transcription factor network for both monohormonal gene expression and proper β-cell physiology.

A previous study has shown that Nkx2.2 interacts with Grg3 at the *Arx* gene and forms a repressive complex to silence *Arx* expression in β-cells (11). We have now found that Grg3 is recruited to the *glucagon* promoter by Nkx6.1 in β-cells to repress *glucagon* expression. We also found that ectopic *Grg3* in α-cells can repress *Arx* and *glucagon*. Although the repression of *Arx* can be explained by the presence of *Nkx2.2* in α-cells to recruit Grg3 to the *Arx* gene, the mechanism of *glucagon* repression by ectopic Grg3 is less clear. One possibility is that the detectable low levels of *Nkx6.1* in αTC1-6 cells may be sufficient to direct Grg3-mediated repression of *glucagon*. Another possibility is that other transcription factors known to interact with Groucho corepressors are already bound to the *glucagon* gene in α-cells. For example, previous studies have determined that FoxA factors bind and activate the *glucagon* gene in α-cells (35,36). Our laboratory has shown that Grg3 and FoxA interact (31), and we now show in βTC6 and αTC1-6 cells that this interaction occurs in α-cells during ectopic *Grg3* expression. Overall, the data show that robust levels of *Grg3* repress α-cell genes.

The data imply that decreased Groucho expression may have detrimental consequences on β-cell establishment in humans. When comparing the ratio of α- to β-cells between *Grg3*$^{+/+}$ and *Grg3*$^{+/-}$ mice, we find fewer insulin-positive β-cells and more glucagon-positive α-cells in *Grg3*$^{+/-}$ pancreata. Of note, patients with type 2 diabetes have been shown to have reduced levels of insulin with an increase in glucagon (45). Although *Grg3*$^{+/-}$ adult mice have normal glucose tolerance, further reduction or impairment of Grg3 repression or metabolic stress may induce a hyperglycemic state. Because Grg1/TLE1 is the predominant Groucho protein expressed in the human islet, our work underscores the importance of direct studies on human cells to test the translational relevance of the animal model studies. A more recent study has notably shown that *TLE1* lies near a newly identified type 2 diabetes susceptibility locus (46). Further studies are warranted to determine whether decreased Grg1/TLE1 expression in human islets results in β-cell-to-α-cell conversion or increases the risk for developing diabetes.

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